Effects of gonadotropin-releasing hormone agonist on brain development and aging: results from two animal models

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Summary

Normal brain maturation is the result of many structural and molecular changes that can be modulated by endocrine variables and is associated with brain plasticity and sex- and age specific differences in cognitive performance. In many species, sexual dimorphisms in brain structure and function have been documented, some of which are present at birth but some of which develop post-natally. Using an ovine model, the work contained in this thesis demonstrates that a peri-pubertal pharmacological blockade of gonadotropin-releasing hormone (GnRH) action (by chronic treatment with a gonadotropin releasing hormone agonist (GnRHa)) results in increased sex-differences in emotional behavior and other cognitive functions. One of the aims of this body of work was to determine what changes in brain structure and function were present in such GnRHa treated animals. The hippocampus is the most investigated brain region with regard to the complex interaction of memory and spatial orientation, functions which are thought to be sexually differentiated.

The aim of the study described in paper I was, therefore, to investigate whether peri-pubertal GnRHa treatment had an effect on a hippocampus dependent cognitive task, namely spatial orientation and whether the pharmacological blockade of GnRH affected expression of hippocampal genes associated with endocrine signaling and synaptic plasticity. The GnRHa treatment had no significant effect on spatial orientation ability although there was a tendency of females to perform better than males; however, GnRHa treatment was associated with significant sex- and hemisphere specific changes in mRNA expression for some of the investigated genes.

The aim of the study described in Paper II, was to investigate the effect of GnRHa treatment on structural development of the ovine brain such as total brain, hippocampus and amygdala volume

using magnetic resonance image (MRI). Analysis revealed highly significant GnRHa treatment effects on the volume of the left and right amygdalae, indicating larger amygdalae in treated animals. Significant sex differences were found for total grey matter and the right amygdala, indicating larger volumes in male compared to female animals. Additionally, we observed a significant interaction between sex and treatment on left amygdala volume, indicating stronger effects of treatment in female compared to male animals.

The aim of the study described in Paper III, was to investigate the molecular mechanisms that underlie these GnRHa-induced morphological changes in the amygdala using Agilent ovine microarray technologies to identify genes affected by GnRHa treatment followed by qRT-PCR to verify the noted changes in gene expression. Gene network analysis was performed to predict the functional impact of the differentially expressed genes. The analysis demonstrated that GnRHa treatment was associated with significant sex- and hemisphere specific differential expression of genes in treated female, but not in treated male animals.

Recent studies have indicated an association between hormones of the hypothalamic–pituitary–gonadal (HPG) axis and cognitive senescence, suggesting that post meno-/andropausal changes in HPG hormones are involved in cognitive and neuropathological changes associated with aging such as in Alzheimer´s Disease (AD). GnRH and luteinizing hormone (LH) have long been shown to have central roles in reproductive physiology, however, GnRH receptors are also highly expressed in brain regions that are affected in AD (temporal-hippocampal cortex and limbic system), and thus age related changes in GnRH signaling could have an impact on amyloid deposits in AD. The aim of the study described in paper IV was therefore to investigate the effect of intervention with a GnRHa on amyloid plaque deposition and GnRH and GnRHR

mRNA expression in a double transgenic mouse model that is predisposed to AD due to the presence of Arctic and Swedish amyloid beta (A4) precursor protein mutations (tg-ArcSwe). Analysis showed that the GnRHa/transgenes treatment clearly affects gene expression both at hormone and receptor level, while the effect GnRHa on amyloid plaque development remained unclear.

Overall, these findings substantiate the need for further studies investigating neurobiological effects of GnRH and the potential neurobiological side effects of GnRHa treatment on the brain in animals and humans.

Sammendrag (Norwegian)

Normal utvikling av hjernen er et resultat av mange strukturelle og molekylære endringer som påvirkes av endokrine variabler og er assosiert med hjernens plastisitet og kjønns – og alders spesifikke forskjeller i kognitive prestasjoner. Seksuell dimorphisme i hjernens struktur og funksjon er dokumentert hos mange arter, noe er til stede ved fødselen mens noe utvikles postnatalt. Ved å bruke en ovin modell, har arbeidet i denne avhandlingen vist at en peri-pubertal farmakologisk blokkering av gonadotropin frisettende hormon (GnRH) med en GnRH agonist (GnRHa), resulterte i økte kjønnsforskjeller i emosjonell adferd og andre kognitive funksjoner. Et av målene var å bestemme forandringer i hjernens struktur og funksjon hos GnRHa behandlete dyr. Hippocampus er det mest undersøkte hjerneavsnittet med tanke på den komplekse interaksjonen som fins mellom hukommelse og orientering i rom (spatial orientation), funksjoner sett på som seksuelt differensierte.

Målet med studiet i artikkel 1 var derfor å undersøke om peri-pubertal GnRHa behandling hadde en effekt på en typisk hippocampus avhengig kognitiv oppgave som orientering i rom, og om den farmakologiske blokkeringen av GnRH påvirket uttrykk av gener i hippocampus assosiert med endokrin signalisering og synaptisk plastisitet. GnRHa behandling hadde ingen signifikant effekt på evnen til orientering i rommet, selv om det var en tendens til at hunndyr var bedre enn hanndyr, men GnRHa behandling var assosiert med signifikante kjønns – og hemisfære spesifikke forandringer i mRNA uttrykk for noen av de undersøkte genene.

Målet med studiet i artikkel 2, var å undersøke effekten av GnRHa behandling på den strukturelle utviklingen av den ovine hjernen. Volum av hele hjernen, hippocampus og amygdala

ble undersøkt ved hjelp av magnetic resonance image (MRI). Det var signifikant økt volum av venstre og høyre amygdala hos behandlete dyr. Det ble funnet signifikante kjønnsforskjeller for total grå substans og høyre amygdala med større volum hos hanndyr enn hunndyr. I tillegg observerte vi en signifikant interaksjon mellom kjønn og behandling når det gjaldt volum av venstre amygdala, med større effekt av behandling hos hunndyr enn hanndyr.

Målet med studiet i artikkel 3 var å undersøke de molekylære mekanismene som ligger bak disse GnRHa- induserte morfologiske forandringene i amygdala. Agilent ovine microarray teknologier ble brukt for å identifisere gener som ble affisert av GnRHa behandling, etterfulgt av qRT-PCR for å verifisere endringene i genekspresjonen. Gen nettverk analyser ble utført for å forutsi mulig funksjonell betydning av genene med endret uttrykk. Analysen viste at GnRHa behandling var assosiert med en signifikant kjønns- og hemisfære spesifikk differensiert ekspresjon av gener i behandlete hunndyr, men ikke i behandlete hanndyr.

Nyere studier har indikert en assosiasjon mellom hormoner i hypothalamus-hypofyse-gonade (HPG) akse og kognitive prestasjoner ved begynnende aldring, hvilket indikerer at forandringer etter meno-/andropausen i HPG hormoner er involvert i kognitive og nevropatologiske forandringer assosiert med aldring som f.eks. i Alzheimers sykdom (AD). Det har vært kjent lenge at GnRH og luteiniserende hormon (LH) har sentrale roller i reproduksjonsfysiologien. Imidlertid er GnRH reseptorer også sterkt uttrykt i hjerneavsnitt som affiseres i AD (temporal-hippocampal cortex og limbiske system), og alders relaterte forandringer i GnRH signalisering kan derfor ha en innvirkning på amyloid avleiringer i AD.

Målet med studiet i artikkel 4 var derfor å undersøke effekten av GnRHa på dannelse av amyloid plakk, GnRH and GnRH reseptor (GnRHR) mRNA ekspresjon i en dobbel transgenisk

musemodell som er predisponert for AD grunnet Arctic and Swedish amyloid beta (A4) precursor protein mutasjoner (tg-ArcSwe). Studiet viste at GnRHa behandlingen i de transgene musene tydelig affiserte genekspresjonen både på hormon – og reseptor nivå, mens effekten av GnRHa på dannelsen av amyloid plakk forble uklar.

Funnene i avhandlingen bekrefter behovet for videre studier for å undersøke nevrobiologiske effekter av GnRH og de potensielle nevrobiologiske bivirkningene av GnRHa behandling på dyr og mennesker.

Abbreviations	
AD	Alzheimer's disease
ADD3	Adducin 3 (gamma)
APOE	Apolipoprotein E
APP	Amyloid beta (A4) precursor protein
AR	Androgen receptor
ARHGAP32	GTPase activating protein 32
ATXN10	Ataxin 10
Aβ	Amyloid beta
BDNF	Brain derived neurotrophic factor
BSA	Bovine serum albumin
CCNE1	Cyclin E1
cDNA	Complementary deoxyribonucleic acid
CPP	Central precocious puberty
cRNA	Complementary Ribonucleic Acid
Cy3	Cyanine 3
Cy5	Cyanine 5
CYP19	Aromatase
DAVID	Database for Annotation, Visualization and Integrated Discovery
ESR1	Estrogen receptor alpha
ESR2	Estrogen receptor beta

FAST	FMRIB's Automated Segmentation Tool
FLIRT	FMRIB's Linear Image Registration Tool
FMRIB	Functional MRI of the Brain,
FSH	Follicle Stimulating Hormone
FSL	FMRIB Software Library
GABA	Gamma-aminobutyric acid
GABARAP	GABA(A) receptor-associated protein
GABRA4	Gamma-aminobutyric acid (GABA) A receptor, alpha 4
GH	Growth hormone
GLMs	General Linear Models
GM	Gray matter
GnRH	Gonadotropin-releasing hormone
GnRHa	Gonadotropin-releasing hormone agonist
GnRHI	Gonadotropin-releasing hormone1
GNRHII	Gonadotropin-releasing hormone2
GnRHR	Gonadotropin-releasing hormone receptor
Gria1	Glutamate receptor AMPA1
Grin1	Glutamate (NMDA) receptor, ionotropic,
HPG	Hypothalamus-pituitary-gonadal axis
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
LH	Luteinizing hormone
LHRH	Luteinizing hormone releasing hormone

LHX5	LIM homeobox 5
MAP4	Microtubule-associated protein 4
MRI	Magnetic resonance image
mRNA	Messenger ribonucleic acid
NCAM1	Neural cell adhesion molecule 1
NGS	Normal goat serum
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RIN	RNA integrity number
ROIs	Regions of interest
SD	Standard deviation
SNTA1	Syntrophin, alpha 1
spn	Spinophilin
Syn1	Synapsin1
TBV	Total brain volume
TTR	Ovis aries transthyretin
VGF	VGF nerve growth factor inducible
WM	White matter

List of papers

Paper I- *Behavioural Brain Research 242(0), 9-16. 4-1-2013.*

Peri-pubertal gonadotropin-releasing hormone analog treatment affects hippocampus gene expression without changing spatial orientation in young sheep.

Nuruddin Syed, Wojniusz Slawomir, Ropstad Erik, Krogenæs Anette, Evans Neil P., Robinson Jane E., Solbakk Anne Kristin, Amiry-Moghaddam Mahmood, and Haraldsen Ira Ronit Hebold Haraldsen

Paper II: *Psychoneuroendocrinology 38, 1994-2002.2013*

Effects of peripubertal gonadotropin-releasing hormone agonist treatment on brain development in sheep - a magnetic resonance imaging study.

Syed Nuruddin, Muriel Bruchhage, Erik Ropstad, Anette Krogenæs, Neil P. Evans, Jane E. Robinson, Tor Endestad , Lars T. Westlye, Cindee Madison and Ira Ronit Hebold Haraldsen.

Paper III: *Psychoneuroendocrinology 38(12), 3115-3127. 2013*

Peri-pubertal gonadotropin-releasing hormone agonist treatment affects sex biased gene expression of amygdala in sheep.

Syed Nuruddin, Brynildsrud Ola Brønstad, Anette Krogenæs, Steven Verhaegen, Neil P. Evans, Jane E. Robinson, Ira Ronit Hebold Haraldsen, Erik Ropstad.

Paper IV: *(submitted)*

Down-regulation of elevated gonadotropin-releasing hormone gene expression in hippocampus in tg-ArcSwe mice following receptor agonist treatment.

Syed Nuruddin, Gry Helen Enger Syverstad, Sveinung Lillehaug, Anette Krogenæs, Erik Ropstad, Trygve B. Leergard, Lars Nilsson, Ira Ronit Hebold Haraldsen, Reidun Torp

Introduction

Brain development is a complex and precisely regulated process that occurs over an extended period of time (Kostovic- and Judaš, 2006; Rubenstein, 2011). This developmental process is characterized by sex specific changes in cognitive performance, behavior and social ability (Bethke et al., 2009; Zeng et al., 2005). These cognitive and behavioral changes are also accompanied by structural and molecular changes in the brain (Dumas, 2005; Luna et al., 2001; Paterson et al., 2006; Shaw et al., 2006). Furthermore, these structural and molecular changes which are important for normal development, may also be associated with increased neuropsychiatric disorders, if disturbed (Anagnostou and Taylor, 2011; Chow et al., 2012). Brain development and functions such as cognitive ability, for example emotional control and spatial orientation, are not only influenced by age, but also sexually differentiated (De Bellis et al., 2001) and are seen in numerous species (Cooke et al., 1998). In humans, the temporal sequence of brain maturation and the formation of functional circuits are sexually differentiated; the subcortical (e.g. striatum) and prefrontal cortical regions develop at different times in boys and girls (Casey and Jones, 2010). These temporal and organizational differences in brain development are thought to potentially result in sex-specific behaviors (Casey and Jones, 2010). It has been proposed that they may underlie sex-associated differences in the risk of developing some neuropsychiatric diseases (Bao and Swaab, 2010). This hypothesis is supported by the observation that the time of onset of neuropsychiatric disorders such as schizophrenia, autism spectrum disorders and Alzheimer's disease (AD), correlates with major endocrine changes during puberty and menopause (Stevens, 2002; Tareen and Kamboj, 2012; Vadakkadath Meethal and Atwood, 2005;). Recently, different stages of development, such as the prenatal and pubertal

periods, have received more attention with regard to their organizational impact on brain development and behavior (Berenbaum and Beltz, 2011). In particular, the pubertal period has been noted as a time during which significant neuronal changes may be correlated with large changes in reproductive hormone production and secretion patterns (Blakemore et al., 2010; Pfaff, 2009; Sisk and Zehr, 2005). The timing and magnitude of these neuronal changes may be important in directing earlier organizational activities within the brain (Kalynn et al., 2013). To investigate underlying pubertal hormonal mechanisms that might underlie sex differences in brain development and cognitive functions, some studies have focused on the modulatory effect of the sex hormone precursor, gonadotropin-releasing hormone (GnRH) on cognition and psychomotoric activity (Bryan et al., 2010; Grigorova et al., 2006).

Gonadotropin- releasing hormone and its receptor

GnRH or GnRHI is a decapeptide, an evolutionary old, 10 amino acid neurohormone that plays an important connective role between the neuronal and endocrine system (Skinner et al., 2009; Tsai, 2006). This first GnRHI isoform was discovered and described in the mammalian brain (Cheung and Wong, 2008). The second type of GnRH or GnRH-II was first identified in the chicken brain and is referred to as chicken GnRH-II (Millar et al., 2001). It is also highly conserved among vertebrates, including mammals (Chen et al., 1998). GnRH-II specifically plays a role as a potent inhibitor of potassium channels in the amphibian sympathetic ganglion, and inhibition of these ion channels facilitates rapid excitatory transmission of conventional neurotransmitters which might provide a general neuromodulatory mechanism for GnRH-II in the nervous system (Millar et al., 2004). In many vertebrate species, a third form of GnRH is

present and is designated as GnRH III. GnRH-III is localized in the terminal part of the olfactory neuronal cells in the brain (Millar et al., 2001).

GnRH receptors have the characteristic features of G-protein coupled receptors (GPCRs). The amino acid sequence of the GnRH receptor was first revealed for the mouse receptor cloned from the pituitary αT3 gonadotrop cell line (Tsutsumi et al., 1992). This sequence was confirmed (Reinhart et al., 1992) and provided the basis for the cloning of GnRH receptors in the pituitary glands of the human (Chi et al., 1993) sheep (Illing et al., 1993) and pig (Weesner and Matteri, 1994) which share over 80% amino acid identity. The identification of structural variants of GnRH, the discovery of their cognate GnRH receptor types in lower vertebrates and some mammals, and the influence of ligands and the intracellular milieu of signaling pathways are providing considerable insight into novel physiological and pathophysiological roles of GnRHs in diverse processes. In human, mouse and some ungulates the GnRH Type II receptor is non-functional as a GPCR. Nevertheless, GnRH II is able to bind the Type I receptor and signal in a manner distinctly different from GnRH I. A detailed molecular delineation of the interaction of GnRH variants and GnRH analogs with GnRH receptors in different cellular environments is contributing to the development of novel GnRH therapeutics.

Functional roles of mammalian GnRH and its receptors

Functions in the reproductive system

It is well known that GnRH is primarily synthesized and secreted by neuroendocrine cells in the preoptic area of the hypothalamus and transported along axons into the hypophyseal median eminence (Harrison et al., 2004) where it is released into the hypophysial portal vasculaturees

GnRH binds to its target, the GnRH receptor (GnRHR) on pituitary gonadotropes cells to stimulate the synthesis and intermittent release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn stimulate gametogenesis and gonadal hormone synthesis (Conn et al., 1986; Kasten et al., 1996; Rama and Rao, 2001). Feedback mechanisms by sex hormones regulate hypothalamic GnRH and pituitary gonadotropin release via homeostatic mechanisms. This hormonal cascade of events constitutes the well-documented hypothalamus-pituitary-gonadal axis (HPG) and this axis plays a pivotal role in the initiation of puberty and controls reproductive and behavioral function (Daniel, et al., 2011). GnRH, and its receptor gene expression in gonadotropes are critical for GnRH signaling and hence for gonadotropin secretion and sexual development. The importance of GnRH for sexual development has been shown by a strong induction of the gene and its receptor during the infantile period, followed by a weaker persistent activation during puberty in the female rat (Plant, 2001; Zapatero-Caballero et al., 2004).

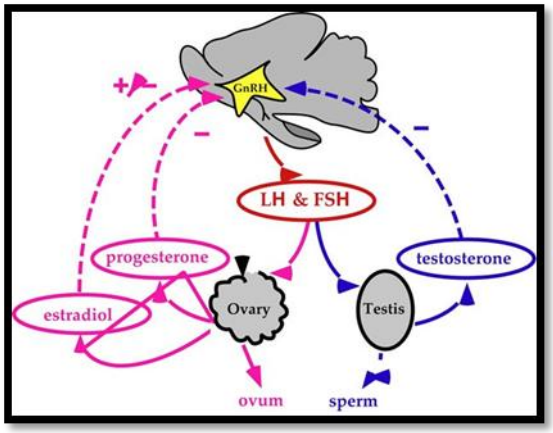

Figure 1: Classical view of the organization of the Hypothalamus pituitary gonadal axis (Source: Catherine rivier laboratory; http://pblcr.salk.edu/research.php)

The expression of GnRH and/or its receptor in gonads (McGuire and Bentley, 2010) may modulate various processes or mechanisms during the fertilization process. The presence of GnRH and GnRH receptors have been shown to play a role in autocrine and paracrine regulation, gonadal steroidogenesis, follicular atresia, and mediation of apoptosis during spermatogenesis, sperm maturation, and fertilization (Ramakrishnappa et al., 2005).

Function in the central nervous system

Whereas the fundamental role of pituitary GnRH receptors in reproductive function is undisputed, their presence in peripheral and central tissues, unrelated to reproduction, is less known and their function poorly understood. GnRH receptors have been reported in the heart, kidney, liver, skin, bladder, hippocampus, amygdala, central grey, cerebellum, and other tissues in various species (Coit et al., 2009; Cheng and Leung, 2005; Dong et al., 2011; Harrison et al., 2004; López de Maturana et al., 2007; Skinner et al., 2009; Xing et al., 2009;). Thus, the expression of both GnRH and its receptor in multiple mammalian non-pituitary tissues and cells suggest numerous and diverse autocrine, paracrine and endocrine extra-pituitary functions. These include neuronal migration during development (Romanelli et al., 2004), neuromodulation in the brain to affect sexual behavior (Millar, 2005), neuronal plasticity in the brain (Schang et al., 2011) and inhibition of gastric acid secretion (Chen et al., 2005), cardiac development and function (Skinner et al., 2009).

Over the last three decades, it has been also shown that GnRH can affect the central nervous system. GnRH has been shown to affect hippocampal (Albertson et al., 2008; Farn et al., 1999; Jennes et al., 1988; Osada and Kimura, 1995; Yang et al., 1999), amygdalar (Albertson et al. , 2008; Jennes et al., 1988), cerebellar (Renaud et al., 1975), preoptic (Pan et al., 1988), and

cortical (Renaud et al., 1975; Xu et al., 2004) neurons in mice, rats and sheep. GnRH projections are wide spread in the brain (Buma, 1989; Richardson et al., 2004); Furthermore, GnRH /GnRHR expression in several areas within the central nervous system provide indication of its more extended role in brain function.

Among the different regions of the central nervous system, the hippocampus consistently expresses high levels of GnRH receptors (Skinner et al., 2009). GnRH receptor-immunoreactive neurons were found within the pyramidal cell layer, dentate gyrus and indusium griseum of the human, mouse and sheep brain (Albertson et al., 2008; He et al., 1999; Kubek et al., 1979; Lu et al., 1999; Osada and Kimura, 1995; Wilson et al., 2006). This region is the most important integrative central nervous area for cognitive, endocrinological and behavioral processes. The long lasting enhancement of synaptic transmission can be induced by activation of GnRH receptors that is mediated by ionotropic glutamate receptors in CA1 pyramidal neurons of rat hippocampal slices. Furthermore, GnRH potentiates the excitability of hippocampal and cortical neurons which are crucially involved in learning and memory (Wang et al., 2010). It has been also reported that hippocampal GnRH receptor-expressing neurons co-express estrogen receptor beta (ERβ) in sheep (Albertson et al., 2008). Interestingly, it has been reported that GnRH is likely to be elevated during the post-menopausal period in such regions (Gore et al., 2004). Therefore, GnRH action on these neurons may contribute to the neurodegenerative pathology that accompanies Alzheimer's disease.

In addition to the presence of extensive GnRHR neurons in hippocampal regions, these neurons are also evident throughout the olfactory system in the rodent (Albertson et al., 2008; Choi et al., 1994; Jennes et al., 1997). These structures include amygdala, mitral cell layers of the olfactory,

accessory olfactory bulbs, piriform cortex and tenia tecta. Among these structures, the amygdala is the region where GnRH receptor expressing neurons are most widely distributed (Albertson et al., 2008). Nevertheless, there are some controversial reports about the distribution of GnRH binding sites in the rat amygdala. Some authors have reported a limited distribution, while others have detected a high density of potential GnRH receptor expressing neurons in the mouse and rat amygdala (Granger et al., 2004; Haour et al., 1987). It is postulated, that GnRH may access these receptors through neurons that project directly to the amygdala (Sanchez and Dominguez, 1995). An increase in plasma gonadotropins after electrical stimulation of the amygdala in rats and cats has been reported, which is attributed to a direct connection between the amygdala and the preoptic area (Layton et al., 1981; Sirett et al., 1986; Velasco and Taleisnik, 1969;). Furthermore, a dense population of GnRHR-immunoreactive neurons was detected throughout the amygdala (Albertson et al., 2008) of adult mice, as with the hippocampus, elevated levels of GnRH in the amygdala provides further support for multifunctional physiological or pathological roles of GnRH. In addition to the olfactory system, GnRH receptor expression in the superior colliculus, red nucleus and cerebellum may also suggest that GnRH modulates motor control (Albertson et al., 2008)

Functional roles of hippocampus and amygdala in cognition

Hippocampus

The hippocampus and amygdala are key structural elements of the limbic system and have been investigated as mediators for learning, memory and emotional regulation and control (Ortu et al., 2013; Phillips et al., 2003). The hippocampal regions (the CA fields, dentate gyrus, and subicular

complex) are parts of a system of anatomically related structures in the medial temporal lobe that is important for generation of new neurons (Kheirbek and Hen, 2011) and contribute to diverse memory formation. The rate of hippocampal neurogenesis is positively correlated to hippocampal-mediated learning abilities (Drapeau et al., 2003) and Hippocampal synaptic plasticity is believed to be the mechanism underlying certain types of learning and memory (Bliss and Collingridge, 1993). In this regard, neuroimaging studies have provided evidence that the hippocampus becomes more active during spatial navigation in humans, and hippocampal morphology can be affected by learning of spatial navigation skills (Mark, 2002). Furthermore, studies have shown that damage to the hippocampus impairs the acquisition and retrieval of information required to navigate in spatial mazes in rats (Jarrard, 1978; Morris et al., 1982). The left hippocampus, in particular, appears to be a key component in the retrieval of spatial memory (Spreng and Mar, 2012), and though actions with other areas of the brain makes memory recall possible.

Amygdala

Extensive empirical evidence suggests that the amygdala is a key region of the brain involved in underpinning emotions (Schaefer and Gray, 2007). Animal models investigation of amygdala function have emphasized its role in emotional learning and demonstrated that the amygdala is critical for the acquisition, storage, and expression of conditioned fear responses (Cahill et al., 1995; Cahill et al., 1999; Maren, 2001). Studies on the cognitive neuroscience of emotion and memory have demonstrated a range of means by which emotion can change the formation and recollection of episodic memory. It has been suggested that emotion, through the amygdala's influence, can alter three components of episodic memory: encoding, consolidation and the

subjective sense of remembering. Although episodic memory critically depends on other brain regions, most notably the hippocampal complex (Eichenbaum, 2002), the amygdala may be important for modulating the neural circuitry. Despite the fact that the amygdala and hippocampus have independent memory systems, their interaction seems to be crucial because an emotional stimulus increases the memorial impact (Phelps, 2004).

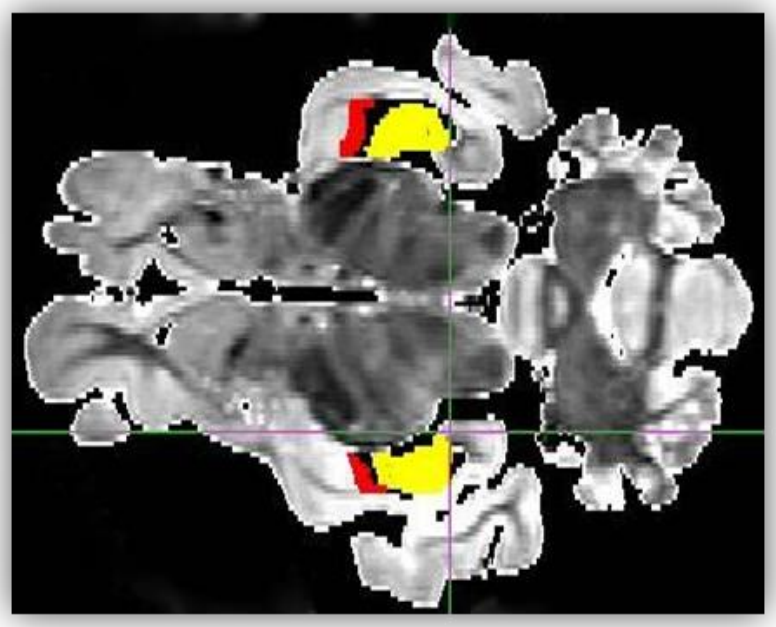

Figure 2: Magnetic resonance images of sheep amygdala and hippocampus in axial view (Source: Nuruddin et al., 2013). Bilateral red colored areas indicate the amygdala and the yellow colored areas indicate the hippocampus.

Role of GnRH and its receptor in cognitive function

In recent years, research has focused on the effects of gonadotropin-releasing hormone (GnRH) and its receptor on sex-specific cognitive and physiological patterns, initiated by the fact that GnRH receptor expression has been found in various brain areas and peripheral tissues unrelated to reproduction (Skinner et al., 2009; Wilson et al., 2006). Results in adult human males and females, as well as in rodents, indicate that blockage of GnRH function using chronic GnRH agonist treatment may lead to significant changes in several cognitive functions. Significant impairments in domains of visuospatial and higher-order executive control functions (Nelson et al., 2008), and an episodic increase of depressive symptoms (Schmidt et al., 2004), have been described in men. In females, GnRH agonist treatment is associated with a decline in working memory (Grigorova et al., 2006; Palomba et al., 2004) and disrupted encoding of episodic verbal memory (Craig et al., 2007). Furthermore, animal models of Alzheimer's disease show that blockade of GnRH signaling by GnRH agonist had positive effects on cognitive function (Bryan et al., 2010). However, these studies have only reported effects in adults. Comparative studies in children and adolescents are lacking (Carel et al., 2009) and it has been suggested that this is due to our insufficient understanding of the biological mechanisms of sex-specific brain development during puberty (Jazin and Cahill, 2010). Recent research has suggested that GnRH plays a vital role in controlling the extent of the specificity of brain function, and changes in the availability of this decapeptide during critical periods of brain development (such as at puberty) may be reflected in altered sex-specific behavioral and physiological patterns (Wojniusz et al., 2011) in later life. Nevertheless, the exact neurobiological mechanism by which GnRH might affect brain development during puberty is not fully understood.

GnRH agonist

Pharmaceutical GnRH agonists (GnRHa) are synthetic peptides that bind to and activate the endogenous GnRHR. GnRH agonists have been developed to be more potent than natural GnRH peptide due to the fact that they have a higher affinity to GnRH receptors and a longer half-life (Conn and Crowley, 1994). The natural GnRH has a shorter half-life because of the rapid cleavage of the bonds between amino acids at positions 5-6, 6-7 and 9-10. Synthetic GnRH agonists are derived from native GnRH by substitution of a D-amino acid for the native L-amino acid position 6 in the decapeptide. This substitution at position 6 helps the agonist resist degradation and increases its half-life and the time of receptor occupancy. Under normal conditions pulsatile secretion of GnRH from the hypothalamus is required to stimulate the release of FSH and LH from the pituitary gland as, upon stimulation, receptors are internalized and the interval between pulses allows receptor concentration to be replenished. A continuous administration (subcutaneous/intramuscular/intranasal) of GnRH agonists (goserelin, leuprolide, nafarelin, buserelin, triptorelin) initially induce a sharp increase in pituitary secretion and serum level of FSH and LH (the flare effect), which stimulates an increase in serum sex steroids (within 3 days of initial treatment). However, continuous stimulation of the pituitary by chronic administration of a GnRH agonist produces an inhibition of the hypophyseal-gonadal axis due to a down-regulation of pituitary receptors for GnRH, and decreased levels of LH, FSH and sex steroids within 2-4 weeks (Schally et al., 2001).

Application of GnRH agonist in adults and their side effects

In adults GnRH- agonists are applied in the treatment of various sex hormone dependent conditions including endometriosis (Rothman and Wierman, 2007), prostate cancer (Tammela,

2004), breast cancer (Mastro et al., 2011) and suppression of the LH surge for use in in-vitro fertilization protocols (Zafeiriou et al., 2000). GnRHa has also been implemented in the treatment of AD patients (Bowen et al., 2004; Wang et al., 2010). The major side effects of GnRHa therapy have been primarily explained as a result of withdrawal of sex hormones. Men with prostatic cancer, receiving GnRHa therapy report physical discomfort, (Potosky et al., 2001) and lowered quality of life (Alibhai et al., 2006), reduced bone mineral density (Diamond et al., 2004), increased risk of cardiovascular events including myocardial infarction and cardiovascular mortality (Bourke et al., 2012; Levine et al., 2010) and decreased cognitive functions (Nelson et al., 2008).

GnRHa treatment in women with breast cancer is also associated with a variety of side effects including hot flushes, decreased libido, mood swings, increased cardiovascular risk and skeletal-related conditions such as bone loss (Tan and Wolff, 2007). A decline in working memory has also been reported in women treated for benign gynecological problems (Grigorova et al., 2006).

Usage of GnRHa in pediatric medicine

In pediatric medicine, GnRHa is mainly used in the treatment of central precocious puberty (CPP) and developmental disorders of the genital system. Puberty is defined as the biological, social and cognitive maturation to adulthood where the individual becomes capable of sexual reproduction to enable fertilization (Marcell, 2007). The pubertal transition is initiated and driven by increased GnRH secretion into the hypophysial portal circulation leading to downstream activation of the HPG axis (Partsch et al., 2002). Precocious puberty (CPP) is defined as the development of pubertal changes, at a younger age than the accepted lower limits for the age of onset of puberty. Specifically, CPP is characterized by early onset of secondary sexual

characteristics in girls who are less than eight years old and in boys who are less than nine years old (Cesario and Hughes, 2007). CPP is associated with mental and behavioral problems ranging from depression, anxiety and eating disorders to increased risk- taking behaviors as well as reduced adult height (AH) due to earlier epiphyseal closure initiated by rising sex hormones (Baker et al., 2012; Carel et al., 2004; Graber et al., 2004; Negriff et al., 2011; Stattin et al., 2011) Nevertheless, whether comorbidity is correlated with the treatment or the condition itself has not been properly investigated. To a lesser degree GnRHa treatment is used in several other diseases including idiopathic short stature, severe hypothyroidism, growth hormone deficiency or congenital adrenal hyperplasia (Carel et al., 2009).

GnRHa treatment in children is generally considered to be safe and well tolerated, No clear evidence of predicted side-effects, such as increased body mass index and reduced bone mineral density, headaches and hot flashes, have been documented (Carel et al., 2009). However, little is known about the ability of GnRHa treatment to prevent or affect development of mental and behavioral problems in children. A tendency towards fewer difficulties in coping with precocious puberty in treated CPP patients vs. untreated has been previously reported (Xhrouet-Heinrichs et al., 1997); however this study was hampered by a very small control group consisting of five untreated CPP patients that were compared to 15 GnRH analog treated CPP girls.

In neuropsychiatric research to investigate GnRH treatment effects in children, one of the main challenges is that study design is limited in such studies. To explore the effects of treatment on behavior, cognitive function and emotion regulation, an ideal design would be a randomized controlled trial (RCT). However, since GnRH agonist treatment in CPP is well established and considered to be somatically advantageous, it is unethical to randomize CPP patients into a non-

treatment group for comparison reasons. In practice, this limits the design possibilities to longitudinal, cross-sectional or case-control studies. A longitudinal design where CPP children are assessed several times; before, under, and after cessation of the treatment, and compared to other types of patients or healthy children, assessed at the same time points, is an acceptable alternative. Nevertheless it is still difficult to interpret whether the observed effects in such studies are a result of the progressing condition or the GnRHa therapy. To address this limitation, it is necessary to use an animal model. In contrast to human models, animal studies provide the opportunity for systematic experimental manipulation of genetic and environmental variables in a RCT setting. Although the applied outcome measures and their neural substrates are not always directly applicable in humans, there are considerable similarities between pubertal and adolescent developmental processes in mammals and humans considering behavioral, neural and hormonal characteristics (Spear, 2004).

Aim of the thesis

Manipulation of GnRH receptors for clinical purposes has been used in adult and pediatric medicine for several decades with the goal of blocking sex hormone production. Existing research has concentrated on the specific effects of GnRHa treatment on the targeted disease or its influence on reproduction, with little focus on potential side effects of this treatment regime on cognitive function, emotion regulation, structural brain organization or cellular and molecular changes during brain development (Carel et al., 2009). These potential side effects of GnRHa treatment are of significant clinical importance, as many neuropsychiatric disorders, behavior and emotional problems are first observed during the peri-pubertal period. The principal aim of this thesis was to investigate effects of peri-pubertal GnRHa treatment on brain development to

shed light both on the potential roles of GnRH and GnRHR in structural, neurobiological and cognitive functions, in brain regions that are unrelated to reproductive function.

Secondary aims were to investigate the effects of GnRHa treatment on

- Endocrine and synaptic plasticity related gene expression levels in the hippocampus and any association with spatial orientation behavior.
- Structural volume of total brain and other regions of interest such as the hippocampus, amygdala, white matter and grey matter.
- Molecular mechanism and gene expression levels in amygdala that might underlie treatment induced structural changes in this structure.
- Expression levels of *GnRH* and its receptor *GnRHR* in the hippocampus as well as beta amyloid (Aβ) deposition in the thalamus, cerebral cortex and hippocampus, in an AD mouse model.

Materials and Methods

Animals and treatment

For paper (I, II, III), the study was conducted at the University of Glasgow's Cochno Research Centre (55° 55'N) and all animals procedures were approved by the University's Welfare and Ethics Committee, and in accordance with Home Office regulations (PPL 60/3826). To eliminate the possible developmental effects of steroid transfer between siblings of different sexes and reduce genetic variation, the whole study was conducted using 46 pairs of same-sex twin lambs (Scottish Mule Texel Cross, 22 female and 24 male). Lambs were born between 17th March and 1st April 2008, and remained with their dams until weaned at about 12 weeks of age. Males and females were maintained separately during the entire study period. Within each set of twins, one was randomly assigned, at birth, to the control (C) and the other to the treatment (T) group. Animals in the treatment group received subcutaneous implants of the GnRH agonist, goserelin acetate (Zoladex®; kindly donated by Astra Zeneca; Macclesfield, UK 3.6 mg) every 4 weeks from 8 weeks of age in males and 28 weeks of age in females because of the sex-specific timing of puberty in this species (Wood and Foster, 1998). The Zoladex® implant was administered in the axillar region, using a 'SafeSystem' needle and syringe. Animals were maintained in accordance with normal husbandry conditions, i.e., on grass, at all times except for periods of behavioral testing at approximately 8, 28 and 48 weeks of age, when they were group housed indoors. Every four weeks, throughout the study, animals were gathered and held, for no more than two hours (with their mothers, prior to weaning), in a specialized sheep handling facility at the University of Glasgow's Cochno Research Center where blood samples were collected from

the jugular vein, physical measurements taken (weight, shoulder height, girth) and agonist administered (where required). To guarantee that GnRHa treatment blocked pubertal development, plasma samples were assayed regularly for testosterone (males) and progesterone (females), monthly measurements of scrotal volume made during the animals' life and the testes or ovaries excised, weighed and histologically evaluated after euthanasia at 12 months of age (March 2009) (Robinson et al., in press)

For paper IV, we used transgenic mice (Tg-ArcSwe) carrying a human AβPP cDNA with the Arctic (E693G) and Swedish (KM670/671NL) mutations and wild-type mice. Animals (Tg-ArcSwe and wild–type mice) were injected subcutaneously with 25 ng/g of the GnRH-agonist Leuprorelin acetate (Procren Depot "AbbVie") dissolved in physiological saline, or vehicle alone. The injections were given once every fourth week from the age of 4 months, before plaque deposition has begun (Lord et al., 2006). Of the animals included in the study, about 20 % died of unknown causes before reaching the age of 12 months. This included both treated and untreated animals, leaving us with the number of animals referred to in Table 1. The remaining animals were anaesthetized using Isofluran Baxter (Isoflo™, Abbot Laboratories Ltd) and sacrificed by decapitation at 12 months, after 8 months of treatment. In addition to this, 4 month-old animals, who did not receive any pharmacological intervention, were sacrificed. Details are shown in Table 1. The tissues from all sacrificed animals were stored at -80 ºC for further use.

Table 1. Animals involved in the paper IV study.

Age (in months)	12	12	4
Treatment ***(Leuprorelin acetate; 25ng/g)***	Treated	Untreated	Untreated
Tg - ArcSwe males	3	6	6
Wild-type males	---	6	6
Tg - ArcSwe females	7	6	6
Wild-type females	---	6	6

--- indicate tissue not analyzed

Methods

The sheep used in the studies of articles I-III were subjected to several behavior experiments at three different time points to assess their cognitive, emotional and behavioral development. The results from all of these behavioral tests are not included in this thesis. In a subsample of 30 animals (16 males and 14 females, half of them treated), spatial orientation, the expression of 17 genes within the hippocampus and the transcription profile within the amygdala (by microarray) were analyzed to ascertain if they were affected by GnRHa treatment or sex. The brains of 41 sheep (17 treated; 10 females and 7 males, and 24 controls; 11 females and 13 males) were used for morphometric analysis of different regions of the brain by using Magnetic resonance image (MRI). The mRNA expression of *GnRH* and *GnRHR* (study-IV) were analyzed by quantitative real time PCR (qRT-PCR) by using hippocampal samples from the Tg-ArcSwe mouse model and its respective controls. Furthermore hippocampus, cerebral cortex and thalamus from these animals were used for imunohistochemical studies of amyloid beta deposition.

Behaviour study - Spatial orientation task

Spatial orientation is an essential cognitive function because most mammals depend on it for finding food, mates, and avoiding becoming prey (Wolbers and Hegarty, 2010). Animal and human studies have demonstrated that robust sex-differences exist in spatial abilities, usually favoring males (Jonasson, 2005; Kerns and Berenbaum, 1991; Terry, 2009). The main goal of this experiment (Paper I) was to explore the sexually differentiated nature of spatial orientation ability and to establish whether pre-pubertal GnRHa treatment interfered with the development of this function.

Spatial orientation was assessed at 48 weeks of age (when males and females had received GnRHa treatment for 40 and 20 weeks respectively) by means of a spatial maze task. The spatial maze setup (Figure 3) was based on the design developed and validated for use in sheep by Lee and colleagues (Lee et al., 2006)

The dividing walls of the maze were made of metal penning that was familiar to the animals and through which the test animals could see the audience pen. The outer walls of the maze arena were solid. For testing, animals were separated into smaller groups and sequentially placed in the audience pen. Individual ‘test’ animals were removed from the audience pen by a trained and familiar handler and calmly led to the entrance of the maze.

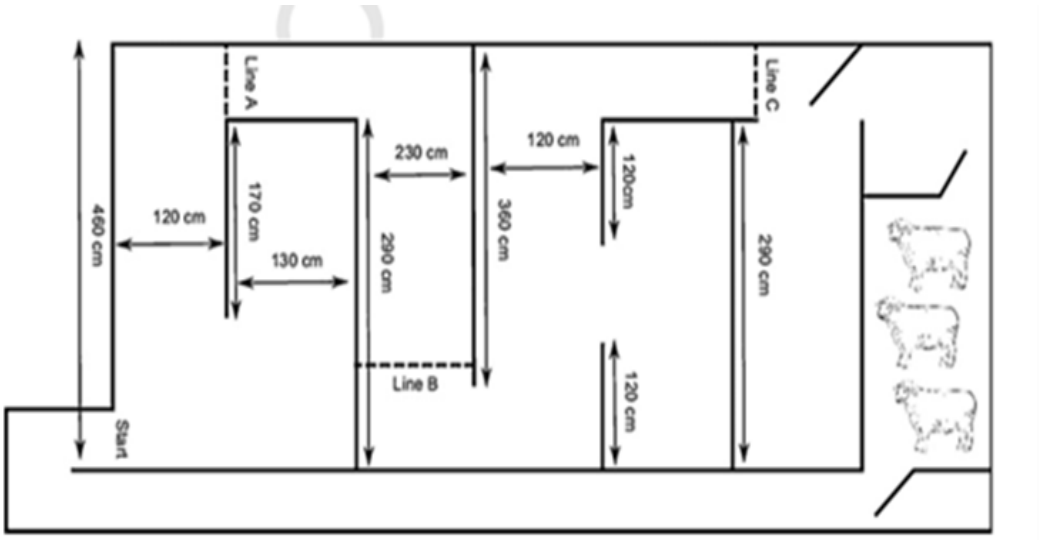

Figure 3: Diagram of the spatial maze used at 48 weeks of age (Paper: I).

