UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Zentrum für experimentelle Medizin
Institut für Neuroanatomie

Institutsdirektorin: Frau Prof. Dr. med. Gabriele Rune

Hippocampal aromatase expression and spine synapse density in *reeler* mutant mouse

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
an der Medizinischen Fakultät der Universität Hamburg

vorgelegt von

Martha Elisabeth Lewerenz
aus Wedel

Hamburg 2018

Angenommen von der Medizinischen Fakultät am: 06.06.2018

Veröffentlicht mit Genehmigung der Medizinischen Fakultät der Universität Hamburg

Prüfungsausschuss, der/die Vorsitzende: Prof. Dr. Gabriele Rune

Prüfungsausschuss, 2. Gutachter/in: Prof. Dr. Christian Hagel

Table of contents

1. Introduction 6
1.1 Overview 6
1.2 The hippocampus 6
1.3 Aromatase and Estrogen 7
1.3.1 Estrogenic effects on the brain 7
1.3.2 Aromatase 7
1.3.3 Steroids are synthesized in the brain 8
1.3.4 Aromatase is regulated by GnRH 9
1.3.5 How is aromatase activity regulated and what is its effect in females and males? 11
1.3.6 Sexual dimorphism regarding synaptic plasticity in response to sexual steroids 12
1.4 *Reeler* and Reelin 13
1.4.1 The *reeler* mutant 13
1.4.2 Role for Reelin during cortical development 13
1.4.3 What if Reelin is missing? Consequences of Reelin deficiency 14
1.4.4 Reelin and synaptic plasticity 16
1.4.5 The need of Reelin from a clinical point of view 17
1.4.6 Reelin signalling pathway 17
1.5 Reelin and E2 crosstalk? 18
1.6 Purpose of work 20
2. Materials and Methods 21
2.1 Materials 21
2.1.1 Instruments and equipment 21
2.1.2 Chemicals 22
2.1.3 Animals 23
2.1.4 Solutions and Buffers 23
2.1.4.1 Perfusion 23
2.1.4.2 Slice Cultures 24
2.1.4.3 Immunohistochemistry 24
2.1.4.4 Western Blot 25
2.1.4.5 PCR 26
2.2 Methods 26
2.2.1 Organotypic hippocampal slice culture 27

2.2.2 Tissue preparation and fixation of brain tissue by perfusion 27
2.2.3 Electron microscopy 28
2.2.4 Calculation of spine synapse density 28
2.2.5 Genotyping 29
2.2.6 Protein Biochemistry 30
2.2.6.1 Preparation of lysate from tissue 30
2.2.6.2 Bradford protein assay 30
2.2.6.3 SDS-Page 31
2.2.6.4 Sample preparation 31
2.2.6.5 Immunoblotting 32
2.2.6.6 Immunodetection 32
2.2.7 Immunohistochemistry 32
2.2.8 Analysis with ImageJ 33
2.2.8.1 Analysis of immunohistochemically stained slices 33
2.2.8.2 Analysis of Immunoblots 33
3. Results 34
3.1 Determination of spine synapse density by electron microscopy 34
3.1.1 Spine synapse density in postnatal mice 36
3.1.1.1 Spine synapse density in postnatal *reeler* compared to WT mice 36
3.1.1.2 Spine synapse density in postnatal *reeler* after estrogen treatment 36
3.1.1.3 Determination of sex steroid responsiveness in male and female hippocampal slice cultures 38
3.1.2 Spine synapse density during development and in adult mice 38
3.1.2.1 Spine synapse density in juvenile *reeler* 39
3.1.2.2 Spine synapse density in adult *reeler* 42
3.2 Aromatase expression in young and adult *reeler* compared to WT 45
3.2.1 Western Blot 45
3.2.2 Immunohistochemistry 47
4. Discussion 51
4.1 Estrogen-induced spine synapse formation? 51
4.2 Disturbed synaptogenesis in *reeler* 51
4.3 Quantification of spine synapses 53
4.3.1 Spines and spine synapses: a small but subtle difference 53
4.4 Is it all about aromatase? 55

4.4.1 Immunoblot assay to determine aromatase expression....55
4.4.2 Visualization of aromatase expression by immunohistochemistry....56
4.5 Impact of cyclicity....56
4.5.1 Steroid- and synaptogenesis: a gender-based issue?....57
4.5.1.1 Estrogen and generation of LTP....58
4.6 Expression and activation of steroid enzymes....58
4.7 Questions and issues to be focused on in future....60
5. Summary....61
6. Zusammenfassung....62
7. References....63
8. Appendix....71
8.1 List of abbreviations....71
8.2 Index of figures and tables....73
8.2.1 Figures....73
8.2.2 Tables....74
8.3 Danksagung....75
8.4 Curriculum vitae....76
8.5 Eidesstattliche Erklärung....77

1. Introduction

1.1 Overview

The ability of the brain to reorganize itself as a function of use was first described by Donald Hebb in 1949 (Hebb 1949). In his description he emphasizes that the brain is not static but rather that synapses and even certain cerebral regions can change. Consequently, the whole structure and morphology of the brain can be altered (Brown and Milner, 2003). This particularly is true for synapses. Generally speaking, a synapse consists of the presynaptic neuron, a postsynaptic neuron and the synaptic cleft in between. The axon contributes the presynapse, while spines are the postsynaptic elements. Synapses are highly dynamic and are formed or disappear upon various stimuli, such as neuronal activity, neurotrophins etc., a process called structural synaptic plasticity.
Synaptic plasticity and non-synaptic plasticity like changes in integration of excitatory and inhibitory synapses by modulating membrane components (Mozzachiodi and Byrne, 2010) are summarized under the umbrella term neural plasticity. Given the fact that the brain and in particular synapses are not static, it needs to be questioned as to what exactly causes the organization of the brain. Regarding neural plasticity aromatase, the final enzyme of steroid biosynthesis plays an important role among other factors; neuroprotective actions and synaptic strengthening are only some of the processes affected by aromatase and steroids.

1.2 The hippocampus

The hippocampus belongs to the limbic system and represents the major part of the archicortex. Phylogenetically, the archicortex is older than the neocortex, therefore they differ with respect to the laminar organization: while the neocortex consists of six different layers, the archicortex is composed of only three distinct layers. The cytoarchitecture divides the hippocampus in three parts: The dentate gyrus (fascia dentata), the cornu ammonis (CA; hippocampus proper) and the subiculum. In frontal sections the typical u-shaped neuronal bands are seen, i.e. the dentate gyrus and the CA-Region. According to different types of neurons, the CA-region can be further divided into the subregions CA1-CA3. Afferent fibers coming from different parts of the brain enter the hippocampus via the tractus perforans where axons build synapses with the dendrites of granule cells of the dentate gyrus. Axons, called mossy fibers leave this region and form synapses with pyramidal neurons of the CA3 region. Branched axons originate from this area, one leaving the hippocampus via the fornix (Commissural fibres) while the other axon makes contact with CA1 pyramidal cells (Schaffer-collaterals). This loop needs to be established during brain development. It is realized by axonal path finding, target layer recognition and synapse formation (Förster et al., 2006). In figure 1.1. a schematic diagram illustrates the anatomy of the rodent's hippocampus and the connections between the particular regions.

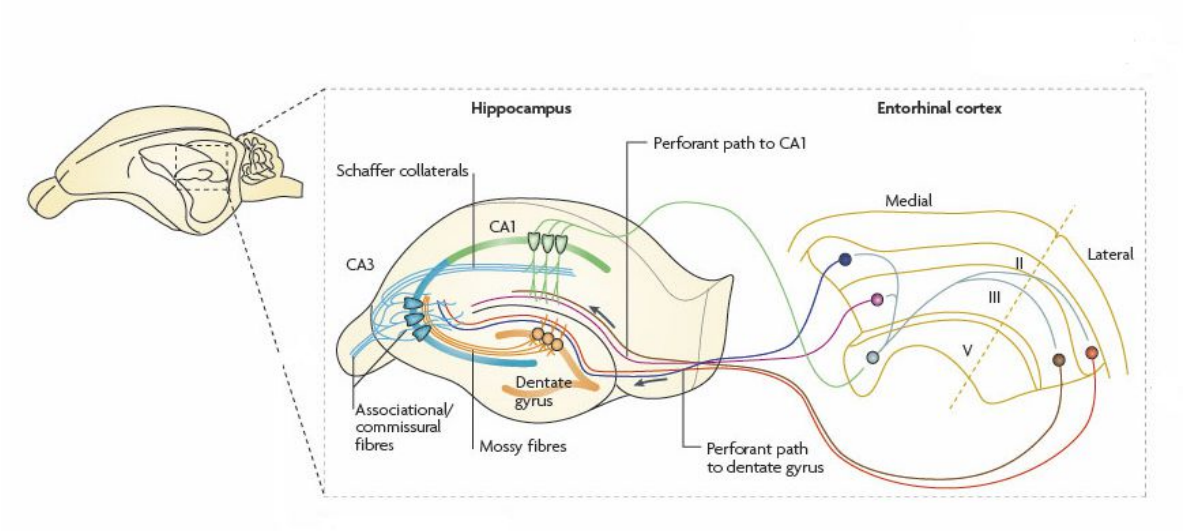


Figure 1.1: Anatomy of the hippocampus
The hippocampal circuit is traditionally shown as a trisynaptic loop. Axons originating from the entorhinal cortex enter the hippocampus via the perforant path and make contact to dendrites of the granule cells of the dentate gyrus. Axons of the granule cells project through the mossy fibres to the apical dendrites of the CA3 pyramidal cells. From here branched axons originate: Schaffer collaterals project to the ipsilateral pyramidal cells while commissural fibres make contact to the contralateral CA3 and CA1 pyramidal cells. In addition, the apical dendrites of the CA1 pyramidal cells are innervated directly from the entorhinal cortex. (Illustration adapted from Neves et al. 2008)

1.3 Aromatase and Estrogen

1.3.1 Estrogenic effects on the brain

Since 1949, neuroscience has advanced rapidly and the development of the brain has been widely explored. In fact, the brain is not completely developed after embryogenesis, but is subject to continuous remodeling. So far it is known that certain regions, such as the hippocampus, are particularly susceptible with respect to structural remodeling; above all, it is certain that steroid hormones like estrogen are necessary for the modification of the neuronal circuits (Rune and Frotscher, 2005). Woolley et alii were able to demonstrate that the densities of spines, which are the postsynaptic elements of synapses, vary during the estrous cycle (Woolley et al. 1990). In fact, low levels of estrogen seem to cause a reduction in spines in the hippocampal CA1 region whereas exogenous application of estrogen induces an increase in the number of synaptic spines (Woolley et al., 1990; Yankova et al., 2001). Additionally, it has been shown by McEwen that the cyclic turnover of synapses in the hippocampus is a function of gonadal estrogen levels in the serum (McEwen 2002).

1.3.2 Aromatase

The enzyme aromatase which is encoded by the gene CYP19A1 belongs to the CYP450-protein family (Lephart et al. ,1996). It is the final enzyme in estrogen synthesis, i.e. it catalyses the conversion of estrogens from androgens. In detail, this conversion is realized by three successive hydroxylations of the 19-methyl-group of androgens, followed by the elimination of one methyl group as formiate and finally the aromatization of the A-ring. Figure 1.2. shows the conversion of testosterone to estrogen.

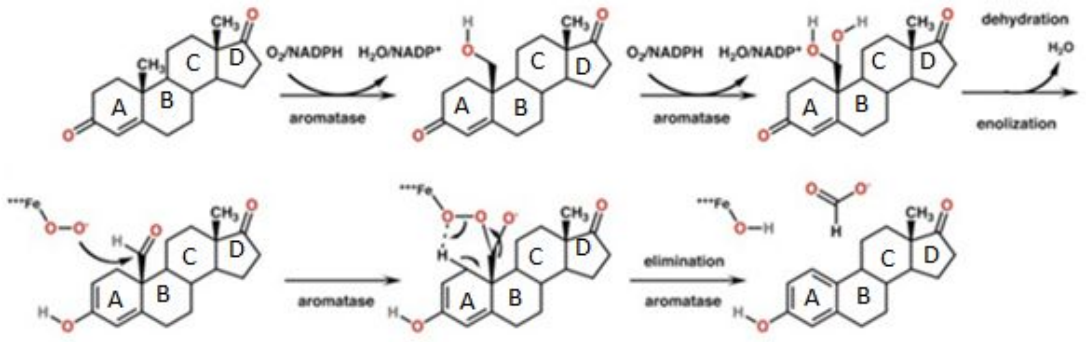


Figure 1.2: Catalysis of estrogens from androgens, realized by the CYP450-protein aromatase.
In general, steroids are composed of four rings; one of them is converted by aromatase in an aromatic state. Biosynthesis is made up of three following steps, first the oxidation of the methyl group, followed by its elimination and finally the aromatisation of ring A. (Illustration adapted from Biegon et al. 2012)

The conversion takes place in multiple tissues; for example, expression of aromatase is found in the gonads, placenta, bone tissue, blood vessel, adipose tissue, breast and endometrial cancer cells. However, aromatase is also needed for the maturation of spermatozoa in males (Carreau et al. 2010). Interestingly, the activity of aromatase depends on its phosphorylation. While it is active in a dephosphorylated state, it is inactive when it is phosphorylated (Balthazart et al. 2001)
Estrogen as a product of aromatase affects neuronal plasticity; this is of special interest with respect to neuronal protection. It is assumed that steroids are needed for the prevention of neurodegenerative diseases such as Morbus Alzheimer or cerebral insults (Behl and Manthey 2000.; Garcia-Segura 2008; McCullough et al. 2003). But, apart from neuronal protection in general, several experimental studies and clinical trials indicate that women who are treated with aromatase inhibitors due to breast cancer show deficits in learning and memory (Shilling et al. 2003; Jenkins et al. 2004, Bayer et al., 2015). However, recent papers challenge this conclusion and strongly recommend further studies to confirm adverse effects of aromatase inhibitors on cognitive function (Jenkins et al. 2006; Phillips et al. 2011) One theory suggests that estrogen is metabolized directly to genotoxic compounds. However, another more likely function of estrogen in the development of breast cancer might be its stimulating effect on genetic mutations as well as on the promotion of these mutated cells.
Based on these hypothesis aromatase inhibitors are increasingly being used in breast cancer therapy (Zhou et al. 2010).
Initially it has been thought that gonadal estrogens influence synaptic activity (McEwen 2002; Woolley et al. 1990). However, within the last few years it became clear that estrogens are locally synthesized, so the brain is a steroidogenic organ as well (Fester et al. 2011). Evidently, aromatase is needed not only for reproduction; it rather makes a contribution to the cognitive state by regulating synaptic activity and plasticity (Garcia-Segura 2008; Rune and Frotscher 2005)

1.3.3 Steroids are synthesized in the brain

Neuronal activity of aromatase was first detected by Naftolin (Naftolin et al. 1971). Several following studies confirmed the existence and activity of aromatase in different species (MacLusky et al. 1986; Schumacher and Balthazart 1987; Shinoda et al. 1994). In 1991, Sanghera et al. already demonstrated immunoreactivity of aromatase in different

areas of the brain of rats (Sanghera et al. 1991); however, it took another ten years to confirm the activity of aromatase in hippocampal neurons. In fact, a *de novo* synthesis of estrogen from cholesterol in hippocampal neurons has been demonstrated for the first time by Prange-Kiel in 2003 (Prange-Kiel et al. 2003). After incubation of hippocampal neurons originating from adult rats under serum- and steroid free conditions, radioimmunoassay could demonstrate a notable amount of estrogen. Furthermore, following experiments using hippocampal slice cultures, originated from postnatal rats, affirmed these findings (Kretz et al. 2004). Additional experiments confirmed the presence and activity of aromatase in certain brain regions, especially in the frontal and temporal lobe (Prange-Kiel et al., 2006). By now it is known that almost every enzyme needed for steroid biosynthesis is not only expressed in the reproductive system, but also in the central nervous system (Compagnone and Mellon 2000; Prange-Kiel et al. 2006).
Typically, aromatase is expressed by pyramidal cells in the hippocampus (Rune and Frotscher 2005) where it is found in the endoplasmatic reticulum, an organelle involved in protein production, processing and transport. However, it has been identified in axons as well as in synapses (Balthazart and Ball 1998).
Based on the fact that steroids are present and synthesized from endogenous precursors in the brain such as cholesterol, Baulieu and Robel established the term Neurosteroids, considering them to be steroids that accumulate in the brain even in the absence of steroidogenic glands (Baulieu and Robel 1990).
The presence of aromatase and hence the synthesis of steroids in the hippocampus is of special interest since this brain region is associated with cognitive skills such as learning and formation of memory. As already mentioned, several publications imply that inhibition of aromatase, which is actually a favored therapy in hormone sensible types of breast cancer in postmenopausal women, leads to reduced cognitive skills, thus learning and memory seem to be negatively influenced (Shilling et al. 2003; Castellon et al. 2004; Jenkins et al. 2004, Bayer et al., 2015). Previous *in vitro* experiments showed that treatment of hippocampal slice cultures with letrozole, a potent, non-steroidogenic, reversible aromatase inhibitor, causes a considerable downregulation of estrogen synthesis. This down regulation, however, is accompanied by a significant reduction of spines and spine synapses. In addition, following experiments have demonstrated that systemic inhibition of aromatase *in vivo* induces spine synapse loss, both in intact and, strikingly, also in ovariectomized animals (Zhou et al., 2010). Accordingly, the turn-over of dendritic spine synapses is at least to some extent controlled by sexual hormones originating from hippocampal neurons. Furthermore, hippocampus-derived estrogens are needed for the formation of synapses or at least for their maintenance.

1.3.4 Aromatase is regulated by GnRH

Gonadotropin-releasing hormone (GnRH) is synthesized from neurons within the hypothalamus and is secreted pulsatile to the hypophysial portal blood stream at the median eminence. Reaching the pituitary gland it binds to its receptor (GnRH-R), leading to the release of gonadotropins like follicle-stimulating-hormone (FSH) and luteinizing hormone (LH). These two hormones are involved in reproductive processes, mediated via estrogen and testosterone originating from the gonads which in turn operate via a negative feedback-mechanism on the secretion of GnRH. (Fig. 1.3.)

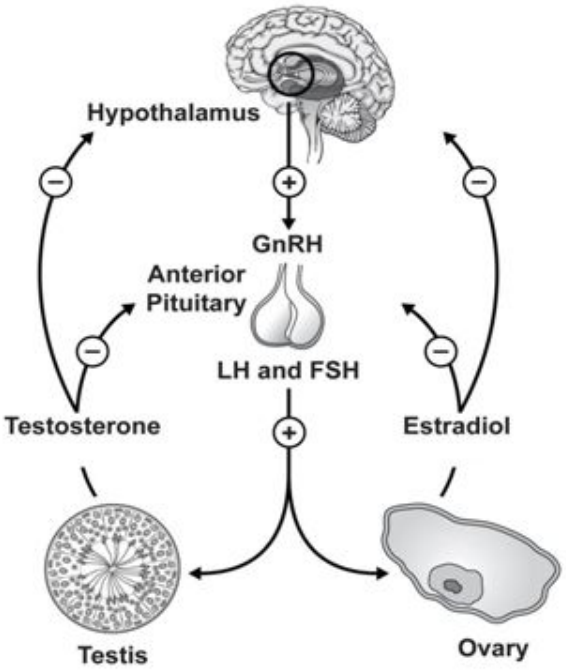


Figure 1.3: Hypothalamo-Pituitary-Gonadal Axis
The hypothalamus produces gonadotropin-releasing hormone (GnRH) which signals through the pituitary gland to produce gonadotropins like Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH). In females, FSH and LH act primarily to activate the ovaries to produce estrogen. In males, LH stimulates the Leydig cells of the testes to produce testosterone and FSH signals through the Sertoli cells to support spermatogenesis. The sex steroid hormones testosterone and estrogen feed back to inhibit the release of GnRH and pituitary secretion of LH and FSH (Illustration adapted from Whirledge and Cidlowski 2010).

The following figure represents chronology of a rodent's estrous cycle. A fine tuned hormonal interaction accounts for periodic ovulation.

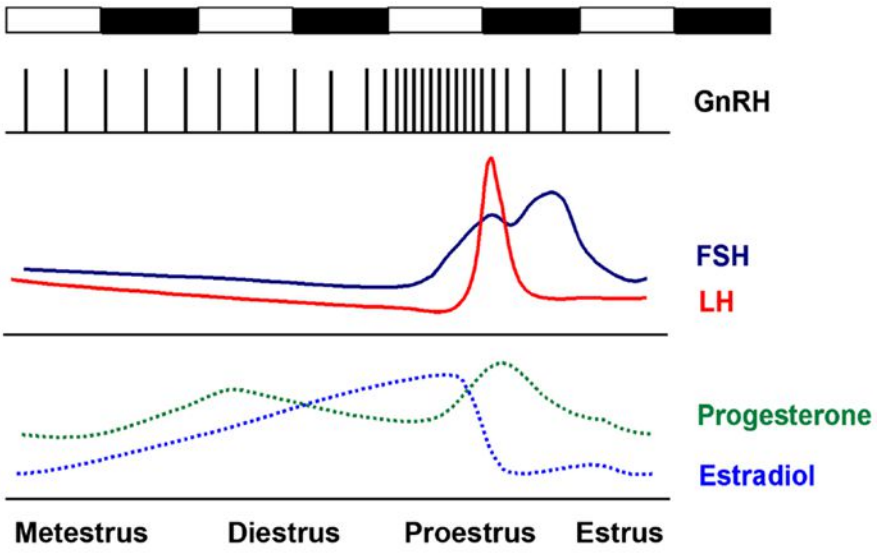


Figure 1.4: The rodent estrous cycle.
Ovulation occurs in mice every 4–5 days. During metestrus and diestrus estrogen concentrations are low but slowly increasing. On the late afternoon of proestrus, elevated estradiol levels induce frequent pulses of GnRH release from the hypothalamus, which induces the LH and FSH peak at approximately the start of the active (dark) period. Ovulation occurs 12-14 hours later (Illustration adapted from Miller and Takahashi 2013).

In addition, GnRH also stimulates the granulosa cells within the ovaries directly to produce estrogen (Parinaud et al. 1988).
Previous experiments showed that GnRH also regulates the *de novo* synthesis of E2 in the hippocampus (Prange-Kiel et al. 2008). Interestingly, GnRH receptors as well as mRNA were found in the hippocampus of rats (Jennes et al. 1997). Moreover, GnRH mRNA is five times higher in the hippocampus compared to the neocortex, suggesting a specific responsiveness in the hippocampus (Prange-Kiel et al. 2008). Furthermore, application of GnRH together with letrozole results in reduced spine synapse density in this area of the brain.
To summarize, the synthesis of estrogen and hence the spine synapse density in the hippocampus is regulated by the cyclic release of GnRH. The exact mechanism, especially how GnRH reaches the hippocampus, still remains to be unraveled.

1.3.5 How is aromatase activity regulated and what is its effect in females and males?

GnRH is important for the regulation of reproductive functions but as we know it is also needed for regulating neurosteroids. GnRH neurons originate from the olfactory placode and migrate to the basal forebrain and finally the hypothalamic region (Cariboni et al. 2005). Along their way, different molecules are involved in the guidance of these neurons to their final destination. Of special interest here is the extracellular matrix protein Reelin, which affects the migration and distribution of the neuroendocrine cells (Cariboni et al. 2005). As it is known *reeler* has a migratory defect of GnRH-neurons (Caviness et al. 1972; Cariboni et al. 2005). This might be an explanation for the reduced fertility; however, the absence of Reelin, and the consequential disorientation of GnRH-neurons has important consequences on the synthesis and effects of neurosteroids like estrogen as well.
As already mentioned, GnRH controls the secretion of sexual steroids via LH and FSH. Basically the release of sexual steroids such as estradiol depends on the activity of aromatase, the final enzyme of estradiol synthesis. The activity of aromatase, in turn, is up to the pulsatile secretion of GnRH. However, the mechanism seems to be opposite in females than in males: while GnRH stimulates the activity of aromatase in the female hippocampus, in males GnRH stimulation does not increase the E2 concentration (own unpublished observations). It rather seems that in males the conversion of testosterone to E2 is suppressed but the conversion to dihydrotestosterone (DHT), a non-aromatizable androgen is stimulated
Furthermore, synaptic plasticity is especially affected in females by inhibition of aromatase and to a lesser extent in males (Fester et al. 2012; Leranth et al. 2004; Vierk et al. 2012). In fact, estrogen has no such effect on synaptogenesis in males as in females. Interestingly, our further experiments showed that correspondent to the effect of estrogen on synaptic plasticity in females, in males it is controlled by testosterone (Unpublished data).
Since aromatase uses testosterone as a substrate for the synthesis of estrogen, the concentrations of these two hormones are contrarious in females and males.
A simplified model of the regulatory circuit of GnRH and the consequences of steroid hormones are shown in figure 1.5.

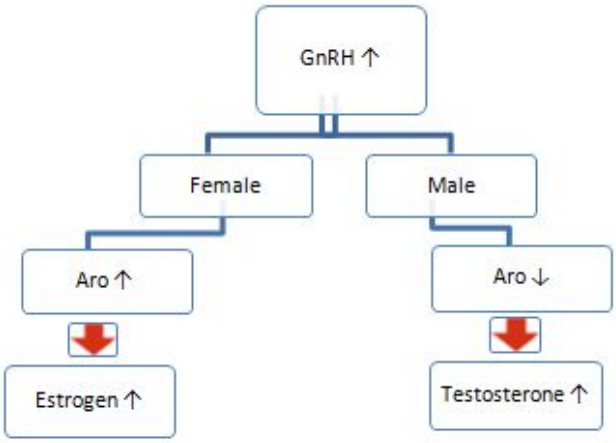


Figure 1.5: Regulation of steroid hormones by GnRH in WT.
GnRH has an opposite effect in males than in females: While in males the activity of aromatase is suppressed, in females it is stimulated. Since Aromatase converts androgens like testosterone into estrogen, steroid hormone levels are contrarious: In males testosterone levels are high while in females estrogen synthesis is increased.

1.3.6 Sexual dimorphism regarding synaptic plasticity in response to sexual steroids

Based on the finding that inhibition of aromatase activity in hippocampal neurons induces spine synapse loss in females (Kretz et al. 2004), we questioned whether aromatase inhibition will influence the generation of long-term-potentiation (LTP). LTP induces the formation of spines (Yuste and Bonhoeffer 2001), basically it is an electrophysiological correlate for synaptic activity. Vierk et alii showed that aromatase inhibition by letrozole impairs LTP, followed by spine and spine synapse loss (Vierk et al. 2012). However, while LTP was abolished in females, it was only impaired by 20% in males following 7 days of treatment with letrozole. Furthermore, stereological counts of spine synapses demonstrated a reduction after one day, proceeding until day 7, subsequent to letrozole treatment in females. In males, however, no reduction has been identified. In addition, in vivo experiments showed that systemic application of letrozole causes synapse loss in female but not in male rats. (Fester et al. 2012).
Recent experiments underline a sexual dimorphism regarding steroid-dependent synaptic plasticity. Removal of gonads induces spine loss in the hippocampus, both in male and female. Interestingly, in females a rescue can be achieved by treatment with estrogen, but in males it has no effect. In contrast, treatment with testosterone induces a rescue effect in males, but not in females (Brandt et al. in revision).
Taken together, several aspects refer to a sexual dimorphism concerning steroid controlled synaptic plasticity. First the regulation of aromatase by GnRH is oppositional. This is consistent with different concentrations of estrogen and testosterone in females and in males. In addition, estrogen has an effect on synaptic plasticity in females but not in males. However, the same holds true for testosterone, which rescues a spine synapse loss after systemic inhibition in males, but not in females.

1.4 *Reeler* and Reelin

1.4.1 The *reeler* mutant

The *reeler* mouse is a naturally-occuring mutant firstly described by Falconer in 1951 (Falconer 1951). The *reeler* mutation is an autosomal recessive trait resulting in an ataxic gait, tremor and dystonic posture (Falconer 1951), starting around 12 days after birth (Curran and D'Arcangelo 1998). The mutant has been studied for more than fifty years in order to elucidate the development of cortical structures and function (D'Arcangelo 2006; Förster et al. 2010; Herz and Chen 2006). It therefore serves as a murine model for neuronal development as well as for several neurological disorders (Rice and Curran 2001).
The gene, which is mutated in *reeler* encodes the extracellular matrix glycoprotein Reelin. A schematic diagram of the protein is shown in figure 1.6.

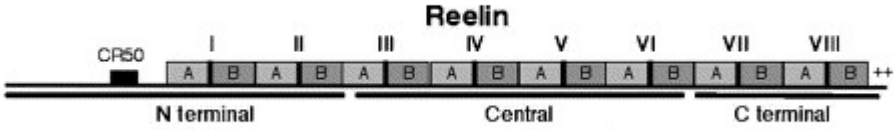


Figure 1.6: Schematic diagram of the extracellular matrix protein Reelin.
Reelin has a molecular weight of 388kDa and consist of eight consecutive repeats (I-VIII), whereas each repeat is build up of two subrepeats, separated by an EGF-like cysteine pattern. After secretion to the extracellular environment it is proteolytically cleaved to three major fragments, the N-terminal, the central and the C-terminal fragments. The N-terminal CR50 epitope is commonly detected by antibodies directed against Reelin (Nakano et al. 2007) (Illustration adapted from D'Arcangelo 2006).

Reelin controls neuronal cell migration and positioning in the developing brain as well as maturation of dendritic spines and extension of dendritic processes later during development (D'Arcangelo 2006; Förster et al. 2006; Frotscher 2010; Herz and Chen 2006; Niu et al. 2008). With Reelin missing, cellular layer formation as well as cell organization in general is disturbed. Leading to an impaired embryonic and postnatal neuronal development (D'Arcangelo 2006; Lambert de Rouvroit and Goffinet 1998) in the adult brain it is involved in synaptic transmission and synaptic plasticity, in addition it is needed for cytoarchitectural stabilization (Frotscher 2010). Viability and fertility is limited in *reeler* (Caviness et al. 1972), the reason, however, is not fully understood yet.
In summary, depending on the age and developmental stage, Reelin fulfils different functions in the brain and its lack results in dramatic disorders, both in rodents and in humans (See below).

1.4.2 Role for Reelin during cortical development

In the last few years, different genes and their products responsible for the correct positioning of neurons have been characterized (For review see Rakic and Caviness 1995); in addition, molecules that provide a correct cell-cell and cell-matrix interaction have been identified. In the hippocampus, Reelin is expressed during cortical development by early-generated Cajal-Retzius-cells (CR) which are located at the marginal zone (future layer I) and possibly controlled by a trophic factor that is secreted by meningeal cells (Bender et al.

2010; Frotscher 1998). Mice lacking Reelin show disorientation and an incorrect laminar organization, whereas CR cells themselves are located properly. This implies that it controls the positioning of neighboring cells.
During embryogenesis Reelin is expressed by CR cells in the cerebral cortex, the telencephalon, the hypothalamus, thalamus, striatum and several other brain areas (Alcantara et al. 1998; Förster et al. 2006). However, after birth the expression by CR cells decreases while expression appeared in GABAergic interneurons. In the adult Reelin is only secreted by GABAergic interneurons (Alcantara et al. 1998).
Neuronal migration coordinated by Reelin is detected in various areas of the brain: the cerebellum, spinal cord, neocortex and the hippocampus (Förster et al. 2006; Frotscher 2010; Herz and Chen 2006; Mariani et al. 1977; Rice and Curran 2001). In the cerebellum the defect causes a dramatic hypoplasia, explaining the motional disorders typical for *reeler* (D'Arcangelo 2006).
Cariboni et alii showed that Reelin is effective as guidance signal for migrating GnRH neurons (Cariboni et al. 2005). These cells, which play an essential role in the reproductive system, originate in the olfactory bulb and are guided by Reelin to the hypothalamic region. Moreover, it was shown that Reelin acts inhibitory in guiding GnRH neurons.
However, apart from its need as a guiding signal, Reelin is indispensible for a controlled layer formation i.e. for the correct formation and positioning of radially migrating principal neurons (D'Arcangelo 2006; Frotscher 1997).

1.4.3 What if Reelin is missing? Consequences of Reelin deficiency

The layering of the hippocampus is arranged in an inside out fashion, which means early-generated neurons rest near the bottom while later-generated neurons have to pass these cell layers in order to settle close to the surface (Förster et al. 2010; Frotscher 1998; Tissir and Goffinet 2003). But how does this layering work? What are the molecular mechanisms providing a controlled lamination? A simplified model is given in figure 1.7.. In brief, specified radial glial cells form a connection between the ventricular zone and the marginal zone with their axons, thus providing a scaffold for radially migrating neurons (Bagirathy et al. 2002). Principal neurons, such as pyramidal cells and granule precursor cells migrate radially up to the surface (Förster 2014; Nadarajah et al. 2003). However, signals originating from the marginal zone are needed for an appropriate migration of neurons.

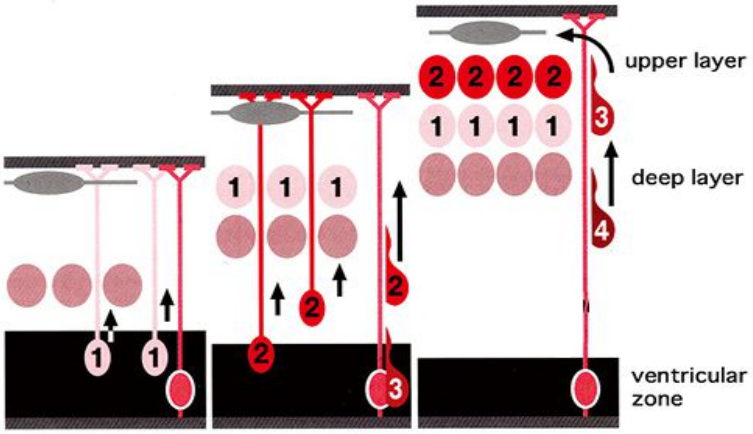


Figure 1.7: Layering of the hippocampus by neuronal migration.
Pyramidal neurons (1) present in the cerebral ventricular zone of the embryonic cerebrum migrate radially toward the superficial layer. Subsequently later generated neurons (2) pass early generated neurons so the cortex is formed in an "inside/out pattern" whereby the earliest neurons formed are placed in the deepest layers and later neurons occupy the more superficial layers. The numbers indicate the birth order. (Illustration adopted from *Research Results at BSI, Laboratory for Developmental Neurobiology (2008),* New Discovery for Mechanism of Neuronal Migration and Morphogenesis, Online: http://www.brain.riken.jp/bsi-news/en/no40/research03.html, Date: 8th July 2014)

Lack of Reelin expression promotes an undefined lamination, the cells are distributed in an unstructured fashion; therefore afferent and efferent fibers are not clearly separated (Förster et al., 2010). In fact, each layer has its specificity, either destined for generating efferents or serving as a destination for afferents. As there is no strict separation between individual layers in Reelin deficient organisms, incoming and outgoing information are not coordinated properly.
In the developing brain, Reelin coordinates the radial outgrowth of glial fibers in order to provide a structured framework for migrating neurons (Tissir and Goffinet 2003). Later-generated cortical neurons can migrate along these fibers and condense in the cortical plate where the population of cells divides and two subgroups are formed. In addition, subplate neurons are stopped by Reelin and therefore cannot reach the marginal zone. Later-generated cells pass the cortical plate, forming an inside-out pattern, characteristic for laminated brain structures. In *reeler*, the preplate will not split into two components, and the cells will be arranged in a disoriented fashion. Due to the lack of the stop signal, subplate cells can also migrate to the marginal zone and later-generated neurons settle before they pass the cortical plate, forming an inverted lamination (Frotscher 2010; Tissir and Goffinet 2003) (Fig. 1.8.).