Each sheep was given 300s to traverse the maze and the test was deemed completed when the animal passed line C (Figure 3). The time taken to reach lines A, B and C were recorded as was the vocalization rate (VR) in each of the three sections of the maze, calculated as a number of vocalizations per min. If an animal did not complete the maze within 300s, it was moved back through the maze and exited through the entrance gate. Animals were tested in the same sequence over three successive trials. Trial one and two were carried out on the same day and trial three was performed on the next day.

Hippocampal gene expression involved in synaptic plasticity and endocrine signaling

In paper I of this thesis, hippocampal gene expression analyses were performed using tissue collected from animals maintained in the model outlined above and the results discussed in the context of spatial orientation, a hippocampus dependent cognitive function (Stella et al., 2012).

Specifically, animals were sacrificed at approximately 50 weeks of age, with an overdose of barbiturate (Somulose 1ml/kg body weight; Decra Veterinary Products, Shrewsbury, UK), decapitated and their brains removed. The hippocampus was then carefully dissected from the right and left hemispheres, and the right and left hippocampi individually trisected, perpendicular to the long axis of the hippocampus, allowing isolation of hippocampal subregions (each containing CA1 to CA3). Samples were immediately frozen in liquid nitrogen and stored at -75^{o}C. Total RNA was isolated from the sub region containing CA1 to CA3, from the anterior section of the hippocampus of each animal, using TRIzol Reagent (InvitrogenTM, Paisley, UK) and processed according to standardized procedures. Samples were analyzed using quantitative real time-PCR (qRT-PCR) in a subset of 30 animals (Texel Cross, 14 female and 16 male). The expression of genes involved in synaptic transmission (*Grin1, Gria1, GABRA4, Syn1, spn, BDNF, VGF*), proliferation and differentiation (*LHX5*), structuring (*NCAM1*), that underlie synaptic plasticity and genes associated with endocrine signaling (*GnRH I, GnRH II, CYP19, AR, GH, ESR1* and *ESR2*) were analyzed.

Postmortem magnetic resonance image (MRI)) for morphometric analysis of global and regional brain volumes

MRI provides many benefits relative to the assessment of the volume of specific brain areas, as it bypasses dissection and photography methods, which carry higher risks of errors in quantitative measurements (Pfefferbaum et al., 2004). In paper II of this thesis, the goal of the experiment was to explore GnRHa treatment effects on global and regional brain volumes. This experiment, was conducted using 41 brains (17 treated; 10 females and 7 males, and 24 controls; 11 females and 13 males) that were perfusion fixed with 4 % paraformaldehyde in 0.5% BSA immediately

following euthanasia, post fixed in 4% PFA overnight and stored in 30% sucrose until examination. In order to minimize movements during scanning, each fixed postmortem specimen was suspended in agar gel, which was placed in a rectangular plastic container. Image acquisition was performed with a 3-T MRI scanner. For MRI preprocessing, the raw dicom files were converted to nifti and inspected for artifacts. Bias correction of the images was done using Freesurfer mri_nu_correct.mni to correct for intensity non-uniformity. Spurious noise outside the brain was removed, and Brain Extraction Tool (BET) (Smith, 2002) part of FMRIB's Software Library (FSL)(Smith et al., 2004) was used to strip residual gel artifacts outside the brain. A set of anatomical regions of interest (ROIs) were manually drawn on the best quality brain MRI, of the chosen sheep, to act as a template. The manual segmentation of the amygdala and the hippocampus was done using FSLView, part of FMRIB's Software Library (FSL) (Smith et al., 2004) by a single rater (Muriel Bruchage) blinded to any subject characteristics. The hippocampal ROI included the cornu ammonis, dentate gyrus and the subiculum, as each of these components show different histological characteristics and topographically well-ordered afferents and efferents (Nolte, 1993). A combination of T1- and T2-weighted data was used to distinguish the anterior boundaries that separate the amygdala from the hippocampus. However, precise delineation of the boundaries is difficult even at a histological level (Bergin, 1994). Therefore, inter-rater reliabilities for each ROI were established by having two raters (Muriel Bruchhage and Syed Nuruddin) independently segment five datasets. Percent inter-rater voxel overlap was 94 % for hippocampus and 97 % for amygdala, indicating high reliability. For tissue segmentation, FMRIB's Automated Segmentation Tool (FAST) (Zhang et al., 2001) was used to automatically segment the T2-weighted MRI volumes of the individual brains into gray matter (GM) and white matter (WM). The underlying method of FAST is based on a hidden Markov

random field model and an associated Expectation-Maximization algorithm. All segmented data were visually inspected for accuracy. Total GM and WM volumes were calculated by multiplying the number of voxels in each of the segmented classes by its voxel resolution. Four datasets (3 untreated males and 1 treated male) were excluded during the automatic tissue segmentation procedure due to imaging artifacts probably related to the formalin fixation of the brain where water mobility can be affected (Tovi and Ericsson, 1992).

Transcription profiling through microarray in amygdala samples

Based on the results from the MRI experiment which suggested changes in amygdala volume, microarray gene expression studies were conducted to explore GnRHa effects in the amygdala at a molecular level. The genome information availability and the parallel development of microarray technology have provided the means to perform global analyses of the expression of thousands of genes in a single assay (Eisen and Brown, 1999; King and Sinha, 2001). The results provide an assessment of the expression levels of the genes included on the microarray in a particular cell, tissue or organ. In Paper III of this thesis, we describe the gene expression profiles of the left and right amygdala using 8 X 15 K Agilent ovine microarrays in 30 same-sex twin lambs (14 female and 16 male), half of which were treated with the GnRHa. Two-color microarray experiments were conducted to identify genes being significantly differentially expressed due to long term peri-pubertal GnRHa treatment. The microarray experiment was conducted as a common reference design, using a reference where target samples were hybridized with a reference sample consisting of equal amounts of total-RNA from all samples. This method reduces human error as each sample is handled the same way and compared against a reference sample which contains many samples pooled (Kendziorski et al., 2005; König et al.,

2004). Total RNA extraction was performed according to the TRIzol manufacturer recommendations (InvitrogenTM, Paisley, United Kingdom), followed by a DNase I treatment (RNase-Free DNase Set, Qiagen, Crawley, UK) for 20 minutes at 25°C. Further RNA purification was conducted using an RNeasy Mini-Kit (Qiagen, Crawley, UK) according to the manufacturer's recommendations. A dye swap design was applied, whereby within each group DNAse treated RNA was randomly labeled with cyanine 3 (Cy3) CTP and cyanine 5 (Cy5) CTP using a Quick Amp Labeling Kit, two-color (5190-0444, New Castle, DE) according to the manufacturers methodology. Following hybridization, washing and drying, the slides were scanned in GenePix® 4000B two-color scanner (Axon instrument, Foster City, CA). Two channel images were imported into Agilent's feature extraction 9.1 software for features (spot) extraction and alignment.

After analysis microarray data have been submitted to the NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE44202. The differential expressions of some of the selected genes from the microarray experiment were confirmed by qRT-PCR. In order to understand the biological significance of microarray results, Gene Ontology (GO) term enrichment analysis of the list of differentially expressed genes was conducted in The Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.7 (Huang et al., 2008). To identify gene orthologs or homologs to sheep genes, we used a translation table provided by Agilent Technologies and converted probe IDs into official gene symbols using an in-house python script. Furthermore, to interpret the biological function of the gene lists, the Ingenuity Pathway Analysis (IPA) version 7.5 (http://www.Ingenuity.com) software packages were used for networking analyses. This software not only generates a

biological network from a list of selected genes, but also provides biological functions and canonical pathways from HUGO ortholog names of the genes that were imported into the program. A network of genes is created when a gene regulates the function of other genes. To develop a network, the IPA software adds other genes and/or different molecules that are linked with two focus genes.

mRNA Expression of GnRHI and GnRHR in the hippocampus of Transgenic mice

In paper IV of thesis, exploration of GnRHa (Leuprorelin acetate) treatment effect on murine gene expression level of *GnRHI* and its receptor in hippocampal samples were performed by using standard qRT-PCR methods from the sample of left hemisphere hippocampus.

Measurement of amyloid-β deposition in cerebral cortex, thalamus and hippocampus using immunohistochemistry

The right hemisphere of hippocampus, cerebral cortex and thalamus were used for immunohistochemistry study. Sagittal cryosections (25 µm; Leica CM3050 S) of tissues were performed. All sections were post–fixed with 4% formaldehyde (PFA) for 5 minutes, pretreated with 80 % formic acid for 2 minutes and 2% H2O2 for 7 minutes. After washing with 10 mM PBS, pre - incubation solution (10% normal goat serum (NGS), 1% bovine serum albumin (BSA), 0.5% Triton X-100 in 10 mM PBS) was applied to the sections for 30 minutes at room temperature. Afterwards, the sections were incubated with an Aβx-40-specific polyclonal primary antibody (0.5 µg/ml; Agrisera, Umeå, Sweden) diluted 1:2000 in primary antibody solution (3% NGS, 1% BSA, 0.5% Triton X-100 in 10 mM PBS) at 4˚C overnight. The antisera was generated and evaluated for specificity as described (Näslund et al., 2000). After washing

steps, the sections were incubated for 1 hour with a biotinylated goat-anti-rabbit antibody (BA-1000, Vector Laboratories, CA, USA) diluted at 1:300 in 3% NGS, 1% BSA, 0.5% Triton X-100 in 10 mM PBS, washed in 10 mM PBS, and afterwards, incubated 1 hour at room temperature with streptavidin-biotinylated horseradish peroxidase complex diluted at 1:100 in 0.5% Triton X-100 in 10 mM PBS. After washing with 10 mM PBS, all sections were incubated with 3,3'-diaminobenzidine tetrahyprochloride (Sigma, MO, USA) for 5 minutes, before 0.1% H2O2 was added to 10 ml DAB solution and applied on the sections until proper labeling was achieved (3 minutes). The sections were briefly rinsed in water, mounted and stored at room temperature.

Then, image acquisition was done by using an automated slide scanner system (Mirax Scan, Carl Zeiss MicroImaging GmbH, Jena, Germany) for acquiring high-resolution TIFF images with a spatial resolution of 0,205 µm/pixel. Images were inspected by virtual microscopy using the Mirax Viewer tool. To compensate for differences in background color intensity following immunohistochemistry, all image histograms were normalized using the match-color algorithm in Adobe Photoshop CS6 with a photomicrograph of a wild-type section as reference (Sedgwick, 2008). Afterwards, quantitative image analysis was performed in three regions of interest (ROIs) using ImageJ 1.46r (http://imagej.nih.gov/ij). The outlines of the cerebral cortex and hippocampus were manually delineated in each section. The cerebral cortex was delineated, in an anterior plane, by a line connecting the rhinal fissure and anterior tip of the external capsule, dorsally by the external surface of the brain, ventrally by the external capsule, and posteriorly by the dorsal subiculum. The delineation of the hippocampus included the three cornu ammonis subfields (CA1, CA2, and CA3), the dentate gyrus, and subiculum, and was defined anterior to the fimbria, dorsally and posterior to the external capsule, and ventral to the thalamus. Images

were binarized by selecting a threshold value in ImageJ which yielded boundary definitions best corresponding to the observed plaque boundaries. The same threshold value was used for all sections. The area of each ROI and the area of labeled objects within each ROI were calculated, and the results expressed as labeled area fraction in percent (labeled area/ ROI area x 100). For each animal the mean area fractions of three sections were used for the final analyses of group variation and standard deviation (s).

Statistical analyses

The analyses were conducted with the goal of exploring the sex-specific effect of GnRHa treatment on various outcome measures. The statistical methods were applied according to the experimental design and data characteristics.

Paper I

Spatial orientation

Times to traverse the spatial maze and vocalization rate at 48 weeks of age were analyzed in the same 30 animals as hippocampal gene expression. All data were logarithmically transformed because of skewed data distribution. Mixed between-within subject ANOVA was used to analyze the change in time and vocalization rate through the three successive trials and to assess the differences between T and C animals. Independent sample t-tests were used for assessment of sex differences. Since gene expression analysis provides only relative values no statistical associations between spatial orientation results and gene expression analyses could be performed.

The analysis of hippocampal gene expression

The ΔCt was calculated from the difference in expression between the gene of interest and mean expression of the two reference genes (*GAPDH* and *SDHA*). In order to assess any effects of treatment the ΔΔCt was calculated as the difference between the ΔCt value of control and treated samples, within sexes and for both hemispheres. Sex differences were analyzed separately for control and treated animals; ΔΔCt was calculated as the difference between male and female ΔCt values. Relative gene expression, expressed as fold change was calculated by using $2^{-\Delta\Delta Ct}$. The log2 transformed fold change values ($2^{-\Delta\Delta Ct}$) were used for statistical analysis. Differences in gene expression between C and T animals, as well as between males and females were evaluated by Wilcoxon signed rank test.

Paper II

MRI study for measurement of global and regional brain volume

The mean and standard deviation (SD) were calculated for the volume of the different regions of interest. Total brain volume, white matter and gray matter volume, as well as the volume of the left and right amygdala and hippocampus, were compared between groups (C vs T), while controlling for sex and treatment group using General Linear Models (GLMs). The sex by treatment interaction term was included in the models, allowing us to test for differential effects of treatment between male and female animals. The null hypothesis of no effects was rejected if $p < 0.05$, Bonferroni adjusted by a factor of 7 (corresponding to a nominal alpha of $p < 0.007$). The assumption of normally distributed residuals was evaluated using a normal quintile (Q-Q) plot and this assumption of normally distributed residuals was met for all parameters. Statistical analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA).

Paper III

Transcription profiling through microarray gene expression experiment

The statistical program R was used to analyze the microarray data. Microarray data generated from Genepix were imported into the Bioconductor package LimmaGUI and corrected for background (Smyth and Speed, 2003). For within- and between-array normalization, print tip Loess and scale were used, respectively (Smyth and Speed, 2003). In order to detect differentially expressed genes across treated and control samples, an empirical Bayes moderated t-test (Smyth and Speed, 2003; Smyth et al., 2005) was applied. The p-values were corrected for multiple testing using the Benjamini-Hochberg (BH) method (Benjamini and Hochberg, 1995), and adjusted p-values <0.05 were selected as differentially expressed genes. The generated gene list was further filtered for genes with low intensity and with small changes in expression. In the averaged normalized MA-plot, the genes with M values between ±0.3 and mean log intensity < 3 were excluded from the gene list.

Validation of microarray analysis by qRT-PCR

qRT-PCR technical replicates of samples were averaged, and expression ratios were calculated by the ΔCt method normalized to the reference genes (*GAPDH* and *ACTB*). Statistical significance of analyses was calculated by using Wilcoxon signed rank test. These statistical analyses were performed in the software JMP 10.0 (SAS Institute Inc, Cary, NC, USA). The Pearson product-moment correlation coefficient was used to analyze the qRT-PCR data. The non–parametric Spearman's p correlation coefficient was used when the outlier was included. The p-values <0.05 were considered statistically significant.

Paper IV

Gene expression in hippocampal samples

The ΔCt was calculated from the difference in expression between the gene of interest and mean expression of the two reference genes. To observe the effect of genetic modification in transgenic mice, defined as the difference between the ΔCt value of WT mice and AD mice samples for both sexes at 4 months and 12 months of ages. In order to assess any effects of treatment at 12 months of age, the ΔΔCt was calculated as the difference between the ΔCt value of untreated AD mice and GnRHa treated AD mice samples, within sexes. Relative gene expression, expressed as fold change was calculated by using $2^{-\Delta\Delta Ct}$. The log2 transformed fold change values ($2^{-\Delta\Delta Ct}$) were used for statistical analysis by applying JMP 10.0 software (SAS Institute Inc, Cary, NC, USA). Differences in gene expression between AD and treated (T) AD animals as well as between WT and AD animals with each sex were evaluated by the Student's t-test ($p < 0.05$).

Measurement of amyloid plaque deposition

Statistical analyses were performed using InStat® (GraphPad Softwear, San Diego, CA, USA). ANOVA assay and paired t‑test used for observation Amyloid plaques deposition measurement between different groups (Control vs treated). A p-value less than 0.05 was considered to indicate a statistically significant difference.

Results; summery of the papers

Paper I

Peri-pubertal gonadotropin releasing-hormone analog treatment affects hippocampus gene expression without changing spatial orientation in young sheep

In this study, we explore the effects of GnRHa treatment on hippocampal gene expression and spatial orientation. This study shows, for the first time, that longtime peri-pubertal GnRHa treatment, in sheep, is associated with significant changes, within the hippocampus, of levels of expression of mRNA transcripts known to be involved in endocrine signaling and synaptic plasticity. Indeed, expression of 12 out of the 16 genes was altered in GnRHa treated sheep compared to controls. It should be noted, that the effects of treatment varied, both between the sexes and the right and left hemispheres. Interestingly, the study showed for the first time that long-time peri-pubertal GnRHa treatment in sheep is associated with significant changes of levels of expression of mRNA transcripts known to be involved in endocrine signaling and synaptic plasticity. This treatment also led to a significant increase of sexually dimorphic expression patterns of genes associated with neuronal plasticity. In contrast, treatment led to a generalized reduction in the levels of gene expression associated with endocrine function. Despite the known importance of the hippocampus for spatial learning and memory, the differences in hippocampal gene expression was not paralleled by differences in performance in a behavioral test of spatial orientation conducted prior to tissue collection. However, while there was a tendency for females to perform better than males in spatial orientation, the differences

were not significant. Although these effects could not be connected to spatial orientation ability, the study underlines the need for further investigations in this area.

Paper II

Effects of peripubertal gonadotropin-releasing hormone agonist treatment on brain development in sheep - a magnetic resonance imaging study

In this Magnetic Resonance Image (MRI) study, effects of peri-pubertal GnRHa treatment on regional brain volumes in an ovine animal model were investigated. Specifically, total brain volume, total white matter and grey matter, as well as hippocampal and amygdala volumes were compared between female and male animals that had been treated with GnRHa during the peri-pubertal period and their respective controls. The results demonstrated significant effects of GnRHa treatment on the volume of the left ($t = -13.76$, $p = 0.0001$) and right ($t = -16.56$, $p = 0.0001$) amygdala, indicating larger amygdala in treated compared to untreated animals. Further, a significant ($t=-3.56$, $p =0.001$) sex by treatment interaction effect on the volume of the left amygdala was observed, indicating that the effect of treatment was stronger in female (Mean volume=50 and SD =1 in mm3) compared to male (Mean volume =48 and SD=1 in mm3) animals. All treatment and treatment/sex effects on regional volumes remained practically unaltered when including total brain volume (TBV) as an additional covariate, significantly larger volumes in male compared to female animals were observed in total GM ($t = -3.05$, $p = 0.004$) and the right amygdala ($t = -5.12$, $p= 0.0001$). There was also a significant sex difference in total WM volume ($t = -2.37$, $p = 0.024$), indicating larger volumes in males compared to females. Sex differences were still significant for total GM ($t= -2.91$, $p=0.006$) and WM ($t=-3.12$, $p=0.004$) when including TBV as an additional covariate. The effects of GnRHa treatment on

amygdala volumes indicate that increasing GnRH concentrations during puberty may have an important impact on normal brain development in mammals.

Paper III

Peri-pubertal gonadotropin-releasing hormone agonist treatment affects sex biased gene expression of amygdala in sheep

In this gene expression study, by using ovine 8 X 15 K Agilent microarrays followed qRT-PCR and gene network analysis, we compared the effects of GnRHa treatment on gene expression levels compared to controls within both sexes. The results revealed that GnRHa treatment was associated with significant sex- and hemisphere specific differential expression of genes. GnRHa treatment was associated with differential expression of 432 ($|\log FC| > 0.3$, adj. p value <0.05) and 46 (p value <0.0.5) genes in the left and right amygdala, respectively, of female animals, relative to the reference sample which consisted of a pooled sample from control and treated animals of both sexes. No genes were found to be differentially expressed as a result of GnRHa treatment in the male animals. Further, gene ontology analysis demonstrated that within the treated female left amygdala sample group the biological function term "microtubuli" was significantly overrepresented among the up-regulated genes. In the down-regulated group, the following terms were significantly overrepresented: "Anti-apoptosis", "mitotic cell cycle", "positive regulation of transcription", "ovulation-cycle process", "mitochondrial envelope" and "ubiquitin binding". In the right part of the amygdala of treated females, two enrichment terms were considered significantly overrepresented: "immunoglobulin-mediated immune response" and "immune response. The results indicated that GnRH may, directly and/or indirectly, be

involved in the regulation of sex- and hemisphere-specific differential expression of genes in the amygdala.

Paper IV

Down-regulation of elevated gonadotropin-releasing hormone gene expression in hippocampus in tg-ArcSwe mice following receptor agonist treatment

Quantitative real time PCR gene expression analysis (qRTPCR) on hippocampal samples demonstrated that GnRHa treatment clearly effects gene expression both at hormone and receptor level. At 12 month of age, both *GnRH I* (p=0.001 in both sexes) and *GnRHR I* (p=0.01 male and p=0.001 female) mRNA expression was significantly elevated in tg-ArcSwe compared to WT in both sexes. Strikingly, after 8 months of treatment with Leuprorelin acetate, the gene expression of both *GnRH I* (p=0.001 male and p=0.003 female) and *GnRHR I* (p=0.001 in both sexes) were significantly down-regulated in both sexes when comparing treated tg-ArcSwe to vehicle treated tg-ArcSwe at the same age. At 4 months no significant differences were observed. Furthermore; Immunohistochemistry and image analysis showed that Leuprorelin acetate treatment had no significant effects in changes of the plaques load in hippocampus, cerebral cortex and thalamus. However, we found a tendency of lower plaque load in the cerebral cortex of treated females (p= 0.0597) compared to female vehicle treated. This study points to the involvement of hormonal changes in AD and the prospect of mitigating these through targeted treatment. Although known to increase in normal aging, this study shows that Gnrh/Gnrhr mRNA expression increases even more with amyloid-β deposition in Tg-ArcSwe mice. Further, treatment with Leuprorelin acetate alters hippocampal gene expression. However, the effect on amyloid plaque development remains uncertain in this model.

Discussion

The diversity of the signaling pathways activated by the type I GnRHR, suggests that GnRHR mediates a variety of functions in mammalian brain development. The role of GnRH in Alzheimer's disease (AD) is also evidenced by a reduction in neurodegenerative disease among prostate cancer patients receiving GnRH agonistic therapy (Almeida et al., 2004). We will discuss the evidence supporting the role of the GnRH hormone and its receptor system in brain development and aging condition by using two different animal models (sheep and tg-ArcSwe mouse). The major benefits of using these two models were to further understanding of this treatment effect in neurodegenerative disorder during pubertal development and during the aging process.

The results presented in this thesis show, for the first time, that long term peri-pubertal GnRHa treatment in sheep affects the expression of hippocampal genes involved in synaptic plasticity and endocrine signaling in sex and brain-laterality specific ways. Despite the known importance of the hippocampus in spatial orientation behavior, the differences in gene expression were not paralleled by differences in performance of a spatial orientation task. Peri-pubertal GnRHa treatment also affected the volume of both hemispheric amygdalae, such that the amygdalae in treated animals were larger compared to controls. This anatomical change in the amygdala was accompanied by sex biased changes in the expression of genes linked to emotional regulation and behavior. Furthermore, the results from the transgenic AD mice model demonstrated that GnRHa treatment induced down-regulation of *GnRHI* and *GnRHR* gene expression in the hippocampus of 12 months old AD mice relative to the vehicle treated AD mice, but this was without effect on AD associated plaque load.

Methodological considerations

Well established methods, such as microarray transcription profile, quantitative reverse transcription PCR (qRT-PCR), Magnetic resonance image (MRI), immunohistochemistry and spatial orientation behavior tests were used to investigate different endpoints. Specific methodological considerations will be discussed below.

Animal models

Despite the fact that GnRHa has been used in adult and pediatric medicine for over 30 years, investigations of GnRH effects on brain development in humans is limited, due to the obvious ethical restrictions. Furthermore, as children e.g. that are treated for precocious puberty, have been subjected to the influence of sex hormones before diagnosed and starting GnRHa treatment, they do not provide an ideal system to differentiate between the effects of the diagnosis in itself and the medication. In contrast, our treated sheep had not been subjected to a classical pubertal increase in GnRH, and represent, therefore, an important clinical model for GnRHa substitution effects.

We used this model for observations of GnRHa treatment effects on brain development. The relatively large number of sheep used in this experiment (46 pairs of same sex twins) allows for good environmental control and reduces the impact of genetic variability and other potentially causal factors such as maternal nutrition and gestation length. Our sheep model provided sufficient tissues for extensive analyses and the simultaneous measurements of DNA, RNA and protein in relatively homogeneous tissue samples. The recent publication of the sheep genome (The International Sheep Genomics Consortium et al., 2010) helps with the identification of

target genes related to phenotypes important in human disease and key to the development of new therapeutic strategies. In addition to benefits of body size with regard to tissue samples post mortem, the large body size in sheep also allows repeated blood sampling. Furthermore, pubertal development, has been well characterized in the sheep (Foster et al. 2002; Smith and Clarke, 2010) and the mechanisms behind sex-differences in the timing of puberty onset and sexual differentiation of reproductive neuroendocrine function are well researched (Wood and Foster, 1992; Wood and Foster, 1998). While the onset of puberty and periodicity of fertility differ between sheep and humans, a similar interplay between neurosecretory cells of the brain and the peripheral sexual organs is well documented in mammals, primates and humans (Wang et al., 2010). Recent studies have also demonstrated that the sheep is a very useful model in research of complex cognitive executive functions (Morton and Avanzo, 2011). An additional major benefit of using sheep as a model for brain development is the fact that sheep have a long period of brain maturation (Wood and Foster, 1998) compared to small laboratory species such as rodents. This make sheep especially suited for assessment of peri-pubertal brain development and brain functions such as cognitive ability. Sheep are also relatively easy to handle and inexpensive.

In addition to a sheep model, a transgenic Alzheimer's (tg-ArcSwe) mouse model was used to observe the impact of GnRHa on AD pathogenesis in mice. There is an urgent need for improved diagnostic tools and a better understanding of the genetic and molecular basis of AD which may lead to more efficient therapeutic interventions. Therefore, genetically altered animal models represent powerful tools for experimental investigations of pathogenic processes, potential development of new diagnostic markers, and identification and evaluation of potential new therapeutic strategies. Several gene mutations associated with familial AD (Schellenberg and

Montine, 2012) have been used to create transgenic mouse models. The first genetic animal model reflecting important characteristics of AD was the PDAPP model carrying the Indiana mutation (p.V717F) (Games et al., 1995). Since then a range of other models have emerged, and there are a considerable number of genetic mouse models available that mimic various aspects of AD (Philipson et al., 2010). None of the animal models, however, can reproduce the full spectrum of histological and biochemical changes seen in AD patients (Duyckaerts et al., 2009) and the selection of appropriate models for different experimental investigations is a considerable challenge and critical for investigational outcomes. For studies of AD, a model should typically express specific hallmarks of AD such as the amount, density, size and detailed morphology of amyloid plaques or neurofibrillary tangles (Philipson et al., 2010). The transgenic tg-ArcSwe mouse model (Lord et al., 2006; Philipson et al., 2009) stands out as a particularly promising model for investigations of intraneuronal Aβ accumulation as well as formation and clearance of amyloid plaques, resembling the human AD brain. The transgene carries both the Arctic mutation (p. E693G) which is located within the Aβ domain of the amyloid-β precursor protein (AβPP) gene and facilitates Aβ protofibril formation (Nilsberth et al., 2001; Philipson et al., 2012) and the Swedish mutation (p. KM670/671NL), which is coupled to increased generation of Aβ peptides (Citron et al., 1992, Mullan et al., 1992). The Swedish AβPP mutation accelerates of the age of AD onset compared to using the Arctic mutation alone (Lord et al., 2006, Rönnbäck et al., 2011). Among dozens of APP-transgenic models, the tg-ArcSwe mouse model is practical to use, considering the early onset of extracellular plaque formation. Senile plaques appear in the brain at an age of 5-6 months (Lord et al., 2006), become highly abundant in regions where the AβPP-transgene is expressed by 12 months of age, but are also present in other brain regions. Compared to other models, e.g. the commonly used Tg2576, phenotype,

variability among tg-ArcSwe mice is modest, both in terms of anatomic distribution and density of deposits in the cerebral cortex and hippocampus. This means that fewer mice are needed to gain statistical power when evaluating novel therapeutics.

It is important to bear in mind that the findings of mice study (Paper IV) should be interpreted very cautiously as we have lost animals and the final number of animals for gene expression and immunohistochemistry analysis was relatively small.

Spatial orientation test

The spatial orientation test has captured a solid position in neuroscience research. This task has been frequently investigated in animal models (Lee et al., 2006; Wolbers and Hegarty, 2010) and has been used mainly on rats, a species endowed with formidable spatial abilities (Olton et al., 1979). Sheep have also previously been shown to be able to learn and improve their performance in a spatial maze test (Edwards et al., 1996; Lee et al., 2006). Spatial orientation tasks facilitate a measurement of working and reference memory based on exploration (Oades, 1982; Morris, 1984). This test also encompasses spatial ability, i.e. the capability to generate and recall spatial information (Voyer et al., 1995), but differs from spatial ability as it also involves the environment and, therefore, the ability of a subject to acquire information about its surroundings and navigate through them (Coluccia and Louse, 2004). It has been reported that pubertal hormones can modulate learning and memory (Sisk and Zehr, 2005); therefore spatial orientation behavior results have been used in Paper I to assess the sex specific GnRHa treatment effect on spatial orientation ability.

Microarray

Over the past decade microarray analysis has gained acceptance as a standard tool for studies in molecular biology. Although it is subject to several considerations, a microarray experiment cannot verify or falsify a null hypothesis, since it is, primarily a screening technique. The use of microarray and fold change has the disadvantage that it needs cut-off values; consequently, it does not include all biologically present genes at any time, as some might have a very small fold-change, while some mRNA have a short half-life due to rapid degradation. In contrast, microarray analysis allows for massive data acquisition in parallel, which increases experimental efficacy, and provides for meaningful comparisons (Schena et al., 1998). The ability to study the behavior of many genes simultaneously is perhaps the greatest advantage of its use. Today's microarray technology is convenient, and since the first reported use of this technology in 1982 (Augenlicht and Kobrin, 1982), the method has improved, and is now considered to provide reliable results. Because of the fact that the microarray experiment itself is a procedure involving multiple steps, the design needs to be strictly standardized. It is also of utmost importance that the starting material is of the highest quality. In order to secure quality of the total RNA, we used standard procedures such as keeping the sample in dry ice before adding it to the Trizol solution. In order to analyze the microarray results, we used Bioconductor package LimmaGUI. One of the most commonly used software tools is limma (Linear model for microarray analysis) which provides data analysis, normalization for cDNA microarray data and analysis of differential expression for multi factor designed experiments. The core of the limma package is an implementation of the empirical Bayes linear modeling approach (Smyth et al., 2005). LimmaGUI provides a point and click interface to the core function of the limma package. For within- and between-array normalization, print tip Loess and scale were used, respectively

(Smyth and Speed, 2003). Normalization is required to adjust for effects which arise from variation in the microarray technology rather than biological differences between the RNA samples or between the printed probes. The differences between the labeling efficiencies or scanning properties of CY3/CY5 dyes or different scanner settings can cause the imbalance between the red and green dyes. Differences between arrays may arise from differences in print quality, from differences in ambient conditions or changes in the scanner settings. Therefore normalization between as well as within arrays were considered. Print tip Loess and scale method has been recommended as routine normalization methods for cDNA arrays (Smyth and Speed, 2003). It corrects the M-values both for sub array spatial variation and for intensity-based trends. We used GO (gene ontology) analysis (Michael et al., 2000) to gain knowledge about the biological process, cellular components and molecular functions of the genes that were identified as being differentially expressed in the microarray experiment. This analysis is widely used for systemic assessment of the key functions and processed enriched in a set of genes identified by transcriptomic analysis. In addition, gene network analysis was used to visualize the biological relationships between genes. Although we used both GO and gene network analysis, it is important to bear in mind that the results of such *in silico* analysis could be due to the many different gene interactions that could be occurring as a result of various experimental variables.

Although, the gold standard for transcriptional assays is still the Northern blot, the time and RNA amounts needed are often beyond what is available, real-time qRT-PCR is the default for validation of microarray results. The qRT-PCR is often referred to as the “gold standard” for gene expression measurements, due to its detection sensitivity, sequence specificity, large

dynamic range, as well as high precision and reproducible quantitation. Therefore, we used qRT-PCR to validate microarray results.

Quantitative real time pcr (qRT-PCR)

In paper I, III and IV, qRT-PCR was used to investigate gene expression changes. The reference genes in relative quantifications were different between the studies included in this thesis. In Paper 1, *GAPDH* and *SDHA* genes were used, in Paper III, *GAPDH* and *ACTB* genes and in Paper IV, *B2m* (beta-2 microglobulin) and *Gusb* (glucuronidase, beta). It has become increasingly clear that expression of the traditionally used reference genes may vary according to tissue, development, disease state or treatment and species. It is, therefore, recommended to test for stability of the reference genes (Ruan and Lai, 2007; Stürzenbaum and Kille, 2001). Although, the expression of no single gene will stay fully stable under all conditions; it is advisable to use more than one reference gene. In order to identify most stable reference genes, recently several software programs have been developed. The one chosen for use in the studies of this thesis, GeNorm, builds on the idea that the expression ratio of two ideal reference genes is identical in all samples. For every gene included in the test, a pairwise variation with all other genes is determined and the gene with the most variation, lowest M-value, is excluded in a stepwise fashion until the two most stable are determined (Vandesompele et al., 2002).

Magnetic resonance image (MRI)

In the MRI study (volumetric study), we utilized a region of interest (ROI) approach. We included seven a priori defined ROIs, and performed seven independent multiple linear regressions. Hence, we adjusted our nominal alpha by a factor of seven according to Bonferroni.

Importantly, Bonferroni correction is most appropriate when analyzing independent measures, and may actually be too conservative when performing several non-independent tests. This is to some degree the case in the present context, where the various volumetric estimates are expected to be correlated. Thus, it is believed that the present correction for multiple comparisons may actually be too conservative rather than too liberal. However, it was preferred to control strictly for Type I errors in the study III. One could argue that Small Volume Correction (SVC at p=.05) could be used on ROIs. SVC is one way of reducing the number of statistical tests by reducing the number of voxels to predefined areas, but this is something very different from the current ROI approach, where SVC is both intuitively and technically inappropriate. Another argument could arise that performing anatomically detailed volumetric measures would be of interest. However, since detailed probabilistic atlases of the sheep brain currently do not exist, we would have to perform full-brain manual labeling of all datasets. This would be extremely time-consuming. Increasing the number of ROIs would also increase the number of statistical tests, effectively reducing our power to detect true differences in the amygdala and hippocampus, which was the main scope of the study because our study using the same cohort of the animals showed sex specific effect of GnRHa treatment on hippocampal gene expression (Paper I) and emotional regulation behavior (Wojniusz, et al., 2011). Furthermore, in multiple linear regression model analysis result, we presented t value for interaction term instead of an F-value. The t-values reflect the effect sizes of the estimated parameter estimates from the multiple regressions. Since we only had two groups, the t-value reflects the sign and strength of the interaction term scales directly with the F-value (or more precisely, t = sqrt(F)), but provides additional information about the direction of the effects, of which the F-value is insensitive. Therefore, the t-value is more informative. Regional brain volumes often scale almost linearly with total brain

volume (TBV), and apparent regional effects may in fact reflect global effects if one fails to account for variability in TBV. Our main hypotheses were related to specific structures including the hippocampus and the amygdala. Thus, we first tested for treatment effects on TBV. Since we did not observe any effects of treatment on TBV, we performed our main regional analysis without adjusting for TBV. However, to rule out that marginal differences in TBV still affected our results, we ran extra analysis with TBV as an additional covariate. Importantly, all reported treatment effects on regional volumes remained practically unaltered. Thus, it indicates that the reported effects are not confounded by more "trivial" global effects.

Immunocytohistochemistry

There are several antibodies specific for different Aβ epitopes available. The Aβx-40-antibody used in immunohistochemistry experiment for paper IV detects a neo-epitope tested for specificity by using ELISA (Näslund et al., 2000). It has previously been demonstrated that Aβ antibodies stain the same, homogeneous population of cored Aβ-plaques in the tg-ArcSwe model, irrespective of whether the antibodies recognize the N- or C-terminal epitopes in Aβ (Lord et al., 2006; Philipson et al., 2009). Thus, it is assumed that the 6E10, Aβx-40 or Aβx-42 antibodies will produce the same labeling pattern and indicate the same Aβ burden in tg-ArcSwe mice, at least at the light-microscopic level. This probably reflects the fact that the Arctic AβPP mutation makes Aβ-peptides far more prone to aggregate, including Aβ1-40 (Nilsberth et al., 2001). Additional evidence of the specificity of the Aβx-40-antibody was provided by an earlier investigation of tg-Swe mice, where two plaque populations were demonstrated, one consisting of diffuse wild-type Aβ plaques which are only Aβ42-immunopositive, and a second of cored plaques which stains with Aβ40- and Aβ42-specific antibodies (Lord et al., 2011). It should

further be noted that antibody 6E10, unlike an Aβx-40 antibody, binds to both AβPP and sAPPβ. However, pretreatment of sections with formic acid as was done in the present study, will dramatically lower signals from AβPP and enhance detection of aggregated and fibrillized Aβ (Chirstenson et al., 2009; Philipson et al., 2009).

Experimental findings

GnRHa treatment impact in spatial orientation as well as hippocampal genes involved in synaptic plasticity and endocrine signaling

While indications of treatment effects on emotion regulation and emotion processing dependent behavior were found in this cohort of GnRHa treated sheep (Wojniusz et al., 2011), no clear effects were observed with regard to cognitive function, spatial orientation ability. In the behavior study of Paper I, the spatial orientation ability task was investigated at 48 weeks of age. Interestingly, at this time point, when animals were well into their adolescence, females seemed to outperform males in the spatial maze task. Despite this, the difference in performance was identical in GnRHa treated and untreated groups. It is challenging to fully explain the fact that females outperformed males at this time point, especially as the opposite is usually found (Jonasson, 2005; Voyer et al., 1995). Nonetheless, it must be underlined that spatial orientation as a complex function that not only encompasses the capability to generate and recall spatial information (i.e. spatial ability), but also involves acquisition of information about the environment and navigation through it (Coluccia and Louse, 2004). Consequently, our findings might be related to the experimental setup, as males and females use different strategies to solve spatial tasks (Rodriguez et al., 2010; Sandstrom et al., 1998; Saucier et al., 2002). Motivational differences might also explain the difference, previous shown by the food acquisition task (FAT)

experiment, where females appeared more eager to rejoin their social group than males (Wojniusz et al., 2011).

Beside this sex difference in spatial orientation ability, one could argue that there is no treatment effect observed due to the low sample number, which could be a valid point. It is important to appreciate that this study was part of a more extensive one. Specifically, the total study sample consisted of 92 animals (44 females and 48 males) and all of them were tested with regard to spatial orientation at three different time points (8, 28, and 48 weeks of age). When the whole sample was analyzed, there were still no significant differences between treated and control animals (Wojniusz et al., 2013). This is particularly interesting in the light of earlier findings on the same sample of sheep that showed the impact of GnRHa treatment on higher order cognitive function. In a food acquisition task treated females were less prone to leave their companions to acquire the food than control females while the opposite was seen in males. Furthermore, treated males displayed more risk taking behavior than control males. The similar pattern was also found in measurements of heart rate variability (HRV), showing a relative increase in HRV in treated males and a relative decrease in treated females; lower HRV indicates poorer ability to regulate emotions (Wojniusz et al., 2011). The treatment effects on emotional regulation were also observed in another experiment measuring psychophysiological motoric activity, where treated males were significantly less affected by separation and movement restriction compared to control males (Evans et al., 2012). Overall, these findings showed that blockage of pubertal hormones with GnRHa treatment may influence development of emotion regulation and decision making. Therefore, it is interesting that peri-pubertal GnRHa treatment did not affect development of spatial orientation, which indicates that pubertal hormones selectively influence

specific cognitive functions. It might be possible that spatial orientation as an example of a fundamental and evolutionary "old" cognitive function and is more likely to be genetically pre-programmed and shaped prenatally; whereas other cognitive functions, e.g. risk taking behavior and emotion regulation, are more susceptible to the influence of pubertal hormones than spatial orientation.

There was a hypothesis that if GnRHa influences the development of spatial orientation, a hippocampus dependent function (O'Keefe and Nadel, 1978), it should have an effect on hippocampal gene expression. Therefore, GnRHa effects on mRNA expressions of hippocampal genes associated with synaptic plasticity and endocrine signaling were explored. The results were viewed in the light of findings from the spatial orientation experiment. Although molecular processes for learning and memory in the brain are not clear, it is reasonable to assume that they are strongly related to synaptic plasticity (Wieraszko, 1998). Genes that were selected to be studied are known to be involved in synaptic transmission, proliferation, differentiation and structuring that underlie synaptic plasticity. While there were no effects of GnRHa on spatial maze performance in the present subsample of 30 animals (half of them treated), significant differences between treated and untreated animals and between the sexes were found with regard to the levels of expression of mRNA transcripts in a number of genes within the hippocampus. This indicates that some other hippocampus dependent functions, which are not directly related to spatial learning, might have been influenced. Since the hippocampus is well known to play a role in emotion regulation, being involved in the inhibition of stress responses (Lopez et al., 1999) and complex regulation of affective states, involving behavioral inhibition and facilitating exploratory rather than defensive patterns of behavior (Gray and McNaughton, 2000), it is

possible that the observed differences in gene expression are related to differences in emotion processing. In any case, such interpretation is at best speculative as it is difficult to establish a direct association between expression levels of particular genes and behavior. Differential mRNA expression is only an intermediate step on the way to protein synthesis which can be more directly related to particular behaviors. Moreover, single genes are rarely responsible for such a complex behavior trait, but they rather operate together with other genes and cellular processes, mutually influencing each other. Consequently, by choosing genes of interest, as has been done in this study, it is possible that other important genes, affected by GnRHa, have been left out. For large scale investigations of gene expression, microarray would be a preferred method, giving a possibility of screening the expression of thousands of genes simultaneously. As the next step, these finding should be validated by qPCR that is a much more sensitive method compared to microarray (Vanguilder et al., 2008). Due to logistical reasons and high costs, microarray investigation was not performed.

GnRHa treatment effect on amygdalar volume of the brain

The sex and hemisphere specific peri-pubertal GnRHa treatment effect on hippocampal gene expression (Paper I) and sex specific emotional regulation behavior that linked to function of amygdala in this sheep model (Wojniusz et al., 2011), let us anticipate that this treatment might have localized effects both in the structure of the hippocampus and amygdala. Magnetic resonance imaging of fixed brains demonstrated GnRHa treatment effects on the volume of the left and right amygdalae, indicating larger amygdalae in treated animals. Further, a significant sex by treatment interaction on the volume of the left amygdala indicated stronger effects of treatment in female compared to male animals. This MRI study also revealed sex differences in

gray matter and right amygdala volume, indicating larger volumes in males compared to females. These results finally indicated that treatment affected the amygdala, but not other major structures of the limbic system. The observation of an effect of GnRHa treatment on amygdala volume is especially interesting as this brain region also expresses a high density of GnRH receptor expressing neurons (Albertson et al., 2008; Granger et al., 2004; Haour et al., 1987), It could be argued however, that sex steroid perturbations could contribute to the observed morphological changes in brain development, as it has been reported that testosterone is positively associated with increased regional volume of the medial amygdala (MeA) and posterodorsal subnucleus (MeApd) of the posterior MeA in males. In addition, rising concentrations of androgen promote excitatory synaptic connectivity (Cooke and Woolley, 2009; Romeo and Sisk, 2001) and functional activity in the amygdala. An MRI study in children and adolescents (males age 6-16 years and females 4-11 years) has also reported that changes in volume and functional activity of the amygdala might be due to excess androgens, estrogens or progestins, deficiency or further exogenous excess of glucocorticoids or some combination of these (Merke et al., 2003). This study focused on the examination of the effect of sex steroids on the growth and development of the amygdala, however possible involvement of GnRH has not been described elsewhere. Thus, volumetric effects of GnRHa treatment on the amygdala suggest that GnRH directly may play a modulatory role in morphology and function of the amygdala. Furthermore, human and animal behavioral studies have shown that amygdala volume is associated with increased anxiety and reactivity (Barrós-Loscertales et al., 2006; De Bellis et al., 2000; MacMillan, 2003; Thomas et al., 2001; Vyas et al., 2006). Thus, GnRHa treatment effects on amygdala structure, as reported in this study by MRI, might be related to the cognitive changes reported in Wojniusz et al., (2011) The significant sex differences in GM and trend

differences for WM volume in this sheep model is in concordance with human studies as sex differences for GM and WM volume during pubertal development has been documented (Peper et al., 2011) where GM and WM volumes were larger in males than females. The sex difference in right amygdala volume, seen in this study, could add further weight to the proposals for sex dependent lateralization of the amygdala (Peper et al., 2011; Schneider et al., 2011). However this result should be interpreted cautiously because male animals reach puberty 20 weeks earlier than female and the male animals had in these studies received GnRHa treatment for a longer duration than females.

The significant treatment by sex interaction on the volume of the left amygdala could indicate a stronger effect of treatment in females compared to males. In imaging studies, sex related hemispheric lateralization of amygdala function in relation to emotion regulation has been reported where the left amygdala has been implicated as dominant in females while the right amygdala was more dominant in males (Cahill et al., 2004; Koscik et al., 2010). We have observed GnRHa treatment affects the volume of the amygdala in both sexes that corresponds to the behavior results where both sexes were affected by treatment. Therefore, the cautious interpretation was necessary and this sex related hemispheric lateralization warrant further studies. In paper I, though it was reported that GnRHa treatment affected hippocampal gene expression involved in synaptic plasticity and endocrine signaling, we expected that treatment could affect hippocampal volumes in this cohort of GnRHa treated sheep. Our data did not show any effects of treatment on hippocampal volume. It could be that treatment affects hippocampal processes on a molecular level without affecting microstructural processes in this region.

Impact of GnRHa treatment on gene expression changes in the amygdala

The morphometric analysis (Paper II) and sex specific emotional regulation behavior results using same cohort of GnRH treated sheep (Wojniusz et al., 2011) triggered us to perform further molecular biology study. Therefore, we used microarray transcription profile to gain further insight into the molecular changes that might underlie the effect of GnRHa treatment in sex-specific cognitive function, as well as structural volume changes in the amygdala. Interestingly, peri-pubertal GnRHa treatment in sheep was associated with significant changes in the levels of expression of mRNA transcripts in the amygdala, the changes being dependent upon sex and hemisphere. The GnRHa treatment effects were only observed in females, and there were a much higher number of differentially regulated mRNA transcripts (432) in the left compared with the right amygdala (46).

This finding indicates a lateral effect of GnRHa treatment on gene expression within the amygdala that might cause, or be involved in, later functional behavioral differences. It is interesting to note that our MRI study (Paper II) also revealed lateral, in addition to, sex effects in amygdala volume after treatment, the left amygdala volume in treated females being much larger than the right amygdala in the females and both left and right amygdala in the treated males. The combination of our morphometric and gene expression studies suggests that GnRHa treatment effects at the level of the amygdala may be more pronounced in females than in males, and also that GnRHa treatment has a lateral effect on the development of the amygdala. Few studies have specifically addressed or described lateralization of amygdala function in either humans or animals. Hemispheric lateralization of amygdala function in relation to emotion regulation has been reported in human imaging studies where the left amygdala has been

implicated as dominant in females (Cahill, 2004; Koscik et al., 2010). To our knowledge, this is the first study that shows a hemispheric lateralization of gene expression levels in the amygdala due to peri-pubertal GnRHa treatment. Although we have found such finding in humans and compared with the present sheep study, such interpretation should be tempered by the fact that sheep and humans differ in terms of reproductive behavior and seasonality, and thus physiological differences in species exist that might preclude extrapolation of such data. Replication of this type of study is also needed to determine the mechanisms by which GnRHa affects amygdala function in sheep.

It is interesting that GnRHa treatment effect on gene expression were only seen in females as the behavioral results with the same cohort of GnRHa treated sheep, has also demonstrated sex-specific effect on behavior and emotional regulation (Wojniusz et al., 2011). As the amygdala plays a central role in emotional processing (Zald, 2003), the present findings could suggest that sex specific gene expression changes due to GnRHa treatment are related to sex-specific emotional responses reported in the previous study (Wojniusz et al., 2011). GnRHa treatment effects on development of the amygdala are also supported by the results of morphometric analysis using MRI (Paper II). Although MRI study demonstrated that GnRHa treatment was associated with increased amygdala volume. However, this effect on volume was not limited to the females and was seen in both sexes (Paper II). Therefore, the sex specific changes in gene expression observed in the present study might indicate that while GnRHa treatment has an effect at a macro-structural level in both sexes, at the molecular level the effect is more pronounced in the female.

Since, we are predicting that gene expression changes in amygdale, due to GnRHa treatment, might be related to cognitive function and structural changes in the localized brain regions, it could be interesting to perform correlation analysis between all these experiments which are parts of a larger study. However, the behavioral task for emotional regulation and control that we refer to (Food Acquisition Task - FAT), although showing clear differences between the groups and sexes, did not discriminate well between the individual sheep, particularly in the treated female group. The main outcome measure of FAT was a probability of leaving the social group to acquire the food. Five out of seven treated female sheep included in this study achieved probability scores of zero, meaning that they did not move away from their companions to find the food on any of the eight trials. Only two out of seven sheep went after the food, one on four out of eight trials (score 0.5) and one on every trial (score 1.0) (Wojniusz et al., 2011). Consequently, it is not statistically meaningful to correlate the FAT scores with gene expression findings. Furthermore; we could not use MRI data for correlation analysis with gene expression data because brain samples were not collected from the same animals.

The amygdala gene expression study (Paper III) clearly showed significant differential expression of some genes within the amygdala, these changes are of particular note as the mRNA extraction was from the entire amygdala and thus any observed changes must represent large scale changes in gene expression. The use of this protocol however means that small changes or region specific changes in gene expression could have been missed, given the heterogeneous nature of this brain structure and thus further targeted studies are necessary.

GnRHa treatment effect on gene expression changes and amyloid plaques deposition

In the final chapter we used transgenic mice model to observe GnRHa treatment effects on brain function in a model of neurodegenerative disease during aging. (Paper IV). This study showed, for the first time, that gene expression of GnRHI and its receptor were not changed at 4 months of age in AD relative to WT mice. However, At 12 month of age both the mRNA expression levels of the hormone and its receptor were elevated in AD relative to WT mice, and thus suggest that GnRH and its receptor might have a functional role in the AD condition. This speculation can be supported by the fact that we have observed Leuprorelin acetate (GnRHa) treatment induces down regulation of GnRH I and GnRHRI gene expression in AD mice relative to WT of the same age. Despite the known importance of the hippocampus in terms of plaque deposition in AD pathology (Reilly et al., 2003), we could not find significant changes in plaque load after 8 months treatment with GnRHa in hippocampus, cerebral cortex and thalamus. The involvement of GnRH in neuropathology such as AD is also supported by recent findings where it has been suggested that GnRH/GnRHR act on hippocampal synaptic activity and is involved directly in controlling central nervous system physiology and pathophysiology of AD (Meethal et al., 2005; Wang et al., 2010). It has also been reported that the hippocampal GnRH system is acutely sensitive to both age and reproductive status, as GnRHR expression is increased in aged rats. (Badr et al.,1988). Therefore, our findings in terms of gene expression are in accordance with these reports.

It is described that GnRHa treatment reduced Aβ concentration in total brain after 2 months of treatment in C57BL/6 mice (Bowen et al., 2004) and in Tg2576, carrying the Swedish AβPP mutation) (Casadesus et al., 2006). Therefore; we anticipated that GnRHa treatment could reduce

Aβ burden in the region of interest (ROIs) that we observed. However, we did not find any significant effects of treatment in terms of plaque deposition and the reason is unclear. It could be speculated that tg-ArcSwe is a stronger genetically driven model than Tg2576. The major limitation of this study was less number of brain samples than originally intended and that the gene expression study was performed for only one hormone and its receptor. Microarray expression study followed by qRT-PCR could provide a clearer picture about the genetic contribution and therapeutic effects in this disease.

Relevance of findings for human medicine

The anatomical and behavioral effects associated with chronic GnRH treatment of sheep during the peri-pubertal period observed in this thesis suggest that interruption of GnRH signaling may have significant effects out with the reproductive system. Given that the peri-pubertal period is a known period of neuronal plasticity, the level of which may decrease in later life stages the long-term nature or permanence of these effects of GnRH treatment are not known and could not be determined from the studies conducted. It would be of great interest to test whether similar effects of peri-pubertal GnRHa treatment are seen in other species and therefore it would be interesting to monitor mood and anxiety parameter in human clinical population that also receive a comparable GnRHa treatment. These parameters are suggested as emotional regulation is controlled at the level of the amygdala and hippocampus (Andari et al. 2012; Campbell et al., 2004), areas in which morphological and molecular changes were seen in the sheep study. GnRHa is used to treat peri-pubertal children for a variety of medical and neuropsychiatric disorders and thus such studies should be feasible although establishing an adequately sized patient cohort may require international collaboration, due to confounding differences associated

with condition and treatment options. The establishment of such a patient cohort however would allow design and development of longitudinal studies to examine the long term effects of GnRHa treatment. In addition such a patient group could be used in studies that are more difficult in animals such as fMRI and cognitive reasoning. Having established the principle that GnRH can have actions outside the reproductive axis, particularly during development the reported expression of receptors in hippocampus and amygdala mean that treatments that affect GnRH signaling may also affect these systems. Thus it would be justified to investigate effects of GnRHa treatment on brain functioning using both animal models and in human populations. The present findings may provide a help for clinicians to take careful consideration when pharmacological blockade of GnRH action is used in treatment of young children.

Furthermore; GnRH agonist has been used for AD condition under the clinical trial Phase III (Clinicaltrial.gov. ALADDIN study—phase III, 2005) by assuming that luteinizing hormone is the cause of Alzheimer's disease. The gene expression findings from transgenic AD mice model reported in this thesis revealed that GnRH/GnRHR might also have a potential influence in AD condition. This finding provides the possibility developing GnRH as a diagnostic marker for AD. Further research in this field in different mice and rat models might provide better information to human medicine.

Conclusions

Overall, the findings suggest that peri-pubertal GnRHa treatment may influence gene expression in hippocampus and amygdala as well as structural morphological changes in amygdala, probably in a sex and side specific manner. The data emphasize that changes in the activity of the hypothalamus-pituitary gonadal axis may also affect other brain functions during sensitive

periods (i.e. perinatal, puberty, and aging). GnRH-agonist treatment affects gene expression both at the hormone and receptor level during a treatment period of 8 months, while the effect on amyloid plaque development is marginal in our tg-ArcSwe mouse model and the reason is unknown. Further study using different advanced experimental conditions will provide better information about possible roles of GnRHI/GnRHR in aging disorder such as AD.

Further studies

To gain more knowledge on the consequence of the documented altered gene expression in hippocampus and amygdala as well as structural changes in amygdala due to GnRHa treatment in different animal models need further research. This will provide knowledge for human medicine considering new therapeutic approaches for the treatment of neurodevelopmental and neurodegenerative diseases.

Examples are:

1. There is a need for more mechanistic and systemic research elucidating GnRHa treatment effect on hippocampus. We used some selective genes for qRT-PCR study from hippocampus sample and found sex and side specific gene expression changes but microarray experiment followed by qRT-PCR, gene ontology, gene network analysis, proteomics and pathway analysis can provide detailed information about mechanism or pathway affected due to GnRHa treatment.

2. In the MRI study, we exclusively defined two region of interest (hippocampus and amygdala) in the limbic system to observe GnRHa treatment effects. Further morphometric analysis of other limbic structures such as anterior cingulate cortex (ACC),

nucleus accumbens, parahippocampal gyrus and orbitofrontal cortex (OFC) will provide more detailed information of relationship between limbic structures changes and emotional regulation behavior due to GnRHa treatment.

3. Although microarray followed by qRT-PCR have been performed in amygdala samples, proteomic studies of genes of interest identified by microarray will aid further understanding of GnRHa treatment effect. Furthermore, given the fact that amygdala is known to be a heterogeneous region, specified subregions should be used for further analyses.

4. Although we have found significant effects of GnRHa treatment in gene expression of GnRHI and its receptor in hippocampal samples of AD mice, our results could not provide us detailed mechanism of GnRH involvement in AD condition. A replication of this transgenic mouse model study with parallel microarray and proteomics analyses may provide understanding the role of GnRH in this disease.

References

Albertson, A.J., Navratil, A., Mignot, M., Dufourny, L., Cherrington, B., Skinner, D.C., 2008. Immunoreactive GnRH type I receptors in the mouse and sheep brain. Journal of Chemical Neuroanatomy 35, 326-333.

Albertson, A. J., Talbott, H., Wang, Q., Jensen, D., Skinner, D., 2008. The Gonadotropin-Releasing Hormone Type I Receptor is Expressed in the Mouse Cerebellum. Cerebellum 7, 379-384.

Alibhai, S.M.H., Gogov, S., Allibhai, Z., 2006. Long-term side effects of androgen deprivation therapy in men with non-metastatic prostate cancer: A systematic literature review. Critical Reviews in Oncology/Hematology 60, 201-215.

Almeida, O.P., Waterreus, A., Spry, N., Flicker, L., Martins, R.N., 2004. One year follow-up study of the association between chemical castration, sex hormones, beta-amyloid, memory and depression in men. Psychoneuroendocrinology 29, 1071-1081.

Andari, E., Schneider, F.C., Mottolese, R.l., Vindras, P., Sirigu, A., 2012. Oxytocin's Fingerprint in Personality Traits and Regional Brain Volume. Cerebral Cortex.

Anagnostou, E., Taylor, M., 2011. Review of neuroimaging in autism spectrum disorders: what have we learned and where we go from here. Molecular Autism 2, 4.

Augenlicht, L.H., Kobrin, D., 1982. Cloning and Screening of Sequences Expressed in a Mouse Colon Tumor. Cancer Research 42, 1088-1093.

Badr, M., Marchetti, B., Pelletier, G., 1988. Modulation of hippocampal LHRH receptors by sex steroids in the rat. Peptides 9, 441-442.

Baker, J.H., Thornton, L.M., Lichtenstein, P., Bulik, C.M., 2012. Pubertal development predicts eating behaviors in adolescence. Int.J.Eat.Disord. 45, 819-826.

Bao, A.M., Swaab, D.F., 2010. Sex Differences in the Brain, Behavior, and Neuropsychiatric Disorders. The Neuroscientist 16, 550-565.

Barrós-Loscertales, A., Meseguer, V., Sanjuán, A., Belloch, V., Parcet, M.A., Torrubia, R., Ávila, C., 2006. Behavioral inhibition system activity is associated with increased amygdala and hippocampal gray matter volume: A voxel-based morphometry study. Neuroimage. 33, 1011-1015.

Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society.Series B (Methodological) 57, 289-300.

Berenbaum, S.A., Beltz, A.M., 2011. Sexual differentiation of human behavior: Effects of prenatal and pubertal organizational hormones. Frontiers in Neuroendocrinology 32, 183-200.

Bergin, P.S., Raymond. A.A., Free. S.L., Sisodiya. S.M., Stevens. J.M., 1994. Magnetic resonance volumetry. Neurology. 44, 1770-1771.

Bethke, A., Fielenbach, N., Wang, Z., Mangelsdorf, D.J., Antebi, A., 2009. Nuclear Hormone Receptor Regulation of MicroRNAs Controls Developmental Progression. Science 324, 95-98.

Blakemore, S.J., Burnett, S., Dahl, R.E., 2010. The role of puberty in the developing adolescent brain. Hum.Brain Mapp. 31, 926-933.

Bliss, T.V.P., Collingridge, G.L., 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361, 31-39.

Bourke, L., Chico, T.J.A., Albertsen, P.C., Hamdy, F.C., Rosario, D.J., 2012. Cardiovascular risk in androgen suppression: underappreciated, under-researched and unresolved. Heart 98, 345-348.

Bowen, R.L., Verdile, G., Liu, T., Parlow, A.F., Perry, G., Smith, M.A., Martins, R.N., Atwood, C.S., 2004. Luteinizing Hormone, a Reproductive Regulator That Modulates the Processing of Amyloid-ßPrecursor Protein and Amyloid-ß Deposition. Journal of Biological Chemistry 279, 20539-20545.

Bryan, K.J., Mudd, J.C., Richardson, S.L., Chang, J., Lee, H.g., Zhu, X., Smith, M.A., Casadesus, G., 2010. Down-regulation of serum gonadotropins is as effective as estrogen replacement at improving menopause-associated cognitive deficits. Journal of Neurochemistry 112, 870-881.

Buma, P., 1989. Characterization of luteinizing hormone-releasing hormone fibres in the mesencephalic central grey substance of the rat. Neuroendocrinology 49, 623-630.

Cahill, L., Uncapher, M., Kilpatrick, L., Alkire, M.T., Turner, J., 2004. Sex-Related Hemispheric Lateralization of Amygdala Function in Emotionally Influenced Memory: An fMRI Investigation. Learn Mem. 11, 261-266.

Cahill, L., Babinsky, R., Markowitsch, H.J., McGaugh, J.L., 1995. The amygdala and emotional memory. Nature 377, 295-296.

Cahill, L., Weinberger, N.M., Roozendaal, B., McGaugh, J.L., 1999. Is the Amygdala a Locus of "Conditioned Fear"? Some Questions and Caveats. Neuron 23, 227-228.

Campbell, S., Marriott, M., Nahmias, C., MacQueen, G.M., 2004. Lower Hippocampal Volume in Patients Suffering From Depression: A Meta-Analysis. American Journal of Psychiatry 161, 598-607.

Carel, J.C., Eugster, E.A., Rogol, A., Ghizzoni, L., Palmert, M.R., on behalf of the members of the ESPE-LWPES GnRH Analogs Consensus Conference Group, 2009. Consensus Statement on the Use of Gonadotropin-Releasing Hormone Analogs in Children. Pediatrics 123, e752-e762.

Carel, J.-C., Lahlou, N., Roger, M., Chaussain, J.L., 2004. Precocious puberty and statural growth. Human Reproduction Update 10, 135-147.

Casey, B.J., Jones, R.M., 2010. Neurobiology of the Adolescent Brain and Behavior: Implications for Substance Use Disorders. Journal of the American Academy of Child & Adolescent Psychiatry 49, 1189-1201.

Casadesus, G., Webber, K.M., Atwood, C.S., Pappolla, M.A., Perry, G., Bowen, R.L., Smith, M.A., 2006. Luteinizing hormone modulates cognition and amyloid-ß deposition in Alzheimer APP transgenic mice. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1762, 447-452.

Cesario, S.K., Hughes, L.A., 2007. Precocious Puberty: A Comprehensive Review of Literature. 36 ed., pp. 263-274.

Chi, L., Zhou, W., Prikhozhan, A., Flanagan, C., Davidson, J.S., Golembo, M., Illing, N., Millar, R.P., Sealfon, S.C., 1993. Cloning and characterization of the human GnRH receptor. Molecular and Cellular Endocrinology 91, R1-R6.

Chen, A., Yahalom, D., Ben-Aroya, N., Kaganovsky, E., Okon, E., Koch, Y., 1998. A second isoform of gonadotropin-releasing hormone is present in the brain of human and rodents. FEBS Letters 435, 199-203.

Chen, L., Sun, X.D., Zhao, J., Yang, A.G., Huang, W.Q., 2005. Distribution, cloning and sequencing of GnRH, its receptor, and effects of gastric acid secretion of GnRH analogue in gastric parietal cells of rats. Life Sciences 76, 1351-1365.

Cheng, C.K., Leung, P.C.K., 2005. Molecular Biology of Gonadotropin-Releasing Hormone (GnRH)-I, GnRH-II, and Their Receptors in Humans. Endocrine Reviews 26, 283-306.

Cheung, L.W.T., Wong, A.S.T., 2008. Gonadotropin-releasing hormone: GnRH receptor signaling in extrapituitary tissues. FEBS Journal 275, 5479-5495.

Choi, W.S., Kim, M.O., Lee, B.J., Kim, J.H., Sun, W., Seong, J.Y., Kim, K., 1994. Presence of gonadotropin-releasing hormone mRNA in the rat olfactory piriform cortex. Brain Research 648, 148-151.

Chow, M.L., Pramparo, T., Winn, M.E., Barnes, C.C., Li, H.R., Weiss, L., Fan, J.B., Murray, S., April, C., Belinson, H., Fu, X.D., Wynshaw-Boris, A., Schork, N.J., Courchesne, E., 2012. Age-

Dependent Brain Gene Expression and Copy Number Anomalies in Autism Suggest Distinct Pathological Processes at Young Versus Mature Ages. PLoS Genet 8, e1002592.

Christenson, D.Z., Bayer, T.A., Wirths, O. 2009. Formic acid is essential for immunohistochemical detection of aggregated intraneuronal Abeta peptides in mouse models of Alzheimer's disease. Brain Res. 1301, 116-25.

Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., Selkoe, D.J. 1992. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360, 672-4.

Clinicaltrial.gov. ALADDIN study—phase III: (2005) antigonadotrop- leuprolide in Alzheimer's disease drug INvestigation(VP-AD-301). http://clinicaltrials.gov/ct2/show/ NCT00231946?term=NCT00231946& rank=1

Coit, V.A., Dowell, F.J., Evans, N.P., 2009. Neutering affects mRNA expression levels for the LH- and GnRH-receptors in the canine urinary bladder. Theriogenology 71, 239-247.

Coluccia, E., Louse, G., 2004. Gender differences in spatial orientation: A review. Journal of Environmental Psychology 24, 329-340.

Conn, P.M., Staley, D., Harris, C., Andrews, W.V., Gorospe, W.C., McArdle, C.A., Huckle, W.R., Hansen, J., 1986. Mechanism of Action of Gonadotropin Releasing Hormone. Annu.Rev.Physiol. 48, 495-513.

Conn, P., Crowley, W.F., 1994. Gonadotropin-releasing hormone and its analogs. Annu.Rev.Med. 45, 391-405.

Cooke, B.M., Woolley, C.S., 2009. Effects of prepubertal gonadectomy on a male-typical behavior and excitatory synaptic transmission in the amygdala. Devel Neurobio 69, 141-152.

Cooke, B., Hegstrom, C.D., Villeneuve, L.S., Breedlove, S.M., 1998. Sexual Differentiation of the Vertebrate Brain: Principles and Mechanisms. Frontiers in Neuroendocrinology 19, 323-362.

Craig, M.C., Fletcher, P.C., Daly, E.M., Rymer, J., Cutter, W.J., Brammer, M., Giampietro, V., Wickham, H., Maki, P.M., Murphy, D.G.M., 2007. Gonadotropin hormone releasing hormone agonists alter prefrontal function during verbal encoding in young women. Psychoneuroendocrinology 32, 1116-1127.

Daniel Alexander Beyer, Feriel Amari, Marc Thill, Askan Schultze-Mosgau, Safaa Al-Hasani, Klaus Diedrich, Georg Griesinger, 2011. Emerging gonadotropin-releasing hormone agonists. Expert Opin.Emerging Drugs 16, 323-340.

De Bellis, M.D., Keshavan, M.S., Beers, S.R., Hall, J., Frustaci, K., Masalehdan, A., Noll, J., Boring, A.M., 2001. Sex Differences in Brain Maturation during Childhood and Adolescence. Cerebral Cortex 11, 552-557.

De Bellis, M.D., Casey, B.J., Dahl, R.E., Birmaher, B., Williamson, D.E., Thomas, K.M., Axelson, D.A., Frustaci, K., Boring, A.M., Hall, J., Ryan, N.D., 2000. A pilot study of amygdala volumes in pediatric generalized anxiety disorder. Biol. Psychiatry. 48, 51-57.

Diamond, T.H., Higano, C.S., Smith, M.R., Guise, T.A., Singer, F.R., 2004. Osteoporosis in men with prostate carcinoma receiving androgen-deprivation therapy. Cancer 100, 892-899.

Dong, F., Skinner, D.C., John Wu, T., Ren, J., 2011. The Heart: A Novel Gonadotropin-Releasing Hormone Target. Journal of Neuroendocrinology 23, 456-463.

Drapeau, E., Mayo, W., Aurousseau, C., Le Moal, M., Piazza, P.V., Abrous, D.N., 2003. Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis. Proceedings of the National Academy of Sciences 100, 14385-14390.

Dumas, T.C., 2005. Developmental regulation of cognitive abilities: Modified composition of a molecular switch turns on associative learning. Progress in Neurobiology 76, 189-211.

Duyckaerts, C., Delatour, B.t., Potier, M.C., 2009. Classification and basic pathology of Alzheimer disease. Acta Neuropathol 118, 5-36.

Edwards, G.R., Newman, J.A., Parsons, A.J., Krebs, J.R., 1996. The use of spatial memory by grazing animals to locate food patches in spatially heterogeneous environments: an example with sheep. Applied Animal Behaviour Science 50, 147-160.

Eichenbaum, H., 2002. *The Cognitive Neuroscience of Memory* NewYork: Oxford University Press.

Eisen, M.B., Brown, P.O., 1999. DNA arrays for analysis of gene expression. in: Sherman, M.W. (Ed.), *Methods in Enzymology, cDNA Preparation and Characterization*, Volume 303 ed. Academic Press, pp. 179-205.

Evans, N.P., Robinson, J.E., Erhard, H.W., Ropstad, E., Fleming, L.M., Haraldsen, I.R.H., 2012. Development of psychophysiological motoric reactivity is influenced by peripubertal

pharmacological inhibition of gonadotropin releasing hormone action-Results of an ovine model. Psychoneuroendocrinology 37, 1876-1884.

Farn, L., Jung-Mou, Y., Jian-Nan, W., Yao-Vhung, C., Yu-Hsun, K., Che-Se, T., San-Nan, Y., 1999. Activation of Gonadotropin-Releasing Hormone Receptors Produces Neuronal Excitation in the Rat Hippocampus. Chinese Journal of Physiology 42, 67-71.

Foster, D.L., Padmanabhan, V., Wood, R.I., Robinson, J.E., 2002. Sexual differentiation of the neuroendocrine control of gonadotropin secretion: concepts derived from sheep models. Reprod Suppl 59, 83-99.

Games, D., Adams, D., Alessandrini, R., Barbour, R., Borthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., Gillespie, F., Guido, T., Hagopian, S., Johnson-Wood, K., Khan, K., Lee, M., Leibowitz, P., Lieberburg, I., Little, S., Masliah, E., McConlogue, L., Montoya-Zavala, M., Mucke, L., Paganini, L., Penniman, E., Power, M., Schenk, D., Seubert, P., Snyder, B., Soriano, F., Tan, H., Vitale, J., Wadsworth, S., Wolozin, B., Zhao, J., 1995. Alzheimer-type neuropathology in transgenic mice overexpressing V717F [beta]-amyloid precursor protein. Nature 373, 523-527.

Gore, A.C., Windsor-Engnell, B.M., Terasawa, E., 2004. Menopausal Increases in Pulsatile Gonadotropin-Releasing Hormone Release in a Nonhuman Primate (Macaca mulatta). Endocrinology 145, 4653-4659.

Graber, J.A., Seeley, J.R., Brooks-Gunn, J., Lewinsohn, P.M., 2004. Is Pubertal Timing Associated With Psychopathology in Young Adulthood? Journal of the American Academy of Child & Adolescent Psychiatry 43, 718-726.

Granger, A., NgÔ-Muller, V., Bleux, C., Guigon, C., Pincas, H., Magre, S., Daegelen, D., Tixier-Vidal, A., Counis, R., Laverriére, J.N., 2004. The Promoter of the Rat Gonadotropin-Releasing Hormone Receptor Gene Directs the Expression of the Human Placental Alkaline Phosphatase Reporter Gene in Gonadotrope Cells in the Anterior Pituitary Gland as well as in Multiple Extrapituitary Tissues. Endocrinology 145, 983-993.

Gray, J.A., McNaughton, N., 2000. The Neuropsychology of Anxiety: An Enquiry into the Functions of the Septohippocampal System, 2nd ed. Oxford University Press, Oxford, UK.

Grigorova, M., Sherwin, B.B., Tulandi, T., 2006. Effects of treatment with leuprolide acetate depot on working memory and executive functions in young premenopausal women. Psychoneuroendocrinology 31, 935-947.

Haour, F., Dussaillant, M., Leblanc, P., Rostène, W., 1987. Demonstration and topographical distribution of LHRH receptors in the central nervous system in the normal and castrated male rat. Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie 305, 41-44.

Harrison, G.S., Wierman, M.E., Nett, T.M., Glode, L.M., 2004. Gonadotropin-releasing hormone and its receptor in normal and malignant cells. Endocrine-Related Cancer 11, 725-748.

He, D., Funabashi, T., Sano, A., Uemura, T., Minaguchi, H., Kimura, F., 1999. Effects of glucose and related substrates on the recovery of the electrical activity of gonadotropin-releasing hormone pulse generator which is decreased by insulin-induced hypoglycemia in the estrogen-primed ovariectomized rat. Brain Research 820, 71-76.

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2008. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat.Protocols 4, 44-57.

Illing, N., Jacobs, G.F.M., Becker, I.I., Flanagan, C.A., Davidson, J.S., Eales, A., Zhou, W., Sealfon, S.C., Millar, R.P., 1993. Comparative Sequence Analysis and Functional Characterization of the Cloned Sheep Gonadotropin-Releasing Hormone Receptor Reveals Differences in Primary Structure and Ligand Specificity among Mammalian Receptors. Biochemical and Biophysical Research Communications 196, 745-751.

Jarrard, L.E., 1978. Selective hippocampal lesions: Differential effects on performance by rats of a spatial task with preoperative versus postoperative training. Journal of Comparative and Physiological Psychology 92, 1119-1127.

Jazin, E., Cahill, L., 2010. Sex differences in molecular neuroscience: from fruit flies to humans. Nat Rev Neurosci 11, 9-17.

Jennes, L., Dalati, B., Conn, P., 1988. Distribution of gonadrotropin releasing hormone agonist binding sites in the rat central nervous system. Brain Research 452, 156-164.

Jennes, L., Eyigor, O., Janovick, J., Conn, P., 1997. Brain gonadotropin releasing hormone receptors: localization and regulation. Recent Progress in Hormone Research 52, 475-490.

Jonasson, Z., 2005. Meta-analysis of sex differences in rodent models of learning and memory: a review of behavioral and biological data. Neuroscience & Biobehavioral Reviews 28, 811-825.

Kalynn, M.S., Heather, A.M.-F., Cheryl, L.S., 2013. Back to the Future: The Organizational-Activational Hypothesis Adapted to Puberty and Adolescence. Hormones and Behaviou 55, 597-604.

Kasten, T.L., White, S.A., Norton, T.T., Bond, C.T., Adelman, J.P., Fernald, R.D., 1996. Characterization of Two New preproGnRH mRNAs in the Tree Shrew: First Direct Evidence for Mesencephalic GnRH Gene Expression in a Placental Mammal. General and Comparative Endocrinology 104, 7-19.

Kendziorski, C., Irizarry, R.A., Chen, K.S., Haag, J.D., Gould, M.N., 2005. On the utility of pooling biological samples in microarray experiments. Proceedings of the National Academy of Sciences of the United States of America 102, 4252-4257.

Kerns, K., Berenbaum, S., 1991. Sex differences in spatial ability in children. Behav Genet 21, 383-396.

Kheirbek, M.A., Hen, R., 2011. Dorsal vs Ventral Hippocampal Neurogenesis: Implications for Cognition and Mood. Neuropsychopharmacology 36, 373-374.

King, H.C., Sinha, A.A., 2001. Gene expression profile analysis by dna microarrays: Promise and pitfalls. JAMA 286, 2280-2288.

König, R., Baldessari, D., Pollet, N., Niehrs, C., Eils, R., 2004. Reliability of gene expression ratios for cDNA microarrays in multiconditional experiments with a reference design. Nucleic Acids Research 32, e29.

Koscik, T., Bechara, A., Tranel, D., 2010. Sex-related functional asymmetry in the limbic brain. Neuropsychopharmacology. 35, 340-341.

Kostovic-, I., Judaš, M., 2006. Prolonged coexistence of transient and permanent circuitry elements in the developing cerebral cortex of fetuses and preterm infants. Developmental Medicine & Child Neurology 48, 388-393.

Kubek, M.J., Wilber, J.F., Leesthma, J.E., 1979. The Identification of Gonadotropin-Releasing Hormone (GnRH) in Hypothalamic and Extrahypothalamic Loci of the Human Nervous System. Horm Metab Res 11, 26-29.

Layton, B.S., Lafontaine, S., Renaud, L.P., 1981. Connections of Medial Preoptic Neurons with the Median Eminence and Amygdala. Neuroendocrinology 33, 235-240.

Lee, C., Colegate, S., Fisher, A.D., 2006. Development of a maze test and its application to assess spatial learning and memory in Merino sheep. Applied Animal Behaviour Science 96, 43-51.

Levine, G.N., D'Amico, A.V., Berger, P., Clark, P.E., Eckel, R.H., Keating, N.L., Milani, R.V., Sagalowsky, A.I., Smith, M.R., Zakai, N., 2010. Androgen-Deprivation Therapy in Prostate Cancer and Cardiovascular Risk: A Science Advisory From the American Heart Association, American Cancer Society, and American Urological Association: Endorsed by the American Society for Radiation Oncology. CA: A Cancer Journal for Clinicians 60, 194-201.

Lord, A., Kalimo, H., Eckman, C., Zhang, X.Q., Lannfelt, L., Nilsson, L.N.G., 2006. The Arctic Alzheimer mutation facilitates early intraneuronal Aβ aggregation and senile plaque formation in transgenic mice. Neurobiology of aging 27, 67-77.

Lord, A., Philipson, O., Klingstedt, T., Westermark, G., Hammarstrom, P., Nilsson, K.P., Nilsson, L.N. 2011. Observations in APP bitransgenic mice suggest that diffuse and compact plaques form via independent processes in Alzheimer's disease. Am. J. Pathol. 178, 2286-98.

López de Maturana, R., Martin, B., Millar, R., Brown, P., Davidson, L., Pawson, A., Nicol, M., Mason, J.I., Barran, P., Naor, Z., Maudsley, S., 2007. GnRH-Mediated DAN Production Regulates the Transcription of the GnRH Receptor in Gonadotrope Cells. Neuromol Med 9, 230-248.

Lopez, J.F., Akil, H., Watson, S.J., 1999. Neural circuits mediating stress. Biol Psychiatry 46, 1461-1471.

Lu, F., Yang, J.M., Wu, J.N., Chen, Y.C., Kao, Y.H., Tung, C.S., Yang, S.N., 1999. Activation of gonadotropin-releasing hormone receptors produces neuronal excitation in the rat hippocampus. Chin J Physiol 42, 67-71.

Luna, B., Thulborn, K.R., Munoz, D.P., Merriam, E.P., Garver, K.E., Minshew, N.J., Keshavan, M.S., Genovese, C.R., Eddy, W.F., Sweeney, J.A., 2001. Maturation of Widely Distributed Brain Function Subserves Cognitive Development. NeuroImage 13, 786-793.

Maren, S., 2001. Neurobiology of pavlovian fear conditioning. Annu.Rev.Neurosci. 24, 897-931.

Mark, G., 2002. Spatial Memory and Hippocampal Function: Where are we now? Psicológica 23, 109-138.

Marcell AV. Adolescence. In: Kliegman RM, Behrman RE, Jenson HB, Stanton BF, eds. Nelson Textbook of Pediatrics. 18th ed. Philadelphia, Pa: Saunders Elsevier; 2007:chap 12.

Mastro, L.D., Levaggi, A., Giraudi, S., Pronzato, P., 2011. Luteinising hormone releasing hormone agonists (LH-RHa) in premenopausal early breast cancer patients: Current role and future perspectives. Cancer Treatment Reviews 37, 208-211.

MacMillan, S., Szeszko. P.R., Moore. G.J., Madden. R., Lorch. E., Ivey. J., Banerjee. S.P., Rosenberg. D.R., 2003. Increased amygdala: Hippocampal volume ratios associated with severity of anxiety in pediatric major depression. J. Child. Adolesc. Psychopharmacol. 13, 65-73

McGuire, N.L., Bentley, G.E., 2010. Neuropeptides in the Gonads: From Evolution to Pharmacology. Frontiers in Pharmacology 1.

Merke, D.P., Fields, J.D., Keil, M.F., Vaituzis, A.C., Chrousos, G.P., Giedd, J.N., 2003. Children with Classic Congenital Adrenal Hyperplasia Have Decreased Amygdala Volume: Potential Prenatal and Postnatal Hormonal Effects. Journal of Clinical Endocrinology & Metabolism 88, 1760-1765.

Meethal, S., Smith, M., Bowen, R., Atwood, C., 2005. The gonadotropin connection in Alzheimer's disease. Endocr 26, 317-325.

Michael, A., Catherine, A.B., Judith, A.B., David, B., Heather, B., Michael, C., Allan, P.D., Kara, D., Selina, S.D., Janan, T.E., Midori, A.H., David, P.H., Laurie, I.-T., Andrew, K.,

Suzanna, L., John, C.M., Joel, E.R., Martin, R., Gerald, M.R., Gavin, S., 2000. Gene Ontology: tool for the unification of biology. Nat. Genet. 25, 25-29.

Millar, R.P., 2005. GnRHs and GnRH receptors. Animal Reproduction Science 88, 5-28.

Millar, R., Lowe, S., Conklin, D., Pawson, A., Maudsley, S., Troskie, B., Ott, T., Millar, M., Lincoln, G., Sellar, R., Faurholm, B., Scobie, G., Kuestner, R., Terasawa, E., Katz, A., 2001. A novel mammalian receptor for the evolutionarily conserved type II GnRH. Proceedings of the National Academy of Sciences 98, 9636-9641.

Millar, R.P., Lu, Z.L., Pawson, A.J., Flanagan, C.A., Morgan, K., Maudsley, S.R., 2004. Gonadotropin-Releasing Hormone Receptors. Endocrine Reviews 25, 235-275.

Morris, R., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. Journal of Neuroscience Methods 11, 47-60.

Morris, R.G.M., Garrud, P., Rawlins, J.N.P., O'Keefe, J., 1982. Place navigation impaired in rats with hippocampal lesions. Nature 297, 681-683.

Morton, A.J., Avanzo, L., 2011. Executive decision-making in the domestic sheep. PloS one 6, e15752.

Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B., Lannfelt, L. 1992. A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat. Genet. 1, 345-7.

Näslund, J., Haroutunian, V., Mohs, R., 2000. Correlation between elevated levels of amyloid¦- β peptide in the brain and cognitive decline. JAMA 283, 1571-1577.

Nilsberth, C., Westlind-Danielsson, A., Eckman, C.B., Condron, M.M., Axelman, K., Forsell, C., Stenh, C., Luthman, J., Teplow, D.B., Younkin, S.G., Naslund, J., Lannfelt, L., 2001. The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced A[beta] protofibril formation. Nat Neurosci 4, 887-893.

Negriff, S., Susman, E., Trickett, P., 2011. The Developmental Pathway from Pubertal Timing to Delinquency and Sexual Activity from Early to Late Adolescence. J Youth Adolescence 40, 1343-1356.

Nelson, C.J., Lee, J.S., Gamboa, M.C., Roth, A.J., 2008. Cognitive effects of hormone therapy in men with prostate cancer. Cancer 113, 1097-1106.

Nolte, J., 1993. Olfactory and limbic systems. In R.Farrell (ed.): The Human Brain. An Introduction to its Functional Anatomy. in: St.Louis: Mosby Year Book (Ed.), pp. 397-413.

Nuruddin, S., Bruchhage, M., Ropstad, E., Krogenæs, A., Evans, N.P., Robinson, J.E., Endestad, T., Westlye, L.T., Madison, C., Haraldsen, I.R.H., 2013. Effects of peripubertal gonadotropin-releasing hormone agonist on brain development in sheep - A magnetic resonance imaging study. Psychoneuroendocrinology 38, 1994-2002.

Oades, 1982. Search strategies on a hoile-board are impaired with ventral tegmental damage: animal model for tests of thought disorder. Society Biological Psychiatry, pp. 243-258.

Olton, D.S., Becker, J.T., Handelmann, G.E., 1979. Hippocampus, space, and memory. Behavioral and Brain Sciences 2, 313-322.

Ortu, D., Skavhaug, I.M., Vaidya, M., 2013. Timescales of Learning in the Basal Ganglia and the Hippocampus. Frontiers in Behavioral Neuroscience 7.

Osada, T., Kimura, F., 1995. LHRH effects on hippocampal neurons are modulated by estrogen in rats. Endocrine journal 42, 251-257.

O'Keefe, J., Nadel, L., 1978. The Hippocampus as a Cognitive Map. Clarendon Press, Oxford, UK.

Palomba, S., Orio, J., Russo, T., Falbo, A., Amati, A., Zullo, F., 2004. Gonadotropin-releasing hormone agonist with or without raloxifene: Effects on cognition, mood, and quality of life. Fertility and Sterility 82, 480-482.

Pan, J.T., Kow, L.M., Pfaff, D.W., 1988. Modulatory actions of luteinizing hormone-releasing hormone on electrical activity of preoptic neurons in brain slices. Neuroscience 27, 623-628.

Partsch, C.J., Heger, S., Sippell, W.G., 2002. Management and outcome of central precocious puberty. Clinical Endocrinology 56, 129-148.

Paterson, S.J., Heim, S., Thomas Friedman, J., Choudhury, N., Benasich, A.A., 2006. Development of structure and function in the infant brain: Implications for cognition, language and social behaviour. Neuroscience & Biobehavioral Reviews 30, 1087-1105.

Peper, J.S., Hulshoff Pol, H.E., Crone, E.A., van Honk, J., 2011. Sex steroids and brain structure in pubertal boys and girls: a mini-review of neuroimaging studies. Neuroscience. 191, 28-37.