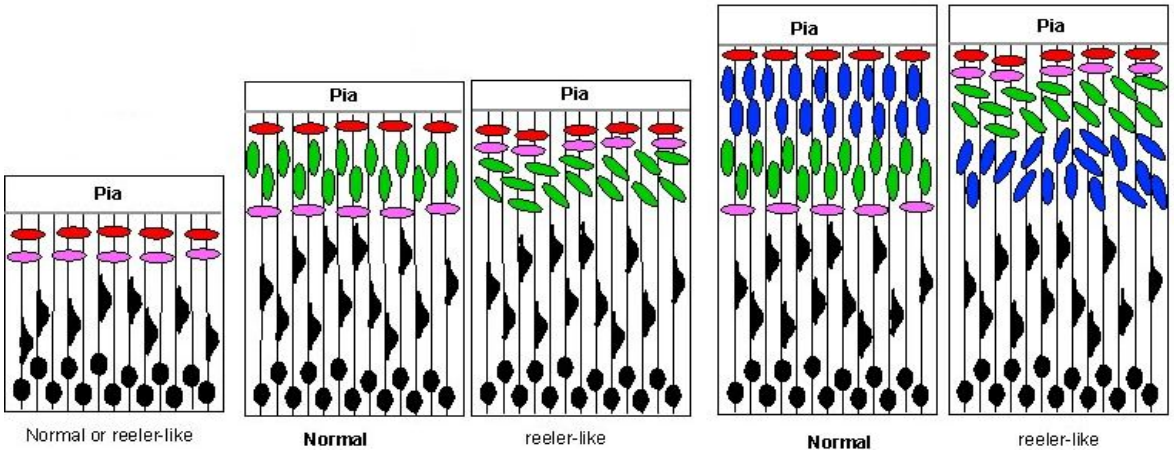


Figure 1.8: Cortical development in normal and *reeler*-like mice at different embryonic stages.
In both genotypes neurons are generated at the ventricular zone and migration is coordinated in the beginning, Reelin positive (red) and Reelin negative (pink) cells form a preplate. Later generated neurons (green) split the preplate, however, due to the lack of Reelin the layering goes awry in *reeler*. Cells are located oblique and the preplate is not divided. A fourth population of cells (blue) generate a typical inside out alignment, however, in *reeler* these cells stay in the deep forming an opposite cell composition. (Illustration adapted from Tissir and Goffinet 2003).

1.4.4 Reelin and synaptic plasticity

In addition to the function of Reelin in cell migration and lamination of neurons, more recent studies point to a role of the protein in synaptic plasticity (Curran and D'Arcangelo 1998; D'Arcangelo 2006; Herz and Chen 2006). Reelin seems to be crucial for dendritogenesis and synaptogenesis, furthermore it affects synaptic function not only during development but in the adult brain as well (Herz and Chen 2006; Niu et al. 2008). Niu et alii were able to demonstrate that Reelin deficiency in adult mice results in a reduced density of dendritic spines in hippocampal CA1 neurons. Fluorescently labeled hippocampal pyramidal neurons were analyzed *in vivo* and in organotypic cultures with the focus on dendritic spines in apical dendrites. Basically they found a reduced density of spines in glutamatergic synapses. Moreover, this reduction can be rescued *in vitro* by addition of recombinant Reelin

All the mechanisms described so far refer to the function of Reelin during embryogenesis and at postnatal ages. However, Reelin has a dual role in the mammalian brain; it also works in the adult brain, where it stabilizes the cortical architecture (Frotscher, 2010). Malfunctioning of Reelin in the mature brain leads to a reduced structural stability of neurons and their processes, thus brain circuitries are disturbed (Chai et al. 2009; Frotscher 2010). Furthermore, the brain is continuously remodeled, there are ongoing dynamic processes making the brain plastic. This is particularly needed in order to cope with new environmental challenges. However, structural changes of synapses and synaptic transmission is a fine tuned process, depending on distinct mechanisms and proteins. Changes in cell shape which is a prerequisite of cell migration and process extension rely on the stabilization of the actin cytoskeleton. Cytoskeletal stabilization and dynamics, in turn, rely on the signaling of Reelin (Förster et al. 2010; Frotscher 2010).

1.4.5 The need of Reelin from a clinical point of view

Unstructured laminar organization, like in *reeler*, prevents proper seperation of incoming afferents and outgoing efferents (Förster et al. 2006). Patients with mesial temporal lobe epilepsy (TLE) show an incorrect layering due to granule cell migration defects, which could result from an aberrant number of Reelin expressing CR-cells (Haas et al. 2002). In addition, in patients suffering from an autosomal recessive form of the developmental disorder called Lissencephaly (LCH) the absence of Reelin has been evidenced (Hong et al. 2000). The clinical picture of LCH is characterized by a mental retardation, an ataxic gait and hypotonic posture (Chang et al. 2007). Histological and cytological analysis showed abnormalities in the cerebellum, brainstem and the hippocampus, furthermore the neuronal migration is impaired, resulting in an agyric or brachygyric cerebral cortex (Hong et al. 2000). The described pathologies can be attributed to two independent mutations in the human gene encoding for Reelin *(RELN),* which is located on chromosome 7q22 (Hong et al. 2000).
Furthermore, Fatemi and co-workers were able to objectify a reduced Reelin expression in patients suffering from schizophrenia and other neuropsychiatric disorders like bipolar disorders, major depression or autism (Fatemi et al. 2000). Assuming that there is a crosstalk between Reelin and estrogen it is important to note that both Reelin and estrogens seem to have a neuroprotective effect in Alzheimers disease (AD) (Garcia-Segura 2008, Prange-Kiel et al. 2016). Furthermore, there is a growing body of evidence which shows that apolipoprotein E (APOE) is involved in the development of late-onset AD (Herz and Chen 2006). APOE and its receptor, Apoer2, as well as Reelin, cholesterol and β-amyloid are modulating synaptic transmission and the activity of *N*-Methyl-D-Aspartat receptors (NMDA receptors, NMDAR). With Reelin missing the activity of GSK3β, a kinase which phosphorylates τ, is down regulated. As a result aggregates of hyperphosphorylated τ are formed (neurofibrillary tangles) most commonly known as primary marker of AD (Weeber et al. 2002).

1.4.6 Reelin signalling pathway

The Reelin signalling cascade is activated by binding of Reelin to apolipoprotein E receptor 2 (Apoer2) and the Very Low Density Lipoprotein receptor (VLDL-R), both members of the lipoprotein receptor family (Bock and Herz 2003; Förster et al. 2010; Herz and Chen 2006; Niu et al. 2008). Clustering of the receptors leads to the recruitment of *Src-familiy-kinases (SFKs)* which activates the intracellular adapter protein *Disabled-1 (DAB1)* by tyrosine phosphorylation (Bock and Herz 2003). Subsequently, different intracellular cascades are initiated, including microtubule stabilization and dynamics as well as actin cytoskeleton rearrangement (For review see Herz & Chen 2006). Furthermore, mediated by the postsynaptic density protein 95 (PSD-5) the activation of SFK by Reelin causes the phosphorylation of NR2 subunits of the NMDA receptor (NMDA-R), resulting in an increased CA2+ influx. This in turn activates the transcription factor *cyclic AMP-response element binding protein (CREB)* which initiates the expression of genes needed for dendritogenesis, synaptogenesis and dendritic spine development (Herz and Chen 2006). In addition, phosphorylation of NR2 subunits prevents the endocytosis of NMDAR, a disadvantageous mechanism known from the development of Morbus Alzheimer (Beffert et al. 2005; Snyder et al. 2005) (See below). Figure 1.9. gives an overview of the Reelin signalling cascade.

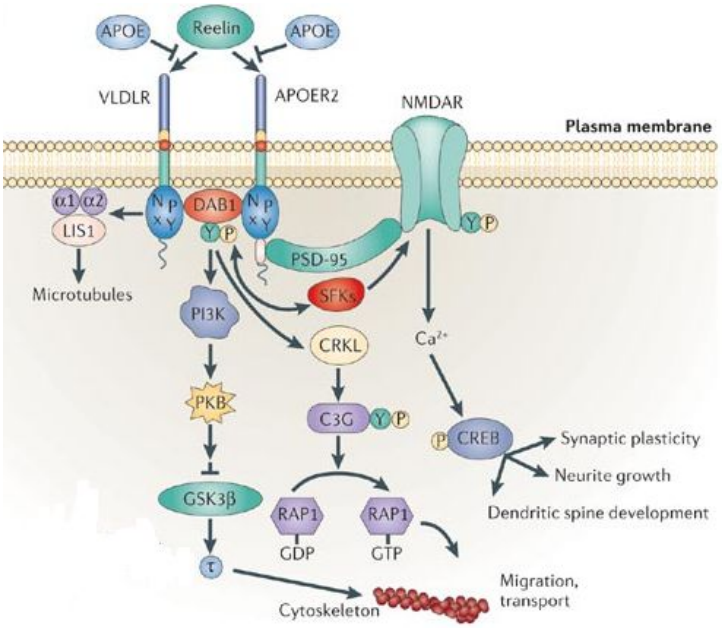


Figure 1.9: Reelin signalling cascade.
Binding of Reelin to its two lipoprotein receptors VLDLR and APOER2 induces the activation of the intracellular adapter protein Disabled 1 (DAB1) by tyrosine phosphorylation. Subsequently, different downstream signalling events of the Reelin signalling cascade are initiated, allowing the cell to migrate to its destination. (Illustration adapted from Herz and Chen 2006.)

The lack of *DAB1* results in a phenotype comparable to *reeler,* called *scrambler* (Sheldon et al. 1997); the same holds true in case VLDLR and Apoer2 are dysfunctional (Trommsdorff et al. 1999). In contrast, if only one receptor is missing or mutated, the effects are quite different (Benhayon et al. 2003; Trommsdorff et al. 1999). In an experimental design using mice deficient for VLDLR, neurons will continue to migrate to the cortical layer I. In contrast, Apoer2 deficiency leads to an accumulation of neurons in the deeper layers. Apparently, each receptor exerts a distinct function: Apoer2 for attracting neurons whereas VLDLR is needed to stop migration (Förster et al. 2010). Furthermore, previous and ongoing experiments by Zhao and colleagues show that Reelin has different functions, either attractive or repellent (Unpublished data). After secretion Reelin is subjected to proteolytic cleavage, generating three major fragments of different sizes (D'Arcangelo 2006; Lambert de Rouvroit et al. 1999). Apparently, larger fragments are not able to diffuse over long distances, but rather stay close to where the protein once was secreted, i.e. the marginal zone. However, smaller fragments can diffuse to the cortical plate, executing its function as a stop signal. Apparently different fragments have different functions; however, the complete mechanism is not fully understood yet.

1.5 Reelin and E2 crosstalk?

The inhibition of aromatase and hence the lowered levels of estrogen causes a reduction of spine synapses in the hippocampus (Zhou et al. 2010). A similar effect was demonstrated regarding spine density (Niu et al. 2008) in the *reeler* mutant*;* Ongoing experiments from our laboratory show that aromatase is reduced in *reeler* possibly due to its migratory defect of GnRH neurons. (Meseke et al. 2016, paper submitted)

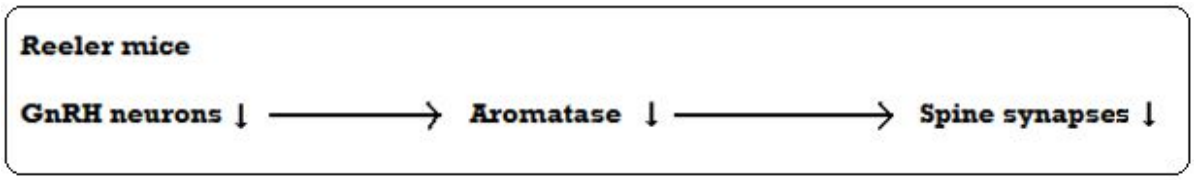


Figure 1.10: Consequences of migration defects of GnRH neurons in *reeler*.
In *reeler* the number of GnRH neurons is reduced due to migratory defects of these neurons in the Hypothalamus (Cariboni et al. 2005). GnRH was shown to stimulate hippocampal estrogen synthesis (Prange-Kiel et al. 2008), which is consistent with reduced aromatase expression in *reeler*. Accordingly estrogen synthesis is reduced as well, resulting in a reduced number of spine synapses.

However, during the last few years it became evident that synaptic plasticity in response to sexual steroids underlies a sexual dimorphism (Vierk et al. 2012).
Considering the interaction of GnRH and aromatase in wild Type (WT) as described earlier, we assume that the migratory defect of GnRH neurons in *reeler* reverses the activity state of aromatase, leading to reduced levels of sexual steroids in males and females. Conclusively, in *reeler* the spine synapse density should be reduced in females as well as in males. As it has been shown in figure 1.5. for WT the analogue is shown in figure 1.11. for *reeler*.

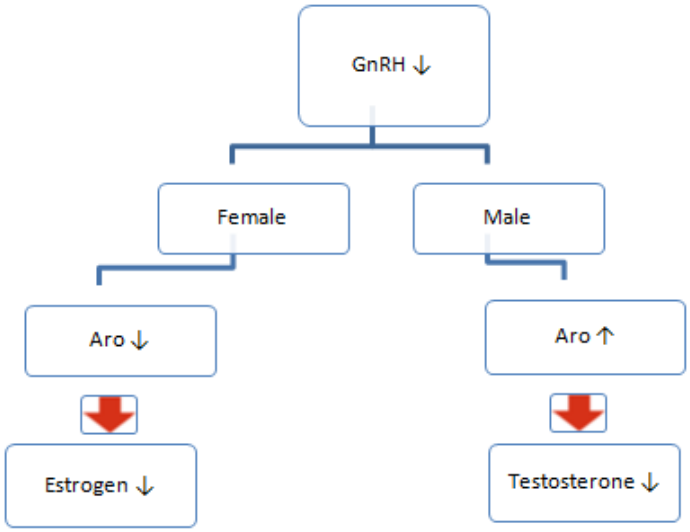


Figure 1.11: Regulation of steroid hormones by GnRH in *reeler*.
As far as it is known *reeler* has a migratory defect of GnRH neurons, resulting in reduced levels of GnRH. Since GnRH regulates the activity of aromatase in *reeler* the hormone levels are in opposition to that in WT: In male WT aromatase is suppressed, in females it has a stimulating effect. However, due to the reduced levels of GnRH the activity state of aromatase is inverted in *reeler,* yielding low levels of testosterone in males as well as low levels of estrogen in females.

Another aspect favouring an interaction between E2 and Reelin is given by the expression pattern of specific estrogen receptors. Preceding studies showed that hippocampal estrogen is able to regulate estrogen receptor (ER) expression (Prange-Kiel et al. 2003). Interestingly, the two isoforms of estrogen receptors (ERα and ERβ) act in an opposite way: While inhibition of estrogen synthesis by letrozole induced a significant decrease in ERα, ERβ was up-regulated. In contrast, treatment of hippocampal cells with estrogen enhances the expression of ERα whereas ERβ was down-regulated.

Studies concerning the expression of ER in the dentate gyrus point to an interaction between Reelin and estrogen (Bender et al. 2010). The expression pattern of estrogen receptors (ER) in the dentate gyrus also revealed an imbalance between the two receptors: While ERα showed a strong expression, ERβ is only slightly expressed. Strikingly, detailed analysis of ERα expressing cells identified them to be Cajal-Retzius (CR) cells, that type of cells known to express Reelin during neuronal development (D'Arcangelo et al. 1995). Supplementation of estrogen to hippocampal slice cultures of postnatal rats induced an increase in Reelin expression by CR cells and inhibition of estrogen synthesis causes a significantly reduced Reelin release. Furthermore, supplementation of estrogen together with estrogen receptor blocker annihilates the release of Reelin (Bender et al. 2010).
Estrogen and Reelin both are ligands for the transmembrane receptor and transcription factor *notch1* (Gaiano 2008; Rizzo et al. 2008). *notch1* is known to be a signalling molecule regulating the morphology and migration of post-mitotic neurons (Hashimoto-Torii et al. 2008). Furthermore, *notch1* binds *in vitro* to d*isabled* which is the Drosophila homologue of the mammalian *DAB1*, the intracellular adapter protein known to be involved in the Reelin signalling pathway (Hashimoto-Torii et al. 2008). In *reeler* the expression of *notch1* is severely reduced, indicating an interaction of Reelin and *notch1* (Hashimoto-Torii et al. 2008; Sibbe et al. 2009). Strikingly, Rizzo et al. were able to show that *notch1* activation can be inhibited by estrogen (Rizzo et al. 2008). In fact, both Reelin and estrogen have the ability to regulate the function of *notch1,* supporting the theory of a Reelin-estrogen cross-talk.

1.6 Purpose of work

In ongoing studies Rune and coworkers found that aromatase expression is reduced in hippocampi of *reeler* mice (unpublished data). In further studies it was also shown that aromatase expression in the hippocampus is mediated by GnRH, which might explain the gonadal cyclicity of spine density in females. *Reeler* mice, in turn, exhibit migratory defects of GnRH neurons, resulting in a reduced number of GnRH neurons in the hypothalamus (Cariboni et al., 2005). Given this background, we speculated that treatment of hippocampal slice cultures with estrogen could rescue spine synapse loss due to reduced aromatase expression in *reeler* mice. To this end, I treated hippocampal slice cultures with estrogen over a period of 8 and 9 days, respectively, and analyzed spine synapse density in these cultures. In addition I questioned what happens during developmental period. Possibly Reelin becomes apparent only later during the development, indicating its necessity for maintenance instead of the formation of synapses during pre- und postnatal development. I therefore performed experiments with juvenile and mature animals as well with respect to spine synapse density in the hippocampal CA1 region. Next to differences concerning the genotype and age I focused on differences between males and females.
Keeping the results of spine synapse density in mind I questioned as to how aromatase is expressed during development. To this end I quantified the expression of aromatase by immunoblotting and immunohistochemistry. Thereby I focused on differences concerning the genotype, age and in particular the gender, since ongoing studies from our own laboratory demonstrated a sexual dimorphism concerning sexual steroid-induced synaptogenesis.