Pfaff, D., 2009. Hormonal Effects on Specific Behaviors, on Global CNS States, and on Human Disease. An Introduction to the Second Edition. in: Editors-in-Chief: Donald, W.P., Arthur, P.A., Susan E.Fahrbach, Anne, M.E.a.R., and Robert, T.R. (Eds.), Hormones, Brain and Behavior (Second Edition) Academic Press, San Diego, p. 1.

Pfefferbaum, A., Sullivan, E.V., Adalsteinsson, E., Garrick, T., Harper, C., 2004. Postmortem MR imaging of formalin-fixed human brain. NeuroImage 21, 1585-1595.

Phelps, E.A., 2004. Human emotion and memory: interactions of the amygdala and hippocampal complex. Current Opinion in Neurobiology 14, 198-202.

Philipson, O., Lord, A., Gumucio, A., O'Callaghan, P., Lannfelt, L., Nilsson, L.N.G., 2010. Animal models of amyloid-βrelated pathologies in Alzheimer's disease. FEBS Journal 277, 1389-1409.

Philipson, O., Hammarström, P., Nilsson, K.P., Portelius, E., Olofsson, T., Ingelsson, M., Hyman, B.T., Blennow, K., Lannfelt, L., Kalimo, H., Nilsson, L.N.G., 2009. A highly insoluble state of Aß similar to that of Alzheimer's disease brain is found in Arctic APP transgenic mice. Neurobiology of aging 30, 1393-1405.

Philipson, O., Lord, A., Lalowski, M., Soliymani, R., Baumann, M., Thyberg, J., Bogdanovic, N., Olofsson, T., Tjernberg, L.O., Ingelsson, M., Lannfelt, L., Kalimo, H., Nilsson, L.N.G.,

2012. The Arctic amyloid--βprecursor protein (AβPP) mutation results in distinct plaques and accumulation of N- and C-truncated Aβ. Neurobiology of aging 33, 1010.

Phillips, M.L., Drevets, W.C., Rauch, S.L., Lane, R., 2003. Neurobiology of emotion perception I: the neural basis of normal emotion perception. Biological Psychiatry 54, 504-514.

Plant, T.M., 2001. Neurobiological Bases Underlying the Control of the Onset of Puberty in the Rhesus Monkey: A Representative Higher Primate. Frontiers in Neuroendocrinology 22, 107-139.

Potosky, A.L., Knopf, K., Clegg, L.X., Albertsen, P.C., Stanford, J.L., Hamilton, A.S., Gilliland, F.D., Eley, J.W., Stephenson, R.A., Hoffman, R.M., 2001. Quality-of-Life Outcomes After Primary Androgen Deprivation Therapy: Results From the Prostate Cancer Outcomes Study. Journal of Clinical Oncology 19, 3750-3757.

Rama, S., Rao, A.J., 2001. Embryo implantation and GnRH antagonists: The search for the human placental GnRH receptor. Human Reproduction 16, 201-205.

Ramakrishnappa, N., Rajamahendran, R., Lin, Y.M., Leung, P.C.K., 2005. GnRH in non-hypothalamic reproductive tissues. Animal Reproduction Science 88, 95-113.

Reilly, J.F., Games, D., Rydel, R.E., Freedman, S., Schenk, D., Young, W.G., Morrison, J.H., Bloom, F.E., 2003. Amyloid deposition in the hippocampus and entorhinal cortex: Quantitative analysis of a transgenic mouse model. Proceedings of the National Academy of Sciences 100, 4837-4842.

Reinhart, J., Mertz, L.M., Catt, K.J., 1992. Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor. Journal of Biological Chemistry 267, 21281-21284.

Renaud, L.P., Martin, J.B., Brazeau, P., 1975. Depressant action of TRH, LH-RH and somatostatin on activity of central neurones. Nature 255, 233-235.

Richardson, H.N., Gore, A.C., Venier, J., Romeo, R.D., Sisk, C.L., 2004. Increased expression of forebrain GnRH mRNA and changes in testosterone negative feedback following pubertal maturation. Molecular and Cellular Endocrinology 214, 63-70.

Robinson, J.E., Evans, N.P., Dumbell, R., Solbakk, A.K., Ropstad, E., Haraldsen, I.R.H., 2013, Effects of inhibition of Gonadotropin Releasing Hormone secretion on the response to novel objects in young male and female sheep. Psychoneuroendocrinology. (In press)

Rodriguez, C.A., Torres, A., Mackintosh, N.J., Chamizo, V.D., 2010. Sex differences in the strategies used by rats to solve a navigation task. J Exp Psychol Anim Behav Process 36, 395-401.

Romanelli, R.G., Barni, T., Maggi, M., Luconi, M., Failli, P., Pezzatini, A., Pelo, E., Torricelli, F., Crescioli, C., Ferruzzi, P., Salerno, R., Marini, M., Rotella, C.M., Vannelli, G.B., 2004. Expression and Function of Gonadotropin-releasing Hormone (GnRH) Receptor in Human Olfactory GnRH-secreting Neurons: An autocrine gnrh loop underlies neuronal migration. Journal of Biological Chemistry 279, 117-126.

Romeo, R.D., Sisk, C.L., 2001. Pubertal and seasonal plasticity in the amygdala. Brain Research 889, 71-77.

Rothman, M.S., Wierman, M.E., 2007. The role of gonadotropin releasing hormone in normal and pathologic endocrine processes. Current Opinion in Endocrinology, Diabetes and Obesity 14.

Ruan, W., Lai, M., 2007. Actin, a reliable marker of internal control? Clinica Chimica Acta 385, 1-5.

Rubenstein, J.L.R., 2011. Annual Research Review: Development of the cerebral cortex: implications for neurodevelopmental disorders. Journal of Child Psychology and Psychiatry 52, 339-355.

Rönnbäck, A., Zhu, S., Dillner, K., Aoki, M., Lilius, L., Naslund, J., Winblad, B., Graff, C. 2011. Progressive neuropathology and cognitive decline in a single Arctic APP transgenic mouse model. Neurobiol. Aging 32, 280-92.

Sanchez, M.A., Dominguez, R., 1995. Differential effects of unilateral lesions in the medial amygdala on spontaneous and induced ovulation. Brain Research Bulletin 38, 313-317.

Sandstrom, N.J., Kaufman, J., Huettel, S.A., 1998. Males and females use different distal cues in a virtual environment navigation task. Brain Res Cogn Brain Res 6, 351-360.

Saucier, D.M., Green, S.M., Leason, J., MacFadden, A., Bell, S., Elias, L.J., 2002. Are sex differences in navigation caused by sexually dimorphic strategies or by differences in the ability to use the strategies? Behav Neurosci 116, 403-410.

Schaefer, A., Gray, J.R., 2007. A Role for the Human Amygdala in Higher Cognition. Reviews in the Neurosciences, 18 ed., p. 355.

Schally, A.V., Comaru-Schally, A.M., Nagy, A., Kovacs, M., Szepeshazi, K., Plonowski, A., Varga, J.L., Halmos, G., 2001. Hypothalamic Hormones and Cancer. Frontiers in Neuroendocrinology 22, 248-291.

Schellenberg, G.D., Montine, T.J., 2012. The genetics and neuropathology of Alzheimer's disease. Acta Neuropathologica 124, 305-323.

Schena, M., Heller, R.A., Theriault, T.P., Konrad, K., Lachenmeier, E., Davis, R.W., 1998. Microarrays: biotechnology's discovery platform for functional genomics. Trends in Biotechnology 16, 301-306.

Schneider, S., Peters, J., Bromberg, U., Brassen, S., Menz, M.M., Miedl, S.F., Loth, E., Banaschewski, T., Barbot, A., Barker, G., Conrod, P.J., Dalley, J.W., Flor, H., Gallinat, J., Garavan, H., Heinz, A., Itterman, B., Mallik, C., Mann, K., Artiges, E., Paus, T., Poline, J.B., Rietschel, M., Reed, L., Smolka, M.N., Spanagel, R., Speiser, C., Ströhle, A., Struve, M., Schumann, G., Böchel, C., 2011. Boys do it the right way: Sex-dependent amygdala lateralization during face processing in adolescents. NeuroImage. 56, 1847-1853.

Schang, A.L., Ngô-Muller, V., Bleux, C., Granger, A., Chenut, M.C., Loudes, C., Magre, S., Counis, R., Cohen-Tannoudji, J., Laverriére, J.N., 2011. GnRH Receptor Gene Expression in the Developing Rat Hippocampus: Transcriptional Regulation and Potential Roles in Neuronal Plasticity. Endocrinology 152, 568-580.

Schmidt, P.J., Berlin, K.L., Danaceau, M.A., 2004. THe effects of pharmacologically induced hypogonadism on mood in healthymen. Archives of General Psychiatry 61, 997-004.

Sedgwick, J., 2008. *Scientific Imaging with Photoshop: Methods, Measurement, and Output*Peachpit Press, California.

Shaw, P., Greenstein, D., Lerch, J., Clasen, L., Lenroot, R., Gogtay, N., Evans, A., Rapoport, J., Giedd, J., 2006. Intellectual ability and cortical development in children and adolescents. Nature 440, 676-679.

Sirett, N.E., Hyland, B.I., Hubbard, J.I., Lapwood, K.R., Elgar, H.J., 1986. Luteinizing Hormone Release in the Anaesthetised Cat Following Electrical Stimulation of Limbic Structures. Neuroendocrinology 42, 128-136.

Sisk, C.L., Zehr, J.L., 2005. Pubertal hormones organize the adolescent brain and behavior. Frontiers in Neuroendocrinology 26, 163-174.

Skinner, D.C., Albertson, A.J., Navratil, A., Smith, A., Mignot, M., Talbott, H., Scanlan-Blake, N., 2009. Effects of Gonadotropin-Releasing Hormone Outside the Hypothalamic-Pituitary-Reproductive Axis. Journal of Neuroendocrinology 21, 282-292.

Smith, J.T., Clarke, I.J., 2010. Seasonal breeding as a neuroendocrine model for puberty in sheep. Molecular and Cellular Endocrinology 324, 102-109.

Smith, S.M., 2002. Fast robust automated brain extraction. Hum.Brain Mapp. 17, 143-155.

Smith, S.M., Jenkinson, M., Woolrich, M.W., Beckmann, C.F., Behrens, T.E.J., Johansen-Berg, H., Bannister, P.R., De Luca, M., Drobnjak, I., Flitney, D.E., Niazy, R.K., Saunders, J., Vickers, J., Zhang, Y., De Stefano, N., Brady, J.M., Matthews, P.M., 2004. Advances in functional and structural MR image analysis and implementation as FSL. NeuroImage 23, Supplement 1, S208-S219.

Smyth, G.K., Michaud, J., Scott, H.S., 2005. Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics 21, 2067-2075.

Smyth, G.K., Speed, T., 2003. Normalization of cDNA microarray data. Methods 31, 265-273.

Spear, L.P., 2004. Adolescent Brain Development and Animal Models. Annals of the New York Academy of Sciences 1021, 23-26.

Spreng, R.N., Mar, R.A., 2012. I remember you: A role for memory in social cognition and the functional neuroanatomy of their interaction. Brain Research 1428, 43-50.

Stattin, H., err, M., koog, T., 2011. Early Pubertal Timing and GirlsGÇÖ Problem Behavior: Integrating Two Hypotheses. J Youth Adolescence 40, 1271-1287.

Stella, F., Cerasti, E., Si, B., Jezek, K., Treves, A., 2012. Self-organization of multiple spatial and context memories in the hippocampus. Neuroscience & Biobehavioral Reviews 36, 1609-1625.

Stevens, J.R., 2002. Schizophrenia: Reproductive Hormones and the Brain. American Journal of Psychiatry 159, 713-719.

Stürzenbaum, S.R., Kille, P., 2001. Control genes in quantitative molecular biological techniques: the variability of invariance. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 130, 281-289.

Tammela, T., 2004. Endocrine treatment of prostate cancer. The Journal of Steroid Biochemistry and Molecular Biology 92, 287-295.

Tan, S.H., Wolff, A.C., 2007. Luteinizing Hormone-Releasing Hormone Agonists in Premenopausal Hormone Receptor-Positive Breast Cancer. Clinical Breast Cancer 7, 455-464.

Tareen, R.S., Kamboj, M.K., 2012. Role of Endocrine Factors in Autistic Spectrum Disorders. Pediatric Clinics of North America 59, 75-88.

Terry, A.V.Jr., 2009. *Spatial Navigation (Water Maze) Tasks.* Methods of Behavior Analysis in Neuroscience. 2nd edition. ed. CRC Press, Boca Raton (FL).

The International Sheep Genomics Consortium, Archibald, A.L., Cockett, N.E., Dalrymple, B.P., Faraut, T., Kijas, J.W., Maddox, J.F., McEwan, J.C., Hutton Oddy, V., Raadsma, H.W., Wade, C., Wang, J., Wang, W., Xun, X., 2010. The sheep genome reference sequence: a work in progress. Animal Genetics 41, 449-453.

Thomas, K.M., Drevets, W.C., Dahl, R.E., Ryan, N.D., Birmaher, B., Eccard, C.H., Axelson, D., Whalen, P.J., Casey, B.J., 2001. Amygdala response to fearful faces in anxious and depressed children. Arch. Gen. Psychiatry. 58, 1057-1063.

Tovi, M., Ericsson, A., 1992. Measurements of T1 and T2 over time in formalin-fixed human whole-brain specimens. Acta Radiol 33, 400-404.

Tsai, P.S., 2006. Gonadotropin-releasing hormone in invertebrates: Structure, function, and evolution. General and Comparative Endocrinology 148, 48-53.

Tsutsumi, M., Zhou, W., Millar, R.P., Mellon, P.L., Roberts, J.L., Flanagan, C.A., Dong, K., Gillo, B., Sealfon, S.C., 1992. Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Molecular Endocrinology 6, 1163-1169.

Vadakkadath Meethal, S., Atwood, C.S., 2005. Alzheimer's disease: the impact of age-related changes in reproductive hormones. CMLS, Cell.Mol.Life Sci. 62, 257-270.

VanGuilder, H.D., Vrana, K.E., Freeman, W.M., 2008. Twenty-five years of quantitative PCR for gene expression analysis. BioTechniques 44, 619-626.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3, research0034.

Velasco, M.E., Taleisnik, S., 1969. Release of Gonadotropins Induced by Amygdaloid Stimulation in the Rat. Endocrinology 84, 132-139.

Voyer, D., Voyer, S., Bryden, M.P., 1995. Magnitude of sex differences in spatial abilities: A meta-analysis and consideration of critical variables. Psychological Bulletin 117, 250-270.

Vyas, A., Jadhav, S., Chattarji, S., 2006. Prolonged behavioral stress enhances synaptic connectivity in the basolateral amygdala. Neuroscience. 143, 387-393.

Wang, L., Chadwick, W., Park, S., Zhou, Y., Silver, N., Martin, B., Maudsley, S., 2010. Gonadotropin-releasing hormone receptor system: modulatory role in aging and neurodegeneration. CNS Neurol Disord Drug Targets 9, 651-660.

Wilson, A.C., Salamat, M.S., Haasl, R.J., Roche, K.M., Karande, A., Meethal, S.V., Terasawa, E., Bowen, R.L., Atwood, C.S., 2006. Human neurons express type I GnRH receptor and respond to GnRH I by increasing luteinizing hormone expression. Journal of Endocrinology 191, 651-663.

Weesner, G.D., Matteri, R.L., 1994. Rapid communication: nucleotide sequence of luteinizing hormone-releasing hormone (LHRH) receptor cDNA in the pig pituitary. Journal of Animal Science 72, 1911.

Wieraszko, A., 1998. Avian Hippocampus as a Model to Study Spatial Orientation-Related Synaptic Plasticity. in: Ehrlich, Y. (Ed.), Molecular and Cellular Mechanisms of Neuronal Plasticity, 446 ed. Springer US, pp. 107-129.

Wojniusz, S., Vögele, C., Ropstad, E., Evans, N., Robinson, J., Sütterlin, S., Erhard, H.W., Solbakk, A.K., Endestad, T., Olberg, D.E., Haraldsen, I.R.H., 2011. Prepubertal gonadotropin-releasing hormone analog leads to exaggerated behavioral and emotional sex differences in sheep. Hormones and Behavior 59, 22-27.

Wojniusz, S., Ropstad, E., Evans, N., Robinson, J., Solbakk, A.K., Endestad, T., Haraldsen, I.R.H., 2013. Sex-specific development of spatial orientation is independent of peripubertal gonadal steroids. Psychoneuroendocrinology 38, 1709-1716.

Wolbers, T., Hegarty, M., 2010. What determines our navigational abilities? Trends in Cognitive Sciences 14, 138-146.

Wood, R.I., Foster, D.L., 1992. Prenatal androgens and the timing of seasonal reproductive transitions in sheep. Biology of Reproduction 47, 389-396.

Wood, R.I., Foster, D.L., 1998. Sexual differentiation of reproductive neuroendocrine function in sheep. Rev Reprod 3, 130-140.

Xhrouet-Heinrichs, D., Lagrou, K., Heinrichs, C., Craen, M., Dooms, L., Malvaux, P., Kanen, F., Bourguignon, J.P., 1997. Longitudinal study of behavioral and affective patterns in girls with central precocious puberty during long-acting triptorelin therapy. Acta Paediatrica 86, 808-815.

Xing, Y., Nakamura, Y., Rainey, W.E., 2009. G protein-coupled receptor expression in the adult and fetal adrenal glands. Molecular and Cellular Endocrinology 300, 43-50.

Xu, C., Xu, X.Z., Nunemaker, C.S., Moenter, S.M., 2004. Dose-Dependent Switch in Response of Gonadotropin-Releasing Hormone (GnRH) Neurons to GnRH Mediated through the Type I GnRH Receptor. Endocrinology 145, 728-735.

Yang, S.N., Lu, F., Wu, J.N., Liu, D.D., Hsieh, W.Y., 1999. Activation of gonadotropin-releasing hormone receptors induces a long-term enhancement of excitatory postsynaptic currents mediated by ionotropic glutamate receptors in the rat hippocampus. Neuroscience Letters 260, 33-36.

Zald, D.H., 2003. The human amygdala and the emotional evaluation of sensory stimuli. Brain. Res. Rev. 41, 88-123.

Zafeiriou, S., Loutradis, D., Michalas, S., 2000. The role of gonadotropins in follicular development and their use in ovulation induction protocols for assisted reproduction. 5, 157-167.

Zapatero-Caballero, H., Sanchez-Franco, F., Fernandez-Mendez, C., García-San Frutos, M., Botella-Cubells, L.M., Fernandez-Vazquez, G., 2004. Gonadotropin-Releasing Hormone Receptor Gene Expression During Pubertal Development of Female Rats. Biology of Reproduction 70, 348-355.

Zeng, Z., Huang, Z.Y., Qin, Y., Pang, H., 2005. Hemolymph Juvenile Hormone Titers in Worker Honey Bees under Normal and Preswarming Conditions. Journal of Economic Entomology 98, 274-278.

Zhang, Y., Brady, M., Smith, S., 2001. Segmentation of brain MR images through a hidden Markov random field model and the expectation-maximization algorithm. Medical Imaging, IEEE Transactions on 20, 45-57.

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Research report

Peri-pubertal gonadotropin-releasing hormone analog treatment affects hippocampus gene expression without changing spatial orientation in young sheep

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HIGHLIGHTS

- GnRHa treatment had sex and side specific effect on hippocampal gene expression.
- The affected genes are associated with endocrine function and plasticity.
- The mRNA expression changes did not reflect performance in spatial orientation.
- Females showed tendency to outperform males in spatial orientation.

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ABSTRACT

Background: Normal brain maturation is the result of molecular changes that can be modulated by endocrine variables associated with brain plasticity and results in sex- and age specific changes in cognitive performance. Using a sheep model, we have previously shown that peri-pubertal pharmacological blockade of gonadotropin releasing hormone (GnRH) receptors results in increased sex-differences in cognitive executive function and emotional control. In this study we explore effects of this treatment regime on hippocampal gene expression and spatial orientation.
Methods: The study was conducted with 30 same-sex twin lambs, half of which were treated with the GnRH analog (GnRHa) goserelin acetate every 4th week, beginning before puberty, until 50 weeks of age. Animals were tested in their spatial orientation ability at 48 weeks of age. Quantitative real time PCR analysis was conducted to examine effects of treatment on the expression of genes associated with synaptic plasticity and endocrine signaling.
Results: GnRHa treatment was associated with significant sex- and hemisphere specific changes in mRNA expression for some of the genes studied. The treatment had no significant effect on spatial orientation. However, there was a tendency that females performed better than males in spatial orientation.
Conclusion: Our results indicate that GnRH directly and/or indirectly, is involved in the regulation of sex- and side-specific expression patterns of genes. Hence, these results should be considered when long-term peri-pubertal GnRHa treatment is used in children.

1. Introduction

Brain development is characterized by sex-specific changes in cognitive performance, behavior and social ability [1,2]. Given the chronology and dynamic nature of such changes, for example the silencing of cognitive differences in late childhood, their

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Table 1
Target genes and real-time amplification products.

Gene symbol	Gene name	Forward primer	Reverse primer	Accession number
GnRHI	Gonadotropin-releasing hormone1	GCTGCGCCCTGGAGGAAAGAG	TCCAGAGCTGCCTTCAGGTCCC	U02517
GNRHII	Gonadotropin-releasing hormone2	GCAGCCCTGCTATGGGCCAC	GCCGTGGGACCGGTGTTGAG	XM_001255677
CYP19	Aromatase	CTGGCCTGGTGCGCATGGT	TGCGCCGCATGAGGGTCAAC	NM_001123000
AR	Androgen receptor	AAGGCCTTGCCTGGCTTCCG	TCCGGGACTTGTGCATGCGG	AF105713
GH	Growth hormone	CCGAAACCATCCCAGCCCCC	AGACACGGTCCGAGGTGCCA	NM_001009315
ESR1	Estrogen receptor alpha	TGCACGCCCCAGCCAACTTC	CGGGAGCCGGGGAACTCTCA	AY033393
ESR2	Estrogen receptor beta	TCAGGCCTGTCGACTGCGGA	GCTCAGGGCACTCAGCAGCA	NM_001009737
BDNF	Brain derived neurotrophic factor	GGGAGCTGAGCGTGTGCGAC	GCTGGCCTTTCGAGACGGGG	NM_001046607
VGF	VGF nerve growth factor inducible	AATCTCTGCTGACCGAGACC	CTCCTGAAGCAGGGAAGCTA	XM_870373
GRin1	Glutamate (NMDA) receptor, ionotropic	GACCACAAGCGCGGACCCAA	ACCAGCCACACGTACCCCGA	AY434689
NCAM1	Neural cell adheshion molecule 1	ACGTGAAACCCACAGCCAAACT	TGCCGGTTCGGCAACACCAA	NM_174399
Gria1	Glumate receptor AMPA1	TGCGGACCACAGAGGAGGGG	TCTCAGGGCGGACCCCTTGG	AY346122
LHX5	LIM homeobox 5	GCAGCAAAGTCTTCCACCTC	CAGGTAGTCGTCCTTGCACA	NM_001102061
Syn1	Synapsin1	GCAAAATTTCCCCAATCCTT	AAAACTTTGAGGGGCTTGGT	NM_174191
SNTA1	Syntrophin, alpha 1	GGTCACCCGTCTGGGGCTCT	GCAATCAGCGGAGGGCCAGG	NM_001075898
spn	spinophilin	CAAGGAGCTCCAGATCAAGC	ACCCCAGTAGCCTTCCAGTT	NM_001103102
GABRA4	Gamma-aminobutyric acid (GABA) A receptor, alpha 4	TTCCTGTGCCTGGCGGCTTG	CCCCCAAATCCAGGACGCAGC	NM_174543

reintroduction with puberty and the decline in cognitive performances during aging [3], it has been proposed that age-related endocrine changes may affect plasticity genes and modulate brain structure and function. Sex differences in hormone sensitivity of such plasticity genes might, therefore, underlie measured sex-specific cognitive differences [1,2].

Sex-specific differences in brain organization and function are seen in numerous species [4]. In humans, the temporal sequence of brain maturation and the formation of functional circuits are sexually differentiated; the subcortical (e.g. striatum) and prefrontal cortical regions develop at different times in boys and girls [5]. These temporal and organizational differences in brain development are thought to result in sex-specific behaviors [5] and it has been proposed that they may also underlie sex-associated differences in the risk of developing some neuropsychiatric diseases [6]. The observation that the time of onset of neuropsychiatric disorders such as schizophrenia, autism and Alzheimer's disease (AD) correlates with major endocrine changes during puberty and menopause [7–9] would also support this hypothesis.

While the primary target of the neuropeptide gonadotropin releasing hormone (GnRH) has historically been considered to be in the pituitary gland, to facilitate the central regulation of reproductive function, recent studies have identified non-reproductive effects of GnRH, including a role in cognitive function [10]. In this regard, GnRH receptors are expressed in regions of the brain not associated with the regulation of reproduction [11] and recent reports have also indicated that therapy with GnRH analogs (GnRHa) may reduce adult neuropsychiatric disease symptoms [12]. Consequently, their use in the treatment of Alzheimer's disease (AD) has been discussed [10]. While it was originally proposed that the cognitive effects of GnRH were indirect and mediated by luteinizing hormone (LH) or gonadal steroids [9], recent findings support a direct GnRH receptor (GnRHR) mediated effect [10].

This study is part of a major project, characterizing the effects of peri-pubertal GnRHa treatment in sheep. Using this novel sheep model, we have demonstrated significant effects of GnRHa treatment on sex-specific cognitive functions and emotional control in young sheep [13,14]. Specifically, GnRHR blockage led to pronounced avoidance behavior in females and exaggerated risk-taking approach behavior in males.

The aim of the present study was two-fold; first to investigate whether pharmacological blockade of GnRH action affected expression of hippocampal genes associated with endocrine signaling and synaptic plasticity and second, to explore the effects of peripubertal GnRHa treatment on a hippocampus dependent cognitive task, namely spatial orientation. We investigated effects of GnRHa treatment on a selection of genes (Table 1) known to be involved in the synaptic transmission (*Grin1*, *Gria1*, *GABRA4*, *syn1*, *spn*, *BDNF*, *VGF*), proliferation and differentiation (*LHX5*) and structuring (*NCAM1*) that underlie synaptic plasticity and genes associated with endocrine signaling (*GnRH I*, *GnRH II*, *CYP19*, *AR*, *GH*, *ESR1* and *ESR2*).[1]

2. Materials and methods

2.1. Animals

All animal procedures were conducted at the University of Glasgow's Cochno Research Centre (55°55′N) following review by the University's Welfare and Ethics Committee and in accordance with Home Office regulations (PPL 60/3826). To eliminate the possible developmental effects of steroid transfer between siblings of different sexes, the study was conducted using 46 pairs of same-sex twin lambs (Scottish Mule Texel Cross, 22 female and 24 male). The study presented here is based on a subsample of 30 animals (14 female and 16 male) that had been tested in their spatial orientation ability and that had their hippocampal gene expression analyzed. Lambs were born between 17th March and 1st April 2008 and remained with their dams until weaned at about 12 weeks of age. Males and females were maintained separately during the entire study period. Within each set of twins, one was randomly assigned, at birth, to the control (C) and the other to the treatment (T) group. Animals in the treatment group received subcutaneous implants of the GnRH analog goserelin acetate (Zoladex, kindly donated by Astra Zeneca; Macclesfield, UK 3.6 mg) every 4 weeks from 8 weeks of age in males and 28 weeks of age in females. The differences in the timing of treatment initiation between males and females, was instigated as puberty in sheep, as in humans, is sexually dimorphic. Opposite to humans, in sheep, male pubertal transition begins earlier than female. This treatment paradigm was designed so that the pharmacological inhibition of GnRH action should begin approximately 2 weeks before the predicted time of onset of pubertal development in both rams and ewes [15]. Blood serum analyses were performed regularly during the animals' life. The analyses confirmed that treatment prevented puberty by complete suppression of the hypothalamus–pituitary–gonadal axis (data not shown).

2.2. Behavioral test: spatial orientation and learning

Animals were tested at about 48 weeks of age, approximately 2 weeks before they were euthanized and tissues, including the brains collected for laboratory analysis. The spatial maze used in this study (Fig. 1) was an adaptation of that used by Lee and colleagues [16]. The dividing walls of the maze were made of metal penning that was familiar to the animals and through which the test animals could see the audience pen. The outer walls of the maze arena were solid. For testing, animals were separated into smaller groups and sequentially placed in the 'audience' pen. Individual 'test' animals were removed from the audience pen by a trained and familiar handler and calmly led to the entrance of the maze. Each animal was given 300 s to traverse the maze and the test was deemed completed when the animal passed the finish line (Fig. 1). One of the T males effectively completed the task but stopped for a while before crossing the finish line increasing his total time to 274 s. After all animals had been tested once, the process was repeated a 2nd time on the same day and for a 3rd time the following day.

[1] All genes symbols and full names are explained in Table 1.

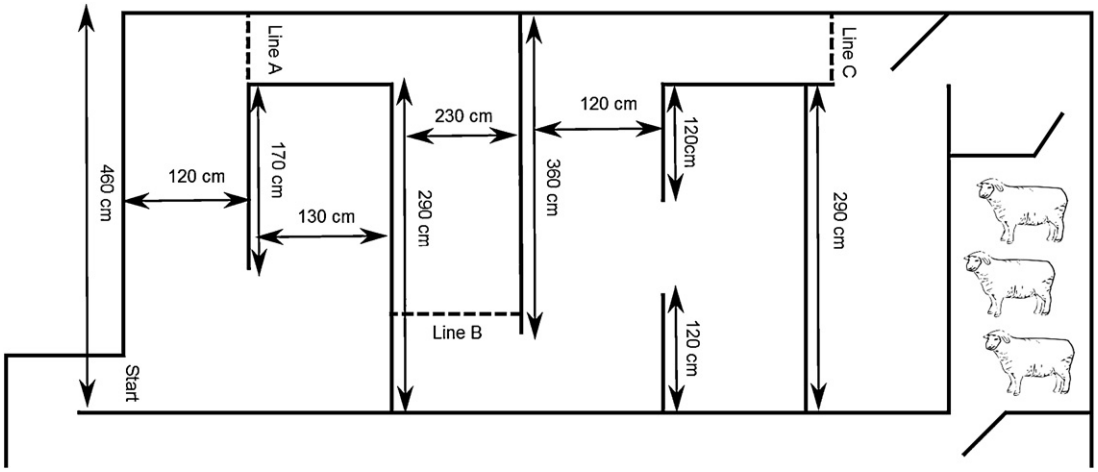

Fig. 1. Spatial maze layout.

2.3. Tissue processing

At approximately 50 weeks of age, animals were sacrificed with an overdose of barbiturate (Somulose 1 ml/kg body weight; Decra Veterinary Products, Shrewsbury UK), decapitated, and the brains removed. After removal, the hippocampus was carefully dissected from the right and left hemispheres, and the right and left hippocampi individually trisected, perpendicular to the long axis of the hippocampus, allowing isolation of the hippocampal subregions (each containing CA1–CA3). Samples were immediately frozen in liquid nitrogen and stored at −80 °C.

2.4. mRNA expression analysis

2.4.1. RNA isolation

Total RNA was isolated from the sub region containing CA1 to CA3, from the anterior section of the hippocampus from each animal, using TRIzol reagent (Invitrogen™, Paisley, UK). For each sample, 27–30 mg of frozen tissues were homogenized in 1 ml of Trizol reagent using MagNA lyser green beads (Roche Diagnostics, Mannheim, Germany) and Retsch MM 301 mixer mill (Retsch GmbH, Haan, Germany). After extraction, RNA pellets were dissolved in 50 μl of RNase-free water (Qiagen, Crawley, UK) followed by DNase I treatment (RNase-free DNase set, Qiagen, Crawley, UK) for 20 min at room temperature, immediately followed by purification using RNeasy mini-kit (Qiagen, Crawley, UK) according to the manufacturer's recommendations. The concentration and quality of the RNA were determined using NanoDrop (Thermo-Scientific, Waltham, MA, USA) and Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. RNA was stored at −75 °C until further processed.

2.5. Primer design and testing

Sixteen genes known to be involved in the regulation of endocrine function and neuronal plasticity were selected for analysis (Table 1). Primer pairs were designed to target cDNA fragments that encompassed at least one intron in the gene sequence to prevent amplification of genomic DNA. Primer sequences of reference genes (*ACTB*, *YWHAZ*, *RPL19*, *GAPDH*, *G6PDH*, and *SDHA*) were obtained from Garcia-Crespo et al. [17].

To find the most stable reference genes, the GeNorm software (Primer design Ltd) was used. Out of six genes tested, *GAPDH* and *SDHA* were the most stable (*M* value < 0.5) and were selected as the reference genes in this study. All primers were optimized with regard to annealing temperature. PCR products were analyzed on an ethidium bromide agarose gel to ensure the expected product size and to check for potential primer artifacts. Amplification efficiency was examined by generating standard curves from 10 fold serial dilutions of pooled cDNA from both control and treated samples, respectively. The primer efficiency for each gene was in the range of 95%–105%.

2.6. Quantitative real time-PCR (qRT-PCR)

cDNA synthesis and real-time PCR was performed using superscript platinum III two-step qRT-PCR with SYBR green (Invitrogen, Paisley, UK) according to the manufacturer's instructions. A Peltier thermal cycler-225 (MJ Research, Waltham, MA, USA) was used to synthesize cDNA and real time-PCR was carried out using a DNA engine thermal cycler with chromo 4 real-time detector (MJResearch, Waltham, MA, USA) operated by the Opticon Monitor 3 software (Bio-Rad Laboratories, CA, USA). Technical duplicates of RNA samples, negative controls without reverse transcriptase of each RNA sample and negative control with no added RNA template underwent cDNA synthesis. 800 ng of total RNA was used to synthesize cDNA (10 min at 25 °C, 50 min at 42 °C and 5 min at 85 °C). The qPCR reaction contained 30 ng of cDNA, 200 nM of each primer pair, 0.5 μl ROX dye (10× diluted), and 12.5 μl qPCR supermix in a final reaction volume of 25 μl. Cycling conditions were 50 °C for 2 min (UDG incubation), 95 °C for 2 min (enzyme activation), followed by 40 cycles of 95 °C for 15 s, 62 °C for 30 s and 72 °C for 30 s. A melting curve from 65 to 90 °C, read for 1 s every 0.3 °C, was included to monitor potential primer dimer formation or products from genomic DNA or other DNA contamination at the end of each run. Negative controls without template and without reverse transcriptase enzyme were included in each run.

2.7. Statistical analyses

2.7.1. Spatial orientation

SPSS version 17 was used for statistical data analysis. Time spent to complete the maze was used as the main outcome measure. The number of vocalizations per minute (*VR*) made by a sheep during the test-period was recorded. All data were logarithmically transformed because of skewed data distribution. Mixed between-within subject analysis of variance was used to analyze the change in time and *VR* through the three successive trials and to assess the differences between T and C animals. Independent sample *t*-tests were used for assessment of sex differences.

2.7.2. qRT-PCR analysis

The ΔCt was calculated from the difference in expression between the gene of interest and mean expression of the two reference genes. In order to assess any effects of treatment the ΔΔCt was calculated as the difference between the ΔCt value of control and treated samples, within sexes and for both hemispheres. Sex differences were analyzed separately for control and treated animals; ΔΔCt was calculated as the difference between male and female ΔCt values. Relative gene expression expressed as fold change was calculated by using $2^{-\Delta\Delta Ct}$. The log 2 transformed fold change values ($2^{-\Delta\Delta Ct}$) were used for statistical analysis by applying JMP 9.0 software (SAS Institute Inc, Cary, NC, USA). Differences in gene expression between control (C) and treated (T) animals as well as between males and females were evaluated by Wilcoxon signed rank test. Cohen's *d* was calculated to estimate the effect sizes.

3. Results

3.1. Spatial maze

A mixed between-within subjects' analysis of variance was conducted to assess the change in spatial performance and in *VR* in C and T animals respectively. No significant interaction effects between T and C groups and spatial performance or *VR* were observed in either males or females. In course of three trials, males improved significantly in their spatial performance, $F(2, 28) = 18.19$, $p < .001$ and their *VR* decreased, $F(2, 28) = 4.14$, $p = .027$. Females also improved significantly in spatial performance, $F(2, 24) = 12.16$, $p = .001$, however no decrease in *VR* was noted $F(2, 24) = 1.78$, $p = .190$. No significant between subjects effects were observed in females $F(1, 12) = 0.11$, $p = .751$. In males, although not significant, there was a tendency that treated males were slower than untreated, $F(1, 14) = 3.24$, $p = .093$. Specific comparisons of C and T

Table 2
The effects of puberty blockage on spatial maze traverse time and vocalization rate (*VR*) in 48 weeks old sheep.

		Males						Females					
		Time	*p*	*d*	*VR*	*p*	*d*	Time	*p*	*d*	*VR*	*p*	*d*
Trial 1	C	3.87 (0.99)	.358	0.48	0.80 (0.98)	.208	0.53	3.36 (0.70)	.719	0.20	1.51 (1.13)	.397	0.12
	T	4.28 (0.70)			1.36 (0.70)			3.52 (0.83)			1.00 (1.04)		
Trial 2	C	2.97 (0.36)	.071	0.98	0.44 (0.83)	.600	0.46	2.61 (0.41)	.751	0.17	1.13 (1.09)	.386	0.23
	T	3.36 (0.44)			0.68 (0.94)			2.71 (0.72)			0.62 (1.05)		
Trial 3	C	2.95 (0.43)	.102	0.87	0.36 (0.66)	.333	0.52	2.66 (0.47)	.974	0.02	1.86 (0.27)	.009	1.08
	T	3.26 (0.24)			0.73 (0.81)			2.65 (0.40)			0.54 (0.94)		

The results are presented as means and standard deviations of total time (s) spent to traverse the maze and number of vocalizations/min (*VR*). All data have been logarithmically transformed. C – control group; T – treatment group; *p* – significance value (independent sample *t*-test); *d* – effect size (*Cohen's d*). Results represent a subsample of 16 males and 14 females that were also analyzed according to hippocampal gene expression. At the time of the test 8 males and 7 females were under treatment with GnRHa, for 40 and 20 weeks respectively.

animals according to the time spent in spatial maze and to the *VR* are shown in Table 2.

Sex differences were tested with independent sample *t*-tests for every trial and group separately. In the C group, although not significant ($p > .008$), there was a tendency that females were completing the maze faster than males on every trial. In the T group the differences in performance were more pronounced; at trial 1, $t(13) = 1.93$, $p = .075$, $d = 1.00$; at trial 2, $t(13) = 2.143$, $p = .052$, $d = 1.11$; at trial 3, $t(13) = 3.61$, $p = .003$, $d = 1.87$. There were no significant sex differences in *VR*s, apart from in C animals at trial 3, when females vocalized significantly more than males $t(13) = 5.61$, $p < .001$, $d = 3.01$ (Table 2).

3.2. qRT-PCR

3.2.1. Effects of treatment on relative gene expression

The effects of GnRHa treatment on the panel of transcripts are summarized in Table 3. The data indicate significant changes in the mRNA levels of 12 out of 16 genes following GnRHa treatment, including genes associated with both endocrine variables and neuroplasticity. With the exception of *CYP19*, effects of treatment on gene expression differed between males and females. Furthermore, with the exception of *CYP19* and *AR*, the direction of change was hemisphere-specific.

Among the studied genes that encoded endocrine variables, GnRHa treatment was associated with lower mRNA expression of *GnRH1* (left hemisphere, females) and *CYP19* (males and females in both hemispheres), *AR* (left and right hemispheres, males) and *GH* mRNA (left hemisphere, females). *ESR1* mRNA expression was only affected by GnRHa treatment in females but the observed effects differed between the two hemispheres, being elevated in the left but reduced in the right hippocampus.

Among the studied genes encoding molecules involved in neuroplasticity, within the males, significant effects of treatment were seen in 4 out of 9 genes. In each case the effect was localized to the right hemisphere. GnRHa treatment was associated with significantly decreased expression levels of *VGF*, *NCAM1* and *LHX5* but an increased expression level of *Gria1*. In the females, the expression of 5 of the studied genes associated with neuroplasticity was affected by GnRHa treatment; in all cases gene expression was increased but the patterns of change were hemisphere dependent. *BDNF*, *Grin1* and *LHX5* were all affected in the right hemisphere, whereas *NCAM1* and *GABRA4* mRNA expression were affected in the left hemisphere.

3.2.2. Effects of sex on bilateral patterns of gene expression in control animals

The relative levels of the studied gene transcripts, within the hippocampus, were compared between the female and male animals as shown in Table 4. Four transcripts (*GH*, *ESR1*, *BDNF*, *LHX5*)

Table 3
Effects of GnRHa treatment on gene expression in left and right hippocampus of male and female sheep.

Gene grouping		Relative gene expression in GnRHa treated animals relative to controls			
		Males		Females	
		Left	Right	Left	Right
Endocrine variables	*GnRH I*	–	–	0.63* (0.21)	–
	GnRH II	–	–	–	–
	CYP19	0.66* (0.22)	0.47* (0.14)	0.71* (0.19)	0.85* (0.31)
	AR	0.68* (0.2)	0.64* (0.17)	–	–
	GH	0.48* (0.35)	–	–	–
	ESR1	–	–	1.90* (0.29)	0.60* (0.31)
	ESR2	–	–	–	–
Neuroplasticity	*BDNF*	–	–	–	1.54* (0.20)
	VGF		0.64* (0.22)	–	–
	Grin1	–	–	–	1.45* (0.15)
	NCAM1	–	0.76* (0.12)	1.53*** (0.08)	–
	Gria1	–	1.3* (0.14)	–	–
	LHX5	–	0.73* (0.24)	–	1.75* (0.25)
	Syn1	–	––	–	–
	Spn	–	–	–	–
	GABRA4	–	–	1.36* (0.14)	–

Gene expression is presented as fold change and values in brackets '( )' are the standard error of the mean. Values higher than 1 indicate increased, values lower than 1 decreased expression in treated relative to control animals. '–' indicates that gene expression was not significantly affected by GnRHa treatment. Significance levels are indicated by asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 4
Effects of sex on gene expression in left and right hippocampus.

Gene grouping		Relative gene expression in females (females/males)			
		Control		Treated	
		Left	Right	Left	Right
Endocrine variables	*GnRH I*	–	–	0.57* (0.21)	0.55* (0.20)
	GnRH II	–	–	–	–
	CYP19	–	–	–	–
	AR	–	–	–	–
	GH	–	2.66* (0.41)	–	–
	ESR1	0.10*** (0.14)	1.46*** (0.12)	–	–
	ESR2	–	–	–	–
Synaptic plasticity	*BDNF*	–	0.72** (0.16)	–	–
	VGF	–	–	1.39* (0.16)	1.60*** (0.12)
	Grin1	–	–	–	–
	NCAM1	–	–	1.53*** (0.08)	1.89*** (0.17)
	Gria1	–	–	–	1.09*** (0.11)
	LHX5	–	0.56** (0.33)	1.46* (0.19)	–
	Syn1	–	–	–	–
	Spn	–	–	1.30* (0.14)	1.26* (0.05)
	GABRA4	–	–	–	1.13* (0.105)

Gene expression is presented as a fold change and values in brackets '( )' indicate standard error of the mean. Values higher and values lower than 1 indicate increased and decreased gene expression in females relative to males, respectively. Missing observation ('–') indicate that gene expression did not differ significantly between males and females. Significance levels are indicated by asterisks: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

showed sexually dimorphic patterns of expression in the control animals. *GH* was seen to be elevated in females relative to males (right hippocampus); whereas *BDNF* and *LHX5* were lower in females compared to males (right hippocampus). *ESR1* was the only gene for which a significant sexually dimorphic pattern of expression was seen in both sides of the hippocampus. Interestingly, the observed pattern of expression differed between the two sides of the hippocampus being higher in samples from the right in females and samples for the left in males.

3.2.3. Effects of sex on bilateral patterns of gene expression in treated animals

One of the most pronounced effect of peri-pubertal GnRHa treatment was the induction of robust bilateral sex differences in the expression of one gene encoding an endocrine variable namely *GnRH*, and 3 genes associated with synaptic plasticity; *VGF*, *NCAM* and *Spn*. In addition a unilateral sex differences were seen in a further 2 genes associated with synaptic plasticity, *Gria1*, and *GABRA4*, in the hippocampi of the GnRHa treated animals. In all cases, the expression levels were higher in females compared to males. In contrast to controls animals, sexual dimorphism was not seen in the expression of *GH*, *ESR1* and *BDNF* mRNA in the GnRHa treated animals (Table 4). The *LHX5* was the only gene in which expression was sexually dimorphic in both the control and GnRHa-treated animals. GnRHa treatment was associated with higher levels of *LHX5* expression in females in the left hemisphere and the loss of a sex difference in gene expression in the right hemisphere.

4. Discussion

This study shows, for the first time, that longtime peri-pubertal GnRHa treatment, in sheep, is associated with significant changes, within the hippocampus, of levels of expression of mRNA transcripts known to be involved in endocrine signaling and synaptic plasticity. Indeed, expression of 12 out of the 16 genes was altered in GnRHa treated sheep compared to controls. It should be noted, that the effects of treatment varied both between the sexes and the right and left hemispheres. Interestingly, GnRHa treatment led to a significant increase of sexually dimorphic expression patterns of genes associated with neuronal plasticity. In contrast, treatment led to a generalized reduction in the levels of gene expression associated to endocrine function. Despite the known importance of the hippocampus for spatial learning and memory the differences in hippocampal gene expressions were not paralleled by differences in performance in a behavioral test of spatial orientation conducted prior to tissue collection.

4.1. Effect of treatment and hemisphere side on gene expression

Hippocampal *GnRH1* gene expression was affected by peri-pubertal GnRHa treatment albeit only unilaterally in females. This result could indicate that *GnRH1* mRNA expression in females might be more sensitive to GnRHa treatment. The observed sex specific down-regulation in *AR* and *GH* mRNA expression in males could reflect the effects of longer exposure to GnRHa. It has been reported in imaging studies that AR gene might play a role in the sexual dimorphism of neurodevelopmental processes such as growth of white matter and cortical maturation during human adolescence [18,19]. This sex specific nature of AR gene functions and its expression in hippocampus due to GnRHa treatment raises the possibility of GnRH involvement in the regulation of *AR* gene expression. GnRHa treatment suppressed aromatase gene *CYP19* in both T males and females, which has been shown to be involved in sexual differentiation, regulation of the reproductive cycle, male reproductive behavior and local conversion of androgen to estrogen, throughout the hippocampus in both sexes [20,21]. This result would strongly suggest that the expression of *CYP19* within the hippocampus is linked to the endocrine changes that occur at puberty and agrees with the view that GnRH may regulate the expression of *CYP19* in hippocampus [22].

Interestingly the effects of the peri-pubertal GnRHa treatment on a large proportion of the studies genes (*GnRH1*, *GH*, *BDNF*, *VGF*, *Grin1*, *Gria1*, *LHX5*, *GABRA4*) were mainly limited to one hemisphere. In only one case were both hemispheres (*ESR1* in females), affected and even in this instance the changes observed in gene expression in the two hemispheres were in opposite directions. This lateralized pattern of change agrees with the view that gene expression is differentially regulated in different hemispheres leading to lateralized hippocampal functions [23].

The side-specific (right) significant down-regulation of plasticity genes (*VGF*, *NCAM1* and *LHX5*) in the male hippocampus suggests that synaptic remodeling and structuring is regulated by GnRH in sex and hemispheric manners for signal transduction cellular migration, proliferation, structuring, synaptogenesis and synaptic

plasticity [24–26]. This possibility is further supported by the noted higher expression of glutamate receptor ionotropic genes (*Gria1*) mRNA on the same side of the hippocampus in T males, as this gene is critical for the maintenance of synaptic plasticity [27]. Thus, the pattern of altered plasticity gene expression in T males relative to C males might be associated with the tendency toward relatively "poorer" performance in the spatial orientation task observed in the T males compared to C males.

In females, however, GnRHa treatment effects on plasticity genes were seen in both the right and left hemispheres. This less strict lateralization of the changes in gene expression agrees with the suggestion that the male brain might be more lateralized than that of the female [28]. Regardless of hemispheric specificity, gene expressions in females tended to be up-regulated by GnRHa treatment. This finding suggests that blocking GnRH action had stimulatory effect on plasticity genes in the female and therefore may have increased the potential for production of proteins that are involved in long-term potentiation, synaptic plasticity shaping and memory formation [25,29,30].

BDNF is a member of the neurotrophin family known to play an important role in maintenance and growth of neurons as well as modulation of synaptic plasticity [31]. Recent studies have shown that *BDNF* increase glutamatergic synaptic transmission and facilitates phosphorylation of the subunit (*Grin1*) of N-methyl-D-aspartate (NMDA) receptors and, thus, enhance long term potentiation (LTP) in the hippocampus [32] by post synaptic mechanism. The higher expression of *BDNF* and *Grin1* mRNA in the T females in the current study suggests that an effect of GnRHa treatment on *BDNF* mediated synaptic activity may occur via a variety of signal cascades; and therefore may be beneficial with regard to development [33] as well as synaptic plasticity. *GABRA4* gene is one of the 16 distinct subunits of GABA (gamma-aminobutyric acid) A receptor which encodes subunit alpha-4. It has been reported that *GABRA4* expression increases at pubertal onset in the mouse hippocampus [34] and higher expression of $GABA_A$ receptors in the hippocampus play a pivotal role in the generation of anxiety [35]. Interestingly, the expression of *GABRA4* was increased in the hippocampus of the GnRHa treated female sheep in the current study and our previously published work with this cohort of animals has documented increased anxiety and poorer emotional control [14] in this group, which would be conducive with a role for *GABRA4* in enhancing inhibitory synaptic transmission. Therefore, the hormonal regulation of *GABRA4* gene expression may be important for understanding the etiology and treatment of anxiety.

4.2. Effect of sex on gene expression

For the first time, we show here that the hippocampal expression of two of the plasticity genes *BDNF*, *LHX5* is sexually dimorphic. Additionally, we documented that *LHX5* expression was sexual dimorphic in the left hemisphere in the GnRHa T group and in the right hemisphere in the C group. The most pronounced sexually dimorphic gene expression patterns were seen in the genes associated with neuronal plasticity, significantly higher expression levels were seen in 6 of the 9 genes in T females relative to T males. This result would imply that pre-pubertal GnRHa treatment increases synaptic plasticity in females relative to males. However, our results do not allow us to conclude finally whether the treatment suppresses the expression of plasticity genes in the male or stimulates their expression in the female because timing of the pubertal transition and, thus, the timing and duration of treatment differed between males and females. Further studies are needed in order to explain the sex specific role of *BDNF* and *LHX5* gene in sheep hippocampus during the pubertal transition.

In the C animals, the expression of two endocrine genes *GH* and *ESR1* were seen to be sexually dimorphic. This pattern of gene expression is in concordance with the literature for *GH* [36] but not for *ESR1* which although dynamic during the peri-pubertal period has not previously been reported to be sexually dimorphic [37]. It should be noted, however, that the later report was in rodents and thus the difference relative to the results of the current experiment could reflect a species difference. Interestingly, in animals in which puberty was blocked the sex differences in *GH* and *ESR1* gene expression were lost suggesting that the sex-differences in gene expression are the result of pubertal endocrine driven events. The lower levels of *GnRH1* mRNA expression in T females than T males could be explained by a greater sensitivity of the female to treatment than males but as noted above it is difficult to speculate as the duration of the treatment differed between males and females.

4.3. Spatial orientation

There has been a considerable debate about the effects of pubertal hormones on the development of cognitive functions, including spatial abilities [38]. Some naturalistic human studies indicate that differences in maturation rate and thus in exposure to sex hormones, may influence development of spatial abilities; early maturation being associated with better spatial abilities [39–41]. However the effects seem to be small and it is difficult to establish whether they are specifically related to sex hormones exposure. Therefore more research is needed to further explore these issues. In present study, although there were no significant effects of treatment on performance in spatial maze, in males, there was a tendency that T animals were slower in completing the spatial maze than the controls during every trial. Whereas it is possible that treatment might have influenced spatial learning in T males, the fact that the improvement from trial to trial was similar in T and C group tells against it. Furthermore, the analysis of the whole sample (92 animals) revealed the same pattern of results but still no significant differences between T and C animals (Wojniusz et al., submitted for publication). This does not prove that the pubertal hormones have no effect on spatial orientation in the developing sheep but if any, the effect is probably small. In the same time it is important to consider whether treatment might have influenced other cognitive functions such as emotion regulation that could indirectly influence animals' performance in the spatial maze. In present experimental design, the main incentive for the sheep to complete the maze as fast as possible was a perspective of joining their peers on the other side of the maze, which would reduce the stress imposed by social separation. In previous experiments conducted on the same sample, treated males were less emotionally affected by social separation than untreated, displaying more uncritical and explorative behavior [14]. Thus, in present experimental context, T males might have been less motivated than C males to complete the maze in fastest possible manner.

In contrary, in females, the maze performance was more similar in T and C group than in males. The fact that T females were treated for 20 weeks shorter than T males may explain the diversity in differences between T and C group in two sexes. It is also interesting those females tended to outperform males in the spatial maze, especially when treatment groups were compared. This sex difference was confirmed in C and T groups after analysis of the whole sample (Wojniusz et al., submitted for publication). Spatial orientation is usually characterized by significant sex differences in the opposite direction [42]. However, the results in spatial tasks can be influenced by the physical test-environment and exposure to stress [43]. In the current study, the audience pen was constantly visible to the animals during conduct of the spatial orientation task; this is likely to have promoted use of landmark based searching strategies, which are described in the literature as advantageous to females [43,44]. In addition, we have previously reported that females were

less prone to leave their companions to acquire food than males, a result that would suggest lower emotional control, while males were more prone to engage in explorative and risk taking behavior [14]. Therefore, the forced separation in our spatial orientation test may have differentially increased stress levels in the female sheep, motivating them to reach the audience pen as quickly as possible. Cortisol measurements, which were significantly higher in females than males, suggest that females were generally more stressed than males [13], however the lack of clear sex differences in *VR* (higher *VR* indicates higher level of stress [45]), especially in the T group tells against it. In summary, the observed sex differences could be related to several interplaying phenomenon; a real sex-difference in spatial orientation abilities, enhanced motivation in females to complete the maze due to higher stress level, or higher tendency in males to engage in explorative behavior while traversing the maze. Finally, our results emphasize the importance of careful consideration of testing environment and choice of task in assessment of the sex differences in spatial orientation, usually referred to context dependency of neuropsychological abilities in humans [46].

5. Conclusions

The results indicate that GnRH directly and/or indirectly, is involved in the regulation of sex- and side-specific expression patterns of genes that encode proteins modulating brain plasticity during the peri-pubertal period. These results have to be taken into consideration when long-term peri-pubertal GnRHa treatment is used in children [47]. The data emphasize that changes in the activity of the hypothalamo-pituitary gonadal axis may also affect other brain functions during sensitive periods (i.e. perinatal, puberty, and menopause). These results could indicate effects of GnRHa on the perception of stress and the role of stress as a motivating factor in the spatial orientation task used in this study. Our findings strongly imply the importance of further studies connected to GnRHa treatment in humans.

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References

[1] Bethke A, Fielenbach N, Wang Z, Mangelsdorf DJ, Antebi A. Nuclear hormone receptor regulation of microrna controls developmental progression. Science 2009;324(5923):95–8.
[2] Zeng Z, Huang ZY, Qin Y, Pang H. Hemolymph juvenile hormone titers in worker honey bees under normal and preswarming conditions. Journal of Economic Entomology 2005;98(2):274–8.
[3] William H. Sex differences in early childhood, adolescence, and adulthood on cognitive tasks that rely on orbital prefrontal cortex. Brain and Cognition 2004;55(1):134–47.
[4] Cooke B, Hegstrom CD, Villeneuve LS, Breedlove SM. Sexual differentiation of the vertebrate brain: principles and mechanisms. Frontiers in Neuroendocrinology 1998;19(4):323–62.
[5] Casey BJ, Jones RM. Neurobiology of the adolescent brain and behavior: implications for substance use disorders. Journal of the American Academy of Child & Adolescent Psychiatry 2010;49(12):1189–201.
[6] Cosgrove KP, Mazure CM, Staley JK. Evolving knowledge of sex differences in brain structure, function, and chemistry. Biological Psychiatry 2007;62(8):847–55.
[7] Ingudomnukul E, Baron-Cohen S, Wheelwright S, Knickmeyer R. Elevated rates of testosterone-related disorders in women with autism spectrum conditions. Hormones and Behavior 2007;51(5):597–604.
[8] Stevens JR. Schizophrenia, reproductive hormones and the brain. The American Journal of Psychiatry 2002;159(5):713–9.
[9] Vadakkadath MS, Atwood CS. Alzheimer's disease: the impact of age-related changes in reproductive hormones. Cellular and Molecular Life Sciences 2005;62(3):257–70.
[10] Wang L, Chadwick W, Park SS, Zhou Y, Silver N, Martin B, et al. Gonadotropin-releasing hormone receptor system: modulatory role in aging and neurodegeneration. CNS & Neurological Disorders – Drug Targets 2010;9(5):651–60.
[11] Skinner DC, Albertson AJ, Navratil A, Smith A, Mignot M, Talbott H, et al. Effects of gonadotrophin-releasing hormone outside the hypothalamic–pituitary–reproductive axis. Journal of Neuroendocrinology 2009;21(4):282–92.
[12] Bryan KJ, Mudd JC, Richardson SL, Chang J, Lee Hg, Zhu X, et al. Down-regulation of serum gonadotropins is as effective as estrogen replacement at improving menopause-associated cognitive deficits. Journal of Neurochemistry 2010;112(4):870–81.
[13] Evans NP, Robinson JE, Erhard HW, Ropstad E, Fleming LM, Haraldsen IRH. Development of psychophysiological motoric reactivity is influenced by peripubertal pharmacological inhibition of gonadotropin releasing hormone action – results of an ovine model. Psychoneuroendocrinology 2012;37(11):1876–84.
[14] Wojniusz S, Vögele C, Ropstad E, Evans N, Robinson J, Sütterlin S, et al. Prepubertal gonadotropin-releasing hormone analog leads to exaggerated behavioral and emotional sex differences in sheep. Hormones and Behavior 2011;59(1):22–7.
[15] Wood RI, Foster DL. Sexual differentiation of reproductive neuroendocrine function in sheep. Reviews of Reproduction 1998;3(2):130–40.
[16] Lee C, Colegate S, Fisher AD. Development of a maze test and its application to assess spatial learning and memory in Merino sheep. Applied Animal Behaviour Science 2006;96(1–2):43–51.
[17] Garcia-Crespo D, Juste R, Hurtado A. Selection of ovine housekeeping genes for normalisation by real-time RT-PCR; analysis of PrP gene expression and genetic susceptibility to scrapie. BMC Veterinary Research 2005;1(1):3.
[18] Perrin JS, Hervé P-Y, Leonard G, Perron M, Pike GB, Pitiot A, et al. Growth of White Matter in the adolescent brain: role of testosterone and androgen receptor. The Journal of Neuroscience 2008;28(38):9519–24.
[19] Raznahan A, Lee Y, Stidd R, Long R, Greenstein D, Clasen L, et al. Longitudinally mapping the influence of sex and androgen signaling on the dynamics of human cortical maturation in adolescence. Proceedings of the National Academy of Sciences 2010;107(39):16988–93.
[20] Ish H, Tsurugizawa T, Ogiue-Ikeda M, Asashima M, Mukai H, Murakami G, et al. Local production of sex hormones and their modulation of hippocampal synaptic plasticity. The Neuroscientist 2007;13(4):323–34.
[21] McCarthy MM. Estradiol and the developing brain. Physiological Reviews 2008;88(1):91–134.
[22] Galmiche G, Richard N, Corvaisier S, Kottler ML. The expression of aromatase in gonadotropes is regulated by estradiol and gonadotropin-releasing hormone in a manner that differs from the regulation of luteinizing hormone. Endocrinology 2006;147(9):4234–44.
[23] Moskal JR, Kroes RA, Otto NJ, Rahimi O, Claiborne BJ. Distinct patterns of gene expression in the left and right hippocampal formation of developing rats. Hippocampus 2006;16(8):629–34.
[24] Dityatev A, Dityateva G, Schachner M. Synaptic strength as a function of post- versus pre-synaptic expression of the neural cell adhesion molecule NCAM. Neuron 2000;26(1):207–17.
[25] Paylor R, Zhao Y, Libbey M, Westphal H, Crawley JN. Learning impairments and motor dysfunctions in adult Lhx5-deficient mice displaying hippocampal disorganization. Physiology & Behavior 2001;73(5):781–92.
[26] Snyder SE, Li J, Salton SRJ. Comparison of VGF and trk mRNA distributions in the developing and adult rat nervous systems. Molecular Brain Research 1997;49(1–2):307–11.
[27] Lee HK, Takamiya K, Han JS, Man H, Kim CH, Rumbaugh G, et al. Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. Cell 2003;112(5):631–43.
[28] Shaywitz BA, Shaywltz SE, Pugh KR, Constable RT, Skudlarski P, Fulbright RK, et al. Sex differences in the functional organization of the brain for language. Nature 1995;373(6515):607–9.
[29] Park CS, Gong R, Stuart J, Tang SJ. Molecular Network and Chromosomal clustering of genes involved in synaptic plasticity in the hippocampus. Journal of Biological Chemistry 2006;281(40):30195–211.
[30] Shen H, Sabaliauskas N, Sherpa A, Fenton AA, Stelzer A, Aoki C, et al. A critical role for $\alpha4\beta\delta$ $GABA_A$ receptors in shaping learning deficits at puberty in mice. Science 2010;327(5972):1515–8.
[31] McAllister AK, Katz LC, Lo DC. Neurotrophins and synaptic plasticity. Annual Reviews of Neuroscience 1999;22(1):295–318.
[32] Jiang X, Tian F, Mearow K, Okagaki P, Lipsky RH. The excitoprotective effect of N-methyl-D-aspartate receptors is mediated by a brain-derived neurotrophic factor autocrine loop in cultured hippocampal neurons. Journal of Neurochemistry 2005;94(3):713–22.
[33] Barnabé-Heider F, Miller FD. Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. The Journal of Neuroscience 2003;23(12):5149–60.
[34] Shen H, Gong QH, Aoki C, Yuan M, Ruderman Y, Dattilo M, et al. Reversal of neurosteroid effects at $\alpha4\beta2\delta$ $GABA_A$ receptors triggers anxiety at puberty. Nature Neuroscience 2007;10(4):469–77.

[35] Gulinello M, Gong QH, Li X, Smith SS. Short-term exposure to a neuroactive steroid increases alpha4 GABA(A) receptor subunit levels in association with increased anxiety in the female rat. Brain Research 2001;910(1–2):55–66.
[36] Donahue CP, Kosik KS, Shors TJ. Growth hormone is produced within the hippocampus where it responds to age, sex, and stress. Proceedings of the National Academy of Sciences 2006;103(15):6031–6.
[37] Wilson ME, Westberry JM, Trout AL. Estrogen receptor-alpha gene expression in the cortex: sex differences during development and in adulthood. Hormones and Behavior 2011;59(3):353–7.
[38] Berenbaum SA, Beltz AM. Sexual differentiation of human behavior: effects of prenatal and pubertal organizational hormones. Frontiers in Neuroendocrinology 2011;32(2):183–200.
[39] Waber DP. Sex differences in cognition: a function of maturation rate? Science 1976;192(4239):572–4.
[40] Hassler M. Maturation rate and spatial, verbal, and musical abilities: a seven-year-longitudinal study. International Journal of Neuroscience 1991;58(3–4):183–98.
[41] Mueller SC, Temple V, Oh E, VanRyzin C, Williams A, Cornwell B, et al. Early androgen exposure modulates spatial cognition in congenital adrenal hyperplasia (CAH). Psychoneuroendocrinology 2008;33(7):973–80.
[42] Jonasson Z. Meta-analysis of sex differences in rodent models of learning and memory: a review of behavioral and biological data. Neuroscience & Biobehavioral Reviews 2005;28(8):811–25.
[43] Platek SM. Merging the “new sciences of the mind” – introduction to special issue on evolutionary cognitive neuroscience. Human Nature – An Interdisciplinary Biosocial Perspective 2007;18(2):85–7.
[44] Rodriguez CA, Torres A, Mackintosh NJ, Chamizo VD. Sex differences in the strategies used by rats to solve a navigation task. Journal of Experimental Psychology – Animal Behavior Processes 2010;36(3):395–401.
[45] Hernandez CE, Matthews LR, Oliver MH, Bloomfield FH, Harding JE. Effects of sex, litter size and periconceptional ewe nutrition on the ewe-lamb bond. Applied Animal Behaviour Science 2009;120(1–2):76–83.
[46] Sandstrom NJ, Kaufman J, Huettel SA. Males and females use different distal cues in a virtual environment navigation task. Cognitive Brain Research 1998;6(4):351–60.
[47] Carel JC, Eugster EA, Rogol A, Ghizzoni L, Palmert MR, On behalf of the members of the ESPE–LWPES GnRH analogs consensus conference group. Consensus statement on the use of gonadotropin-releasing hormone analogs in children. Pediatrics 2009;123(4):e752–62.

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Effects of peripubertal gonadotropin-releasing hormone agonist on brain development in sheep— A magnetic resonance imaging study

The Sex On Brain European Research Group — SOBER
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Sheep

Summary In many species sexual dimorphisms in brain structures and functions have been documented. In ovine model, we have previously demonstrated that peri-pubertal pharmacological blockade of gonadotropin releasing hormone (GnRH) action increased sex-differences of executive emotional behavior. The structural substrate of this behavioral alteration however is unknown. In this magnetic resonance image (MRI) study on the same animals, we investigated the effects of GnRH agonist (GnRHa) treatment on the volume of total brain, hippocampus and amygdala.

In total 41 brains (17 treated; 10 females and 7 males, and 24 controls; 11 females and 13 males) were included in the MRI study. Image acquisition was performed with 3-T MRI scanner. Segmentation of the amygdala and the hippocampus was done by manual tracing and total gray and white matter volumes were estimated by means of automated brain volume segmentation of the individual T2-weighted MRI volumes. Statistical comparisons were performed with general linear models.

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Highly significant GnRHa treatment effects were found on the volume of left and right amygdala, indicating larger amygdalae in treated animals. Significant sex differences were found for total gray matter and right amygdala, indicating larger volumes in male compared to female animals. Additionally, we observed a significant interaction between sex and treatment on left amygdala volume, indicating stronger effects of treatment in female compared to male animals. The effects of GnRHa treatment on amygdala volumes indicate that increasing GnRH concentration during puberty may have an important impact on normal brain development in mammals. These novel findings substantiate the need for further studies investigating potential neurobiological side effects of GnRHa treatment on the brains of young animals and humans.

1. Introduction

Pronounced neurobiological differences exist between males and females beyond the traditional domain of reproduction, including brain anatomy and function (Keller and Menon, 2009; Tomasi and Volkow, 2011). In humans, sex differences have been documented in the limbic system (Filipek et al., 1994; Caviness et al., 1996; Goldstein et al., 2001), and sex differences in the brain correlate with emotional and facial processing, working memory and spatial learning ability (George et al., 1996; Speck et al., 2000; Killgore et al., 2001; Andreano and Cahill, 2009).

To gain an understanding of the hormonal mechanisms that might underlie sex differences in cognitive functions, studies have investigated the modulatory effects of gonadotropin-releasing hormone (GnRH) and its agonist (GnRHa) (Grigorova et al., 2006; Bryan et al., 2010; Wojniusz et al., 2011) on physiological and cognitive parameters (Skinner et al., 2009; Wojniusz et al., 2011; Evans et al., 2012). GnRH is a decapeptide neurohormone that plays a key role in the reproductive axis, ultimately modulating the release of gonadal steroid hormones. Its receptor (GnRHR) has been found in various brain regions, including the frontal cortex, the olfactory bulb and the limbic system (Albertson et al., 2008; Skinner et al., 2009; Chu et al., 2010; Rosati et al., 2011; Schang et al., 2011). Importantly, not all of these brain regions have roles in the control of reproductive function, suggesting that GnRH is involved in other processes and, as such, may modulate sex differences in emotional processing and cognitive functions.

In this regard, GnRHa treatment has been shown to influence visuospatial and higher-order executive control functions (Casadesus et al., 2006; Bryan et al., 2010). To date, effects of GnRHa on cognition have only been reported in adults and studies that delineate the possible effects of peri-pubertal GnRHa treatment on brain development in children and adolescents are lacking. During critical stages of development, structural and functional neuronal circuits that follow sex-specific spatiotemporal patterns are built (Casey and Jones, 2010) and it has been proposed that disturbances of this maturational process might cause abnormal behavioral and cognitive function in later life (Sisk and Foster, 2004; Casey and Jones, 2010). Despite the widespread use of GnRHa for treatment purposes, ranging from central precocious puberty, gonadal protection for children undergoing chemotherapy, congenital adrenal hyperplasia to autism (Carel et al., 2009), the mechanisms by which GnRHa treatment may interfere with the maturation of fronto-temporal-limbic circuits and corresponding cognitive functions are not understood.

In order to investigate possible sex specific effects of peripubertal GnRHa treatment on brain development, we have developed a novel ovine model, chosen because of its relatively long period of brain maturation compared to other model species, e.g. rodents. To assess possible effects of GnRHa treatment on brain morphology, we combined this model with postmortem magnetic resonance imaging (MRI), to accurately measure global and regional brain volumes. MRI has the advantage that it bypasses conventional dissection and photography methods, which carry higher risks of introducing errors in quantitative measurements (Pfefferbaum et al., 2004). In previous studies, we have shown different effects of GnRHa treatment in this animal model, specifically, sex specific effect on behavior and emotion regulation (Wojniusz et al., 2011) and gene expression in hippocampus without changing spatial orientation ability (Nuruddin et al., 2013). Although emotion regulation may be more closely linked to the amygdala and spatial navigation to the hippocampus; we anticipated that GnRHa treatment might have localized effects in both structures.

2. Materials and methods

2.1. Animals

All animal procedures were conducted at the University of Glasgow's Cochno Research Centre (55°55′N) following review by the University's Welfare and Ethics Committee and in accordance with Home Office regulations (PPL 60/3826). To eliminate developmental effects of steroid transfer between siblings of different sexes, the study was conducted using forty six pairs of same-sex twin lambs (Scottish Mule Texel Cross, 22 female and 24 male). Lambs were born between 17th March and 1st April 2008 and remained with their dams until weaned at about 12 weeks of age. Males and females were maintained separately during the entire study period. Within each set of twins, one was randomly assigned, at birth, to the control (C) and the other to the treatment (T) group. Although sheep and humans have different timing of onset of puberty and periodicity of fertility, a common interaction between neurosecretory cells of the brain and the peripheral sexual organs is well documented in vertebrates (humans, primates and other mammalsl including sheep) (Wang et al., 2010). Intracerebral blockage of GnRH isoforms in both sheep and human will therefore suppress the hypothalamic-pituitary-gonadal axis, stop production of gonadal hormones in sheep as effectively as in humans, arresting sexual maturity in both species. In our study animals in the treatment group received subcutaneous implants of

the GnRH agonist Goserelin acetate (Zoladex; kindly donated by Astra Zeneca; Macclesfield, UK 3.6 mg) every 4 weeks from 8 weeks of age in males and 28 weeks of age in females. The difference in the timing of treatment initiation between males and females is necessary as onset of puberty in sheep, as in humans, is sexually dimorphic (Wood and Foster, 1998). Opposite to humans, ovine pubertal transition begins at about 10 weeks in males, and in females 20 weeks later (Claypool and Foster, 1990; Foster and Ryan, 1979). Our treatment protocol ensures that pharmacological inhibition of GnRH action begins approximately 2 weeks before the predicted time of onset of pubertal development in both rams and ewes. To confirm that GnRHa treatment blocked pubertal development, blood serum analyses were performed regularly during the animals' life. In addition, after death at 12 months of age, testes and ovaries were excised and weighed and histologically evaluated. The analyses confirmed that treatment prevented puberty by complete

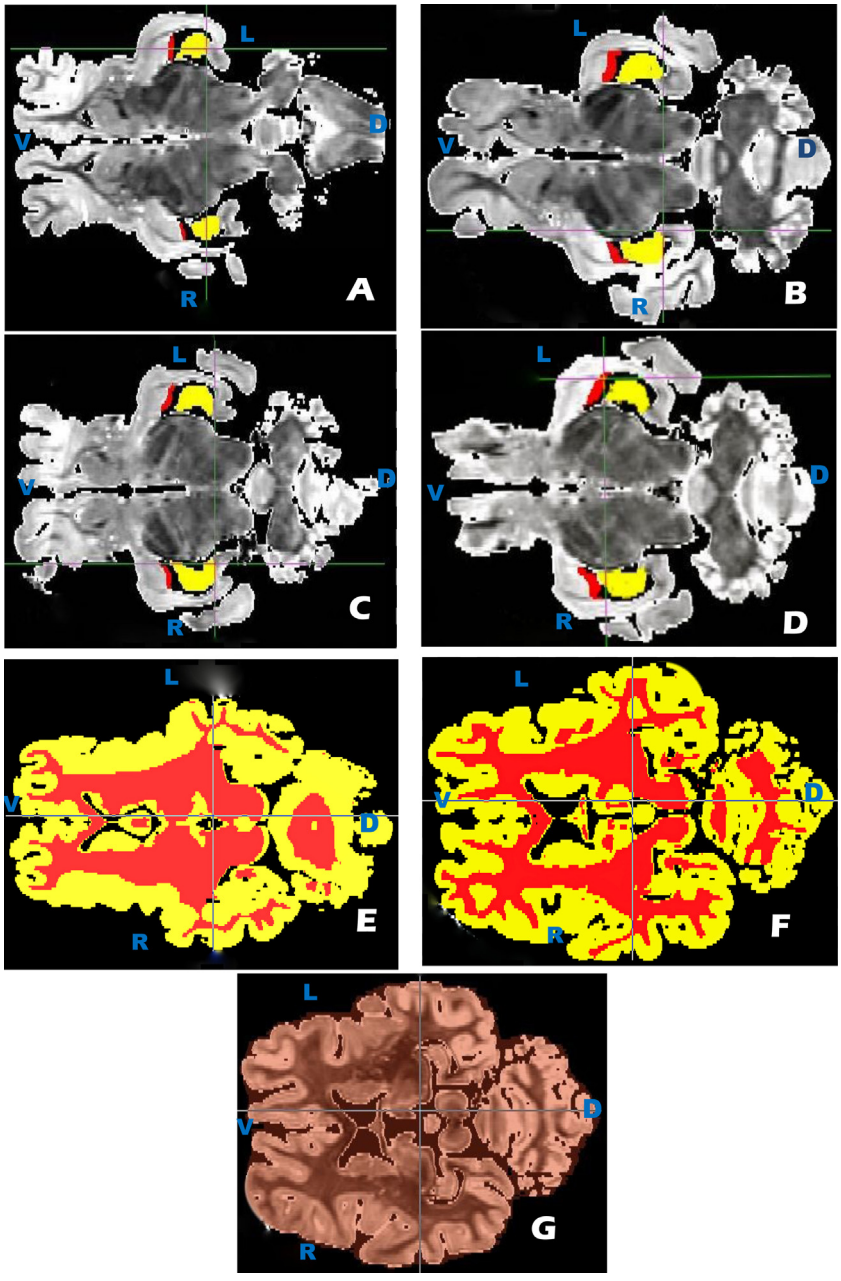

Figure 1 Magnetic resonance images including automatic and manual segmentations in axial view. Orientation of the picture are the following: V = ventral, D = dorsal, L = left, R = right. (A)—(D) represents magnetic resonance images of manual and automatic segmentation: (A) female untreated, (B) female treated, (C) male untreated, (D) male treated. Bilateral red colored areas indicate the amygdala and the yellow colored areas indicate the hippocampus. (E) and (F) represent automatic tissue segmentation images: (E) males, (F) females. The red colored area represents white matter, the yellow colored area represents gray matter; (G) represents an image of the manually drawn mask of the total brain. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

suppression of the hypothalamic-pituitary-gonadal axis (for more details see: Wojniusz et al., 2011).

2.2. Specimen collection

At approximately 50 weeks of age, animals were sacrificed with an overdose of barbiturate (Somulose 1 ml/kg body weight; Decra Veterinary Products, Shrewsbury UK), decapitated, and brains perfused, with 4% formaldehyde, through the carotid artery (bilaterally) using a peristaltic pump. In total 41 brains (17 treated; 10 females and 7 males, and 24 controls; 11 females and 13 males) were fixed in this way. These brains were stored in a 4% neutral buffered formalin solution and were the brains used for the MRI study.

2.3. Postmortem specimen preparation

To minimize movement during scanning, each postmortem specimen was suspended in agar, which was placed in a rectangular plastic container. The agar was semi-translucent, thus permitting gross land marking before scanning. Landmarks for scanning were drawn on each container and helped obtain the same alignment across scanning sessions.

2.4. MRI acquisition

Imaging was performed at the Interventional Centre (Oslo University Hospital, Oslo, Norway) using a 3T Philips Achieva Scanner (Release 2.5.3, Philips Healthcare, Netherlands) and a dedicated solenoid rat coil (signal to noise: 1064). Structural T1- and T2-weighted MRI data were obtained by acquiring a series of sagittal IR-spoiled GRE sequences with the following parameters: for the T1-weighted image; field of view (FOV): 90 mm × 90 mm × 72 mm, 144 slices of 0.5 mm with matrix size 180 × 180, bandwidth 95.8 Hz, inversion time = 450 ms, repetition time (TR)/echo time (TE) = 11/3.1 ms with segmented linear k-space filling (20 lines) and 20 repeated acquisitions to improve SNR with a total scan duration of 18 min. Voxel size was fixed to 0.5 mm × 0.5 mm × 0.5 mm. For the T2-weighted image; FOV: 90 mm × 90 mm × 72 mm, 144 slices of 0.5 mm with matrix size 180 × 179, bandwidth 137.0 Hz, inversion time = 450 ms, TR/TE = 2000/200 ms, voxel size was fixed to 0.5 mm × 0.5 mm × 0.5 mm and total scan duration was 34 min. Volumes covered the whole brain, cerebellum and brain stem. T1- and T2-weighted volumes were resampled to 0.23 mm × 0.23 mm × 0.5 mm on the scanner.

2.5. MRI preprocessing

The raw DICOM files were converted to NIFTI and inspected for artifacts. Bias correction of the images was done using Freesurfer mri_nu_correct.mni to correct for intensity non-uniformity. Spurious noise outside the brain was removed, and Brain Extraction Tool (BET) (Smith, 2002), part of FMRIB's Software Library (FSL) (Smith et al., 2004) was used to strip residual gel artifacts outside the brain. A set of anatomical regions of interest (ROIs) were manually drawn on the best quality MRI brain of the sheep chosen to act as the template. These ROIs were then aligned to each individual's native space by first using FLIRT (Jenkinson and Smith, 2001) to calculate the affine transform of each individual brain to the template brain using 12 dof, and minimizing correlation ratio. Next, non-linear warp fields generated by FNIRT (Andersson et al., 2007a, 2007b) using a 6 mm warp resolution were calculated and inverted. The inverse warp was applied to the template-drawn ROI, followed by application of the inverse affine transform, bringing the template-drawn region of interest into the individual subject space. The ROI in subject space was then hand edited. Inter-rater reliability was calculated by comparing the percent overlap between two raters. Finally, the number of voxels of each ROI was extracted and multiplied with voxel size for use in statistical models.

2.6. Region of interest definition/selection and inter-rater reliability

Segmentation of the amygdala and the hippocampus was done by manual tracing using FSLView, part of FMRIB's Software Library (FSL) (Smith et al., 2004) by a single rater (M.B.) blinded to any subject characteristics. The hippocampal ROI included the cornu ammonis, dentate gyrus and the subiculum, as each of these components show different histological characteristics and topographically well-ordered afferents and efferents (Nolte, 1993). A combination of T1- and T2-weighted data was used to distinguish the anterior boundaries that separate the amygdala from the hippocampus. However, precise delineation of the boundaries is difficult even at a histological level (Bergin et al., 1994). Therefore, inter-rater reliabilities for each ROI were established by having two raters (M.B. and S.N.) independently segment five datasets. Percent inter-rater voxel overlap was 94% for hippocampus and 97% for amygdala, indicating high reliability.

2.7. Tissue segmentation

TBV was calculated using manually segmented brain masks covering both the gray and white matter, ventricular compartments and the cerebellum. FMRIB's Automated Segmentation Tool (FAST) (Zhang et al., 2001) was used to automatically segment the T2-weighted MRI volumes of the individual brains into gray matter (GM) and white matter (WM), while correcting for spatial intensity variations (bias field or RF in homogeneities). The underlying method of FAST is based on a hidden Markov random field model and an associated Expectation-Maximization algorithm. All segmented data were visually inspected for accuracy. Total GM and WM volumes were calculated by multiplying the number of voxels in each of the segmented classes by its voxel resolution. Four datasets (3 untreated males and 1 treated male) were excluded during the automatic tissue segmentation procedure due to imaging artifacts probably related to the formalin fixation of the brain (Tovi and Ericsson, 1992).

2.8. Statistical analysis

The mean and its standard deviation (SD) were calculated for the volume of the different ROIs. TBV, WM and GM volume, as well as the volume of the left and right amygdala and hippocampus, were compared between groups (control vs

treated), while controlling for sex and treatment group using General Linear Models (GLMs). The sex by treatment interaction term was included in the models, allowing us to test for differential effects of treatment between male and female animals. The null hypothesis of no effects was rejected if $p < 0.05$, Bonferroni adjusted by a factor of 7 (corresponding to a nominal alpha of $p < 0.007$). The assumption of normally distributed residuals was evaluated using a normal quintile (Q—Q) plot and this assumption of normally distributed residuals was met for all parameters. Statistical analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA).

3. Results

MR images including example automatic and manual segmentations in difference data sets are shown in Fig. 1. The raw mean volumes of each ROI by group and sex and in total are summarized in Table 1. Significant effects of GnRHa treatment (Table 1) were observed on the volume of the left ($t = -13.76$, $p = 0.0001$) and right ($t = -16.56$, $p = 0.0001$) amygdala, indicating larger amygdalae in treated compared to untreated animals. Further, a significant ($t = -3.56$, $p = 0.001$) sex by treatment interaction effect on the volume of the left amygdala was observed, indicating that the effect of treatment was stronger in female (mean volume = 50 and SD = 1 in mm^3) compared to male (mean volume = 48 and SD = 1 in mm^3) animals. All treatment and treatment*sex effects on regional volumes remained practically unaltered when including TBV as an additional covariate (results shown in Supplementary Table 1). Table 1 also shows the effect sizes and the corresponding p-values for each of the estimated parameters from the GLMs. Briefly, significantly larger volumes in male compared to female animals were observed in total GM ($t = -3.05$, $p = 0.004$) and the right amygdala ($t = -5.12$, $p = 0.0001$). There was also a marginally significant sex difference in total WM volume ($t = -2.37$, $p = 0.024$), indicating larger volumes in males compared to females. Sex differences were still significant for total GM ($t = -2.91$, $p = 0.006$) and WM ($t = -3.12$, $p = 0.004$) when including TBV as an additional covariate.

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4. Discussion

In the present MRI study, we have reported sex differences in the volume of specific regions of the brain and effects of peripubertal GnRHa treatment on regional brain volumes in an ovine animal model. Specifically, total brain volume, total WM and GM, as well as hippocampal and amygdala volumes were compared between female and male animals that had been treated with GnRHa during the peripubertal period and their respective controls. The results demonstrated strong and specific GnRHa treatment effects on the volume of left and right amygdalae, indicating larger amygdalae in treated animals. Further, we found a significant sex by treatment interaction on the volume of the left amygdala, indicating stronger effects of treatment in female compared to male animals. Also, we have demonstrated sex differences in total GM and right amygdala volume, indicating larger volumes in male compared to female animals. As far as we know, this is the first study demonstrating effects on MRI derived regional brain volumes in GnRHa treated sheep. The interpretations and implications of these novel findings will be discussed below.

4.1. GnRHa treatment effects on amygdala volume

We observed a specific effect of pharmacological hypothalamus-pituitary-gonadal axis (HPG) blockage on amygdala volume, indicating larger volumes in GnRHa treated compared to untreated animals. This pattern of change indicates that GnRHa treatment did not affect the development of all areas within the limbic system but rather modulated the development of specific limbic structures. It should be noted that the experimental design does not allow differentiation between direct effects of GnRHa treatment or secondary effects such as the blockade of the reproductive axis with its consequent reduction in gonadal steroid secretion.

Previously published work has indicated that a number of factors could contribute to the observed morphological differences, for example, steroid hormones. Specifically, testosterone has been shown positively associated with increased regional volume of the medial amygdala (MeA) and posterodorsal subnucleus (MeApd) of the posterior MeA in males. In addition rising concentrations of androgen promote excitatory synaptic connectivity (Cooke and Woolley, 2009; Romeo and Sisk, 2001) in the amygdala.

It has previously been demonstrated, with this cohort of GnRHa treated sheep that GnRHa treatment had a sex specific effect on behavior and emotion regulation. Specifically, female animals in which the pubertal transition was blocked by GnRHa treatment displayed pronounced avoidance behavior, less emotional control capability or more anxiety behavior, whereas male animals exhibit risk-taking and exaggerated approach behavior (Wojniusz et al., 2011). The results of this study indicate that GnRHa treatment was also associated with increased amygdala volume in these animals. In line with these findings, amygdala volume in humans has been shown to be associated with both anxiety and depression during childhood and adolescence (Thomas et al., 2001), and children diagnosed with anxiety disorder show a larger and more reactive amygdala as well as greater processing bias for negative information (De Bellis et al., 2000; MacMillan et al., 2003; Barrós-Loscertales et al., 2006). Furthermore, an MRI study including children and adolescents (males age 6—16 years and females 4—11 years) has reported that changes in volume and functional activity of the amygdala might be due to excess androgens, estrogens or progestins; endogenous deficiency or exogenous excess of glucocorticoids; or some combination of these (Merke et al., 2003). This study focused on the examination of the effect of sex steroids on the growth and development of the amygdala, however possible involvement of GnRH has not been described elsewhere. Thus, volumetric effects of GnRHa treatment on the amygdala suggest that GnRH might also play role in the growth, development and functional activity of the amygdala.