2. Materials and Methods

2.1 Materials

2.1.1 Instruments and equipment

Aluminium foils	Stock, UKE
Centrifuge	HETTICH
Chemiluminescence camera	FUSION SL2, VILBER
CO_2-Incubator, 37°C	HERAEUS
Cover Slips	MARIENFELD
Cutfix Surgical Disposable Scalpel	BRAUN
Diamant Knife	DIATOM
Disinfectant	BODE
Dissection tools	F.S.T.
Document foils	LEITZ
Electronic microscope	CM 100, PHILIPS, PW 602
Embedding forms	PLANO
ep T.I.P.S. Standard, different sizes	EPPENDORF AG
Falcon Multiwell Cell Culture Plate	BECTON DICKINSON LAB
Falcon tubes, 15 ml, 50 ml	GREINER BIO-ONE
Forceps	MERCK
Freezer (-25°C, -80°C)	LIEBHERR
Fridge (4°C)	BOSCH/LIEBHERR
Gelatin capsule	PLANO
Glass slide	ASSISTANT
Gloves, different sizes	KIMBERLY-CLARK
Heidemann spatula	AESCULAP DE
Incubator, 37°C	MEMMERT
Laser Scanning Microscope	AXIOVERT 100 M ZEISS
Light-optical microscope	AXIOVERT 25 ZEISS
Microtome Blade S35	FEATHER
Microtome	REICHERT-JUNG
Millicell-CM	MILLIPORE
Pipettes, 100ml and 2500ml	EPPENDORF AG
Pipettes 2µ, 20µ, 100µ, 200µ and 1000 µl	GILSON
One-time Cuvettes	ROTH
Parafilm	PECHINEY PLASTIC PACKAGING
Pasteur pipette	ASSISTANT
Photographic paper	TETENAL
Pipette tips for Pipetus 5, 10 und 25 ml	BECTON DICKINSON LAB
Pipette tips	EPPENDORF AG
Pipetus Akku	HIRSCHMANN
Pulp	WEPA
Safety cabinet class 2	HERAEUS
Scales	METTLER
Scissors	FINE SCIENCE TOOLS
Syringes, different sizes	BRAUN
Tissue Chopper	H.SAUER
Ultramicrotome	REICHERT-JUNG
Tissue	WEPA

Tubes, 1, 2 and 5 ml	EPPENDORF AG
Vibraxer	EDMUND BÜHLER
Vortex Genie 2	SCIENTIFIC INDUSTRIES
Wet Chamber	SELF-MADE, UKE

2.1.2 Chemicals

10 x PCR buffer	INVITROGEN
5fold DNA Loading Buffer blue	BIOLINE
Ammonium persulfate (APS)	SIGMA
Aqua ad iniectabilia	BAXTER
Bio Rad Protein Assay	BIO-RAD LAB. GMBH
Bovine serum albumin (BSA)	SIGMA
Bromophenol blue	MERCK
Carbonic acid	SOL SPA
Chemiluminescence substrates	
ECL (PierceECL Western Blotting Substrate)	THERMO SCIENTIFIC
Pico (Super Signal West Pico)	THERMO SCIENTIFIC
Femto (Super Signal West Femto)	THERMO SCIENTIFIC
Dako Mounting Medium	DAKO CYTOMATION
4',6-diamidino-2-phenylindole (DAPI)	SIGMA
Diluent	DAKO CYTOMATION
Disodium phosphate	MERCK
Dithiothreitol (DTT) 0,1M	INVITROGEN
dNTP-Mix	FERMENTAS
Ethanol, 100%	MERCK
Ethanol, 70 %, 96 %	Chemistry, UKE
Ethidium bromide	SERVA
Ethylendiamintetraacetat (EDTA)	MERCK
Glucose, 50 %	FRESENIUS KABI
Glutaraldehyde , 25%	MERCK
Glutaraldehyde solution, 25%	MERCK
Glycerin	SIGMA
Glycin	ROTH
Hanks Balanced Salt Solution (HBSS)	GIBCO
Horse serum	GIBCO
Hydrochloric acid	MERCK
Lead citrate	MERCK
L-Glutamine, 200 mM	SIGMA
Methanol	J.T. BAKER
MgCl2 50mM	INVITROGEN
Milk powder	HEIRLER
Minimal Essential Medium	GIBCO
Monosodium phosphate	MERCK
Natriumphosphate buffer	MERCK
NP40 Alternative	CALBIOCHEM
Osmiumtetroxide	ROTH
Oxygen	SOL SPA
Page Ruler Plus Prestained Marker	FERMENTAS
PBS-Tablets	MERCK
PhosStop	ROCHE

Ponceau-Rot	MERCK
Primer for PCR, Concentration 10µM	EUROFINS MWG/OPERON
Protease-Inhibitoren-Mix Complete	ROCHE
Protein Block	DAKO CYTOMATION
Rotiphorese-Gel 30 % (Acrylamid)	ROTH
SDS (Sodium dodecylsulfate)	FLUKA
Sodium bicarbonate solution, 7,5%	MERCK
Sodium deoxycholat	SIGMA
Sodium hydroxide	MERCK
Sucrose	MERCK
Taq DNA Polymerase 5U/µl	INVITROGEN
TBE-Puffer 10x	USB
TEMED	SIGMA
Tissue Tek	SAKURA
TRIS	ROTH
Triton-X	SIGMA
Tween	SIGMA

2.1.3 Animals

Postnatal, young adult and adult *reeler* mice (P5-7, 4-5 weeks and 9-14 weeks, respectively; B6C3Fe a/a-Reln<rl>/J;) were obtained from the *Zentrale Versuchstierhaltung, Universitätsklinikum Hamburg Eppendorf (UKE)*. For control group C57/Bl6 mice (Charles River, Germany) were used. The animals were maintained under controlled conditions following the institutional guidelines for welfare. Water and food were available ad libitum. All experiments were performed according to prevailing legal requirements. The initial experiments, using postnatal animals, were performed independent of gender. The following experiments, however, were performed considering both age and gender. Table 2.1 shows animals and amounts used in experiments.

Table 2.1: Animals used for Electron microscopy, Immunohistochemistry and Immunoblot

	electron microscopy					**IHC**		**WB**	
	4-5 Weeks		**9-14 Weeks**		**postnatal (day 5-7)**	**5-6 weeks**			
	male	female	male	female		male	female	male	female
B6C3e a/a Reln<rl>J	3	3	3	3	3	3	3	3	3
C57/Bl6	3	3	3	3	3	3	3	3	3

2.1.4 Solutions and Buffers

2.1.4.1 Perfusion

PBS (Phosphate buffered saline)
For 500ml

- 500 ml distilled water (pH = 7,4)
- 1 tablet of PBS
- Titration with NaOH (1mol/L) and HCl (1mol/L), respectively, until pH = 7,4

Phosphate buffer (PB) 0,1M
For 100 ml:
- 10 ml 0,5 M NaH2PO4
- 10 ml 0,5 M Na2HPO4
- ad distilled water up to 100 ml
- Titration with NaOH (1mol/L) and HCl (1mol/L), respectively, until pH = 7,4

Glutaradehyde solution in 0,1 M PB
For 300 ml:
- 30 ml 0,5 M NaH2PO4
- 30 ml Na2HPO4
- 30 ml Glutaradehyde 25 %
- ad distilled water until 300 ml
- Titration with NaOH (1mol/L) and HCl (1mol/L), respectively, until pH = 7,4

Paraformaldehyde (PFA) 4%
- 4g PFA dissolved in 100ml PBS

2.1.4.2 Slice Cultures

Preparation medium for slice cultures
- 50 ml Minimal Essential Medium (MEM) 2-fold concentrated
- 1 ml L-Glutamine, 200 mM
- 48 ml distilled water
- Titration with NaOH (1mol/L) and HCl (1mol/L) respectively, until pH = 3,5

Incubation medium for slice cultures
- 25 ml Minimal Essential Medium (MEM), 2-fold concentrated
- 1 ml L-Glutamine, 200 mM
- 580 µl Sodium Bicarbonate Solution, 7,5 %
- 25 ml Hanks Balanced Salt Medium, (HBSS)
- 25 ml Horse serum
- 1 ml Penicillin-Streptomycin solution
- 1250 µl glucose, 50%
- 19 ml distilled water
- Titration with NaOH (1mol/l) and HCl (1mol/l), respectively, until pH = 7, 25

2.1.4.3 Immunohistochemistry

PBS (see above)
TBS
For 1000ml:
- Tris-HCl
- NaCl
- dissolved in 850ml aqua dest
- Titration with NaOH (1mol/L) and HCl (1mol/L), respectively, until pH = 7,4
- ad aqua dest up to 1000ml

Triton-X 0,5%
- 1000ml TBS, 1-fold concentrated
- 5ml Triton-X

4`, 6-Diamidino-2phenylindol (DAPI)
- 50µl dissolved in 50ml PBS

2.1.4.4 Western Blot

Wet Blot Buffer (1x)
- 2,9g Glycin
- 5,8g Tris
- 3,7mL SDS 10%
- 200mL Methanol
- Ad 1000ml H_20

5x Laemmli Samplebuffer:
- 1, 54 Dithiothreitol (DTT) (≙0,5M)
- 2,0g SDS (≙10%)
- 8,0ml 1M Tris-HCl, pH 6,8 (≙0,4M)

This mixture yields about 10,5ml. It takes about 30 minutes until it is dissolved, afterwards it has to be filled up with Glycerin 49,5% to 20ml. Finally a bit of bromophenol blue has to be added.

10x Laemmli electrophoresis running buffer
- 30g Tris
- 144g Glycin
- 10g SDS
- ad 1000ml H2O

RIPA Buffer
- 150mM NaCl
- 50mM Tris pH 7,5
- 1% NP 40 Alternative
- 0,1% SDS
- 0,5% Sodium deoxycholate
- 5mM EDTA

Before usage ad tablets of protease inhibitors: PhosStop (1:10) and Complete (1:25)

Blocking solution (Immundetection)
5 % (w/v) milk powder in PBS-Tween

PBS-Tween 20 (PBS/0,3% Tween 20)
- 30ml Tween 10%
- 100ml 10X PBS pH 7, 4
- ad 1000ml H20

Table 2.2: Antibodies used for Immunohistochemistry and Immunoblot

Primary AB	Host	Dilution	Incubation time and temperature	Reference
Anti-aromatase-AB (Madrid)	Mouse	1:200	overnight, 4°C	ACRIS (Herford)
Anti-GapDH-AB	Mouse	1:20000	overnight, 4°C	Applied Biosystem/ Amb ion
Secondary AB				
Anti-Mouse-HRP	Monkey	1:2500	2h, room temp.	Jackson ImmunoResearch Laboratories
Anti-Rabbit Alexa 488	Goat	1:500	2-3h, room temp.	Jackson ImmunoResearch Laboratories

2.1.4.5 PCR

DNA Digesting Buffer
- 0,5% Sodium Dodecyl Sulfate (SDS)
- 10mM Tris-HCl
- 3,6mM CaCl2

10x TBE Stock solution
- 0,89M Tris
- 0,89 M boric acid
- 0,02 M EDTA

Agarose Gel
For 150ml:
- 150 ml TBE Buffer
- 3g agarose
- 3µl ethidium bromide

Primer

Based on data from D'Arcangelo et al., 2008 the following primers were used for amplification (Eurofins mwg/operon, Concentration: 10µM):

Table 2.3: Primer used for amplification (1+2: WT; 1+3: *reeler*)

	NAME	SEQUENCE	APPLICATION
1	WT forward	5'-TAA-TCT-GTC-CTC-ACT-CTG-CC-3'	Genotyping
2	WT reverse	5´-CTA-CAC-AGT-TGA-CAT-ACC-TTA-ATC-TAC-3'	Genotyping
3	*reeler* reverse	5'-ACT-TGC-ATT-AAT-GTG-CAG-TGT-TGT-C -3'	Genotyping

2.2 Methods

Three different methods were used to analyze activities and effects of aromatase in the brain of *reeler* in comparison to WT as well as with respect to a sexual dimorphism.
Initially the synaptic density was evaluated by electron microscopy in mice of different ages with the focus on estrogenic effects on synaptic plasticity. Next the expression of

aromatase was determined at first by western blotting and secondly by immunofluorescence.

2.2.1 Organotypic hippocampal slice culture

To examine postnatal mice organotypic slice cultures were prepared. Organotypic hippocampal slice cultures provide an eminent preparation of central nervous tissue for exploring the structural and physiological features of neuronal cells within their native three-dimensional environment. It is a straightforward method to obtain tissue slices from neonatal animals (Fuller and Dailey 2007)
30 postnatal *reeler* or WT mice aged five to seven days were dissected. Prior to preparation every littermate was observed systematically concerning its motion pattern to identify *reeler* phenotype. However, to verify the genotype a small piece of the tail was obtained for genotyping (see below).
Dissection of hippocampi and subsequent preparation of organotypic slice cultures were carried out according to Stoppini (Stoppini et al. 1991). Mice were decapitated under semi-sterile conditions and the skin covering the calvarium was removed carefully. The skull was opened with a median-sagittal cut, using sharp scissors. Next the brain was carefully dissected with a *Heidemannspatel* and placed on a small sponge saturated with phosphate buffered saline (PBS). The brainstem and the cerebellum were removed and the brain was cut centrically in a median plane. The hippocampus became visible and could be dissected gently. Perpendicularly to its longitudinal axis, slices were obtained (375 µm) using a McIllwain tissue chopper (Sauer, USA). Subsequently slices were immediately placed in preparation medium. Three to four neighboring slices, each containing CA1 - CA3 and dentate gyrus (DG) regions, were chosen from every hippocampus and placed on moistened membrane inserts in a Petri dish filled with incubation medium. Slices were maintained at 37°C and 5% CO_2 during incubation period. After a preculturing period of four days the medium was supplemented for 7 days either with estrogen at a concentration of 10^{-7} M (Sigma, Deisenhofen) or with pure water (Control). The incubation medium with or without estrogen was changed every second day.

2.2.2 Tissue preparation and fixation of brain tissue by perfusion

Adult and young adult (5-6 weeks) animals were prepared for immunohistochemistry, electron microscopy and western blotting. In order to guarantee an ideal preservation of cellular and sub-cellular structures the animals has to be perfused to fixate the brain tissue.
The mouse was placed in a little box and narcotized with a gas mixture of oxygen and carbonic acid. In order to ensure anesthesia pure carbonic acid was used. As soon as the mouse was completely anesthetized it could have been placed on a little metal table and the mouse was fixed with tape to the table. In order to clean the mouse, the animal´s abdomen and thorax was moistened with ethanol. The abdominal and the thoracic cavity were opened carefully with small scissors by a median cut after cutting through the fur. The heart became visible and a sample of blood was obtained from within the heart to measure estradiol serum levels.
After the animals preparation the vena cava or the right ventricle was transected and perfusion was started. According to the following procedure different solutions are used, that is for Electron Microscopy GA 2,5% and for Immunohistochemistry PFA 4%. For Western Blotting, however, no fixation is recommended.
To prevent air within the perfusion system the flow was started before the needle was introduced into the left ventricle. At first blood was washed out with Phosphate Buffered

Saline (PBS). Assuming blood volume of a mouse account for about 25% of its weight, we used three times this quantity as buffering solution. To guarantee proper fixation a following perfusion with phosphate buffer was accompanied in case fixation was realized by GA 2,5%. Subsequently the animal was slowly perfused either with GA 2,5% or with PFA 4% until fixation was completed. For later analysis the ovaries and testis were removed and placed in fixation medium.
The skull of the mouse was opened carefully with a sagittal and a frontal cut. After opening the calvarium the brain became visible and could be obtained by using a *Heidemannspatel.*
For immunohistochemistry and electron microscopy each brain was placed in a Falcon tube filled with fixation medium and kept at 4°C for ongoing fixation. For western blotting hippocampi were isolated and shock-frozen in liquid nitrogen and stored at –80°C until further analysis.
Blood samples were centrifugated for a few seconds and also kept at 4°C. The following day samples were centrifugated at 5000rpm for 10 minutes. Serum was separated and kept at -22°C until further analysis.

2.2.3 Electron microscopy

Hippocampal organotypic slice cultures and hippocampal slices of adult and juvenile animals were further processed for electron microscopy (EM). The use of an electron microscope can reveal structures of smaller objects, since it uses an electronic beam. Electrons have a wavelength about 100.000 times shorter than visible light photons. This feature makes electron microscopy superior to the common light microscopy.
Culturing period of hippocampal slice cultures was completed by fixation of the slices with formaldehyde (4% in PBS). Prior to this process the culturing medium was evacuated and slices were washed with phosphate buffer twice and with PBS once. After fixation slices were washed again with phosphate buffer. Slices were post-fixed for two hours with 1% OsO4 diluted in 0,1M phosphate buffer with sucrose.
To minimize damage to the tissue all slices were firstly dehydrated in graded ethanol (35%, 50%, 70%, 96%, 100% always 15minutes) then dehydrated in propylene oxide and lastly dehydrated in propylene oxide and glycid ether first at a ratio 1:1, then 1:3, supplemented by 2,4,6-Tris(dimethylaminomethyl)phenol 2% serving as an accelerator. Finally probes were embedded in glycid ether and kept at 60°C for eight hours enabling them to polymerize. Afterwards slices were trimmed in order to obtain the isolated CA1 region. For this reason semi thin slices were cut and stained providing a better orientation in the tissue.
Trimmed blocks were cut in ultrathin sections (90nm) using a Reichert-Jung OmU3 ultramicrotome and placed on grids. About 5 consecutive slices were chosen from each block. Staining was performed by uranyl acetate followed by lead citrate. Electron micrographs were produced at 6600x magnification. Disturbances such as large blood vessels or interfering dendrites were intentionally avoided.

2.2.4 Calculation of spine synapse density

To avoid biases the observer was blinded to the experimental group.
The disector technique, established by Sterio in 1984 (Sterio 1984), was used for analysis of EM images. This technique allows the observer to make unbiased estimates of particles in a determined volume. Two pictures covering corresponding neuropil fields were compared by using a reference grid superimposed on the EM prints.