Table 1 Mean volume (mm^3) within each anatomical region of interest (ROIs) per group, sex and results from multiple linear regressions.

	Female						Male						Total						Multiple Linear Regression											
	Untreated ($n = 11$)		Treated ($n = 10$)		All ($n = 21$)		Untreated ($n = 13$)		Treated ($n = 7$)		All ($n = 20$)		Untreated ($n = 24$)		Treated ($n = 17$)		All ($n = 41$)		Sex		Treatment		Sex* treatment		Full model					
ROI	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	t	p	t	p	t	p	F	R^2		
Hippocampus left	785	89	763	72	774	80	780	147	814	160	792	148	782	121	784	115	783	117	−0.86	0.394	−0.60	0.553	0.72	0.477	0.25	0.02		
Hippocampus right	793	110	789	94	791	100	824	147	792	183	813	156	810	130	790	132	801	129	−0.05	0.959	0.50	0.617	−0.32	0.755	0.18	0.01		
Amygdala left	29	3	50	1	39	11	32	3	48	1	38	8	31	3	49	1	38	10	2.02	0.051	**−13.76**	0.0001	**−3.56**	0.001	197.40	0.94		
Amygdala right	23	2	43	2	32	11	30	3	49	1	37	10	27	5	45	4	34	10	**−5.12**	0.0001	**−16.56**	0.0001	−0.86	0.398	223.55	0.95		
White matter (WM)	29,349	1755	27,706	4969	28,566	3655	32,887	1327	31,800	1663	32,615	1443	31,195	2355	28,876	4628	30,317	3525	−2.37	0.024	0.65	0.523	0.26	0.794	6.53	0.37		
Gray matter (GM)	45,193	2332	41,009	10,531	43,201	7563	50,844	4052	52,212	3520	51,186	3859	48,142	4360	44,210	10,355	46,654	7353	**−3.05**	0.004	−0.38	0.705	1.24	0.225	5.86	0.35		
TBV	103,974	7235	103,876	6489	103,928	6717	110,884	5998	109,169	9525	110,198	7416	107,579	7365	106,229	8184	106,986	7665	−1.54	0.131	0.52	0.606	−0.35	0.725	2.66	0.18		

Mean volume (mm^3) within each regions of interest (ROIs) per group and sex, and in total. White matter (WM) and gray matter (GM) were missing for one untreated male and three treated male animals. TBV: total brain volume as estimated using the manually segmented brain masks. In multiple linear regression analysis, the parameter estimates denote the effect size and the statistical significance of the parameter estimates from linear regressions with number of voxels in each region of interest (ROI) as independent variable, sex and treatment group as fixed factors. The sex*treatment interaction term was included in the models. F and R^2 reflect the full model fit. ***Bold***: significant associations at $p < 0.05$, Bonferroni corrected. *Underlined*: significant associations at $p < 0.05$, uncorrected. White matter (WM) and gray matter (GM) volumes were missing for five datasets. For sex, negative t values indicate larger volumes in males compared to females. For treatment, negative values denote larger volumes in treated compared to non-treated animals. For the sex by treatment interaction term, negative values indicate stronger effects of treatment in female compared to male animal.

Animal research also suggests an association between hypertrophy and increased reactivity of the amygdala (Vyas et al., 2006). The GnRHa treatment effect on the volume of the amygdala observed in the present study, therefore, might be related to cognitive changes reported in our previous behavioral data (Wojniusz et al., 2011) and by extension, anxiety and mood parameter changes should be monitored in clinical human populations following GnRHa treatment.

4.2. Sex differences in brain volumes

Sex specific volumetric differences in global and regional brain structures can be important indicators for the development of specific behaviors and diseases (Filipek et al., 1994; Caviness et al., 1996; Allen et al., 2002; Giedd, 2004; Sowell et al., 2007). This is of interest as sexual maturation is linked with changes in brain morphometry in normal developing children and adolescents (Giedd et al., 1997, 2006; Wilke et al., 2007; Neufang et al., 2009). For example, the hippocampi in young females have been shown to be larger compared to males (8—18 years of age) (Filipek et al., 1994; Giedd et al., 1996), and larger amygdalae have been shown in young male compared to female individuals (7—11 years of age) (Caviness et al., 1996; Giedd et al., 1997). Furthermore, extensive evidence has demonstrated that the hippocampi in males and females differ significantly in their anatomical structure, their neuroanatomical makeup and their reactivity to stress (Juraska, 1991; Madeira and Lieberman, 1995). In contrast to human studies, our results show no significant sex difference in hippocampal volume in sheep.

Human studies have also shown sex differences for GM and WM volume during pubertal development (Peper et al., 2011) where GM and WM volumes were larger in males than females. The observed significant sex differences for global GM and the trend differences for WM in the present study are, thus, in concordance with human studies. The sex difference in right amygdala volume, seen in this study, could add further weight to the proposals for sex dependent lateralization of the amygdala (Peper et al., 2011; Schneider et al., 2011). However, it is worth issuing a note of caution relative to this possibility, as while the male and female subjects were age-matched, the interpretation of sex differences is complex because male sheep reach puberty some 20 weeks earlier than female sheep and, thus, the male animals had received GnRHa treatment for a longer duration than females. The results, nevertheless, showed a significant treatment by sex interaction on the volume the left amygdala, which would indicate a stronger effect of treatment in females compared to males.

Sex related hemispheric lateralization of amygdala function in relation to emotion regulation has been reported in imaging studies where the left amygdala has been implicated as dominant in females while the right amygdala was more dominant in males (Cahill et al., 2004; Koscik et al., 2010). The present results indicate effects of GnRHa treatment in both male and female animals. Interestingly, this corresponds with the fact that behavior was also affected in both sexes of the treatment group (Wojniusz et al., 2011). Final interpretations regarding the observed sex by treatment interaction should be performed with caution and warrant replication in future studies.

In a recent study, we found that GnRHa treatment affects hippocampal synaptic plasticity and endocrine signaling gene expression without changing spatial orientation ability which is a hippocampus-related function (Nuruddin et al., 2013), thus we could expect that this treatment could affect hippocampal volumes in the same animals. Our data show no effects of treatment on hippocampal volume. The reason for this is unknown but it could be that treatment affects hippocampal processes at the molecular level without affecting microstructural processes in this region. An important limitation, using this technique/approach to assess brain morphology is that the exact neurobiological substrates of the volumetric effects of the GnRHa treatment are still unknown.

It could be speculated that increase in pubertal GnRH, androgen and estrogen release might affect the growth and development of the amygdala during puberty; or possibly that GnRH along with sex hormones alter the neural substrates of the circuitry involved in growth and development of amygdala and other brain structures. These speculations can be supported by the fact that GnRH and sex hormones receptors are abundant in limbic regions particularly in the amygdala and hippocampus (Simerly et al., 1990; Beyenburg et al., 2000; Skinner et al., 2009). Further studies will therefore be required in which histological, cellular, and genetic analysis such as gene expressions and proteomics are combined in order to identify the molecular biological mechanisms that underlie these GnRHa-induced morphological changes in the amygdala.

4.3. Conclusion

Our results demonstrate that GnRHa treatment modulates specific facets of the development of the amygdala, as indicated by increased amygdala volume in treated compared to untreated animals. These novel findings demonstrate that gonadotropin releasing hormone concentrations during puberty are important for normal brain development. Pharmacological manipulation of gonadotropin releasing hormone action in clinical settings using young patients should therefore be accompanied by carefully monitoring for neurobiological side effects.

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Conflict of interest

The authors have nothing to disclose.

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References

Albertson, A.J., Navratil, A., Mignot, M., Dufourny, L., Cherrington, B., Skinner, D.C., 2008. Immunoreactive GnRH type I receptors in the mouse and sheep brain. J. Chem. Neuroanat. 35, 326—333.

Allen, J.S., Damasio, H., Grabowski, T.J., 2002. Normal neuroanatomical variation in the human brain: an MRI-volumetric study. Am. J. Phys. Anthropol. 118, 341—358.

Andersson, J.L.R., Jenkinson, M., Smith, S., 2007a. Non-linear Optimization. FMRIB Technical Report TR07JA1.

Andersson, J.L.R., Jenkinson, M., Smith, S., 2007b. Non-linear Registration, aka Spatial Normalisation. FMRIB Technical Report TR07JA2.

Andreano, J.M., Cahill, L., 2009. Sex influences on the neurobiology of learning and memory. Learn. Mem. 16, 248—266.

Barrós-Loscertales, A., Meseguer, V., Sanjuán, A., Belloch, V., Parcet, M.A., Torrubia, R., Ávila, C., 2006. Behavioral inhibition system activity is associated with increased amygdala and hippocampal gray matter volume: a voxel-based morphometry study. Neuroimage 33, 1011—1015.

Bergin, P.S., Raymond, A.A., Free, S.L., Sisodiya, S.M., Stevens, J.M., 1994. Magnetic resonance volumetry. Neurology 44, 1770—1771.

Beyenburg, S., Watzka, M., Clusmann, H., Blümcke, I., Bidlingmaier, F., Elger, C.E., Stoffel-Wagner, B., 2000. Androgen receptor mRNA expression in the human hippocampus. Neurosci. Lett. 294, 25—28.

Bryan, K.J., Mudd, J.C., Richardson, S.L., Chang, J., Lee, H.g., Zhu, X., Smith, M.A., Casadesus, G., 2010. Down-regulation of serum gonadotropins is as effective as estrogen replacement at improving menopause-associated cognitive deficits. J. Neurochem. 112, 870—881.

Cahill, L., Uncapher, M., Kilpatrick, L., Alkire, M.T., Turner, J., 2004. Sex-related hemispheric lateralization of amygdala function in emotionally influenced memory: an fMRI investigation. Learn. Mem. 11, 261—266.

Carel, J.C., Eugster, E.A., Rogol, A., Ghizzoni, L., Palmert, M.R., on behalf of the members of the ESPE-LWPES GnRH Analogs Consensus Conference Group, 2009. Consensus statement on the use of gonadotropin-releasing hormone analogs in children. Pediatrics 123, e752—e762.

Casadesus, G., Webber, K.M., Atwood, C.S., Pappolla, M.A., Perry, G., Bowen, R.L., Smith, M.A., 2006. Luteinizing hormone modulates cognition and amyloid-β deposition in Alzheimer APP transgenic mice. Biochim. Biophys. Acta 1762, 447—452.

Casey, B.J., Jones, R.M., 2010. Neurobiology of the adolescent brain and behavior: implications for substance use disorders. J. Am. Acad. Child Adolesc. Psychiatry 49, 1189—1201.

Caviness, V.S., Kennedy, D.N., Richelme, C., Rademacher, J., Filipek, P.A., 1996. The human brain age 7—11 years: a volumetric analysis based on magnetic resonance images. Cereb. Cortex 6, 726—736.

Chu, C., Xu, B., Huang, W., 2010. GnRH analogue attenuated apoptosis of rat hippocampal neuron after ischemia-reperfusion injury. J. Mol. Histol. 41, 387—393.

Claypool, L.E., Foster, D.L., 1990. Sexual differentiation of the mechanism controlling pulsatile secretion of luteinizing hormone contributes to sexual differences in the timing of puberty in sheep. Endocrinology 126, 1206—1215.

Cooke, B.M., Woolley, C.S., 2009. Effects of prepubertal gonadectomy on a male-typical behavior and excitatory synaptic transmission in the amygdala. Dev. Neurobiol. 69, 141—152.

De Bellis, M.D., Casey, B.J., Dahl, R.E., Birmaher, B., Williamson, D.E., Thomas, K.M., Axelson, D.A., Frustaci, K., Boring, A.M., Hall, J., Ryan, N.D., 2000. A pilot study of amygdala volumes in

pediatric generalized anxiety disorder. Biol. Psychiatry 48, 51—57.

Evans, N.P., Robinson, J.E., Erhard, H.W., Ropstad, E., Fleming, L.M., Haraldsen, I.R.H., 2012. Development of psychophysiological motoric reactivity is influenced by peripubertal pharmacological inhibition of gonadotropin releasing hormone action-Results of an ovine model. Psychoneuroendocrinology 37, 1876—1884.

Filipek, P.A., Richelme, C., Kennedy, D.N., Caviness, V.S., 1994. The young adult human brain: an MRI-based morphometric analysis. Cereb. Cortex 4, 344—360.

Foster, D.L., Ryan, K.D., 1979. Endocrine mechanisms governing transition into adulthood: a marked decrease in inhibitory feedback action of estradiol on tonic secretion of luteinizing hormone in the lamb during puberty. Endocrinology 105, 896—904.

George, M.S., Ketter, T.A., Parekh, P.I., Herscovitch, P., Post, R.M., 1996. Gender differences in regional cerebral blood flow during transient self-induced sadness or happiness. Biol. Psychiatry 40, 859—871.

Giedd, J.N., 2004. Structural magnetic resonance imaging of the adolescent brain. Ann. N.Y. Acad. Sci. 1021, 77—85.

Giedd, J.N., Castellanos, F.X., Rajapakse, J.C., Vaituzis, A.C., Rapoport, J.L., 1997. Sexual dimorphism of the developing human brain. Prog. Neuropsychopharmacol. Biol. Psychiatry 21, 1185—1201.

Giedd, J.N., Clasen, L.S., Lenroot, R., Greenstein, D., Wallace, G.L., Ordaz, S., Molloy, E.A., Blumenthal, J.D., Tossell, J.W., Stayer, C., Samango-Sprouse, C.A., Shen, D., Davatzikos, C., Merke, D., Chrousos, G.P., 2006. Puberty-related influences on brain development. Mol. Cell. Endocrinol. 254—255, 154—162.

Giedd, J.N., Vaituzis, A.C., Hamburger, S.D., Lange, N., Rajapakse, J.C., Kaysen, D., Vauss, Y.C., Rapoport, J.L., 1996. Quantitative MRI of the temporal lobe, amygdala, and hippocampus in normal human development: ages 4—18 years. J. Comp. Neurol. 366, 223—230.

Goldstein, J.M., Seidman, L.J., Horton, N.J., Makris, N., Kennedy, D.N., Caviness, V.S., Faraone, S.V., Tsuang, M.T., 2001. Normal sexual dimorphism of the adult human brain assessed by in vivo magnetic resonance imaging. Cereb. Cortex 11, 490—497.

Grigorova, M., Sherwin, B.B., Tulandi, T., 2006. Effects of treatment with leuprolide acetate depot on working memory and executive functions in young premenopausal women. Psychoneuroendocrinology 31, 935—947.

Jenkinson, M., Smith, S., 2001. A global optimisation method for robust affine registration of brain images. Med. Image Anal. 5, 143—156.

Juraska, J.M., 1991. Sex differences in 'cognitive' regions of the rat brain. Psychoneuroendocrinology 16, 105—119.

Keller, K., Menon, V., 2009. Gender differences in the functional and structural neuroanatomy of mathematical cognition. Neuroimage 47, 342—352.

Killgore, W.D.S., Oki, M., Yurgelun-Todd, D.A., 2001. Sex-specific developmental changes in amygdala responses to affective faces. Neuroreport 12, 427—433.

Koscik, T., Bechara, A., Tranel, D., 2010. Sex-related functional asymmetry in the limbic brain. Neuropsychopharmacology 35, 340—341.

MacMillan, S., Szeszko, P.R., Moore, G.J., Madden, R., Lorch, E., Ivey, J., Banerjee, S.P., Rosenberg, D.R., 2003. Increased amygdala: hippocampal volume ratios associated with severity of anxiety in pediatric major depression. J. Child Adolesc. Psychopharmacol. 13, 65—73.

Madeira, M.D., Lieberman, A.R., 1995. Sexual dimorphism in the mammalian limbic system. Prog. Neurobiol. 45, 275—333.

Merke, D.P., Fields, J.D., Keil, M.F., Vaituzis, A.C., Chrousos, G.P., Giedd, J.N., 2003. Children with classic congenital adrenal hyperplasia have decreased amygdala volume: potential prenatal and postnatal hormonal effects. J. Clin. Endocrinol. Metab. 88, 1760—1765.

Neufang, S., Specht, K., Hausmann, M., Güntürkün, O., Herpertz-Dahlmann, B., Fink, G.R., Konrad, K., 2009. Sex differences and the impact of steroid hormones on the developing human brain. Cereb. Cortex 19, 464—473.

Nolte, J., 1993. Olfactory and limbic systems. In: Farrell, R. (Ed.), The Human Brain. An Introduction to its Functional Anatomy. Mosby Year Book, St. Louis, pp. 397—413.

Nuruddin, S., Wojniusz, S., Ropstad, E., Krogenæs, A., Evans, N.P., Robinson, J.E., Solbakk, A.K., Amiry-Moghaddam, M., Haraldsen, I.R.H., 2013. Peri-pubertal gonadotropin-releasing hormone analog treatment affects hippocampus gene expression without changing spatial orientation in young sheep. Behav. Brain Res. 242, 9—16.

Peper, J.S., Hulshoff Pol, H.E., Crone, E.A., van Honk, J., 2011. Sex steroids and brain structure in pubertal boys and girls: a mini-review of neuroimaging studies. Neuroscience 191, 28—37.

Pfefferbaum, A., Sullivan, E.V., Adalsteinsson, E., Garrick, T., Harper, C., 2004. Postmortem MR imaging of formalin-fixed human brain. Neuroimage 21, 1585—1595.

Romeo, R.D., Sisk, C.L., 2001. Pubertal and seasonal plasticity in the amygdala. Brain Res. 889, 71—77.

Rosati, F., Sturli, N., Cungi, M.C., Morello, M., Villanelli, F., Bartolucci, G., Finocchi, C., Peri, A., Serio, M., Danza, G., 2011. Gonadotropin-releasing hormone modulates cholesterol synthesis and steroidogenesis in SH-SY5Y cells. J. Steroid Biochem. Mol. Biol. 124, 77—83.

Schang, A.L., Ngö-Muller, V., Bleux, C., Granger, A., Chenut, M.C., Loudes, C., Magre, S., Counis, R., Cohen-Tannoudji, J., Laverriére, J.N., 2011. GnRH receptor gene expression in the developing rat hippocampus: transcriptional regulation and potential roles in neuronal plasticity. Endocrinology 152, 568—580.

Schneider, S., Peters, J., Bromberg, U., Brassen, S., Menz, M.M., Miedl, S.F., Loth, E., Banaschewski, T., Barbot, A., Barker, G., Conrod, P.J., Dalley, J.W., Flor, H., Gallinat, J., Garavan, H., Heinz, A., Itterman, B., Mallik, C., Mann, K., Artiges, E., Paus, T., Poline, J.B., Rietschel, M., Reed, L., Smolka, M.N., Spanagel, R., Speiser, C., Ströhle, A., Struve, M., Schumann, G., Böchel, C., 2011. Boys do it the right way: sex-dependent amygdala lateralization during face processing in adolescents. Neuroimage 56, 1847—1853.

Simerly, R.B., Swanson, L.W., Chang, C., Muramatsu, M., 1990. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J. Comp. Neurol. 294, 76—95.

Sisk, C.L., Foster, D.L., 2004. The neural basis of puberty and adolescence. Nat. Neurosci. 7, 1040—1047.

Skinner, D.C., Albertson, A.J., Navratil, A., Smith, A., Mignot, M., Talbott, H., Scanlan-Blake, N., 2009. Effects of gonadotrophin-releasing hormone outside the hypothalamic-pituitary-reproductive axis. J. Neuroendocrinol. 21, 282—292.

Smith, S.M., 2002. Fast robust automated brain extraction. Hum. Brain Mapp. 17, 143—155.

Smith, S.M., Jenkinson, M., Woolrich, M.W., Beckmann, C.F., Behrens, T.E.J., Johansen-Berg, H., Bannister, P.R., De Luca, M., Drobnjak, I., Flitney, D.E., Niazy, R.K., Saunders, J., Vickers, J., Zhang, Y., De Stefano, N., Brady, J.M., Matthews, P.M., 2004. Advances in functional and structural MR image analysis and implementation as FSL. Neuroimage 23, 208—219.

Sowell, E.R., Peterson, B.S., Kan, E., Woods, R.P., Yoshii, J., Bansal, R., Xu, D., Zhu, H., Thompson, P.M., Toga, A.W., 2007. Sex differences in cortical thickness mapped in 176 healthy individuals between 7 and 87 years of age. Cereb. Cortex 17, 1550—1560.

Speck, O., Ernst, T., Braun, J., Koch, C., Miller, E., Chang, L., 2000. Gender differences in the functional organization of the brain for working memory. Neuroreport 11, 2581—2585.

Thomas, K.M., Drevets, W.C., Dahl, R.E., Ryan, N.D., Birmaher, B., Eccard, C.H., Axelson, D., Whalen, P.J., Casey, B.J., 2001. Amygdala

response to fearful faces in anxious and depressed children. Arch. Gen. Psychiatry 58, 1057—1063.

Tomasi, D., Volkow, N.D., 2011. Laterality patterns of brain functional connectivity: gender effects. Cereb. Cortex 22, 1455—1462.

Tovi, M., Ericsson, A., 1992. Measurements of T1 and T2 over time in formalin-fixed human whole-brain specimens. Acta Radiol. 33, 400—404.

Vyas, A., Jadhav, S., Chattarji, S., 2006. Prolonged behavioral stress enhances synaptic connectivity in the basolateral amygdala. Neuroscience 143, 387—393.

Wang, L., Chadwick, W., Park, S., Zhou, Y., Silver, N., Martin, B., Maudsley, S., 2010. Gonadotropin-releasing hormone receptor system: modulatory role in aging and neurodegeneration. CNS Neurol. Disord. Drug Targets 9, 651—660.

Wilke, M., Krägeloh-Mann, I., Holland, S., 2007. Global and local development of gray and white matter volume in normal children and adolescents. Exp. Brain Res. 178, 296—307.

Wojniusz, S., Vögele, C., Ropstad, E., Evans, N., Robinson, J., Sütterlin, S., Erhard, H.W., Solbakk, A.K., Endestad, T., Olberg, D.E., Haraldsen, I.R.H., 2011. Prepubertal gonadotropin-releasing hormone analog leads to exaggerated behavioral and emotional sex differences in sheep. Horm. Behav. 59, 22—27.

Wood, R.I., Foster, D.L., 1998. Sexual differentiation of reproductive neuroendocrine function in sheep. Rev. Reprod. 3, 130—140.

Zhang, Y., Brady, M., Smith, S., 2001. Segmentation of brain MR images through a hidden Markov random field model and the expectation-maximization algorithm. IEEE Trans. Med. Imaging 20, 45—57.

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Peri-pubertal gonadotropin-releasing hormone agonist treatment affects sex biased gene expression of amygdala in sheep

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Summary The nature of hormonal involvement in pubertal brain development has attracted wide interest. Structural changes within the brain that occur during pubertal development appear mainly in regions closely linked with emotion, motivation and cognitive functions. Using a sheep model, we have previously shown that peri-pubertal pharmacological blockade of gonadotropin releasing hormone (GnRH) receptors, results in exaggerated sex-differences in cognitive executive function and emotional control, as well as sex and hemisphere specific patterns of expression of hippocampal genes associated with synaptic plasticity and endocrine signaling. In this study, we explored effects of this treatment regime on the gene expression profile of the ovine amygdala.

The study was conducted with 30 same-sex twin lambs (14 female and 16 male), half of which were treated with the GnRH agonist (GnRHa) goserelin acetate every 4th week, beginning before puberty, until approximately 50 weeks of age. Gene expression profiles of the left and right amygdala were measured using 8 × 15 K Agilent ovine microarrays. Differential expression of selected genes was confirmed by qRT-PCR (Quantitative real time PCR). Networking analyses and Gene Ontology (GO) Term analyses were performed with Ingenuity Pathway Analysis (IPA), version 7.5 and DAVID (Database for Annotation, Visualization and integrated Discovery) version 6.7 software packages, respectively.

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GnRHa treatment was associated with significant sex- and hemisphere-specific differential patterns of gene expression. GnRHa treatment was associated with differential expression of 432 ($|\log FC| > 0.3$, adj. p value <0.05) and 46 (p value $<0.0.5$) genes in the left and right amygdala, respectively, of female animals, relative to the reference sample which consisted of all a pooled sample from control and treated animals of both sexes. No genes were found to be differentially expressed as a result of GnRHa treatment in the male animals.

The results indicated that GnRH may, directly and/or indirectly, be involved in the regulation of sex- and hemisphere-specific differential expression of genes in the amygdala. This finding should be considered when long-term peri-pubertal GnRHa treatment is used in children.

1. Introduction

Brain development is a complex and precisely regulated process that occurs over an extended period of time (Kostovic and Judaš, 2006; Rubenstein, 2011). This developmental process underlies changes in complex cognitive skills and emotional control behavior, and is accompanied by structural and molecular changes in the brain (Dumas, 2005; Luna et al., 2001; Paterson et al., 2006; Shaw et al., 2006). Indeed, recent studies have described how the transcriptome of the brain follows a sequential pattern of change over the course of pre- and postnatal development (Fu et al., 2011; Liu et al., 2012). These structural and molecular changes within the brain, while important for the development of normal function, may also be associated with the increased prevalence of neuropsychiatric disorders (Anagnostou and Taylor, 2011; Chow et al., 2012) seen at this developmental stage. Brain development and functions such as cognitive ability, e.g. emotional control are, in addition to being influenced by developmental age, also sexually differentiated (De Bellis et al., 2001). Sex differences in structural and functional brain areas, along with changes in hormone levels, such as occur at the pubertal transition, are also closely related to sex differences in behavior and the prevalence of neuropsychiatric disorders such as autism spectrum and anxiety related disorders (Bao and Swaab, 2010) which would again suggest a potential causative link.

Given that the pubertal transition is driven by changes in secretion of the hormone gonadotropin releasing hormone (GnRH), to identify hormonal mechanisms that might underlie sex differences in brain development and cognitive function, several studies have investigated the modulatory effect of GnRH (Bryan et al., 2010; Grigorova et al., 2006; Wojniusz et al., 2011) and it's agonist (GnRHa) on psychomotoric reactivity, cognitive parameters and gene expression in the hippocampus (Evans et al., 2012; Nuruddin et al., 2013; Wojniusz et al., 2011). GnRH is synthesized in specialized neurons in the brain and released into the hypophysial portal blood vessels to act on receptors in the anterior pituitary gland (Millar, 2005). GnRH has been known to be central to the regulation of reproductive physiology and sexual behavior for decades but recent studies, have reported GnRH receptor (GnRHR) expression in a number of non-reproductive tissues including the heart (Dong et al., 2011), bladder (Coit et al., 2009), skin (Reichler et al., 2008) and in malignant cells (Harrison et al., 2004). The precise role of GnRH in these locations is not known, but it suggests that GnRH might have important physiological functions beyond the traditional ascribed role in reproductive physiology. Several studies have indicated that GnRHR is expressed in regions of the brain, such as the hippocampus, cortex and amygdala, which are not closely linked with reproductive function. These brain areas are rather involved in cognitive functions, for example; memory, emotions and learning (Albertson et al., 2008; Chu et al., 2010; Schang et al., 2011; Skinner et al., 2009; Wilson et al., 2006). Interestingly, long term GnRHa treatment has been reported to lead to impairment of higher-order executive control functions, the processing of visuo-spatial information (Nelson et al., 2008) and working memory (Bryan et al., 2010; Grigorova et al., 2006) in adult humans that suggests a physiological role for GnRH in these brain areas. Similarly, in animal models of Alzheimer's disease (AD), improvements in cognitive function have been reported due to GnRHa treatment (Bowen et al., 2004; Casadesus et al., 2006) and these studies, in combination with others, have led to clinical interest in GnRHa therapy for this condition (Doraiswamy and Xiong, 2006). Although there are a growing number of publications that have described cognitive effects of GnRHa treatment in adults, there is a lack of knowledge about possible extra-gonadal effects from GnRHa treatment in children, and safety concerns were raised in the recently published 'Consensus Statement on the Use of Gondotropin-Releasing Hormone Analogues in children' (Carel et al., 2009). During the peripubertal stage, brain maturation is characterized by the development of sex-specific functional circuits that follows a distinct spatio-temporal pattern (Casey and Jones, 2010), and it has been hypothesized that any disturbances of this maturational process might lead to altered behavior and cognitive functions later in life (Casey and Jones, 2010; Sisk and Foster, 2004). In pediatric medicine the main indications for GnRHa treatments are central precocious puberty, idiopathic short stature, protection of the testes and ovaries in children undergoing cancer chemotherapy, and early onset gender identity disorder (Carel et al., 2009; Delemarre-van de Waal and Cohen-Kettenis, 2006). Despite the fact that GnRHa has been used in adult and pediatric medicine for over 30 years, knowledge on how GnRHa treatment may interfere with maturation of the brain, and specifically the fronto-temporal-limbic system and development of cognitive function and behavior is still very limited. Due to the obvious ethical restrictions, investigation of GnRH effects on brain development in humans is limited. Furthermore, as children that are treated for precocious puberty have been subjected to the influence of sex hormones before GnRHa treatment, they do not provide an ideal system with which to differentiate between the effects of central precocious puberty and the medication itself.

Consequently an animal model where animals are not subjected to a pubertal increase in GnRH represents a better model. As part of a large ongoing study, we have begun to characterize gonadal and extra-gonadal effects of peripubertal GnRHa treatment, in an ovine animal model. To date we have demonstrated that long term peri-pubertal GnRHa treatment impacts on sex-specific brain development, and affected the regulation of behavior and cardiac function in young animals (Wojniusz et al., 2011). In addition, at 48 weeks of age, female sheep in which puberty was blocked displayed increased anxiety and pronounced avoidance behavior, tended to stay close to their companions in the audience pen and were much less prone to engage in food seeking behavior. If they sought food, it was more often the nearby and visible hay than the remotely placed and out of sight pellets, whereas males in which puberty was similarly blocked revealed risk-taking and exaggerated behavior and were highly motivated by the possibility of obtaining a high reward food source that they could not see. This exaggerated ''maleness'' indicates improved emotional control as a result of GnRHa treatment, compared to their same sex twins (Wojniusz et al., 2011). These findings provided evidence that blockade of GnRH's actions during peripubertal development may lead to sex-specific cognitive differences, a finding that is of great medical interest. A structural morphology study, using post mortem magnetic resonance image (MRI) of the brains of these same sheep, demonstrated that GnRHa-treatment was associated with increased volume of the amygdala in both males and females, with a specific sex by treatment interaction whereby the left amygdala in the treated females was significantly larger than in treated males (Nuruddin et al., in press). The observation of an effect of GnRHa treatment on amygdala volume is supported by the fact that this brain region also expresses a high density of GnRH receptor expressing neurons (Albertson et al., 2008; Granger et al., 2004; Haour et al., 1987), although a secondary effect cannot be ruled out. The amygdala plays a fundamental role in emotional regulation, anxiety and fear (Maren and Quirk, 2004; Millan, 2003; Thomas et al., 2001). In human neuroimaging studies, it has been shown that amygdala volume is associated with anxiety and depression in children and adults (Morris et al., 1998; Thomas et al., 2001). Animal studies have also revealed an association between hypertrophy of the amygdala and increased reactivity (Vyas et al., 2006). Based on previous findings from our ovine model, the present study aimed to investigate gene expression profiles in the amygdala, in order to gain further insight into the molecular changes might underlie the effect of GnRHa treatment in sex-specific cognitive function, as well as structural volume changes in the amygdala.

2. Materials and methods

2.1. Animals

All animal procedures were conducted at the University of Glasgow's Cochno Research Centre (55°55′N) following review by the University's Welfare and Ethics Committee, and in accordance with Home Office regulations (PPL 60/3826). To eliminate the possible developmental effects of steroid transfer between siblings of different sexes and reduce genetic variation, the study was conducted using 15 pairs of same-sex twin lambs (Scottish Mule Texel Cross, 7 female and 8 male). Lambs were born between 17th March and 1st April 2008, and remained with their dams until weaned at about 12 weeks of age. Males and females were maintained separately during the entire study period. Within each set of twins, one was randomly assigned, at birth, to the control (C) and the other to the treatment (T) group. Although the timing of the onset of puberty differs between sheep and humans, a similar interplay between neurosecretory cells of the brain and the peripheral sexual organs is well documented in vertebrates (mammals, primates and humans) (Wang et al., 2010). The first GnRH isoform was discovered in the mammalian brain, the same forms of GnRH are present in most vertebrate species (Burgus et al., 1972; Matsuo et al., 1971; Millar, 2005). Intracerebral blockage of such GnRH isoforms in both sheep and humans results in sexual immaturity in both species. GnRH agonists can be used to block the hypothalamic-pituitary-gonadal axis, inhibiting the production of gonadal hormones in sheep as effectively as in humans. GnRHa initially induces a sharp increase in serum concentrations of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), known as the 'flare effect' that leads to an increase in serum sex steroids (within 3–4 days) but this is followed by down-regulation of GnRH receptors resulting in suppression of gonadotropins and sex steroid secretion within 2 weeks (Schally et al., 2001). In our experiment, animals in the treatment group received subcutaneous implants of the GnRH agonist, goserelin acetate (Zoladex®; kindly donated by Astra Zeneca; Macclesfield, UK 3.6 mg) every 4 weeks from 8 weeks of age in males and 28 weeks of age in females. The Zoladex® implant comes within and was administered in the axillar region, using a 'SafeSystem' needle and syringe. The differences in the timing of treatment initiation between males and females was chosen because puberty in sheep, as in humans, is sexually dimorphic, and this treatment paradigm was designed so that the pharmacological inhibition of GnRH action should begin approximately 2 weeks before the predicted time of onset of pubertal development in both rams and ewes (Wood and Foster, 1998). Animal were maintained in accordance with normal husbandry conditions, i.e., on grass, at all times except for periods of behavioral testing, approximately 8, 28 and 48 weeks of age, when they were group housed indoors. Every four weeks, throughout the study, animals were gathered and held, for no more than 2 h (with their mothers, prior to weaning), in a specialized sheep handling facility at the University of Glasgow's Cochno Research Centre where blood samples were collected from the jugular vein, physical measurements taken (weight, shoulder height, girth) and agonist administered (where required). To guarantee that GnRHa treatment blocked pubertal development, plasma samples were assayed regularly for testosterone (males) and progesterone (females), monthly measurements of scrotal volume made during the animals' life, testes or ovaries were excised, weighed and histologically evaluated after euthanasia at 12 months of age. The analysis confirmed that treatment prevented puberty by complete suppression of the hypothalamo-pituitary-gonadal axis (for more details see: Wojniusz et al., 2011).

2.2. Tissue processing

At approximately 50 weeks of age, over a 2 day period, animals were moved to the postmortem facility at the Veterinary School, University of Glasgow where they were group housed until individually isolated, euthanized with an overdose of barbiturate (Somulose 1 ml/kg body weight; Decra Veterinary Products, Shrewsbury UK), decapitated and the brains removed for sample collection by experienced neuroscientists. The interval, across all animals, between euthanasia and tissue collection was less than 10 min.

The amygdala samples were carefully dissected from the right and left hemispheres. A total of 60 amygdala samples (30 controls, 30 treated, 7 female and 8 male pairs for each hemisphere) were collected. All samples were immediately frozen in liquid nitrogen and stored at −80 °C.

2.3. RNA extraction and microarray strategy

Total RNA was extracted from the left and right amygdala samples for microarray and quantitative real time polymerase chain reaction (qRT-PCR) analysis. For RNA extraction the 15−31 mg of frozen tissues were kept on dry ice and resuspended in 1 ml TRIzol reagent (Invitrogen™, Paisley, United Kingdom). Homogenization was carried out using MagNA Lyser Green Beads (Roche Diagnostics, Mannheim, Germany) and Retsch MM 301 mixer mill (Retsch GmbH, Haan, Germany). Total RNA extraction was performed according to the TRIzol manufacturer recommendations (Invitrogen™, Paisley, United Kingdom), followed by a DNase I treatment (RNase-Free DNase Set, Qiagen, Crawley, UK) for 20 min at 25 °C. Further RNA purification was conducted using an RNeasy Mini-Kit (Qiagen, Crawley, UK) according to the manufacturer's recommendations. After purification, the samples were eluted in 50 μl RNase-free water (Qiagen, Crawley, UK) and aliquoted in 2 samples for microarray and qRT-PCR analyses. The concentration and quality of the RNA were determined using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively.

Four samples had an RNA integrity number (RIN) value ranging from 5.1 to 5.5 and were excluded from further analysis: The remaining RNAs had RIN values of 7.0−8.7. In total 56 samples, corresponding to 8 groups (male/female, treatment/control and left/right amygdala) with 7 replicas ($n = 7$), were used for microarray target preparation and hybridization. A reference design was used for this microarray experiment where target samples were hybridized with a reference sample. This method reduces human error as each sample is handled the same way and compared against a reference sample which contains many samples pooled (Kendziorski et al., 2005; König et al., 2004). To ensure sufficient reference sample for all the arrays and for PCR, the reference sample was made by taking 1500 ng RNA from each sample. As the RNA concentration for the samples varied, the volumes of the samples had to be adjusted to make sure that the same amount of each sample was added to the pool. This pooled reference sample (concentration of 399.6 ng/μl) was aliquoted and stored at −70 °C until used to compare the variation in mRNA expression among groups. A dye swap design was applied, whereby within each group DNAse treated RNA was randomly labeled with cyanine 3 (Cy3) CTP and cyanine 5 (Cy5) CTP using a Quick Amp Labeling Kit, two-color (5190-0444, New Castle, DE) according to the manufacturers methodology. The labeled RNA was purified with the Qiagen Rneasy kit (Valencia, CA) according to Agilent's revision of the Qiagen protocol (as shown in the quick Amp kit protocol) except that the micro centrifugation spins were performed at room temperature instead of 4 °C. The resulting labeled cRNA was analyzed with the NanoDrop spectrophotometer, and the specific activities and the yields of cRNAs calculated; these ranged from 9.25 to 28.54 pmol Cy3/Cy5 per μg cRNA. The labeled cRNA was stored at −80 °C until use.

2.4. Microarray hybridization

Microarray hybridization was performed according to protocols supplied by Agilent. In brief 300 ng of each labeled cRNA was fragmented and mixed with hybridization buffer using the Agilent gene expression hybridization kit. These samples were then applied to a sheep 8 × 15 K array slide (Agilent 019921), containing eight arrays with 15,208 oligomers with a length of 60 bases, and hybridized at 65 °C for 17 h at 10 rpm. The arrays were washed, dried, stabilized, and scanned with a GenePix® 4000B two-color scanner (Axon instrument, Foster City, CA). Two channel images were imported into Agilent's feature extraction 9.1 software for features (spot) extraction and alignment.

2.5. Statistical analysis of microarray

The statistical program R was used to analyze the microarray data. Microarray data generated from Genepix were imported into the Bioconductor package LimmaGUI and corrected for background (Smyth and Speed, 2003). For within- and between-array normalization, print tip Loess and scale were used, respectively (Smyth and Speed, 2003). In order to detect differentially expressed genes across treated and control samples, an empirical Bayes moderated t-test (Smyth and Speed, 2003; Smyth et al., 2005) was applied. The p-values were corrected for multiple testing using the Benjamini−Hochberg (BH) method (Benjamini and Hochberg, 1995), and adjusted p-values <0.05 were selected as differentially expressed genes. The generated gene list was further filtered for genes with low intensity and with small changes in expression. In the averaged normalized MA-plot, the genes with M values between ± 0.3 and mean log intensity < 3 were excluded from the gene list.

2.6. Data deposition

Microarray data have been submitted to the NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE44202. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44202).

2.7. Gene Ontology

In order to understand the biological significance of the results, Gene Ontology (GO) term enrichment analysis of the list of differentially expressed genes was conducted in The Database for Annotation, Visualization and Integrated

Discovery (DAVID) 6.7 (Huang et al., 2008). To identify gene orthologs or homologs to sheep genes, we used a translation table provided by Agilent Technologies and converted probe ids into official gene symbols using an in-house python script. Results were split in a 4-leveled hierarchy: 1 — Male/Female, 2 — Left/Right part of amygdala, 3 — Treated/control, and finally 4 — up-regulated and down-regulated genes. Only those genes showing an absolute value of fold change log ratio greater than 0.3 ($|\log FC| > 0.3$) and with adjusted p-values < 0.05 were included for functional clustering. Clustered terms were considered significant if they showed an EASE score < 0.01. Clearly overlapping concepts were omitted.

2.8. Gene networking analysis

In order to interpret the biological function of the gene lists, the Ingenuity Pathway Analysis (IPA) version 7.5 (http://www.Ingenuity.com) software packages was used for networking analyses. This software not only generates a biological network from a list of selected genes, but also provides biological functions and canonical pathways from HUGO ortholog names of the genes that were imported into the program. A network of genes is created when a gene regulates the function of other genes. To develop a network, the IPA software adds other genes and/or different molecules that are linked with two focus genes.

2.9. Quantitative real-time PCR validation (qRT-PCR)

To validate changes in gene transcription identified by the microarray experiment, the relative mRNA expression levels of 10 genes (Table 1), selected based on changes seen in the left amygdala of treated sheep, were verified by qRT-PCR using the same pooled and target RNA samples. For quantification of mRNA transcripts, primers were designed using the Primer3Plus software, a web-based primer designing tool (Untergasser et al., 2007). Primer sequences of reference genes (*ACTB*, *YWHAZ*, *RPL19*, *GAPDH*, *G6PDH*, and *SDHA*) were obtained from Garcia-Crespo et al. (2005). Primers were synthesized by Sigma—Aldrich (St. Louis, MO, USA) and their Specificities were checked using nucleotide BLAST and primer BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To find the most stable reference genes, GeNorm software (Primer design Ltd) was used. Out of six genes tested, *GAPDH* and *ACTB* were the most stable (M value < 0.5) and were selected as the reference genes in this study. All primers were optimized with regard to annealing temperature. PCR products were analyzed on an ethidium bromide agarose gel to ensure the expected product size and to check for potential primer artifacts. Amplification efficiency was examined by generating standard curves from 10 fold serial dilutions of pooled cDNA from both control and treated samples, respectively. The primer efficiency for each gene was in the range of 95—103%.cDNA synthesis and quantitative real-time PCR were performed using superscript III platinum Two-Step qRT-PCR kit with SYBR Green (Invitrogen, Carlsbad, CA, USA) according to the manufacturers protocol. A Peltier Thermal Cycler-225 (MJ Research, Waltham, MA, USA) was used to synthesize cDNA and q-RT-PCR was run with an ABI PRISM 7900 Sequence Detector System (PE Applied Biosystems, USA). Technical duplicates of RNA samples, negative controls without reverse transcriptase of each RNA sample and negative control with no added RNA template underwent cDNA synthesis. 1 μg of total RNA was used to synthesize cDNA (10 min at 25 °C, 50 min at 42 °C and 5 min at 85 °C). The reaction mixture (20 μl) contained 3 μl (15 ng) of cDNA, 10 μl of Platinum SYBR Green qPCR Super Mix-UDG, 0.2 μl of ROX reference dye, 1 μl each of 10 μmol forward and reverse primers and 4.8 μl of DNase/RNase-free water (Invitrogen). Cycling conditions were as follows: Initial denaturation at 95 °C for 2 min; 40 amplification cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. Each assay was performed in quadruplicate, and three negative controls were run for every assay: no template (sample lacking cDNA), no reverse transcriptase, and no RNA in reverse transcriptase reaction. The absence of primer-dimers, genomic DNA, and other DNA contaminations was also monitored during the experiment by including melting curve analysis (ABI PRISM 7900 manufacturer's recommended default settings) at the end of each run.

2.10. qRT-PCR data analysis

qRT-PCR technical replicates of samples were averaged, and expression ratios were calculated by the delta Ct method normalized to the reference genes (*GAPDH* and *ACTB*). Statistical significance of analyses was calculated by using Wilcoxon signed rank test. These statistical analyses were performed in the software JMP 10.0 (SAS Institute Inc, Cary, NC, USA). The Pearson product-moment correlation coefficient was used to analyze the qRT-PCR data. The non-parametric Spearman's p correlation coefficient was used when the outlier was included. p-Values <0.05 were considered statistically significant.

3. Results

3.1. Transcription analysis through microarray profiling

Microarray data analysis revealed that a large number of genes ($n = 432$; adjusted $p < 0.005$) were differentially regulated in the samples collected from the left amygdala of the treated females, compared to the reference sample. Of these genes, 133 were up- and 299 down-regulated. Forty six (adjusted $p < 0.05$) genes were also found to be differentially expressed in the samples collected from the right amygdala of the treated female group, compared to the reference sample; 33 being up- and 13 down-regulated. Interestingly, there was no significant difference in gene expression in samples collected from either side of the amygdala of the treated males, relative to the reference sample.

Comparison of untreated samples with the reference sample, separately for sex and hemisphere, revealed significant differences in gene expression for the control female left ($n = 43$) and control male left ($n = 45$) samples, but not for control female right and control male right. The numbers of genes that exhibited significant differential expression across multiple groups are shown in Fig. 1. The intersections

Table 1 Sequences of genes primer for qRT-PCR and correlation analysis between microarray and qRT-PCR results.

Gene symbol	Gene full name	Forward primer	Reverse primer	Product length	Accession number	Correlation coefficients (CO)		Fold changes (FC) in gene expression[a]			
						qRT-PCR vs microarray		qRT-PCR		Microarray	
						CO	*p*-Value	FC	*p*-Value	FC	*p*-Value[b]
ATXN10	Ataxin 10	CTGAAGAGCCCGGAATTGGT	ACACAGGGGCATCATCCTTG	130	XM_004007655	0.821	0.001	3.63	0.001	1.41	0.03
GABARAP	GABA(A) receptor-associated protein	GCTGTACCAGGAACACCATGA	CAGCAGCTTCACAGACCGTA	84	XM_004012641	0.95	0.01	3.45	0.01	1.31	0.019
APP	Amyloid beta (A4) precursor protein	GTGCAGAATGGGAAGTGGGA	TCAGGGTAGACTTCCTGGCA	98	XM_004002800	0.891	0.02	4.01	0.01	1.47	0.044
TTR	Ovis aries transthyretin	TGCAGAGGTGGTGTTCACAG	CTCACTCCTTGGGACTGCTG	116	NM_001009800	0.94	0.001	−0.61	0.02	−0.25	0.049
APOE	Apolipoprotein E	GAGGCGAAGAACGCGGTTG	CTCCATATCCGCCTGGCATC	101	NM_173991	0.913	0.001	2.80	0.001	1.55	0.038
CCNE1	Cyclin E1	ACTTCTGTACCCACACGCTG	TTGCCCAGTTCAGTACAGGC	89	XM_004015131	0.946	0.001	−0.69	0.031	−0.80	0.000
ARHGAP32	GTPase activating protein 32	CGACGATTCTCCCAGCTCTC	TGAGTGACCGACTCTGGACT	71	XM_004019539	0.871	0.03	−0.85	0.001	−0.81	0.006
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	AACAACTGCACCACCAGTCA	CTGCCTCTGGGCATTTCAGA	148	XM_004007382	0.903	0.001	−0.68	0.01	−0.51	0.000
MAP4	Microtubule-associated protein 4	TAGGAACCTGTCCACACCCT	CTCAAGTCAGCTGGTGCAGA	108	XM_004018497	0.92	0.001	5.65	0.001	1.71	0.000
ADD3	Adducin 3 (gamma)	TTCTCCTCCCCTCAGTCTCG	GTCTGTACAGGCTGGCAAGT	119	XM_004020184	0.851	0.03	−0.82	0.001	−0.52	0.006

CO values indicate Pearson product-moment correlation coefficient between qRT-PCR and microarray for 10 genes.

[a] Fold changes were calculated by comparison of the mean expression values in treated female left amygdala group to reference group (pooled RNA samples from all groups). Negative values indicate a decrease in expression in treated female left amygdala group.

[b] *p*-Values were calculated using the Benjamini–Hochberg (BH) method (Benjamini and Hochberg, 1995) for multiple testing in microarray data. In the qRT-PCR analysis, all genes were normalized to *GAPDH* and *ACTB* genes.

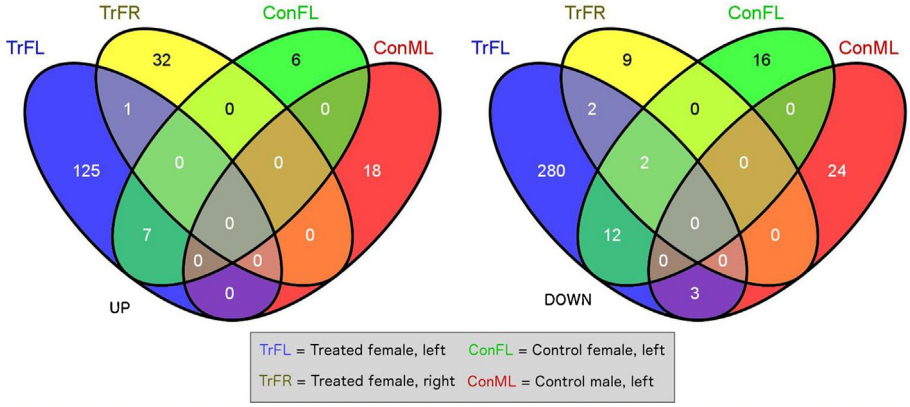

Figure 1 Venn diagrams showing the number of genes identified as differentially expressed. Section UP contained all the significant upregulated genes in different groups; section DOWN contained all the significant downregulated genes across the groups. Both figures also show the overlap of genes between different groups of samples. The intersections of the circle indicate the number of genes in common between contrasts. ConFL and Con ML indicates control female left and control male left amygdala respectively as well as TrFL and TrFR indicates treated female left and right respectively.

in Fig. 1 show the number of differentially expressed genes that were common across the different samples. The complete list of up- and down regulated genes in the different samples can be found in Supplementary Table 1.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2013.09.011.

3.2. Gene Ontology

Clusters of functional enrichment terms that met the criteria stated in the materials and methods section were only present in the treated female group and are presented in Table 2. Results were most striking relative to the samples collected from the left amygdala; in the treated females the biological function term "microtubuli" was significantly over represented among the up-regulated genes. In the down-regulated group, the following terms were significantly over represented: "Anti-apoptosis", "mitotic cell cycle", "positive regulation of transcription", "ovulation-cycle process", "mitochondrial envelope" and "ubiquitin binding". In the right side of the amygdala of treated females, two enrichment terms were considered significantly over represented: "immunoglobulin-mediated immune response" and "immune response". There were no clusters of GO enrichment terms meeting the criteria in any other groups.

3.3. Network analysis of differentially expressed genes in the left and right sides of amygdala in GnRHa treated females

Gene network analyses on the differentially expressed genes were carried out using the Ingenuity Pathway Analysis platform. For the samples collected from the left amygdala of the GnRHa treated females this resulted in five networks with a score $>$ 10. Functions in the top two networks were associated with neurological disease, cancer and cardiovascular disease (score = 39) and cell death, hereditary disease, and neurological disease (score = 28). These networks had 8 genes in common and were merged (Fig. 2). The up-regulated gene, amyloid beta (A4) precursor protein (*APP*), and the down regulated gene, ubiquitin C (*UBC*), formed prominent central nodes in this merged network.

For the samples collected from the right side of the amygdala of the GnRHa treated females a network with functions associated with cell death, cellular development, and cell growth and proliferation was identified (score = 22) (network shown in Supplementary Figure 1). In this network the up- and down-regulated focus genes occupied a more peripheral position, whilst *APP* and beta-estradiol, nodes added by IPA, took up a central position.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2013.09.011.

3.4. qRT-PCR

Data from qRT-PCR and microarray showed a high degree of concordance with a correlation coefficient (r_s) estimated to be 0.964 (Table 1).

4. Discussion

This study shows, for the first time, that long-term peri-pubertal GnRHa treatment in sheep is associated with significant changes in the levels of expression of mRNA transcripts in the amygdala, the changes being dependent upon sex and hemisphere from which samples were collected. Effects of GnRHa treatment were only observed in females, and there were a much higher number of differentially regulated mRNA transcripts (432) in the left compared with the right amygdala (46). The interpretation and implication of this novel finding are discussed below.

4.1. Sex specific effect of GnRHa treatment on gene expression in the amygdala

The observation that gene expression within the amygdala was only affected by GnRHa treatment in the females indicates that GnRHa treatment affects development of specific

Table 2 Over represented Gene Ontology (GO) groups determined by the DAVID 6.7 software.

Left/right	Up/down	Category	Term	Genes	*p*-Value
Left	Up	Cellular component	Microtubuli	*ARL2, NEIL2, KEAP1, GABARAP, TUBB, APP, TBCB, MAP1LC3A, MAP4, TUBA1A, MAP7D1, EMD, TUBB3, NUDC, TUBA1C*	6.44E−05
Right	Up	Biological process	Immunoglobulin-mediated immune response	*C1QB, C3, INPP5D, HLA-DMA, CD74*	3.18E−06
Right	Up	Biological process	Immune response	*IGL@, C3, CTSS, HLA-B, HLA-DMA, CCL4, FTH1, IFI35, PSMB8, CD74, CYBA, C1QB, CEACAM8, CTSC, INPP5D*	3.87E−12
Left	Down	Biological process	Anti-apoptosis	*SGK3, VHL, SKP2, SON, SQSTM1, VEGFA, GLO1, UBC, PRNP, RPS27A, API5, IFI6, UBA52*	1.34E−04
Left	Down	Biological process	Mitotic cell cycle	*YEATS4, CCNK, MAP2K1, HAUS2, SKP2, LRRCC1, EML4, CCNE1, INHBA, PSMB4, CDKN1B, NIPBL, PSMB6, NOLC1, PSMD2, MAPRE2, UBC, UBA52, RPS27A*	3.30E−05
Left	Down	Biological process	Positive regulation of transcription	*YEATS4, PPARA, HMGB2, VHL, MSTN, ARNTL, MED13, CCNE1, INHBA, NCOA3, SQSTM1, PAX8, VEGFA, GTF2A2, UBB, TCF4, UBA52, RPS27A, MYSM1*	0.004
Left	Down	Biological process	Ovulation-cycle process	*CCNE1, INHBA, VEGFA, MSTN, FANCG, SIRT1*	0.003
Left	Down	Cellular component	Mitochondrial envelope	*UQCRC2, NDUFAF4, SUOX, USP30, NDUFB5, COX11, CHCHD3, CPT1A, NDUFS4, COX2, TOMM70A, RHOT1, MTOR, AGK, MDH2*	0.006
Left	Down	Molecular function	Ubiquitin binding	*SQSTM1, BRE, AGL, UIMC1*	0.008

Enriched Gene Ontology terms calculated using the differentially expressed genes in treated female group samples (left and right) relative to reference samples (combination of all groups, male, female, control, treated, left and right amygdala). Clearly overlapping enrichment terms were collapsed into one group. Only those terms with an EASE score of <0.01 were considered significant. Modified from output in DAVID 6.7 (Huang et al., 2008).

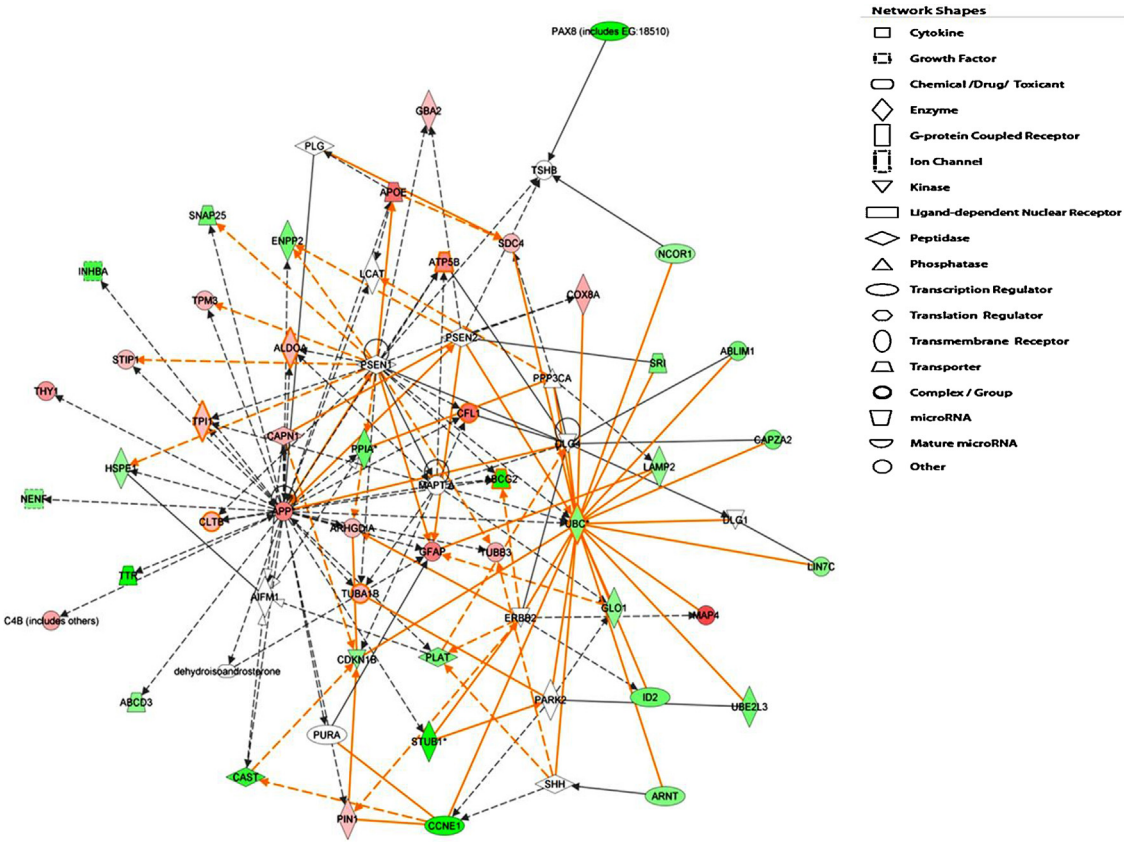

Figure 2 Ingenuity pathway analysis (IPA) was used to identify the connection between genes associated with GnRHa treatment in left amygdala. The analysis identified a network in which up regulated gene APP and down regulated genes UBC occupied central positions. Pink: up regulated genes. Green: down regulated genes. Shapes represent different molecule types as illustrated in the right panel. The intensity of the node (gene) color represents the degree of up-regulation and down-regulation where Pink: upregulated genes and Green: down regulated genes. Nodes (Genes) with uncolored background were not identified as differentially expressed in our experiment and were integrated into the computationally generated network on the basis of evidence stored in IPA knowledge memory indicating a relevance to this network. Solid lines denote protein—protein interactions and dashed lines denote regulation relationships. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

limbic structures in the amygdala in a sex specific manner. Our previously published work, with the same cohort of GnRHa treated sheep, has demonstrated that GnRHa treatment also had a sex-specific effect on behavior and emotional regulation. Specifically, female animals in which the pubertal transition was blocked by GnRHa treatment, displayed pronounced avoidance behavior, less emotional control capability or more anxiety behavior, whereas male animals exhibited risk-taking and exaggerated approach behaviors (Wojniusz et al., 2011). As the amygdala plays a central role in emotional processing (Zald, 2003), the present findings could suggest that sex specific gene expression changes due to GnRHa treatment are related to sex-specific emotional responses reported in the previous study (Wojniusz et al., 2011). The possibility that GnRHa treatment affected development of the amygdala is also supported by the results of morphometric analysis (using MRI) of the brains of these animals as this demonstrated that GnRHa treatment was associated with increased amygdala volume, however, this effect on volume was not limited to the females and was seen in both sexes (Nuruddin et al., in press). The sex specific changes in gene expression observed in the present study might indicate, therefore, that while GnRHa treatment has an effect at a macro-structural level in both sexes, at the molecular level the effect is more pronounced in the female. The present results, however, do not allow us to make definitive conclusions as the timing of the pubertal transition, and thus, the timing and duration of treatment differed between males and females in the current study.

4.2. Lateral effect of GnRHa treatment on gene expression in the amygdala

Interestingly, we observed a greater number of changes in gene (mRNA) expression in the left (438) compared to the right amygdala (46) in the GnRHa treated females lambs. This finding indicates a lateral effect of GnRHa treatment on gene expression within the amygdala that might cause or be involved in later functional behavioral differences. It is interesting to note that our MRI study (Nuruddin et al., in press) also revealed lateral in addition to sex effects in amygdala volume after treatment, the left amygdala volume in treated female being much larger than the right amygdala in the females and both left and right amygdala in the treated

males. The combination of our morphometric and gene expression studies, suggests that GnRHa treatment effects at the level of the amygdala may be more pronounced in females than males, and also that GnRHa treatment has a lateral effect on the development of the amygdala. Few studies have specifically addressed or described lateralization of amygdala function in either humans or animals. Hemispheric lateralization of amygdala function in relation to emotion regulation has been reported in human imaging studies where the left amygdala has been implicated as dominant in females (Cahill, 2006; Koscik et al., 2010). To our knowledge, this is the first study that shows a hemispheric lateralization of gene expression levels in the amygdala due to peri-pubertal GnRHa treatment. It is interesting to speculate that the hemispheric and sex-biased changes in gene expression levels in the amygdala, as a result of GnRHa treatment, might be related to the cognitive changes that we have previously reported (Wojniusz et al., 2011). These data further support the concept of hemispheric lateralization of amygdala function in sheep. However, such interpretation should be tempered by the fact that sheep and humans differs in terms of reproductive behavior and seasonality and thus physiological species differences exist that might preclude extrapolation of such data. Replication of this type of study is also needed to determine the mechanisms by which GnRHa affects amygdala function in sheep and to study anxiety and mood parameters in humans subject to such treatments. In our study, we used GO analysis (Michael et al., 2000) to gain knowledge about the biological process, cellular components and molecular functions of the genes that exhibited differential expression between hemispheres, sexes or as a result of treatment. This analysis is widely used for systemic assessment of the key functions and processed enriched in a set of genes identified by transcriptomic analysis. In addition; gene network analysis was used to visualize the biological relationships between genes which were differentially expressed as determined by the microarray analysis. These analyses indicated that relative to gene expression in the left amygdala the up-regulated APP and down-regulated UBC genes formed central nodes. This is of interest as a recent report has linked APP with proteins constituting the cytoskeleton network polymers, actin and microtubulin (Henriques et al., 2010) and our GO analysis also revealed that the biological function term 'microtubule' was significantly over represented among the up-regulated genes. While it is important to bear in mind that the results of such in silico analysis could be due to the many different gene interactions that could be occurring as a result of various experimental variables, the observed changes in APP gene expression are of functional interest. The APP gene encodes a transmembrane protein that is cleaved by β and γ reductase enzymes to generate amyloid beta (Aβ), the deposition of which occurs in the plaques characteristically seen in Alzheimer's disease patients (Sambamurti et al., 2002). Further, the APP gene has been implicated in detrimental changes in cognitive function associated with the amygdala, such as anxiety, fear and emotional problems, both in human Alzheimer patients and in a mice model of Alzheimer's disease (España et al., 2010). APP and human presenilin (APP/PS1) mutant mice also express abundant extracellular plaques, synaptic dysfunction and loss, astrocytosis, activation of microglia and cognitive deficits (Games et al., 1995). Recently, the morphological basis for amygdala-dependent cognitive impairment was studied using transgenic mice that express a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin1 (Ps1-d39) (Knafo et al., 2009). The results demonstrated that auditory fear conditioning, a learning task that depends on the amygdala, was clearly impaired in APP/PS1 mice (Knafo et al., 2009). The authors also found morphological alterations in the dendrites and spines of neurons in the amygdala. Spines are the main postsynaptic elements of excitatory synapses in the brain and are fundamental in memory, learning and cognition (Lamprecht and LeDoux, 2004). Thus, morphological changes in the dendrites and spines could underlie the cognitive impairment observed in the APP mutant mice. Additional rodent studies have also reported significantly increased expression of APP and Aβ in the basolateral amygdala, in response to environmental stressors and persistent anxiety and it has been suggested that these might be predisposing factor for Alzheimer's disease pathogenesis. Collectively these findings suggest that the amygdala is significantly and consistently affected by Aβ both in patients with AD and in mouse models of this disease and that the amygdala is a central participant in the pathology of AD (Unger et al., 1991). Furthermore that the frequent emotional, psychological and memory disturbances observed during AD may be due to molecular and structural changes in the amygdala which could be linked to hormonal change. Given the evidence of a role for APP within the amygdala during AD pathogenesis, it is perhaps surprising that the expression and functional involvement of the APP gene at the time of the pubertal transition, another time of known cognitive change, has not been described elsewhere. Further studies on *APP* gene function in the peri-pubertal stage are therefore warranted.

The other central node within the functional network derived from our results was Ubiquitin C. Studies that have investigated the role of ubiquitin molecules on target proteins suggest that these molecules play a role in numerous cellular processes including cell cycle regulation, signal transduction, gene transcription and synaptic plasticity (Hegde, 2010; Soh et al., 2010; Wickliffe et al., 2009). Recently, it has been suggested that in Alzheimer's disease, amyloid beta disrupts BDNF-mediated retrograde signaling by alterations to ubiquitin homeostasis (Poon et al., in press). This provides a functional connection between *APP*, neurotropic and ubiquitin genes and potentially links them with periods of known cognitive change.

It could be speculated that an increase in pubertal GnRH, androgen and estrogen release might affect the growth and development of the amygdala during puberty, or possibly, that GnRH along with sex hormones alter the neural substrates of the circuitry involved in growth and development of amygdala, as well as other brain structures. These speculations can be supported by the fact that GnRH and sex hormones receptors are abundant in limbic regions particularly in the amygdala and hippocampus (Beyenburg et al., 2000; Skinner et al., 2009). Our results clearly show differential expression of some genes within the amygdala, these changes are of particular note as mRNA extraction was from the entire amygdala and thus any observed changes must represent large scale changes in gene expression. Conversely, however, the use of this protocol means that small changes or

region specific changes in gene expression could have been missed, given the heterogeneous nature of this brain structure, therefore, further more targeted studies are necessary. Histological, cellular and proteomics studies should be combined in order to gain more insight into the molecular biological mechanisms that underlie these GnRHa-induced structural (Nuruddin et al., in press) and sex and side biased differential expression mRNA transcript.

4.3. Conclusion

Our results demonstrated that GnRHa treatment modulated specific facets of the development of amygdala, as indicated by sex and hemisphere specific gene expression in GnRHa treated compared to control animals. These novel findings demonstrate that changes in GnRH concentrations during puberty are likely to affect normal brain development and should be borne in mind when pharmacological blockade of GnRH action is used for treatment of young children.

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Conflict of interest

The authors have nothing to disclose.

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References

Albertson, A.J., Navratil, A., Mignot, M., Dufourny, L., Cherrington, B., Skinner, D.C., 2008. Immunoreactive GnRH type I receptors in the mouse and sheep brain. J. Chem. Neuroanat. 35, 326—333.

Anagnostou, E., Taylor, M., 2011. Review of neuroimaging in autism spectrum disorders: what have we learned and where we go from here. Mol. Autism. 2, 2—4.

Bao, A.M., Swaab, D.F., 2010. Sex differences in the brain, behavior, and neuropsychiatric disorders. Neuroscientist 16, 550—565.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Statist. Soc. B (Methodological) 57, 289—300.

Beyenburg, S., Watzka, M., Clusmann, H., Blümcke, I., Bidlingmaier, F., Elger, C.E., Stoffel-Wagner, B., 2000. Androgen receptor mRNA expression in the human hippocampus. Neurosci. Lett. 294, 25—28.

Bowen, R.L., Verdile, G., Liu, T., Parlow, A.F., Perry, G., Smith, M.A., Martins, R.N., Atwood, C.S., 2004. Luteinizing hormone, a reproductive regulator that modulates the processing of amyloid-β precursor protein and amyloid-β deposition. J. Biol. Chem. 279, 20539—20545.

Bryan, K.J., Mudd, J.C., Richardson, S.L., Chang, J., Lee, H.g., Zhu, X., Smith, M.A., Casadesus, G., 2010. Down-regulation of serum gonadotropins is as effective as estrogen replacement at improving menopause-associated cognitive deficits. J. Neurochem. 112, 870—881.

Burgus, R., Butcher, M., Amoss, M., Ling, N., Monahan, M., Rivier, J., Fellows, R., Blackwell, R., Vale, W., Guillemin, R., 1972. Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF). Proc. Natl. Acad. Sci. U.S.A. 69, 278—282.

Cahill, L., 2006. Why sex matters for neuroscience. Nat. Rev. Neurosci. 7, 477—484.

Carel, J.C., Eugster, E.A., Rogol, A., Ghizzoni, L., Palmert, M.R., on behalf of the members of the ESPE-LWPES GnRH Analogs Consensus Conference Group, 2009. Consensus statement on the use of gonadotropin-releasing hormone analogs in children. Pediatrics 123, e752—e762.

Casadesus, G., Webber, K.M., Atwood, C.S., Pappolla, M.A., Perry, G., Bowen, R.L., Smith, M.A., 2006. Luteinizing hormone modulates cognition and amyloid-β deposition in Alzheimer APP transgenic mice. Biochim. Biophys. Acta 1762, 447—452.

Casey, B.J., Jones, R.M., 2010. Neurobiology of the adolescent brain and behavior: implications for substance use disorders. J. Am. Acad. Child Adolesc. Psychiatry 49, 1189—1201.

Chow, M.L., Pramparo, T., Winn, M.E., Barnes, C.C., Li, H.R., Weiss, L., Fan, J.B., Murray, S., April, C., Belinson, H., Fu, X.D., Wynshaw-Boris, A., Schork, N.J., Courchesne, E., 2012. Age-dependent brain gene expression and copy number anomalies in autism suggest distinct pathological processes at young versus mature ages. PLoS Genet. 8, e1002592.

Chu, C., Xu, B., Huang, W., 2010. GnRH analogue attenuated apoptosis of rat hippocampal neuron after ischemia-reperfusion injury. J. Mol. Histol. 41, 387—393.

Coit, V.A., Dowell, F.J., Evans, N.P., 2009. Neutering affects mRNA expression levels for the LH- and GnRH-receptors in the canine urinary bladder. Theriogenology 71, 239—247.

De Bellis, M.D., Keshavan, M.S., Beers, S.R., Hall, J., Frustaci, K., Masalehdan, A., Noll, J., Boring, A.M., 2001. Sex differences in brain maturation during childhood and adolescence. Cereb. Cortex 11, 552—557.

Delemarre-van de Waal, H., Cohen-Kettenis, P.T., 2006. Clinical management of gender identity disorder in adolescents: a protocol on psychological and paediatric endocrinology aspects. Eur. J. Endocrinol. 155, S131—S137.

Dong, F., Skinner, D.C., John Wu, T., Ren, J., 2011. The heart: a novel gonadotrophin-releasing hormone target. J. Neuroendocrinol. 23, 456—463.

Doraiswamy, P.M., Xiong, G.L., 2006. Pharmacological strategies for the prevention of Alzheimer's disease. Expert Opin. Pharmacother. 7, 1—10.

Dumas, T.C., 2005. Developmental regulation of cognitive abilities: modified composition of a molecular switch turns on associative learning. Progr. Neurobiol. 76, 189—211.

España, J., Giménez-Llort, L., Valero, J., Miñano, A., Rábano, A., Rodriguez-Alvarez, J., LaFerla, F.M., Saura, C.A., 2010. Intraneuronal β-amyloid accumulation in the amygdala enhances fear and anxiety in Alzheimer's disease transgenic mice. Biol. Psychiatry 67, 513—521.

Evans, N.P., Robinson, J.E., Erhard, H.W., Ropstad, E., Fleming, L.M., Haraldsen, I.R.H., 2012. Development of psychophysiological motoric reactivity is influenced by peripubertal pharmacological inhibition of gonadotropin releasing hormone action—results of an ovine model. Psychoneuroendocrinology 37, 1876—1884.

Fu, X., Giavalisco, P., Liu, X., Catchpole, G., Fu, N., Ning, Z.B., Guo, S., Yan, Z., Somel, M., Pääbo, S., Zeng, R., Willmitzer, L., Khaitovich, P., 2011. Rapid metabolic evolution in human prefrontal cortex. Proc. Natl. Acad. Sci. U.S.A. 108, 6181—6186.

Games, D., Adams, D., Alessandrini, R., Barbour, R., Borthelette, P., Blackwell, C., Carr, T., Clemens, J., Donaldson, T., Gillespie, F., Guido, T., Hagopian, S., Johnson-Wood, K., Khan, K., Lee, M., Leibowitz, P., Lieberburg, I., Little, S., Masliah, E., McConlogue, L., Montoya-Zavala, M., Mucke, L., Paganini, L., Penniman, E., Power, M., Schenk, D., Seubert, P., Snyder, B., Soriano, F., Tan, H., Vitale, J., Wadsworth, S., Wolozin, B., Zhao, J., 1995. Alzheimer-type neuropathology in transgenic mice overexpressing V717F [beta]-amyloid precursor protein. Nature 373, 523—527.

Garcia-Crespo, D., Juste, R., Hurtado, A., 2005. Selection of ovine housekeeping genes for normalisation by real-time RT-PCR; analysis of PrP gene expression and genetic susceptibility to scrapie. BMC Vet. Res. 1, 3.

Granger, A., Ngô-Muller, V., Bleux, C., Guigon, C., Pincas, H., Magre, S., Daegelen, D., Tixier-Vidal, A., Counis, R., Laverriére, J.N., 2004. The promoter of the rat gonadotropin-releasing hormone receptor gene directs the expression of the human placental alkaline phosphatase reporter gene in gonadotrope cells in the anterior pituitary gland as well as in multiple extrapituitary tissues. Endocrinology 145, 983—993.

Grigorova, M., Sherwin, B.B., Tulandi, T., 2006. Effects of treatment with leuprolide acetate depot on working memory and executive functions in young premenopausal women. Psychoneuroendocrinology 31, 935—947.

Harrison, G.S., Wierman, M.E., Nett, T.M., Glode, L.M., 2004. Gonadotropin-releasing hormone and its receptor in normal and malignant cells. Endocr.-Relat. Cancer 11, 725—748.

Haour, F., Dussaillant, M., Leblanc, P., Rostène, W., 1987. Demonstration and topographical distribution of LHRH receptors in the central nervous system in the normal and castrated male rat. C. R. Acad. Sci. III 305, 41—44.

Hegde, A.N., 2010. The ubiquitin-proteasome pathway and synaptic plasticity. Learn. Mem. 17, 314—327.

Henriques, A.G., Vieira, S.I., Da Cruz e Silva, E., Da Cruz e Silva, O., 2010. Aβ promotes Alzheimer's disease-like cytoskeleton abnormalities with consequences to APP processing in neurons. J. Neurochem. 113, 761—771.

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2008. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44—57.

Kendziorski, C., Irizarry, R.A., Chen, K.S., Haag, J.D., Gould, M.N., 2005. On the utility of pooling biological samples in microarray experiments. Proc. Natl. Acad. Sci. U.S.A. 102, 4252—4257.

Koscik, T., Bechara, A., Tranel, D., 2010. Sex-related functional asymmetry in the limbic brain. Neuropsychopharmacology 35, 340—341.

Kostovic, I., Judaš, M., 2006. Prolonged coexistence of transient and permanent circuitry elements in the developing cerebral cortex of fetuses and preterm infants. Dev. Med. Child Neurol. 48, 388—393.

Knafo, S., Venero, C., Merino-Serrais, P., Fernaud-Espinosa, I., Gonzalez-Soriano, J., Ferrer, I., Santpere, G., DeFelipe, J., 2009. Morphological alterations to neurons of the amygdala and impaired fear conditioning in a transgenic mouse model of Alzheimer's disease. J. Pathol. 219, 41—51.

König, R., Baldessari, D., Pollet, N., Niehrs, C., Eils, R., 2004. Reliability of gene expression ratios for cDNA microarrays in multiconditional experiments with a reference design. Nucleic Acids Res. 32, e29.

Lamprecht, R., LeDoux, J., 2004. Structural plasticity and memory. Nat. Rev. Neurosci. 5, 45—54.

Liu, X., Somel, M., Tang, L., Yan, Z., Jiang, X., Guo, S., Yuan, Y., He, L., Oleksiak, A., Zhang, Y., Li, N., Hu, Y., Chen, W., Qiu, Z., Pääbo, S., Khaitovich, P., 2012. Extension of cortical synaptic development distinguishes humans from chimpanzees and macaques. Genome Res. 22, 611—622.

Luna, B., Thulborn, K.R., Munoz, D.P., Merriam, E.P., Garver, K.E., Minshew, N.J., Keshavan, M.S., Genovese, C.R., Eddy, W.F., Sweeney, J.A., 2001. Maturation of widely distributed brain function subserves cognitive development. NeuroImage 13, 786—793.

Maren, S., Quirk, G.J., 2004. Neuronal signalling of fear memory. Nat. Rev. Neurosci. 5, 844—852.

Matsuo, H., Baba, Y., Nair, R.M.G., Arimura, A., Schally, A.V., 1971. Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. Biochem. Biophys. Res. Commun. 43, 1334—1339.

Michael, A., Catherine, A.B., Judith, A.B., David, B., Heather, B., Michael, C., Allan, P.D., Kara, D., Selina, S.D., Janan, T.E., Midori, A.H., David, P.H., Laurie, I.-T., Andrew, K., Suzanna, L., John, C.M., Joel, E.R., Martin, R., Gerald, M.R., Gavin, S., 2000. Gene Ontology: tool for the unification of biology. Nat. Genet. 25, 25—29.

Millan, M.J., 2003. The neurobiology and control of anxious states. Prog. Neurobiol. 70, 83—244.

Millar, R.P., 2005. GnRHs and GnRH receptors. Anim. Reprod. Sci. 88, 5—28.

Morris, J.S., Friston, K.J., Büchel, C., Frith, C.D., Young, A.W., Calder, A.J., Dolan, R.J., 1998. A neuromodulatory role for the human amygdala in processing emotional facial expressions. Brain 121, 47—57.

Nelson, C.J., Lee, J.S., Gamboa, M.C., Roth, A.J., 2008. Cognitive effects of hormone therapy in men with prostate cancer. Cancer 113, 1097—1106.

Nuruddin, S., Bruchhage, M., Ropstad, E., Krogenæs, A., Evans, N.P., Robinson, J.E., Endestad, T., Westlye, L.T., Madison, C., Haraldsen, I.R.H., in press. Effects of peripubertal gonadotropin-releasing hormone agonist on brain development in sheep—a magnetic resonance imaging study. Psychoneuroendocrinology, http://dx.doi.org/10.1016/j.psyneuen.2013.03.009 (in press).

Nuruddin, S., Wojniusz, S., Ropstad, E., Krogenæs, A., Evans, N.P., Robinson, J.E., Solbakk, A.K., Amiry-Moghaddam, M., Haraldsen, I.R.H., 2013. Peri-pubertal gonadotropin-releasing hormone analog treatment affects hippocampus gene expression without changing spatial orientation in young sheep. Behav. Brain Res. 242, 9—16.

Paterson, S.J., Heim, S., Thomas Friedman, J., Choudhury, N., Benasich, A.A., 2006. Development of structure and function in the infant brain: implications for cognition, language and social behaviour. Neurosci. Biobehav. Rev. 30, 1087—1105.

Poon, W.W., Carlos, A.J., Aguilar, B.L., Berchtold, N.C., Kawano, C., Zograbyan, V., Yaopruke, T., Shelanski, M., Cotman, C.W., in press. Aβ oligomers impair BDNF retrograde trafficking by down-regulating ubiquitin-C-terminal hydrolase, UCH-L1. J. Biol. Chem. (in press).

Reichler, I.M., Welle, M., Eckrich, C., Sattler, U., Barth, A., Hubler, M., Nett-Mettler, C.S., Jöchle, W., Arnold, S., 2008. Spaying-induced coat changes: the role of gonadotropins, GnRH and GnRH treatment on the hair cycle of female dogs. Vet. Dermatol. 19, 77—87.

Rubenstein, J.L.R., 2011. Annual Research Review: development of the cerebral cortex: implications for neurodevelopmental disorders. J. Child Psychol. Psychiatry 52, 339—355.

Sambamurti, K., Greig, N., Lahiri, D., 2002. Advances in the cellular and molecular biology of the beta-amyloid protein in Alzheimer's disease. Neuromol. Med. 1, 1—31.

Schang, A.L., Ngô-Muller, V., Bleux, C., Granger, A., Chenut, M.C., Loudes, C., Magre, S., Counis, R., Cohen-Tannoudji, J., Laverriére, J.N., 2011. GnRH receptor gene expression in the developing rat hippocampus: transcriptional regulation and potential roles in neuronal plasticity. Endocrinology 152, 568—580.

Shaw, P., Greenstein, D., Lerch, J., Clasen, L., Lenroot, R., Gogtay, N., Evans, A., Rapoport, J., Giedd, J., 2006. Intellectual ability and cortical development in children and adolescents. Nature 440, 676—679.

Sisk, C.L., Foster, D.L., 2004. The neural basis of puberty and adolescence. Nat. Neurosci. 7, 1040—1047.

Skinner, D.C., Albertson, A.J., Navratil, A., Smith, A., Mignot, M., Talbott, H., Scanlan-Blake, N., 2009. Effects of gonadotrophin-releasing hormone outside the hypothalamic-pituitary-reproductive axis. J. Neuroendocrinol. 21, 282—292.

Schally, A.V., Comaru-Schally, A.M., Nagy, A., Kovacs, M., Szepeshazi, K., Plonowski, A., Varga, J.L., Halmos, G., 2001. Hypothalamic hormones and cancer. Front. Neuroendocrinol. 22, 248—291.

Smyth, G.K., Michaud, J., Scott, H.S., 2005. Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics 21, 2067—2075.

Smyth, G.K., Speed, T., 2003. Normalization of cDNA microarray data. Methods 31, 265—273.

Soh, U.J., Dores, M.R., Chen, B., Trejo, J., 2010. Signal transduction by protease-activated receptors. Br. J. Pharmacol. 160, 191—203.

Thomas, K.M., Drevets, W.C., Dahl, R.E., Ryan, N.D., Birmaher, B., Eccard, C.H., Axelson, D., Whalen, P.J., Casey, B.J., 2001. Amygdala response to fearful faces in anxious and depressed children. Arch. Gen. Psychiatry 58, 1057—1063.

Unger, J.W., Lapham, L.W., McNeill, T.H., Eskin, T.A., Hamill, R.W., 1991. The amygdala in Alzheimer's disease: neuropathology and Alz 50 immunoreactivity. Neurobiol. Aging 12, 389—399.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A.M., 2007. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. 35, W71—W74.

Vyas, A., Jadhav, S., Chattarji, S., 2006. Prolonged behavioral stress enhances synaptic connectivity in the basolateral amygdala. Neuroscience 143, 387—393.

Wang, L., Chadwick, W., Park, S., Zhou, Y., Silver, N., Martin, B., Maudsley, S., 2010. Gonadotropin-releasing hormone receptor system: modulatory role in aging and neurodegeneration. CNS Neurol. Disord. Drug Targets 9, 651—660.

Wickliffe, K., Williamson, A., Jin, L., Rape, M., 2009. The multiple layers of ubiquitin-dependent cell cycle control. Chem. Rev. 109, 1537—1548.

Wilson, A.C., Salamat, M.S., Haasl, R.J., Roche, K.M., Karande, A., Meethal, S.V., Terasawa, E., Bowen, R.L., Atwood, C.S., 2006. Human neurons express type I GnRH receptor and respond to GnRH I by increasing luteinizing hormone expression. J. Endocrinol. 191, 651—663.

Wojniusz, S., Vögele, C., Ropstad, E., Evans, N., Robinson, J., Sütterlin, S., Erhard, H.W., Solbakk, A.K., Endestad, T., Olberg, D.E., Haraldsen, I.R.H., 2011. Prepubertal gonadotropin-releasing hormone analog leads to exaggerated behavioral and emotional sex differences in sheep. Horm. Behav. 59, 22—27.

Wood, R.I., Foster, D.L., 1998. Sexual differentiation of reproductive neuroendocrine function in sheep. Rev. Reprod. 3, 130—140.

Zald, D.H., 2003. The human amygdala and the emotional evaluation of sensory stimuli. Brain Res. Rev. 41, 88—123.

IV

Down-regulation of elevated gonadotropin-releasing hormone gene expression in hippocampus of tg-ArcSwe mice following receptor analog treatment

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Abstract

Research on Alzheimer's disease (AD) has indicated an association between hormones of the hypothalamic–pituitary–gonadal (HPG) axis and cognitive senescence, indicating that post meno-/andro-pausal changes in HPG axis hormones are implicated in neuropathological changes in AD. Studies of transgenic mice with AD pathologies have led to improved understanding of the pathophysiological processes underlying AD. The aim of this study was to investigate the effect of a Gonadotropin releasing hormone analog (Gnrh-a; Leuprorelin acetate) on amyloid plaque deposition and mRNA expression of Gnrh and its receptor (Gnrhr) in transgenic mice carrying the Arctic and Swedish amyloid-β precursor protein (AβPP) mutations (tg-ArcSwe).

Tg-ArcSwe mice were injected subcutaneously with the Gnrh-a once every fourth week from the age of 4 months until 12 months. Quantitative real time PCR for gene expression was performed in hippocampus, while immunohistochemistry analysis was performed on hippocampus, cerebral cortex, and hypothalamus. In both sexes, Gnrh-a treatment caused significant down-regulation of Gnrh and Gnrhr expression in tg-ArcSwe relative to vehicle treated tg-ArcSwe at the same age. Furthermore, immunohistochemistry and quantitative image analysis showed no significant changes in the plaque load after Gnrh-a treatment in hippocampus and thalamus. However, plaque load in the cerebral cortex of treated females tended to be lower than in female vehicle-treated mice. The present study points to the involvement of hormonal changes in AD and the prospect of mitigating these through targeted treatment. Although known to increase in normal aging, our study shows that *Gnrh/Gnrhr* mRNA expression increases even more with amyloid-β deposition in Tg-ArcSwe mice. Further, treatment with Leuprorelin acetate alters hippocampal gene expression both at the hormone and receptor level. However, the effect on amyloid plaque development remains uncertain in this model.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia. The disease affects more than 35 million people worldwide [1] and each year 4.6 million new cases are diagnosed [2]. Although much is known about the neuropathology of AD, the etiology remains unclear and currently there is no cure for this neurodegenerative disease.