Only those synapses were counted being present on picture one, but not on picture two, or vice versa. The following criteria define a spine synapse: It is composed of (1) a postsynaptic density, (2) presynaptic vesicles, (3) a synaptic cleft and (4) pre- and postsynaptic membranes (Kahle W., 2001). To be counted as a spine synapse all these specific criteria must be fulfilled.
At least eight neuropil fields were photographed on each electron microscopic grid. With a minimum of three grids of each section containing two pairs of consecutive, serial ultrathin sections each animal (two Hippocampi) provided at least 8 x 3 x 2 = 48 neuropil fields. Each pair (photos of reference and look-up sections) of photographs represented an 8 x 8 x 0,1µm = 6,4µm³ volume. As already mentioned from each mouse 48 photographs were analyzed, representing a volume of at least 307, 2 µm³.

2.2.5 Genotyping

Postnatal *reeler* mice (day 5-7) cannot be distinguished phenotypically from WT mice. To differentiate between WT, homozygous and heterozygous *reeler* the genotype was determined. Genotyping includes tissue preparation, amplification of DNA by PCR and separation and classification of different DNA-strands.
Genomic DNA from mouse tail biopsies was extracted and amplified by PCR. According to previous reports genomic DNA of homozygous *reeler* mice gives rise to 368bp fragments, whereas in WT the DNA fragment is 266bp, considering the commonly used primers. Three animals were chosen for subsequent experiments for each genotype.
Fermentation: Each biopsy was dissolved in 100µl of buffer solution to be subsequently fermented by 5µl Proteinkinase K (recombinant, PCR Grade, Roche). The incubation at 55°C lasted at least eight hours.
DNA Extraction: To stop fermentation samples were heated to 94°C for 10 minutes. For extraction samples were centrifugated at 14000rpm for 3min, the supernatant was transferred to a new test tube and DNA was precipitated with 20µl 5M NaCl and 450µl ethanol (100%; ice-cold). Probes were then centrifugated at 14000rpm for 15min at 4°C and the supernatant was removed. Further on the probes were washed with 500µl ethanol (70%), centrifugated at 14000rpm for 5min and the supernatant was removed. This washing process was repeated three times. The probes were air-dried for at least one hour to be finally dissolved in 50µl pure water.
Polymerase Chain Reaction: In order to guarantee an effective reaction, at least 50µl of PCR-Mastermix is needed.

Table 2.4: Polymerase Chain Reaction, contents of Mastermix

genomic DNA	3µl
forward primer (10mM)	1,5µl
reverse primer (10mM)	1,5µl
dNTPs (10mM each)	2µl
10 x PCR buffer	5µl
$MgCl_2$ (50mM)	5µl
Taq-Polymerase (5u/µl)	0,2µl
with water ad 50µl	

Primer: Primers named in table 2.3. (see above) were used for amplification.

Depending on different primer combinations two different PCR programs were applied considering each primer's working optimum (Table 2.5.).

Table 2.5: PCR-Conditions

CYCLES	TEMPERATURE	TIME	PROCESS
1	94°C	60s	Initiation
39	94°C	30s	Denaturation
	50°C WT * 49°C *reeler*	30s	Annealing
	72°C	30s	Elongation
1	72°C	10min	

* = Difference

Probes were kept at 4°C or stored at -20°C until further processing. PCR-products were analyzed by gel electrophoresis.
After charging with 10µl loading dye the probes were loaded on agarose gels. Gel was run for 1h (120V) and a picture was taken for documentation and analysis.

2.2.6 Protein Biochemistry

Immunoblot is an analytical technique enabling the detection and expression of specific proteins in a sample of tissue homogenates.
To examine expression of aromatase in the hippocampus of *reeler* an immunoblot was performed, using monoclonal mouse anti-aromatase antibodies. Concerning expression of aromatase differences between *reeler* and WT as well as between females and males were analyzed.

2.2.6.1 Preparation of lysate from tissue

Hippocampi and gonads were dissected and immediately shock-frozen in liquid nitrogen. Until further processing tissues were stored at -80°C. When thawed at 4°C, the probes were homogenized manually with RIPA-buffer. To prevent dephosphorylation PhosStop was added, a tablet containing a blend of phosphatase inhibitors. In addition a mixture of proteinase inhibitors was added to the buffer directly before use. A glaspotter was used for homogenization in case the tissue was too dense like in the ovaries, or syringes with very thin canulas. Samples were incubated on ice for 20 minutes and then centrifugated at 13.000rcf for 20 minutes at 4°C. The supernatant was collected, shock frozen in liquid nitrogen and stored at - 80°C until further processing. For determination of protein concentration we partitioned 10µL of the supernatant separately.

2.2.6.2 Bradford protein assay

The aim of this assay is to measure the quantity of all proteins within different probes. To this end a series of protein standards was prepared, using Bovine Serum Albumin (0,5µg/µL). The protein was diluted in distilled water to final concentrations of 0µg,

1,25µg, 2,5µg, 5µg and 10µg. To make allowance to the RIPA-buffer which might influence the probes we added 1µL of RIPA- buffer to every protein standard.
Of each hippocampal and ovarian probe 1µL was diluted in 19µL distilled water. 1mL of Bradford reagent was added to the standards and to the probes, subsequently extinction was measured and concentration determined.

2.2.6.3 SDS-Page

Sodium Dodecyl Sulfate Polyacrylamid Gel Electrophoresis (SDS-Page) is used to separate proteins depending on their molecular weight. The detergens SDS allows a separation only based on the size by binding and saturating the proteins. The binding creates negatively charged Protein-SDS-complexes, whereby the charge of the proteins becomes negligible. Two different gels are needed: one to accumulate the proteins in the beginning and a second one to subdivide them. Based on experience aromatase gets separated the best using a 10% polyacrylamid gel.

Table 2.6: Recipes for four gels for gelelectrophoresis

	Accumulating gel	**Separating gel**
H2O	5,5ml	8ml
Acrylamid 30%	1,7ml	6,6ml
1,5M Tris pH 8,8	-----	5ml
0,5M Tris pH 6,8	2,5ml	----
10% SDS	100µl	200µl
Bromophenol blue	100µl	----
TEMED	10µl	8µl
10% APS	100µl	200µl

As ammonium persulfate and TEMED initiate polymerization separating gel needs to be poured into the caster quickly. Addition of water atop makes the gel distribute equally. After an hour the water can be removed and accumulating gel can be added. To create sample wells a comb has to be inserted. After another half an hour the comb is ready to be removed and the gels are placed in the electrophoresis chamber. Laemmli running buffer was added around the sample plates to generate current flow.

2.2.6.4 Sample preparation

In total 40µg of protein was used. According to calculated concentration probes were diluted with the appropriate amount of distilled water and Laemmli sample buffer. Proteins become denatured by heating them up to 95°C for 5 minutes. Following a brief centrifugation probes were loaded into the gel. To determine molecular size of the proteins the Page Ruler Plus Prestained Marker (Fermentas) was used. In addition to the hippocampal samples we inserted a sample of an ovary as well. This was aiming to have a control sample. It took about 40 minutes until the relevant proteins were separated at a current of 180V.

2.2.6.5 Immunoblotting

Proteins were transferred from the gels to nitrocellulose membranes by electroblotting. Gels were removed and placed between filter papers and sponges which were drenched in wet blot buffer. Before usage buffer was replenished with cold water and methanol. Buffer was filled into the blotting hardware as well as a magnetic stir bar and an ice cube in order to guarantee cold temperature. Blotting was realized at a voltage of 100V for 80 minutes.

2.2.6.6 Immunodetection

Subsequently membrane was stained with Ponceau S (Sigma) to assess transfer of proteins from gel to membrane. To block unspecific binding sites blots were treated for one hour with 5% (w/v) milk powder in 0,3% PBS-Tween. The blots were incubated over night at 4°C with primary antibody (SM2222P Aromatase Antibody) in the appropriate dilution blocking solution. For every membrane about 3mL is needed. As a control and to determine total quantity of proteins an antibody against GAPDH was used as well.
The next day, blots were washed with PBS-Tween three times, and incubation with secondary antibody was started. (Goat-Anti-Mouse-HRS, diluted in blocking solution). Conjugation of the antibody to a Horse Radish Peroxidase (HRS) enables detection by chemiluminescence. After an hour blots were washed again with PBS-Tween and antibody-protein-complexes were detected by chemiluminescence. This phenomenon describes reaction of two reactants, peroxidase and luminal. Peroxidase is linked to a second antibody and luminal is part of the detection kit. For detection ImmobilonWestern (Millipore, Billerica, USA) and Millipore high sens (Millipore, Billerica, USA) were used.
After 5 minutes aromatase is detectable. GAPDH, however, only needs about 2 minutes. The radiation can be detected by luminous exposure. Exposure time for aromatase was varied between 1 and 15 minutes, however, best results were received after 10 minutes. Creation and documentation of digital images from chemiluminescence is realized by a Fusion SL (Vilber Lourmat, Eberhardzell).

2.2.7 Immunohistochemistry

In this study immunhistochemistry was aimed to assess expression of aromatase in *reeler* in comparison to WT. Basically immunohistochemistry is an immunological reaction between antigen and antibody, with aromatase being the antigen reacting with an anti-aromatase-antibody. The antigen-antibody-complex is visualized by binding of a secondary fluorophore-carrying antibody to initially formed antigen-antibody-complex. Using ultraviolet light the degree of linking can be determined.
After fixation and dissection (see above) the hippocampi were postfixed with PFA 4% for 2 days, cryoprotected (25% sucrose in PBS) for another 2 days, shock-frozen in methyl-butan in liquid nitrogen and stored at -80°C until further processing. Without thawing the brains were cryotomed in slices of 20µm thickness. At least five slices of the left and five slices of the right hippocampus were obtained of each brain and mounted on glass slides. Slices were air-dried for at least half an hour and stored in a box at -20°C until further processing.
For immunohistochemistry slices were thawed, air-dried and washed three times for 10 minutes with PBS. To achieve permeability, sections were washed with the detergent Triton-X, 0,5% diluted in TBS, three times for 10 minutes. Subsequently they were washed again with PBS. To block unspecific binding sites Protein block was mounted on the

slides. It was removed carefully with a facial tissue after 30 minutes. Thereafter primary antibody (Anti-Aromatase-Antibody, diluted in Antibody Background Reducing Medium, 1:200, 50µL/slice) was applied. For about 24 hours the sections were incubated in a wet chamber at 4°C.
The next day, the primary antibody was removed carefully with a tissue and sections were washed three times for 10 minutes with PBS. Next the secondary antibody (Goat anti rabbit, diluted in Antibody Background Reducing Medium, 1:500, 50µL/slice) was applied for 2-3 hours at Room temperature (RT) and deprived from light. From now on every of the following steps was performed under light deprived conditions. After incubation sections were washed again and treated with the DNA-staining solution 4,6-diamidino-2-phenylindol (DAPI) for six minutes, 70µl on each slide. After a final washing with PBS the samples were embedded with fluorescent mounting medium (Dako, Hamburg) and coverslipped. All experiments were accompanied by a negative control, i.e. no primary antibody was used.
Results were viewed and photographed using a Leica Axiovert fluorescence microscope or a laser scanning microscope (SP2 Leica). For analyzing the intensity of aromatase expression the CA1 region was identified. With a 63-fold amplification about 18 pictures from every slice were taken.

2.2.8 Analysis with ImageJ

To analyze Western Blots as well as immunohistochemical stainings ImageJ was used. ImageJ is a Java-based, picture processing computer program developed at the National Institute of Health in the USA.

2.2.8.1 Analysis of immunohistochemically stained slices

To analyze immunohistochemically stained slices of the hippocampi pictures were taken using a Confocal Fluorescent Microscope. To evaluate immunological reaction between aromatase and its antigen we used ImageJ, which translates the fluorescence into different levels of grey varying from 0 to 255. Accordingly, the Integrated Density [IntDent] can be calculated. The Integrated Density describes the mean grey value of a selected area, hence in this experiment the intensity of aromatase expression.

2.2.8.2 Analysis of Immunoblots

Immunoblots were analyzed by means of an examination of the individual bands. In fact, the band will be translated into a U-shaped curve, representing the intensity of staining. The area under the curve which is the Integrated Density can be determined. The Integrated Density, in turn, refers to individual aromatase expression.

3. Results

The aim of this study was to verify spine synapse density in male and female *reeler* mice of different ages. In addition, expression of aromatase protein was analyzed with respect to (1) a sexual dimorphism and (2) differences between *reeler* and Wild Type (WT) mice.
With the aid of stereological methods I determined spine synapse density in the hippocampal CA1 region of *reeler* and WT. For analyzing expression of aromatase I performed western blots as well as immunohistochemical stainings of hippocampal slice cultures.

3.1 Determination of spine synapse density by electron microscopy

I compared two parallel electron microscopic pictures with the focus on spine synapses. By counting only those synapses detectable on picture one but not on picture two I made sure the same synapse is not counted twice. A spine synapse has to meet specific criteria to be counted as described earlier (see chapter Materials and Methods, section 2.2.4.). Intact synapses have been identified in every experimental group. A representative spine synapse is shown in figure 3.1.. Figure 3.2. exemplifies two consecutive pictures covering the same area illustrating how synapses were counted.

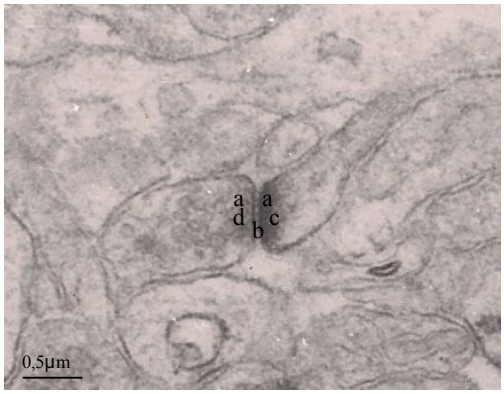


Figure 3.1: Spine synapse
A spine synapse is determined by following criteria: a) pre- and postsynaptic membrane, b) synaptic cleft, c) postsynaptic density, d) presynaptic vesicles (Kahle and Frotscher 2001). The synapse shown meet all required criteria.

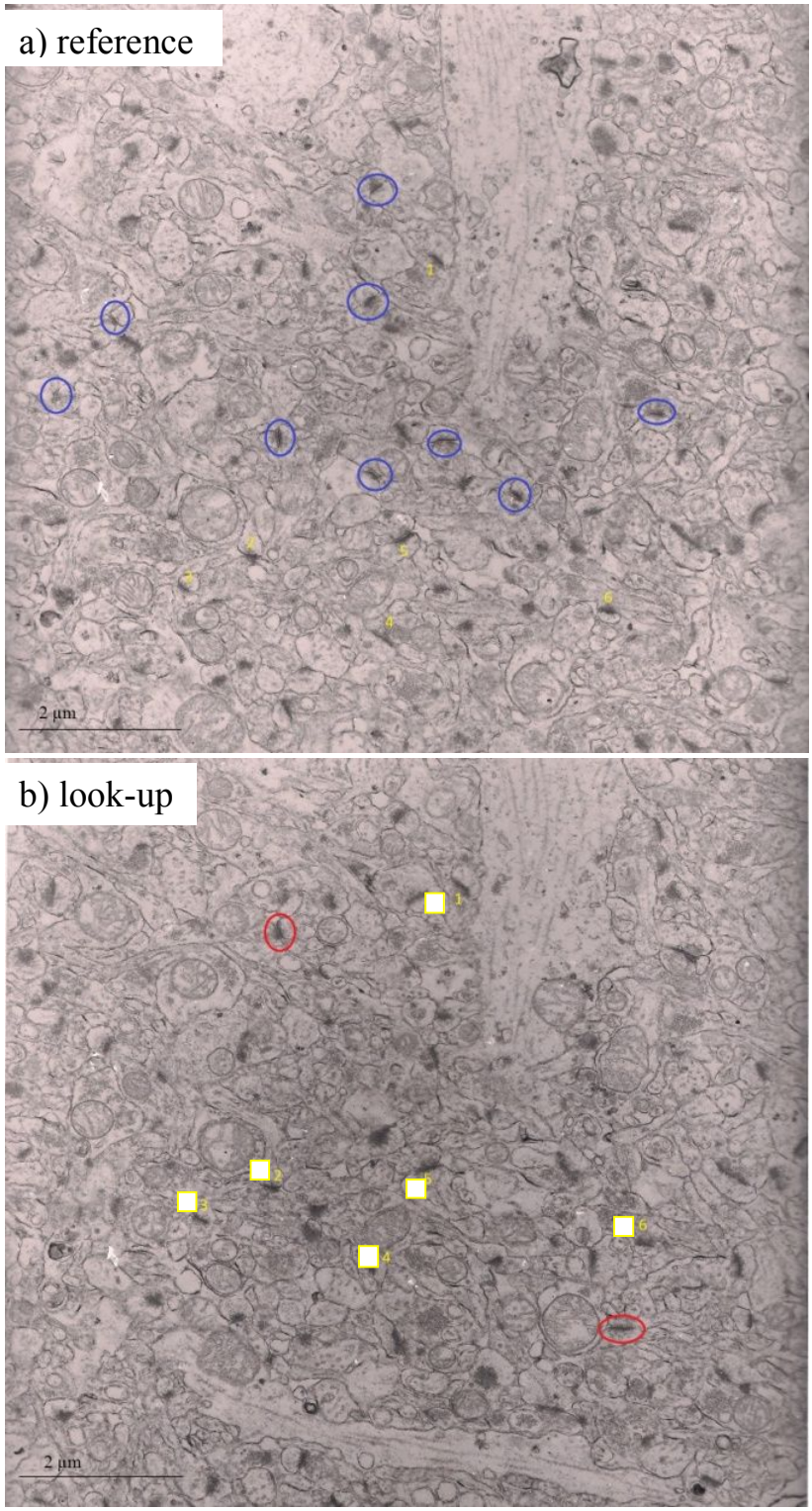


Figure 3.2: Two electron micrographs covering corresponding neuropil fields
The EM pictures illustrate the same area of two consecutive, serial sections depicted from hippocampal CA1 region. Picture **a** shows reference section, picture **b** shows look-up section. Only those synapses were counted visible exclusively in one section (marked with blue circles in the reference section, red in the look-up section). Yellow squares represent synapses observed on both sections.