It is assumed that AD pathogenesis perturbs signal transduction pathways and that this contributes to neurodegeneration. Alterations in different neurotransmitter systems are well established, perhaps most clearly affecting cholinergic and glutamatergic pathways [3]. Misprocessing of amyloid-β precursor protein (AβPP) plays a pivotal role in inherited forms of AD [4], but oxidative, inflammatory [5] and hormonal processes [6,7] might additionally and significantly contribute to the pathogenesis.

Hormonal mechanisms underlying AD development have gained renewed interest. It was recently reported that gonadotropin-releasing hormone (Gnrh) induces adult neurogenesis in several brain regions typically afflicted by AD neuropathology [8]. Furthermore, Gnrh agonist (Gnrh-a) therapy has been shown to decelerate aging in animals [8] and reduce the risk of developing AD in prostate cancer patients [9].

Gnrh is a decapeptide hormone. There are three forms of Gnrh (1, 2 and 3), of which Gnrh1 and 2 are present in reptiles, birds and mammals. Gnrh1 and 2 are not only expressed in the hypothalamus but also in other brain regions e.g. caudate nucleus, hippocampus and amygdala [10–12]. Based on the wide distribution of Gnrh, this hormone is likely to have roles beyond its endocrine function, possibly serving as a neurotransmitter or a modulator of neuroplasticity [13–15]. There is only one single functional Gnrh receptor (Gnrhr) in most species, including mice.

Gnrhr is a unique rhodopsin-like G protein-coupled receptor that appears to mediate a wide variety of Gnrh1 and 2 signaling mechanisms [16].

Furthermore, recent research has elucidated cognitive and physiological effects of Gnrh [17–19]. Results from mice and human studies of both sexes indicate that modulation of Gnrh and its receptor using a Gnrh analog (Gnrh-a) may lead to significant changes in cognitive functions [17, 20, 21].

The hippocampal region is most vulnerable to AD and turns out to be particularly rich in Gnrh receptors [12, 16]. Therefore in this study, we have focused on Gnrh and receptor gene regulation in the hippocampus.

It is proposed that estrogen has a modifying effect on Alzheimer's disease [22] and as Gnrh is elevated post-menopause due to the loss of estrogen feedback, Gnrh may have a direct effect on neurodegeneration [12]. Therefore, the aim of our study was to investigate the effect of Leuprorelin acetate, a Gnrh-analog, on Gnrh and Gnrhr mRNA-expression, as well as on amyloid-β (Aβ) deposition in transgenic mice expressing amyloid-β precursor protein (AβPP) mutations with the Arctic and Swedish mutations (Tg-ArcSwe), an established model of AD [23,24].

2. Materials and methods

Animal model:

In this study we used transgenic mice carrying a human AβPP cDNA with the Arctic (E693G) and Swedish (KM670/671NL) mutations. The animals were housed under standard conditions (12 h dark/light cycles) with unrestricted access to food and water, and sacrificed after 4 or 12 months. All animal procedures were in accordance with the National Institutes of Health Guide

for the care and use of laboratory animals (FELASA) and approved by the Biological Research Ethics Committee in Norway (FOTS/3209).

Animals (Tg-ArcSwe and wild–type mice) were injected subcutaneously with 25ng/g of the Gnrh-analog Leuprorelin acetate (Procren Depot "AbbVie") dissolved in physiological saline, or vehicle alone. The injections were given once every fourth week from the age of 4 months, before plaque deposition has begun [24]. Of the animals included in the study, about 20 % of the animals died of unknown causes before reaching the age of 12 months. This included both treated and untreated animals, leaving us with the number of animals referred to in table 1. The remaining animals were sacrificed at 12 months, after 8 months of treatment, and used for quantitative real time PCR (qRT-PCR) and immunohistochemical investigations. In addition to this, gene expression was analyzed with qRT-PCR in 4 months-old animals who did not receive any pharmacological intervention. Details are shown in Table 1.

Tissue processing:

Animals were anesthetized using Isofluran Baxter (Isoflo™, Abbot Laboratories, Abbot Park, IL, USA) and sacrificed at 4 or 12 months by decapitation. The brain was extracted, and divided into its two hemispheres and freezed using Nordfjord® cool spray (Norden Olje, Ski, Norway) and dry ice. The tissue was stored at -80 ºC until further use. The right hemisphere was used for immunohistochemistry, while hippocampus from the left hemisphere was used for qRT-PCR.

RNA Isolation:

Total RNA was isolated from the hippocampus with TRIzol Reagent (InvitrogenTM, Paisley, UK). For each sample, 11-14 mg of frozen tissue was homogenized in 1 ml of Trizol reagent using MagNA Lyser Green Beads (Roche Diagnostics, Mannheim, Germany) and Retsch MM

301 mixer mill (Retsch GmbH, Haan, Germany). Purified RNA was dissolved in 50 µl of RNase-free water (Qiagen, Crawley, UK) followed by DNase I treatment (RNase-Free DNase Set, Qiagen, Crawley, UK) for 20 minutes at room temperature, immediately followed by purification using RNeasy Mini-Kit (Qiagen, Crawley, UK) according to the manufacturer's recommendations. The concentration and quality of the RNA were determined using NanoDrop (Thermo-Scientific, Waltham, MA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. RNA was stored at -80^{o}C until being further processed.

Quantitative RT-PCR (qRT-PCR):

To predict the most stable reference gene under the present study condition [25], the GeNorm-method was used. GeNorm human detection kit and software was obtained from PrimerDesign Ltd (Southampton, UK). Of the 6 reference genes tested phosphoglycerate kinase 1 (*Pgk1*), ribosomal protein S18 (*Rps18*), beta-2 microglobulin (*B2m*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), glucuronidase, beta (*Gusb*), transferrin receptor (*Tfrc*), the two most stable genes; *B2m* and *Gusb* were selected as reference genes in this study.

Genes included in the gene expression analyses are listed in Table 2. Primer sequences for *Gnrh1, Gnrhr, B2m* and *Gusb* were designed by using primer-3 plus, a web-based primer designing tool [26]. All primers were synthesized by Sigma-Aldrich (St.Louis, MO, USA). Specificities of all primer pairs were checked using nucleotide BLAST and primer BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Primer annealing temperature and cDNA concentration were optimized before the experiment was carried out. All products were run on an agarose-ethidium bromide gel as to verify distinct bands of a size matching the intended product in the absence of primer-dimer formations.

cDNA synthesis and qRT-PCR were performed using superscript III platinum Two-Step qRT-PCR kit with SYBR Green (Invitrogen, Carlsbad, CA, USA) according to the protocol recommended by the manufacturer. A Peltier Thermal Cycler-225 (MJ Research, Waltham, MA, USA) was used to synthesize cDNA, and qRT-PCR was done with an ABI PRISM 7900 Sequence Detector System (PE Applied Biosystems, CA, USA). Technical duplicates of RNA samples, negative controls without reverse transcriptase as well as negative controls devoid of an RNA template all underwent cDNA synthesis. 1 µg of total RNA was used to synthesize cDNA (10 min at 25°C, 50 min at 42°C and 5 min at 85°C). The reaction mixture (20 µl) contained 3 µl of cDNA (15 ng), 10 µl of Platinum SYBR Green qRT-PCR Super Mix-UDG, 0.2µl of ROX reference dye, 1µl of 10µmol of forward and 1µl of 10 µmol of reverse primers, and 4.8 µl of DNase/RNase-free water (Invitrogen). Cycling conditions were: initial denaturation at 95°C for 2 min; 40 amplification cycles were at 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s. Each assay was performed in quadruplicate, and three negative controls were run for every assay: no template (sample lacking cDNA), no reverse transcriptase, and no RNA in reverse transcriptase reaction. The absence of primer-dimers, genomic DNA, and other DNA contaminations was also monitored by melting curve analysis at the end of each run (ABI PRISM 7900 manufacturer's recommended default settings).

qRT-PCR analysis:

The ΔCt was calculated from the difference in expression between the gene of interest (*Gnrh1 and Gnrhr*) and mean expression of the two reference genes (*Gusb and B2m*). To investigate the effect of genetic modification in transgenic mice at 4 months and 12 months, the ΔΔCt was calculated by the difference between the ΔCt value of wild type-mice (WT-mice) and tg-ArcSwe mice samples, within both sexes. Furthermore, in order to assess any effects of treatment at 12

155 months of age, the ΔΔCt was calculated as the difference between the ΔCt value of vehicle-
156 treated tg-ArcSwe mice and Leuprorelin acetate treated tg-ArcSwe mice samples, within both
157 sexes. Relative gene expression expressed as fold change was calculated by using $2^{-\Delta\Delta Ct}$.

158 ***Immunohistochemistry:***

159 Sagittal cryosections (25 µm; Leica CM3050 S) from the right hemisphere were stored at -20 ºC.
160 All sections were post–fixed with 4% formaldehyde (PFA) for 5 minutes, pretreated with 80 %
161 formic acid for 2 minutes and 2% H_2O_2 for 7 minutes. After washing with 10 mM PBS, pre -
162 incubation solution (10% normal goat serum (NGS), 1% bovine serum albumin (BSA), 0.5%
163 Triton X-100 in 10 mM PBS) was applied to the sections for 30 minutes at room temperature.
164 Afterwards, the sections were incubated with an Aβx-40-specific polyclonal primary antibody
165 (0.5 µg/ml; Agrisera, Umeå, Sweden) diluted 1:2000 in primary antibody solution (3% NGS, 1%
166 BSA, 0.5% Triton X-100 in 10 mM PBS) at 4˚C overnight. The antisera was generated and
167 evaluated for specificity as described [23,27].

168 After washing steps, the sections were incubated for 1 hour with a biotinylated goat-anti-rabbit
169 antibody (BA-1000, Vector Laboratories, CA, USA) diluted at 1:300 in 3% NGS, 1% BSA,
170 0.5% Triton X-100 in 10 mM PBS, washed in 10 mM PBS, and afterwards, incubated 1 hour at
171 room temperature with streptavidin-biotinylated horseradish peroxidase complex diluted at 1:100
172 in 0.5% Triton X-100 in 10 mM PBS. After washing with 10 mM PBS, all sections were
173 incubated with 3,3'-diaminobenzidine tetrahyprochloride (Sigma, MO, USA) for 5 minutes,
174 before 0.1% H_2O_2 was added to 10 ml DAB solution and applied on the sections until proper
175 labeling was achieved (3 minutes). The sections were briefly rinsed in water, mounted and stored
176 at room temperature.

Image acquisition and quantitative analyses:

An automated slide scanner system (Mirax Scan, Carl Zeiss MicroImaging GmbH, Jena, Germany) was used for acquiring high-resolution TIFF images with a spatial resolution of 0.205 µm/pixel. Images were inspected by virtual microscopy using the Panoramic viewer software (3D Histech, Budapest, Hungary). Using the export functionality of the Mirax Viewer, images were scaled 1:16 (to a spatial resolution of 3.28 µm/pixel). To compensate for differences in background color intensity following immunohistochemistry, all image histograms were normalized using the match-color algorithm in Adobe Photoshop CS6 with a photomicrograph of a wild-type section as reference [28]. Afterwards, quantitative image analysis was performed in three regions of interest (ROIs) using ImageJ 1.46r (http://imagej.nih.gov/ij). The outlines of the ROIs (cerebral cortex, thalamus, and hippocampus) were manually delineated in each section. Anterior delineation in the cerebral cortex was a line connecting the rhinal fissure and anterior tip of the external capsule. Dorsally it was delineated by the external surface of the brain, ventrally by the external capsule, and at posterior level by the dorsal subiculum. The anterior, ventral, and dorsal boundaries of the thalamus were defined by the surrounding white matter in the fimbria, internal capsule and cerebral peduncle and the hippocampus, while the posterior boundary was approximated by connecting the brachium of the superior colliculus and cerebral peduncle with a curved line encompassing geniculate nucleus. This delineation may also include the subthalamic nuclei. The delineation of the hippocampus included the three cornu ammonis subfields (CA1, CA2, and CA3), the dentate gyrus, and subiculum, and was at anterior level defined against the fimbria, dorsally and posteriorly against the external capsule, and ventrally against the thalamus. Images were binarized by selecting a threshold value in Image J, which yielded boundary definitions best corresponding to the observed plaque boundaries. The same

threshold value was used for all sections. The area of each ROI and the area of labeled objects within each ROI were calculated, and the as area fraction in percent (labeled area/ ROI area x 100) was used as a measure of plaque load. For each animal the mean area fractions of three sections were used for the final analysis.

Statistical analysis:

For immunohistochemistry, ANOVA assay and paired t-test in InStat® (GraphPad Softwear, San Diego, CA, USA) were used to analyze amyloid plaque deposition in different groups (Control vs treated). A P-value less than 0.05 was considered to indicate a statistically significant difference.

For gene expression, the log2 transformed fold change values ($2^{-\Delta\Delta Ct}$) were used for statistical analysis by applying JMP 10.0 software (SAS Institute Inc, Cary, NC, USA). Differences in gene expression were evaluated by Wilcoxon signed rank test.

3. Results

Gene expression:

To investigate the association between *Gnrh1/Gnrhr* and AD we assessed the gene expression levels in hippocampus of tg-ArcSwe mice at 4 months and 12 months. In addition, we analyzed the gene expression level at 12 months after 8 months of treatment with Leuprorelin acetate (25 ng/g). The results are presented in Figure 1. Our gene expression analyses show that at 12 months of age, both *Gnrh1* ($p<0.001$ in both sexes) and *Gnrhr* ($p<0.01$ male and $p<0.001$ female) mRNA expression were significantly elevated in tg-ArcSwe compared to age-matched WT in both sexes. Furthermore, gene expression analysis of 12 months old WT mice relative to 4 months old WT mice revealed elevated expression of Gnrh1 (fold change 2.56, $p<0.05$ for male

and fold change 2.33, $p<0.05$ for female) and Gnrhr (fold change 3.21, $p<0.05$ for male and fold change 3.56, $p<0.05$ for female).Strikingly, after 8 months of treatment with Leuprorelin acetate, gene expression of both *Gnrh1* ($p<0.001$ male and $p<0.01$ female) and *Gnrhr* ($p<0.001$ in both sexes) were down-regulated comparing Leuprorelin acetate-treated tg-ArcSwe to vehicle-treated tg-ArcSwe at the same age. At 4 months no significant differences were observed. All fold change values, standard errors and p-values are presented in Table 2.

Immunohistochemistry:

The amount of Aβx-40 labeling was determined by quantitative image analysis. Estimates of relative amyloid-beta (Aβ) deposition in cerebral cortex, hippocampus and thalamus of 12 months-old tg-ArcSwe mice are listed in Figure 2. The average plaque load in the hippocampus of 12-month-old female and male tg-ArcSwe mice that received Leuprorelin acetate (7 female and 3 male mice), compared to untreated sex- and age-matched tg-ArcSwe mice (4 female and 6 male mice), did not significantly differ (Figure 3). However, we found a tendency of lower plaque load in the cerebral cortex of treated females ($p=0.06$) compared to female vehicle-treated. Additional comparisons of plaque load in cerebral cortex and thalamus in the same animal groups did not reveal any differences.

4. Discussion

This study provides the first evidence that *Gnrh1* and *Gnrhr* are significantly up-regulated in 12 months old tg-ArcSwe mice compared to WT. We further demonstrate that treatment with Leuprorelin acetate effectively down regulates *Gnrh1* and *Gnrhr* mRNA expression in tg-ArcSwe mice relative to age-matched vehicle-treated tg-ArcSwe mice. Interestingly, at young age (4 months old animals) there was no significant differences in *Gnrh1* and *Gnrhr* mRNA

expression between transgenic and WT-mice (prior to treatment and onset of plaque deposition in this animal model). Nevertheless, despite the known importance of the hippocampal plaque deposition in AD pathology [29], we did not find any significant change in hippocampal plaque load after 8 months treatment with Leuprorelin acetate between the groups. Furthermore, we did not observe quantitative differences in plaque load in the cerebral cortex and thalamus between the groups. The interpretation and implication of these findings are discussed below.

It has been reported that the hippocampal Gnrh system is acutely sensitive to both age and reproductive status, as Gnrhr expression is increasing in ageing rats [30]. This result is in concordance with our study in mice, in which we found increased mRNA expression of *Gnrh1* and *Gnrhr* in 12 months WT compared to 4 months WT. Our findings of increased *Gnrh1/Gnrhr* expression in 12 months old AD-mice relative to age-matched WT show that *Gnrh1/Gnrhr* expression increases even further in AD-mice. This is in line with the idea that Gnrh/Gnrhr contributes to the development of AD pathology and studies reporting that Gnrh/Gnrhr influences hippocampal synaptic activity and impacts central nervous system physiology as well as pathophysiology [16, 31]. However, it remains to resolve whether this is a direct influence of Aβ-pathology or a consequence of secondary pathology, e.g. micro-/astrogliosis with release of substances that increase *Gnrh/Gnrhr* expression in hippocampus.

The potential of Gnrh-a treatment was first demonstrated in a mouse study in which treatment lowered hippocampal plaque load and prevented AD-related cognitive dysfunctions [32,33]. Additionally, it has been described that Gnrh-a treatment reduced Aβ concentration in total brain after 2 months of treatment in C57BL/6 mice [32]. This finding suggests that Gnrh-a treatment decreases Aβ levels by suppressing serum gonadotropins [32]. Further, luteinizing hormone (LH) promoted AβPP-processing towards the amyloidogenic pathway in a neuroblastoma cell line, as

evidenced by increased Aβ-formation and secretion [32]. Another study, in which very old mice (Tg2576, carrying the Swedish AβPP mutation) were treated with a Gnrh-a for two months, resulted in decreased hippocampal Aβ-deposition and improved cognitive functions among the aged transgenic mice [33]. Gnrh-a therapy reduced LH by down-regulating Gnrhr expression in the anterior pituitary [34]. Because individuals with AD show a two-fold increase in circulating gonadotropins (LH and Follicle stimulating hormone) compared with age-matched controls, gonadotropins have received increased attention over the last years [35–37]. Mechanistically, Gnrh has been suggested to promote the reactivation of mitotic signaling pathways that occurs early in AD pathogenesis. Although LH might mediate these effects, it is also possible that Gnrh-a treatment mediates its effects directly [38].

Based on these findings, we expected that Leuprorelin acetate would modulate *Gnrh1* and *Gnrhr* expression in tg-ArcSwe, and also exert an impact on amyloid plaque load in the animals. However, we did not record any significant differences in plaque load between groups after 8 months of treatment, although we observed a tendency of decreased cortical plaque load in treated females compared to vehicle-treated controls. Our recent study of plaque load variability in Tg-ArcSwe mice [23] indicates that it would have been possible to detect a major change in plaque load , while smaller changes might have been missed due to insufficient sample size. The fact that tg-ArcSwe is a stronger genetically driven model than, e.g., Tg2576 might also explain differences in therapeutic effects. The modifying effect of Leuprorelin acetate could be relatively weaker than the (trans)-genetic effect in tg-ArcSwe and thereby difficult to detect.

In this study we used an Aβx-40 antibody to assess amyloid burden. It has previously been demonstrated that Aβ antibodies stain the same, homogeneous population of cored Aβ-plaques in the tg-ArcSwe model, irrespective of whether the antibodies recognize the N- or C-terminal

epitopes in Aβ [23,24]. Therefore in tg-ArcSwe mice the 6E10, Aβx-40 or Aβx-42 antibodies will produce similar labeling pattern and indicate the same Aβ burden, at least at the light-microscopic level. This probably reflects the fact that the Arctic AβPP mutation makes Aβ-peptides far more prone to aggregate, including Aβ1-40 [23, 39].

The present gene expression analyses were restricted to a single hormone and its receptor. Obviously, this confers a limitation on the conclusions that can be made. The present findings warrant further studies using microarray gene expression profiling along with proteomics analyses to gain a deeper insight in the hormonal processes that affect the development of AD.

In conclusion, *Gnrh1* and its receptor are overexpressed in 12 months old tg-ArcSwe compared to WT. Leuprorelin acetate treatment was shown to affect the expression of the gene encoding the hormone receptor as well as the gene encoding the hormone itself, consistent with down-regulation of endogenous endocrine systems. The present study brings to the fore the involvement of hormonal changes in AD and the prospect of mitigating these through targeted treatment.

5. Acknowledgement

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References

1. Wimo A, Jönsson L, Bond J, Prince M, Winblad B (2013) The worldwide economic impact of dementia 2010. Alzheimers Dement 9: 1-11.

2. Wimo A, Winblad B, Jönsson L (2007) An estimate of the total worldwide societal costs of dementia in 2005. Alzheimers Dement 3: 81-91.

3. Francis PT, Parsons CG, Jones RW (2012) Rationale for combining glutamatergic and cholinergic approaches in the symptomatic treatment of Alzheimer¢s disease. Expert Rev Neurotherapeutics 12: 1351-1365.

4. Selkoe DJ (1997) Alzheimer's Disease--Genotypes, Phenotype, and Treatments. Science 275: 630-631.

5. Verri M, Pastoris O, Dossena M, Aquilani R, Guerriero F, et al. (2012) Mitochondrial alterations, oxidative stress and neuroinflammation in Alzheimer's disease. Int J Immunopath Ph 25: 345-353.

6. Bowen RL (2001) Sex hormones, amyloid protein, and alzheimer disease. JAMA 286: 790-791.

7. Hogervorst E, Bandelow S, Combrinck M, Smith AD (2004) Low free testosterone is an independent risk factor for Alzheimer's disease. Exp Gerontol 39: 1633-1639.

8. Zhang G, Li J, Purkayastha S, Tang Y, Zhang H, et al. (2013) Hypothalamic programming of systemic ageing involving IKK-[bgr], NF-[kgr]B and GnRH. Nature 497: 211-216.

9. D'Amico AV, Braccioforte MH, Moran BJ, Chen MH (2010) Luteinizing-hormone Releasing Hormone Therapy and the Risk of Death From Alzheimer Disease. Alzheimer Dis Assoc Disord 24: 84-89

10. Nuruddin S, Wojniusz S, Ropstad E, Krogenæs A, Evans NP, et al. (2013) Peri-pubertal gonadotropin-releasing hormone analog treatment affects hippocampus gene expression without changing spatial orientation in young sheep. Behav Brain Res 242: 9-16.

11. Nuruddin S, Krogenæs A, Brynildsrud OB, Verhaegen S, Evans NP, et al. (2013) Peri-pubertal gonadotropin-releasing hormone agonist treatment affects sex biased gene expression of amygdala in sheep. Psychoneuroendocrinology. In press

12. Skinner DC, Albertson AJ, Navratil A, Smith A, Mignot M, et al. (2009) Effects of Gonadotrophin-Releasing Hormone Outside the Hypothalamic-Pituitary-Reproductive Axis. J Neuroendocrinol 21: 282-292.

13. Gault PM, Maudsley S, Lincoln GA (2003) Evidence that gonadotropin-releasing hormone ii is not a physiological regulator of gonadotropin secretion in mammals. J Neuroendocrinol 15: 831-839.

14. Millar RP (2005) GnRHs and GnRH receptors. Anim Reprod Sci 88: 5-28.

15. Stevenson TJ, Hahn TP, MacDougall-Shackleton SA, Ball GF (2012) Gonadotropin-releasing hormone plasticity: A comparative perspective. Front Neuroendocrin 33: 287-300.

16. Wang L, Chadwick W, Park S, Zhou Y, Silver N, et al. (2010) Gonadotropin-releasing hormone receptor system: modulatory role in aging and neurodegeneration. Cns Neurol Disord-DR 9: 651-660.

17. Bryan KJ, Mudd JC, Richardson SL, Chang J, Lee Hg, et al. (2010) Down-regulation of serum gonadotropins is as effective as estrogen replacement at improving menopause-associated cognitive deficits. J Neurochem 112: 870-881

18. Evans NP, Robinson JE, Erhard HW, Ropstad E, Fleming LM, et al. (2012) Development of psychophysiological motoric reactivity is influenced by peripubertal pharmacological inhibition of gonadotropin releasing hormone action-Results of an ovine model. Psychoneuroendocrinology 37: 1876-1884.

19. Wojniusz S, Vögele C, Ropstad E, Evans N, Robinson J, et al. (2011) Prepubertal gonadotropin-releasing hormone analog leads to exaggerated behavioral and emotional sex differences in sheep. Horm Behav 59: 22-27

362 20. Grigorova M, Sherwin BB, Tulandi T (2006) Effects of treatment with leuprolide acetate
363 depot on working memory and executive functions in young premenopausal women.
364 Psychoneuroendocrinology 31: 935-947.

365 21. Nelson CJ, Lee JS, Gamboa MC, Roth AJ (2008) Cognitive effects of hormone therapy in
366 men with prostate cancer. Cancer 113: 1097-1106.

367 22. Simpkins JW, Perez E, Xiaofei W, ShaoHua Y, Yi W, et al. (2009) Review: The potential for
368 estrogens in preventing Alzheimer's disease and vascular dementia. Ther Adv Neurol Disord 2:
369 31-49.

370 23. Lillehaug S, Syverstad GH, Nilsson LNG, Bjaalie JG, Leergaard TB, et al. (2013) Brainwide
371 distribution and variance of amyloid-beta deposits in tg-ArcSwe mice. Neurobiol Aging,
372 dx.doi.org/10.1016/j.neurobiolaging.2013.09.013

373 24. Lord A, Kalimo H, Eckman C, Zhang XQ, Lannfelt L, et al. (2006) The Arctic Alzheimer
374 mutation facilitates early intraneuronal Aß aggregation and senile plaque formation in transgenic
375 mice. Neurobiol Aging 27: 67-77.

376 25. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, et al., (2002) Accurate
377 normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal
378 control genes. Genome Biol 3: research0034.

379 26. Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, et al. (2007) Primer3Plus, an
380 enhanced web interface to Primer3. Nucleic Acids Res 35: W71-W74.

381 27. Näslund J, Haroutunian V, Mohs R (2000) Correlation between elevated levels of amyloid ß-
382 peptide in the brain and cognitive decline. JAMA 283: 1571-1577.

383 28. Sedgewick J (2008) Scientific Imaging with Photoshop: Methods, Measurement, and
384 Output. Peachpit Press, California. ISBN-10: 0321514335.

385 29. Reilly JF, Games D, Rydel RE, Freedman S, Schenk D, et al. (2003) Amyloid deposition in
386 the hippocampus and entorhinal cortex: Quantitative analysis of a transgenic mouse model. Proc
387 Natl Acad Sci 100: 4837-4842.

30. Badr M, Marchetti B, Pelletier G (1988) Modulation of hippocampal LHRH receptors by sex steroids in the rat. Peptides 9: 441-442.

31. Meethal S, Smith M, Bowen R, Atwood C (2005) The gonadotropin connection in Alzheimer¢s disease. Endocrine 26: 317-325.

32. Bowen RL, Verdile G, Liu T, Parlow AF, Perry G, et al. (2004) Luteinizing Hormone, a Reproductive Regulator That Modulates the Processing of Amyloid-β Precursor Protein and Amyloid-β Deposition. J Biol Chem 279: 20539-20545.

33. Casadesus G, Webber KM, Atwood CS, Pappolla MA, Perry G, et al. (2006) Luteinizing hormone modulates cognition and amyloid-b deposition in Alzheimer APP transgenic mice. BBA-Mol Basis Dis 1762: 447-452.

34. Marlatt MW, Webber KM, Moreira PI, Lee H, Casadesus G, et al. (2005) Therapeutic Opportunities in Alzheimer Disease: One for all or all for One? Curr Med Chem 12: 1137-1147.

35. Verdile G, Laws SM, Henley D, Ames D, Bush AI, Ellis KA, Faux NG, Gupta VB, Li QX, Masters CL, Pike KE, Rowe CC, Szoeke C, Taddei K, Villemagne VL, Martins RN (2012) Associations between gonadotropins, testosterone and [beta] amyloid in men at risk of Alzheimer/'s disease. Mol Psychiatry (In press).

36. Rodrigues MA, Verdile G, Foster JK, Hogervorst E, Joesbury K, Dhaliwal S, Corder EH, Laws SM, Hone E, Prince R, Devine A, Mehta P, Beilby J, Atwood CS, Martins RN (2008) Gonadotropins and Cognition in Older Women. J Alzheimers Dis 13: 267-274.

37. Barron A, Verdile G, Martins R (2006) The role of gonadotropins in alzheimer's disease. Endocrine 29: 257-269.

38. Wilson AC, Salamat MS, Haasl RJ, Roche KM, Karande A, et al. (2006) Human neurons express type I GnRH receptor and respond to GnRH I by increasing luteinizing hormone expression. J Endocrinol 191: 651-663.

39. Nilsberth C, Westlind-Danielsson A, Eckman CB, Condron MM, Axelman K, et al. (2001) The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced A[beta] protofibril formation. Nat Neurosci 4: 887-893.

Figure Captions:

Figure 1: mRNA expression levels across the age and groups

----Figure 1----

Hippocampal expression of Gnrh1 and Gnrhr transcripts in male and female tg-ArcSwe mice related to control groups. Data are represented as fold change and standard error mean (SE). The fold change values are derived from relative quantification and normalization to the expression of two reference genes (B2m and Gusb). Genes with values higher than 1 indicate increased mRNA expression, values lower than 1 decreased mRNA expression in target group relative to control group. Light grey bars indicate fold changes of gene expression at 4 month of age AD-mice relative to age-matched WT-mice; Grey bars indicate fold change of gene expression level in 12 month old AD-mice relative to age-matched WT-mice. Black bar indicate fold changes in gene expression in 12-month-old Gnrh-a treated AD-mice relative to vehicle treated AD-mice; within both sexes. Significant (Wilcoxon signed rank test, $p<0.05$) changes in gene expression are indicated by asterisks.

Figure 2: Immunohistochemistry image for Aβ-deposits across different groups

……Figure 2……..

*Immunohistochemistry for amyloid (*Aβx-40*) in the right hemisphere of male and female mouse brain. Aβ-deposits are found throughout the cerebral cortex (Cx) and hippocampus (Hc) in all animals regardless of treatment. Enlarged images E-H show morphological details in the hippocampus. Insets show sections from wild-type animals of corresponding genders demonstrating that background staining was low.*

Figure 3 : Effect of Gnrh-analog treatment on Aβ-deposits across different regions of the brain

……..Figure 3……

Aβ-beta deposition in the cerebral cortex, hippocampus and thalamus of the treated (tre-)and untreated (untre-) tg-ArcSwe mice. For each animal the mean area fractions of three sections were used for the final analysis. Results are presented with means and standard error means (SEM). Unt-untreated females (○) and males (□), Tre-treated females (●) and males (□).There was no significant change between the groups, but a trend towards lower plaque deposition in treated female cerebral cortex (p=0.06) compared to vehicle treated controls.

Table 1. Animals involved in the study.

Age (in months)	12	12	4
Treatment ***(Leuprorelin acetate; 25ng/g)***	Treated	Untreated	Untreated
Tg - ArcSwe males	3	6*	6
Wild-type males	-	6	6
Tg - ArcSwe females	7	6*	6
Wild-type females	-	6	6

** A subset of these animals were anatomically characterized in reference [23]*

Table 2. Primer sequences for target and stable reference genes used in the study.

Gene abbreviation	**Forward primer**	**Reverse primer**	**Product length**	**Accession number**
Gnrh1	GCTCCAGCCAGCACTGGTCCTA	TGATCCACCTCCTTGCCCATCTCTT	100	NM_008145.2
Gnrhr	ATTAGCCTGGACCGCTCCCTGG	CATTGCGAGAAGACTGTGGGCCC	182	NM_010323
B2m	CTTCAGTCGTCAGCATGGCTCGT	TTTCTGGATAGCATACAGGCCGGC	83	NM_009735.3
Gusb	AAGGCGCTGGACGGACTGTGG	AGACTGGGCCCGACTCCCGTA	109	NM_010368.1

Table 3. Gene expression results across different groups, at different age.

	Age (months)	Sex	**Gnrh1**			**Gnrhr**		
			FC	**SEM**	**p-value**	**FC**	**SEM**	**p-value**
ArcSwe-mice relative to Wild-type mice	4	M	0.98	0,14	0.65	1.30	0.23	0.57
	4	F	1.23	0.26	0.54	0.98	0.11	0.46
	12	M	2.89	0.23	<0.001	2.43	0.28	<0.01
	12	F	2.00	0.11	<0.001	1.60	0.10	<0.001
Treated relative to untreated ArcSwe-mice	12	M	0.46	0.14	<0.001	0.33	0.15	<0.001
	12	F	0.67	0.15	<0.01	0.51	0.19	<0.001

T = treatment with Leuprorelin acetate, VT = vehicle treated, FC = fold change, SEM = standard error mean.

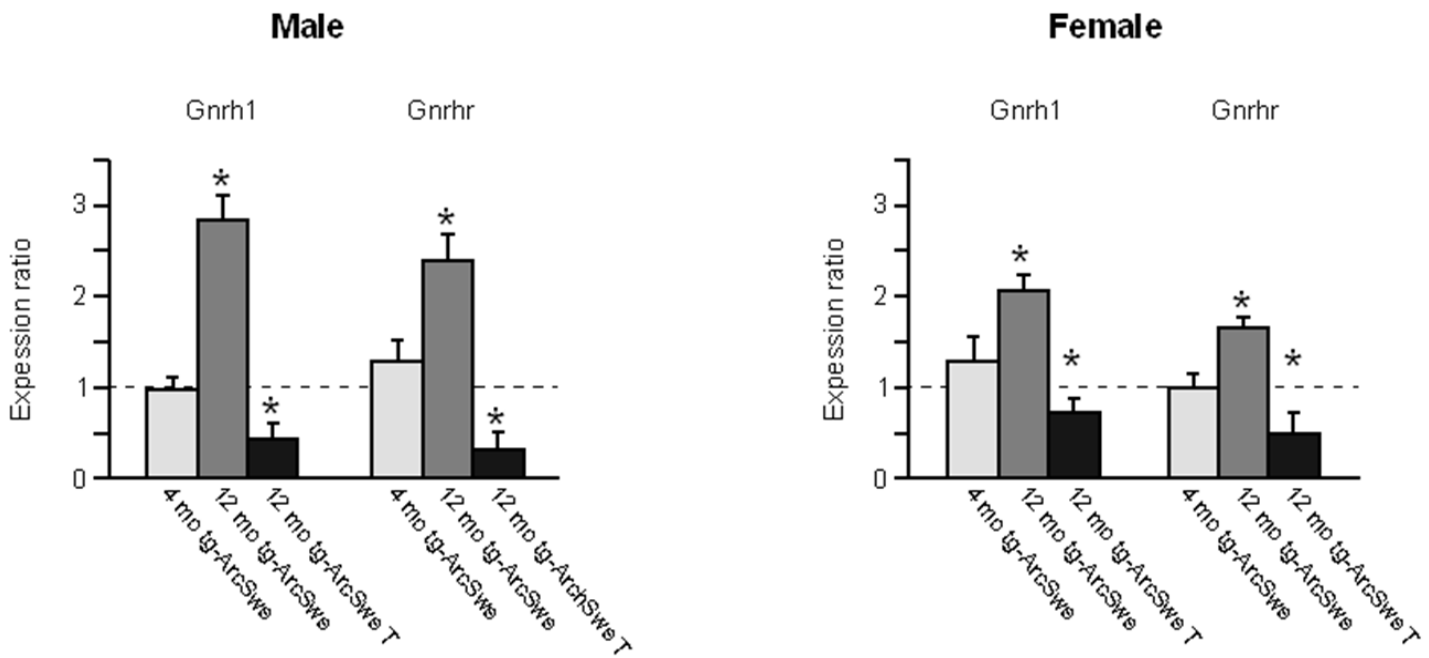
Male
Gnrh1
Gnrhr
Expession ratio
0
1
2
3
*
4 mo tg-ArcSwe
12 mo tg-ArcSwe
12 mo tg-ArcSwe T
Female
Gnrh1
Gnrhr
Expession ratio
4 mo tg-ArcSwe
12 mo tg-ArcSwe
12 mo tg-ArcSwe T

Figure:1

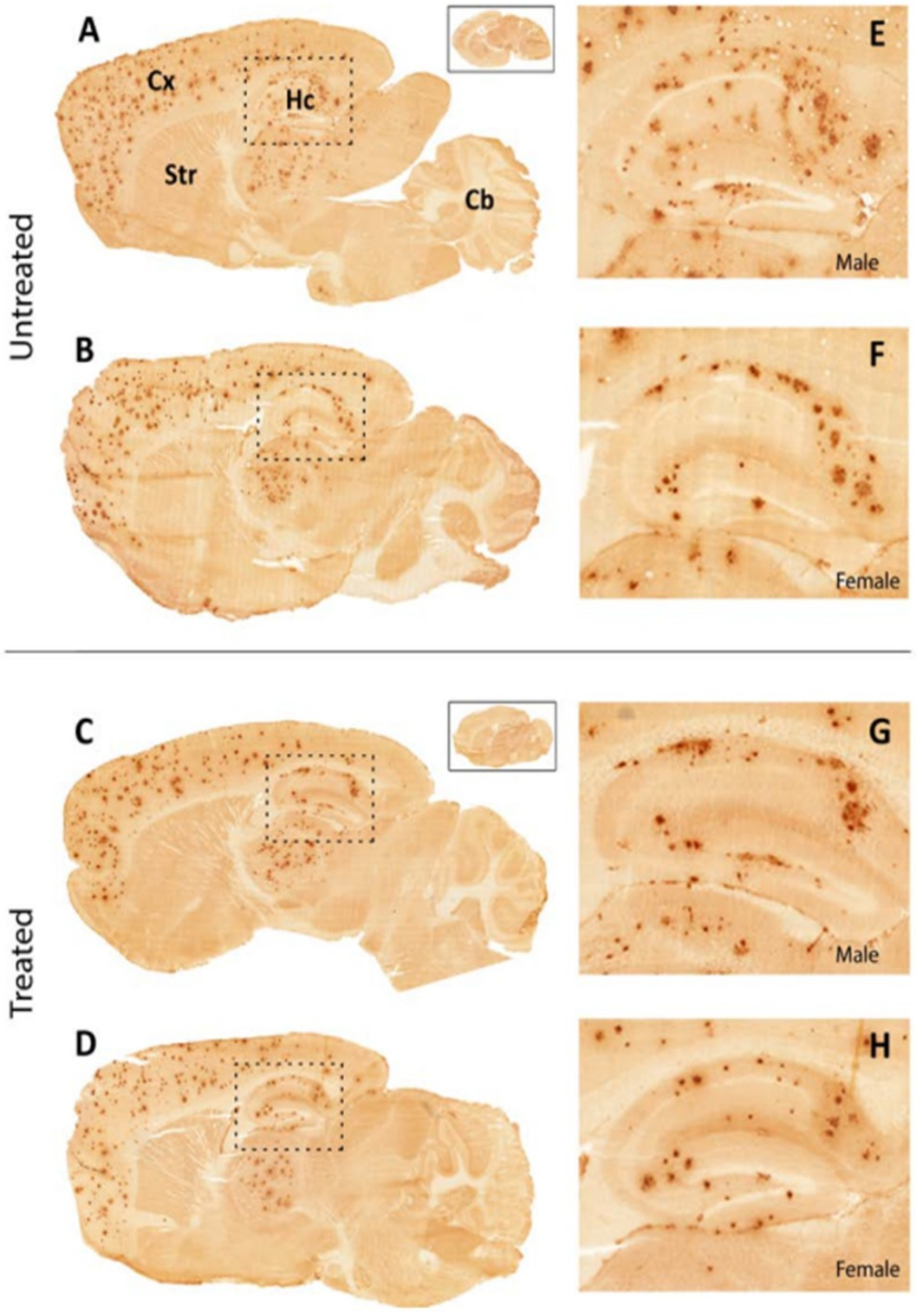
A
Cx
Hc
Str
Cb
E
Male
Untreated
B
F
Female
C
G
Male
Treated
D
H
Female

Figure:2

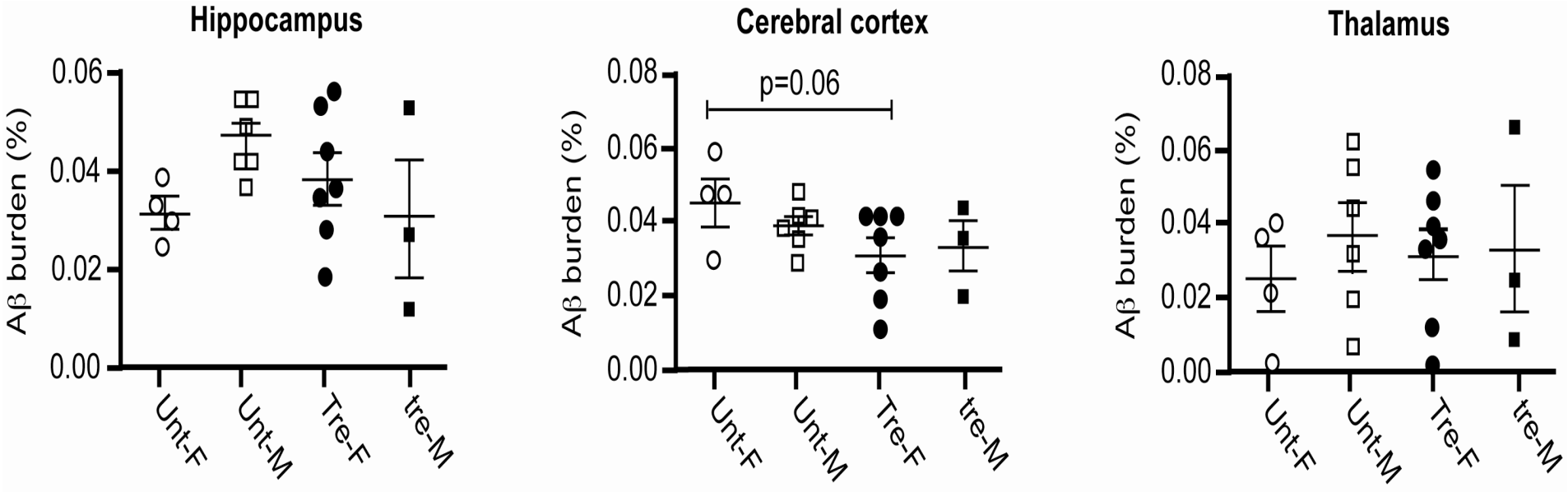
Hippocampus
Aβ burden (%)
0.06
0.04
0.02
0.00
Unt-F
Unt-M
Tre-F
tre-M
Cerebral cortex
p=0.06
0.08
0.06
0.04
0.02
0.00
Thalamus

Figure: 3

ERRATA

1. Page 17: ‘Kalynn et al., 2013’ should read ‘Schulz et al., 2013’, first names (i.e.., Kalynn, Heather, Cheryl) were used, rather than surnames. In the reference list, it should be: Schulz, K.M., Molenda-Figueira, H.A., Sisk, C.L., 2013. Back to the Future: The Organizational-Activational Hypothesis Adapted to Puberty and Adolescence. Hormones and Behaviou 55, 597-604.
2. Page 19: Daniel et al., 2011’ should be ‘Beyer et al., 2011’. In reference list it should be : Beyer, D. A., Amari, F., Thill, M., Schultze-Mosgau, A., Al-Hasani, S., Diedrich, K., Griesinger, G., 2011. Emerging gonadotropin-releasing hormone agonists. Expert Opin.Emerging Drugs 16, 323-340.