Results of synpase counting were tested for significance with the aid of the spreadsheet *Microsoft Excel* and the statistical program *SPSS*. As it is common with statistical analysis α-error was set at 0,05 so significance is provided if p-value < 0,05.

3.1.1 Spine synapse density in postnatal mice

According to recent literature spines are reduced in postnatal heterozygous and especially in homozygous reeler mutants (Niu et al. 2008). To verify the assumption that a reduction of spines has an impact on the entire spine synapses I determined spine synapse density in postnatal homozygous reeler.

3.1.1.1 Spine synapse density in postnatal *reeler* compared to WT mice

First I determined the number of spine synapses in hippocampal slice cultures of postnatal *reeler* and WT mice. Examination was performed after a preculturing period of 4 days and a culturing period of 7 days. Indeed spine synapse density is reduced in *reeler*, however only a slight reduction has been seen. Figure 3.3. shows on the abscissa the groups examined (WT and *reeler*) while the ordinate represents the relative amount of spine synapses determined in a volume of 6,4µm³. WT is defined as 100%, a reduction of 9% was found in *reeler*. Statistical analysis by a student's-t-test did not yield any significant difference.

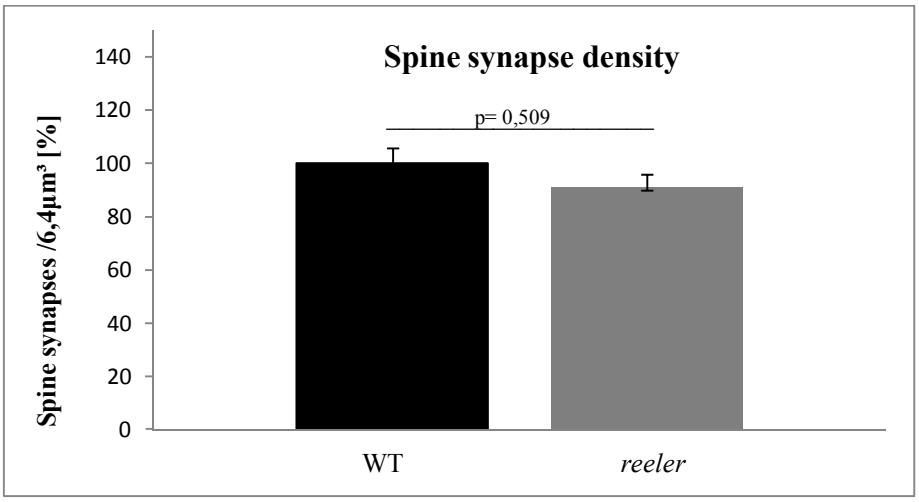


Figure 3.3: Spine synapse density in hippocampal slice cultures of postnatal *reeler*
Electron micrographs obtained from hippocampal slice cultures of postnatal WT and *reeler* were compared and statistically analyzed. Controls (WT) are defined as a 100%. In *reeler* the spine synapse density was ~9% lower, hence no significant difference was found by student´s-t-test. Averaged absolute values: WT= 14, 22 spine synapses and *reeler* = 12, 94 spine synapses per 6,4µm³**.** Both groups: n=9, mean ± SEM

3.1.1.2 Spine synapse density in postnatal *reeler* after estrogen treatment

In previous experiments spine synapse loss was induced *in vitro* and *in vivo* using a reversible non-steroidal aromatase inhibitor (letrozole). Interestingly these effects can be rescued by application of 17ß-estradiol (Kretz et al., 2004).
However, we have shown that exogenously applied estrogen does not induce spine synapse formation as long as estrogen synthesis is not lowered within the cell (Kretz et al. 2004). Figure 3.4. shows spine synapse density in postnatal WT before and after treatment with estrogen. Hippocampal slices of WT mice were treated with estrogen at a dose of 100nM. On the abscissa of figure 3.4. both groups are compared, the ordinate represents relative values of spine synapses. A small increase is seen regarding spine synapse density after

estrogen treatment. However, statistical analysis showed that estrogen at a dose of 100nM did not cause a significant effect with respect to spine synapse density.

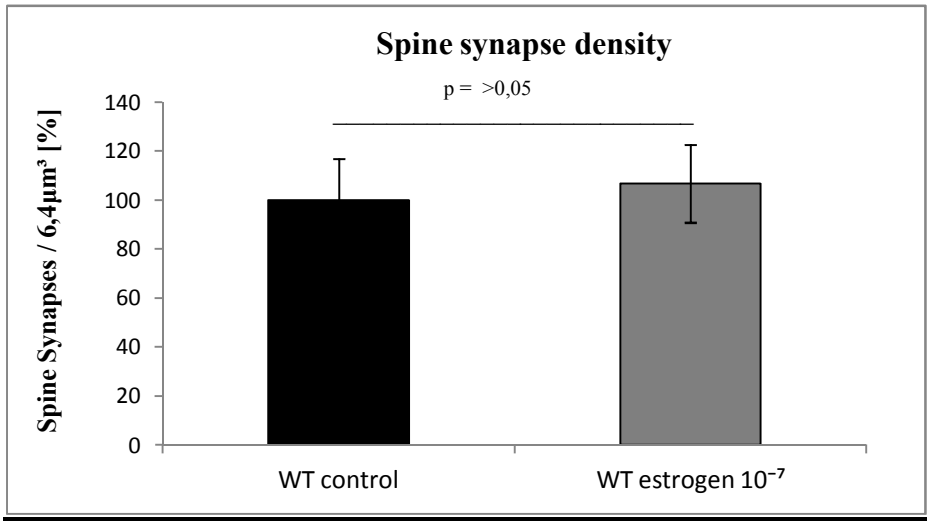


Figure 3.4: Spine synapse density in hippocampal slice cultures in postnatal WT after estrogen treatment
Synapses were quantified in the stratum radiatum of the hippocampal CA1 region before and after stimulation with estrogen at a dose of 10^{-7}M. After stimulation a slight increase of 7% of spine synapses was seen (control = 100%, both groups n= 9, mean ±SEM); (Data kindly provided from previous studies from our own laboratory, Kretz et al., 2004).

Referring to the data from Niu and co-workers (Niu et al. 2008) with synapses being reduced when estrogen is missing we hypothesized that treatment with estrogen might rescue this reduction. To this end I stimulated hippocampal slice cultures of postnatal *reeler* with estrogen. After a preculturing period of 4 days slices were treated with estrogen at a dose of 100nM. Results of stereological counting are shown in figure 3.5.. Values are shown for *reeler* prior to treatment on the left, on the right values are shown following estrogen treatment of *reeler* organotypic slice cultures. However, statistical analysis by student´s-t-test did not show a significant difference between both groups.

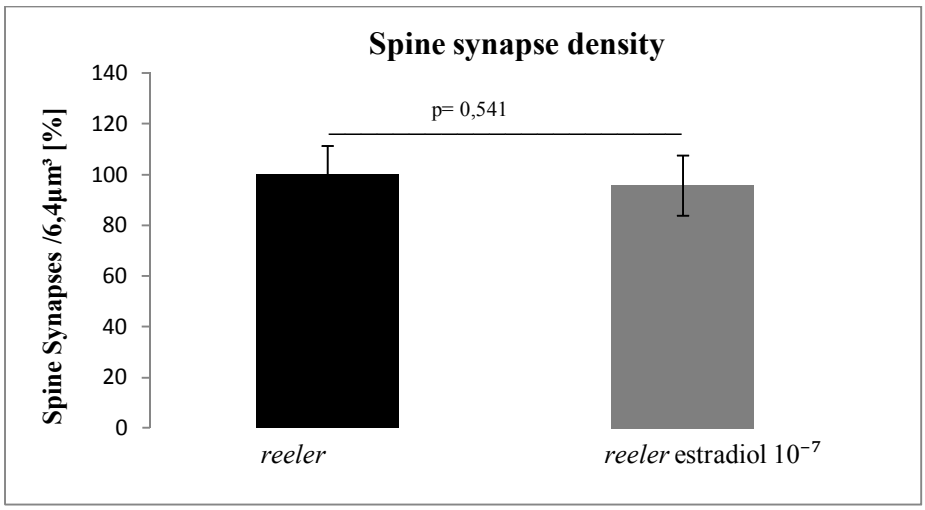


Figure 3.5: Spine synapse density in hippocampal slice cultures of postnatal reeler in response to estrogen treatment
Hippocampal slice cultures of postnatal *reeler* were stimulated with estrogen. After a preculturing period of 4 days slices were treated for 7 days with estrogen at a dose of 10^{-7}M. No significant increase regarding the spine synapse density was found after stimulation. There was rather a slight decrease of 4% in the stimulated group compared to the control group (Control [*reeler*] = 100%). Averaged absolute values: *reeler* =12, 94 spine synapses, *reeler* 10^{-7}= 11, 89 spine synapses**.** Both groups: n=9, mean ± SEM, student´s-t-test.

3.1.1.3 Determination of sex steroid responsiveness in male and female hippocampal slice cultures

Recent experiments demonstrated a sexual dimorphism concerning the synaptic plasticity in response to sexual steroids (Biamonte et al. 2009; Vierk et al. 2012). Spine synapse density is reduced by 26% in females 7 days after treatment with the aromatase inhibitor letrozole. However, no such effect was found in males (Vierk et al. 2012). For this reason I questioned whether effects of estrogen stimulation differ between male and female *reeler* with regard to spine synapse density. In the view of gender differences I analyzed the data again, that is WT and *reeler* group were subdivided according to the sex. However, no significant difference was found. Neither in male nor in female *reeler* an increase was brought about after stimulation. Figure 3.6. illustrate spine synapse density of all four groups.

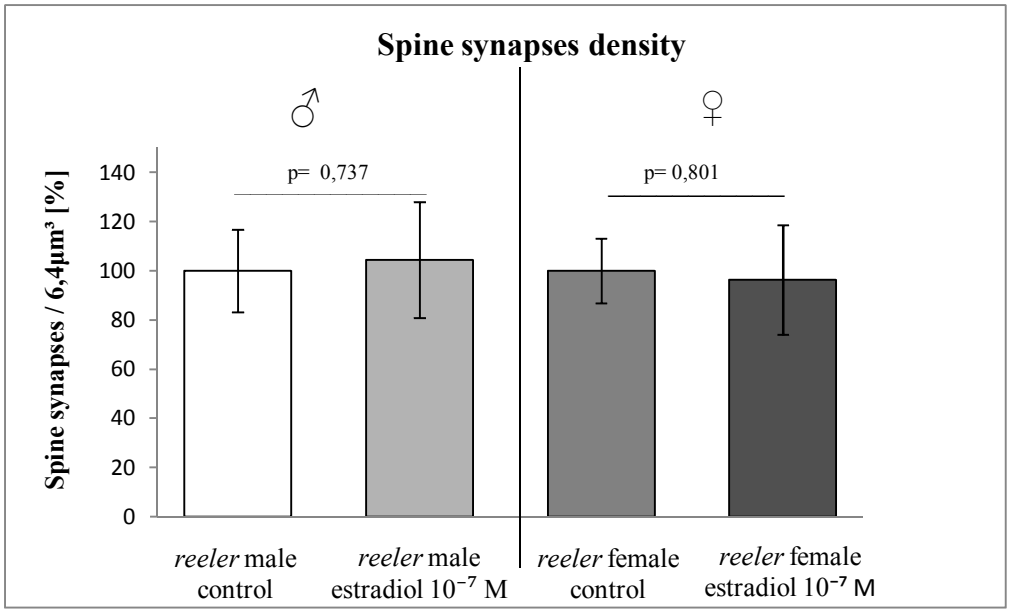


Figure 3.6: Spine synapses in postnatal reeler in males and females separately in response to estrogen stimulation

Neither in male nor in female *reeler* a significant increase or decrease was noticeable. Hippocampal slice cultures were stimulated with estrogen for 7 days after a preculturing period of 4 days. By the aid of stereological methods spine synapse density was determined. In both genders untreated samples were used as a control=100%. In males an increase of 4,49% was found, in females a reduction by 3,7%. Averaged absolute values of spine synapses: *reeler* male control=7,42, *reeler* male estradiol=7,75, *reeler* female control=6,75, *reeler* female estradiol=6,50. In all groups n=3, mean ± SEM. Statistical analysis: ANOVA, Fisher´s least significant difference (LSD)

3.1.2 Spine synapse density during development and in adult mice

Results of postnatal animals did not show a decrease of spine synapses, as it has been contrarily reported in other studies for numbers of spines (Niu et al. 2008). Therefore, external application of estrogen could not initiate a rescue effect in spine synapse number. I questioned whether Reelin might be needed later during development, namely instead of the formation rather for the maintenance of synapses since Reelin has been reported to influence dendritic growth, spine genesis (Niu et al., 2004) or a regulator of both the number and strength of pre- and postsynaptic connections (Niu et al. 2008; for review: Wasser and Herz, 2017). To address this issue I determined spine synapse density in

juvenile (4 weeks) and adult *reeler* using unbiased stereological methods to describe what happens during development. Next to age related differences I focused on the genotype, this means I compared *reeler* with WT animals. Ongoing experiments point to gender-related differences concerning steroidogenic effects on synaptogenesis so I examined differences referring to the gender as well.

3.1.2.1 Spine synapse density in juvenile *reeler*

At first pubescent *reeler* and WT were compared, independently of the sex. Figure 3.7. shows spine synapse density in the hippocampal CA1 region of *reeler* and WT four weeks of age. Hippocampal slices were generated and processed for electron microscopy. Spine synapses were counted in a volume of 6,4µm³ by stereological methods, in total 7 animals were used per group so I had 14 hippocampi to compare (n=14). Results displayed in figure 3.7. show a highly significant reduction (p=0,0022) of spine synapses in four weeks old *reeler* compared to WT of the same age.

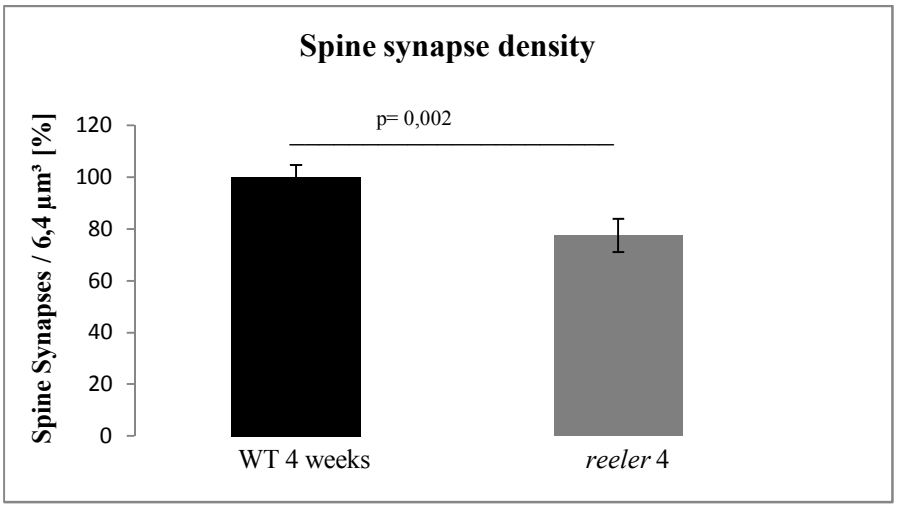
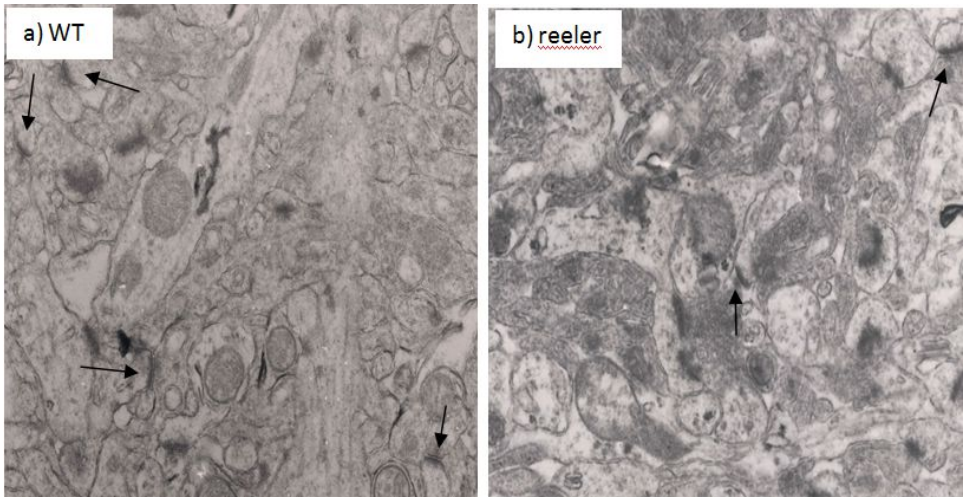


Figure 3.7: Spine synapse density is significantly reduced in 4-week-old reeler
Electron micrographs of hippocampal slice cultures were analyzed and spine synapse densities calculated. WT served as control (=100%). In *reeler* (=77,5%) a highly significant reduction of 22,5% was seen. Absolute values were for WT 131,9 spine synapses and for *reeler* 102,2 spine synapses. Represented are mean percentages of spine synapses ±SEM, n=14, student´s-t-test. 4W= four weeks.

As previously stated female and male WT mice respond differently to aromatase inhibition and estrogen stimulation. Considering this I determined spine synapse density again according to the sex that is for female and male *reeler* separately. A reduction of spine synapses in both sexes was found, which was statistically significant in each case. Figures 3.8. and 3.9. illustrate results of pubescent male and female *reeler*.

(A)



(B)

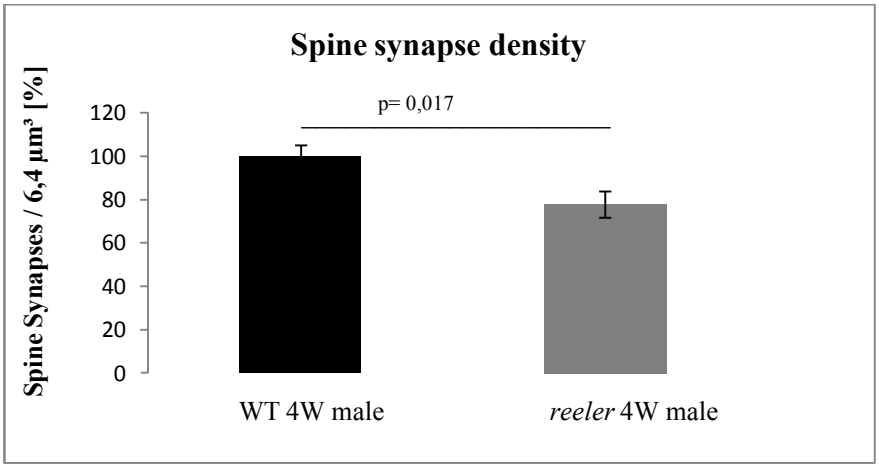
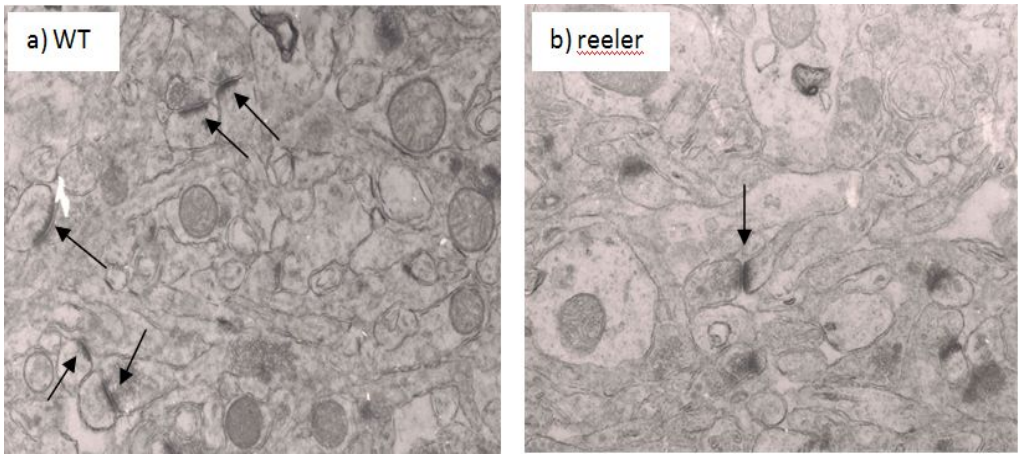


Figure 3.8: Differences between male pubescent *reeler* and WT
(A) Small sections of EM pictures obtained from male WT (a) and *reeler* (b) hippocampus are shown. On the left one can see a small section depicted from male four week old WT. On the right an exemplary section originating from male four week old *reeler*. Arrows indicate spine synapses. In WT spine synapse density is higher which correlates with the quantitative analysis.
(B) Results of synapse counting obtained from electronic micrographs of male hippocampal slices were analyzed. A significant reduction was found regarding spine synapse density in juvenile male *reeler*. Compared to control (WT= 100%) a reduction of 22% was found. Absolute averaged values were 131,8 spine synapses for WT male and 102,6 for *reeler* male. Shown are mean values ±SEM, n=6, student-t-test, 4W= four weeks.

(A)



(B)

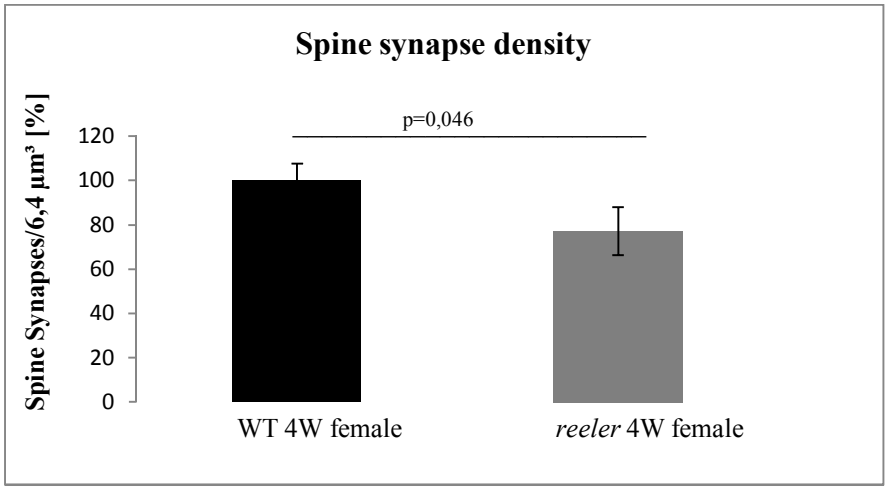


Figure 3.9: Differences between female juvenile *reeler* and WT
(A) Selective sections of EM pictures originating from female WT (a) and *reeler* (b) hippocampus are shown. Spine synapses are labeled with arrows. On the left female 4 week old WT is shown, on the right female 4 week old *reeler*. While in WT five synapses were identified in *reeler* only one spine synapse is detectable in this section.
(B) Quantitative analysis was performed for electronic micrographs from female hippocampal slices. Like in males in female pubescent *reeler* a significant reduction of 23% was found. Absolute averaged values were 132 spine synapses for WT female and 101,8 for *reeler* female, WT female=100%, mean ± SEM, n=8, student´s-t-test, 4W=four weeks.

Summarized, in both sexes a reduction of spine synapses was found in pubescent *reeler* compared to WT. This reduction was statistically significant. Figure 3.10. summarizes data determined for pubescent animals.

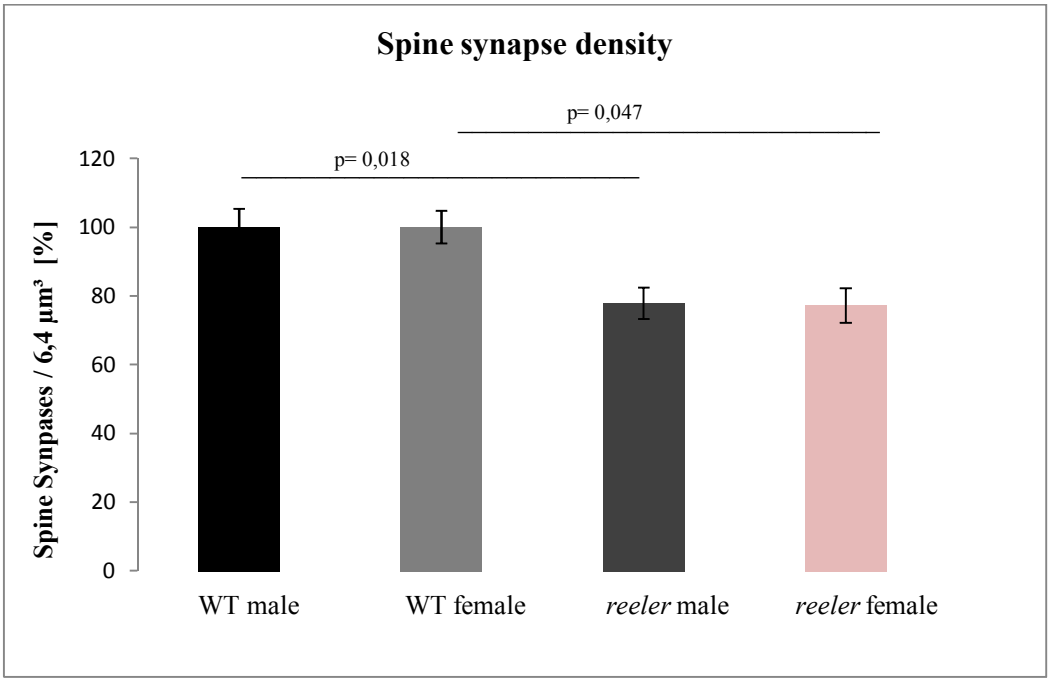


Figure 3.10: Spine synapse density is reduced in pubescent *reeler*
Hippocampal slice cultures of *reeler* and WT mice of both sexes were obtained and electronic micrographs produced. The number of spine synapses was analyzed in the CA1 region of the hippocampus. Significant reductions were seen in *reeler* both in males and in females. WT male provide the control (100%), WT female was almost identical compared to WT male; however, in male and female *reeler* spine synapse density was significantly reduced. Spine synapse density was ~22% lower in *reeler* males compared to WT males and ~23% in *reeler* females compared to WT females. Absolute averaged values: WT male 131,8 spine synapses, WT female: 132 spine synapses, *reeler* male: 102,6 spine synapses, *reeler* female: 101,8 spine synapses. ANOVA and post-hoc LSD.

3.1.2.2 Spine synapse density in adult *reeler*

Spine synapse density is reduced in pubescent *reeler* mice, both in females and in males. A major concern of this work is the effect of Reelin regarding synaptogenesis. Theoretically, Reelin is needed for the formation of spine synapses. However, it might be needed alternatively or additionally for the maintenance of spine synapses. To compare both theories I determined spine synapse density in adult animals as well. The procedure and experimental setup was exactly the same like in pubescent *reeler*.
In figure 3.11. relative spine synapse density in adult *reeler* is illustrated. On the left on the abscissa relative values are shown for WT while on the right relative results for *reeler* are represented. On the ordinate relative spine synapses per 6,4µm³ are shown. The difference of 2% between the two groups was statistically not significant.

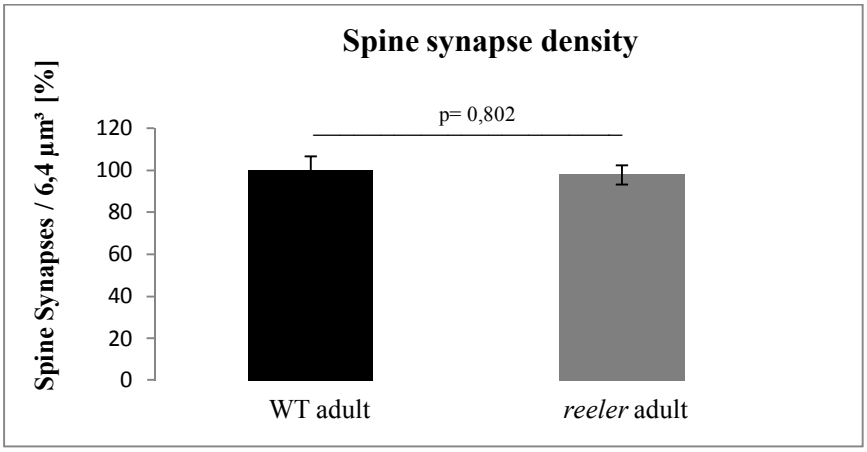
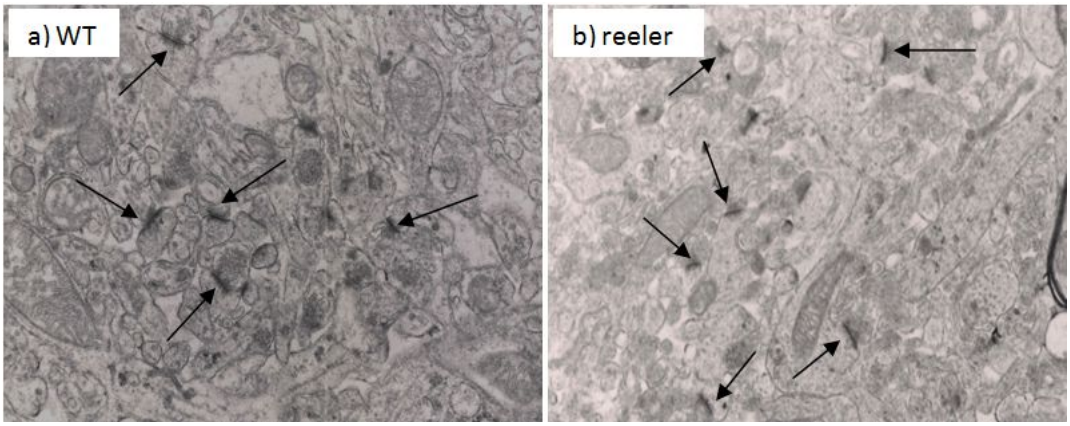


Figure 3.11: Spine synapse density in adult reeler
Electron micrographs obtained from hippocampal slices of adult WT and adult *reeler* were analyzed relating to spine synapse density. No reduction of spine synapses was found in *reeler*, difference was 2%. Shown are relative values, control is provided by WT. Averaged absolute values: WT: 119,5 spine synapses**,** *reeler*: 117 spine synapses**.** Shown are spine synapse densities ±SEM, n=12, student´s-t-test.

With the focus on sex-specific differences no significant reduction was found neither in male nor in female animals as it is shown in figure 3.12. and 3.13..

(A)



(B)

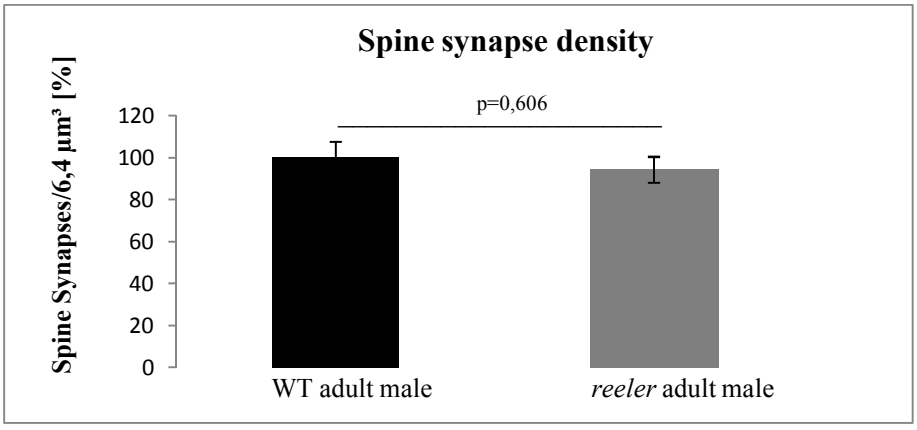
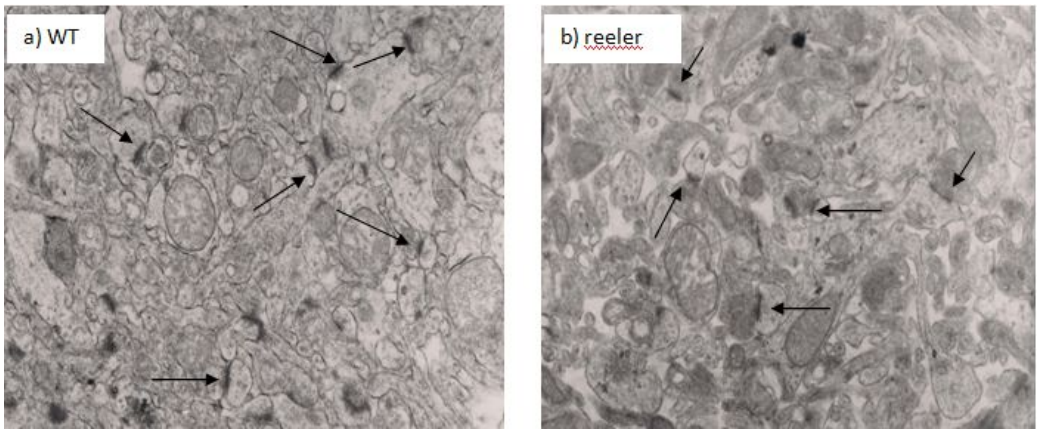


Figure 3.12: Spine synapse density in adult male *reeler* and WT
(A) Selective sections of EM pictures obtained from adult male WT (a) and *reeler* (b) hippocampus are shown. Spine synapses are labeled with arrows. On the left male WT is shown, on the right male *reeler*. In these sections six synapses were seen and counted in *reeler*, in WT five synapses were identified.
(B) Quantitative analysis of spine synapses in males is shown. Evaluation focusing on spine synapse density in males was only performed to challenge sex-specific differences. Compared to WT no significant difference was found in male adult *reeler*, spine synapse density is reduced by 6%. Averaged absolute values: WT adult male: 130,6 spine synapses, *reeler* adult male: 123,3 spine synapses. Shown are relative spine synapse densities ±SEM, n=6, student´s-t-test

(A)



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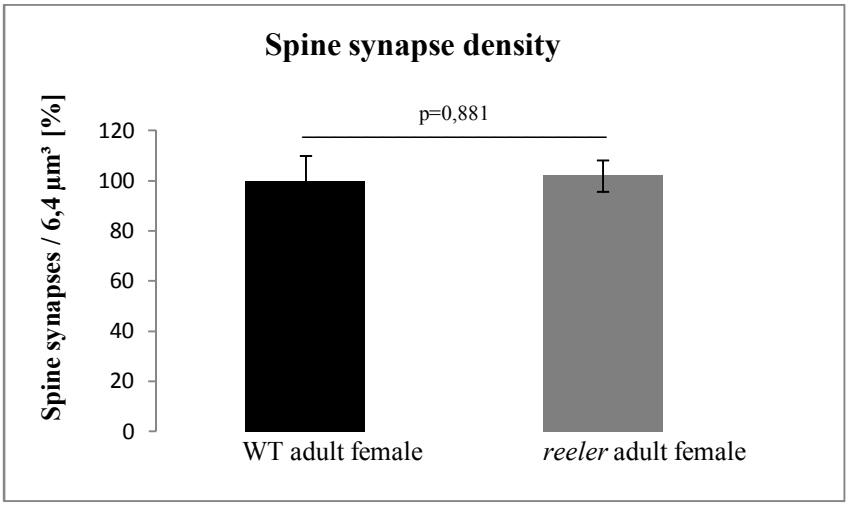


Figure 3.13: Spine synapse density in adult female *reeler* and WT
(A) Selective sections of EM pictures obtained from adult female WT (a) and *reeler* (b) hippocampus are shown. Spine synapses are labeled with arrows. On the left female WT is shown, on the right female *reeler*. In these sections five synapses were seen and counted in *reeler*, in WT six synapses were identified.
(B) Again, spine synapse counting was statistically quantified. Like in males spine synapse density was determined for females separately to elucidate sex-specific differences. No significant difference was found in female adult *reeler*. Compared to WT a slight increase of 2% was found. Absolute averaged values: WT adult female: 108,5 spine synapses, *reeler* adult female: 110,6 spine synapses. Shown are relative spine synapse densities ±SEM, n=6, student´s-t-test.

To summarize, results gained from adult *reeler* showed no difference between both genotypes regarding spine synapse density, neither in males nor in females. Figure 3.14. summarizes data for adult animals.

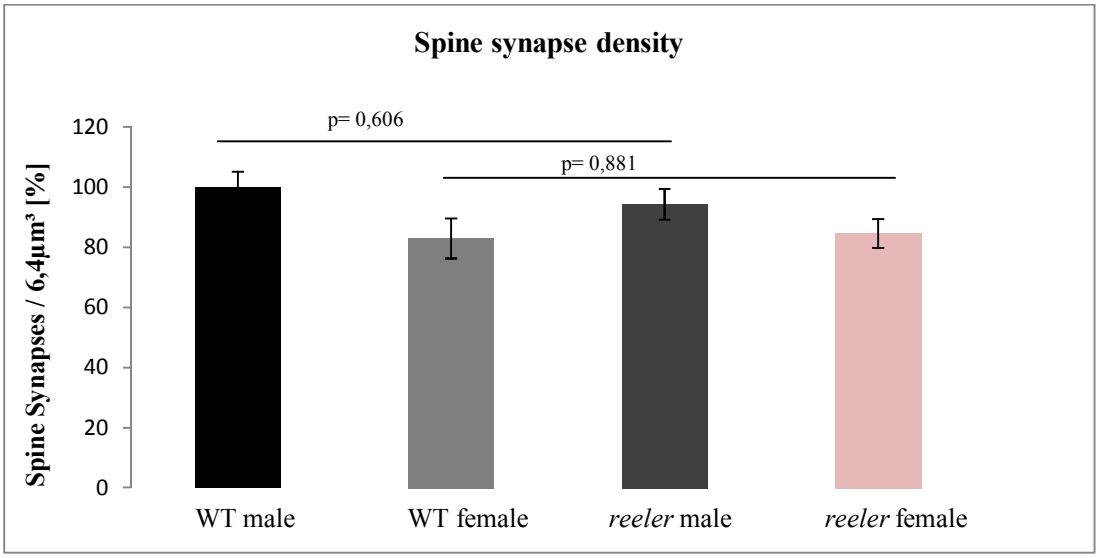


Figure 3.14: Summary of all data gathered from adult animals. Spine synapse density is not reduced in adult *reeler*
The number of spine synapses was determined in the CA1 region of the hippocampus of WT and *reeler*. Next to the genotype the focus was on gender differences. No significant reductions were seen in *reeler* compared to WT, neither in males nor in females. In addition, in both genotypes no significant reduction was found between males and females. WT male provided control (100%), in female WT spine synapse density was 17% less compared to WT male. In male *reeler* a difference of 5% was found, compared to WT male. Difference between WT female and *reeler* female was 2%. Difference between *reeler* male and *reeler* female was 10%. Indeed, no difference was statistically significant. Averaged absolute values: WT male: 130,6, WT female: 108,5, *reeler* male: 123,3, *reeler* female: 110,67. Shown are relative values ±SEM, n=6, ANOVA and post-hoc LSD.

3.2 Aromatase expression in young and adult *reeler* compared to WT

Ongoing experiments showed a sexual dimorphism with respect to synaptogenesis in response to neurosteroids. Therefore I analyzed the expression of aromatase, the final enzyme in the synthesis of neurosteroids, in developing *reeler*. I implemented an immunoblot using the whole homogenized hippocampus; in addition, I determined the rate of expression in the hippocampus by immunohistochemistry. In both cases results were analyzed by using ImageJ to determine integrated density, which is the product of the area at a set threshold and the mean grey value, representing quantitatively aromatase expression. Following statistical analysis was performed using *SPSS*. As it is common with statistical analysis α-error was set at 0,05 so significance is provided if p-value < 0,05.

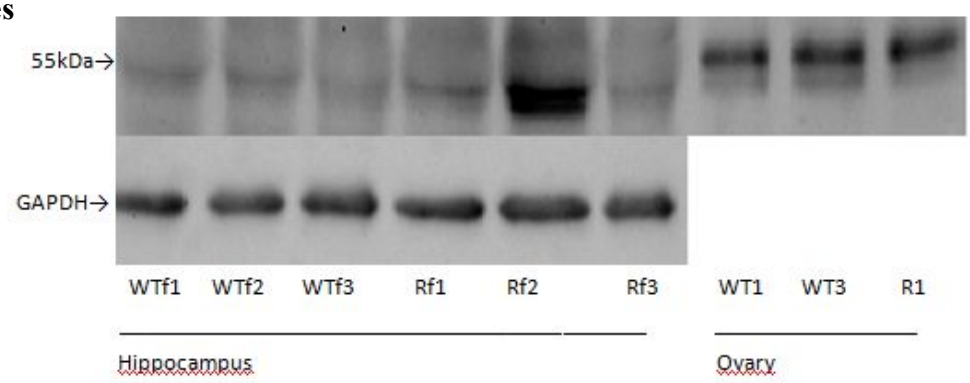
3.2.1 Western Blot

I prepared hippocampi of six *reeler* and six WT, both groups were composed of three females and three males. Experiments were performed three times to guarantee reproducibility. Next to the antibody detecting aromatase an antibody against GAPDH was used as well to determine whole amount of protein. In addition, the ovaries were prepared serving as control since aromatase is usually highly expressed within these organs. Aromatase has a molecular weight of 55kDa and all of the immunoblots produced banding patterns around that size for every animal. However, some of the bands were not well-defined and show rather two bands which complicated the analysis of the blots with ImageJ. In addition, one animal gave rise to a very strong signal in both experiments. It is

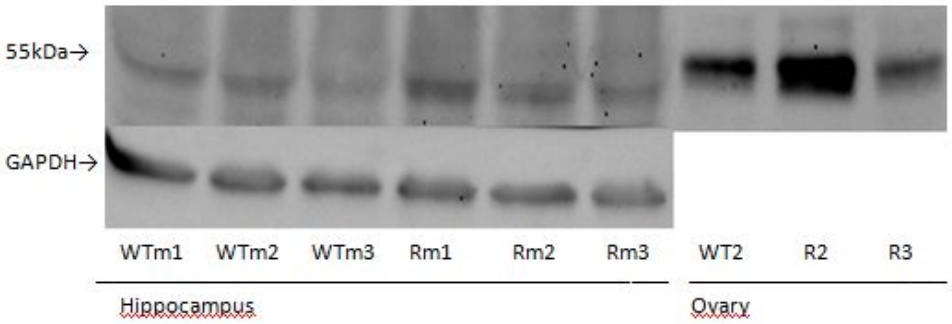
important to bear these two aspects in mind while analyzing and quantifying the immunoblots. In figure 3.15. an immunoblot and the quantitative analysis is shown.

(A)

a) females



b) males



(B)

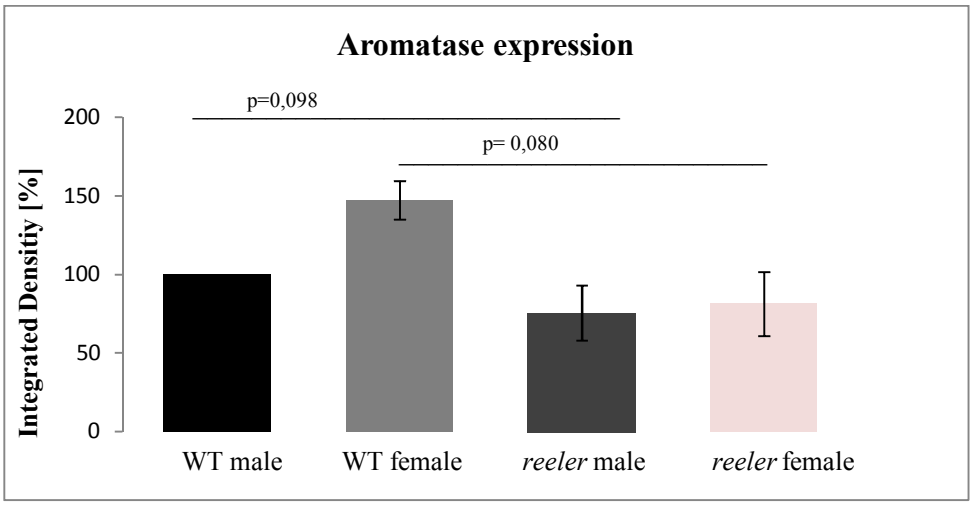


Figure 3.15: Western blot analysis of aromatase expression
(A) Western Blot attested hippocampal aromatase expression (55kDa) in female (a) and male (b) *reeler*. Plotted are three female WT (WTf1-3) and *reeler* (Rf1-3) in line 1. Line 2 shows three male WT (WTm1-3) and *reeler* (Rm1-3). In addition, ovaries were prepared just as hippocampal tissues to provide a control. In line 1 ovarian aromatase expression is plotted originating from two WT and one *reeler* while in line 2 only one WT but two *reeler* bands are shown. The band is doubled in some samples. In addition, Rf2 shows a very prominent signal, both in the hippocampus and the ovary. Both factors influenced the quantitative analysis. **(B)** Quantitative analysis of two blots shows higher expression of aromatase in WT, both in males and in females. However, a statistical difference was found only between WT female and *reeler* male. Shown are mean values as a percentage [Integrated Density %] ± SEM, n=6, ANOVA and post-hoc Bonferroni (WTm=100%, WTf=147,28%, Rm=75,44%, Rf=81,09%; Mean differences (MD): WT male-WT female: -0,5784; Confidence Interval (CI)[-1,28; 0,12]; WT male - *reeler* male MD: 0,42, CI [-0,28; 1,12]; WT male - *reeler* female MD: 0,22, CI [-0,472; 0,92]; *reeler* male - *reeler* female MD: -0,19, CI [-0,89; 0.50]; WT female - *reeler* female MD: 0-8026, CI [0,11; 1,50]).

By focusing on males and females separately one can see in both genders a reduced aromatase expression, however, statistically not significant. Figure 3.16. shows the quantification of aromatase expression in WT males and females compared to *reeler* mice.

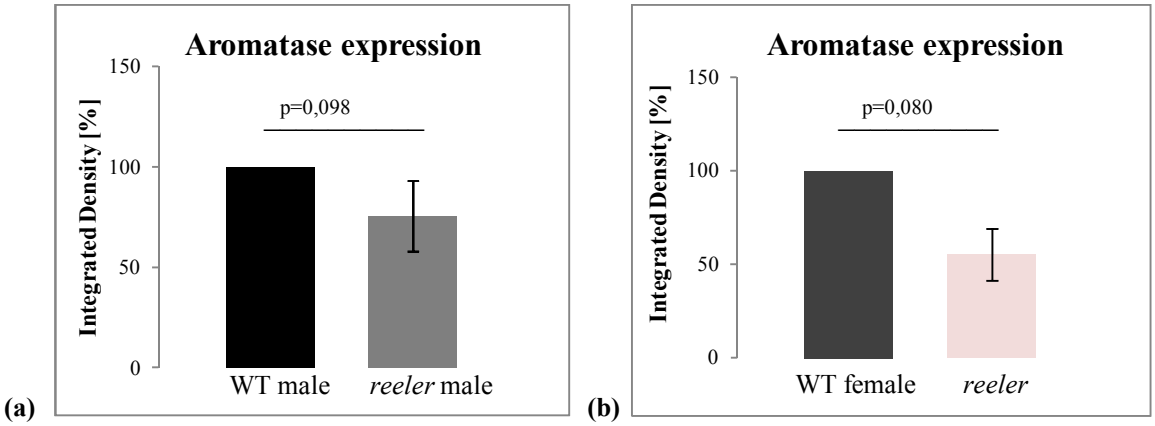


Figure 3.16: Western blot analysis of aromatase expression in males and females separately
a) Quantitative analysis of two western blots shows reduced aromatase expression in 5-6 weeks old male and **b)** female *reeler* compared to WT. Aromatase expression is reduced about 24% in males (WT male: 100%; *reeler* male: 75,45%) and about 45% in females (WT female: 100%; *reeler* female: 55,08%).
In both cases differences were not statistically significant. Shown are mean values as a percentage [Integrated Density %] +SEM, n=6, t-test, p=0,098 (males) and p=0,080 (females).

3.2.2 Immunohistochemistry

For immunohistochemistry aromatase was labeled using a primary anti-aromatase-antibody and a secondary, fluorescent antibody. During examination by laser scanning microscopy the differences between *reeler* and WT became evident on the first sight. As demonstrated in figure 3.17. in WT cell layers were arranged properly and the CA1 region, the region of interest, was detectable easily. In contrast, in *reeler* no cellular organization was seen and cell layers were rather promiscuously. In addition, total numbers of cells were frequently reduced in *reeler*. Therefore I counted cells of each picture and related staining intensity to cell counts aiming for standardized values. Next to aromatase staining, DNA within the cell nuclei was stained with 4',6-diamidino-2-phenylindole (DAPI) to facilitate orientation and objectify specific morphology. In figure 3.17. representative excerpts of CA1 showing differences between WT and *reeler* are illustrated.

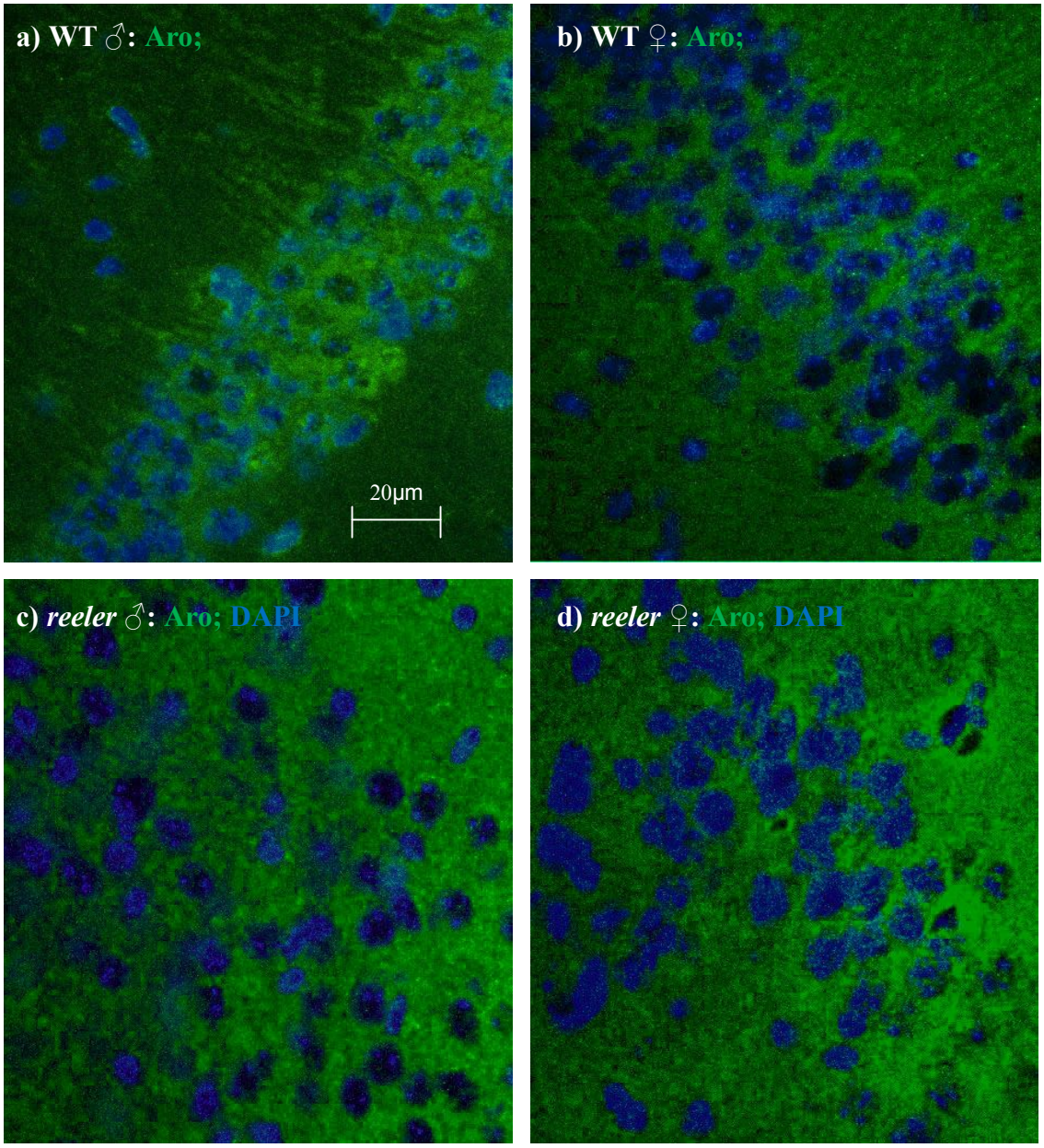


Figure 3.17: Aromatase immunoreactivity in the CA1 region of hippocampal slices
Hippocampal slices of WT and *reeler* were immunohistochemically stained to make aromatase detectable by confocal laser scanning microscopy. Cell nuclei were stained with DAPI to visualize morphology. Picture a and b show representative sections originating from WT (a=male, b=female) while c and d represents *reeler* (c=male, d=female). In all experimental groups aromatase staining was detectable, however in *reeler* staining was less selective i.e. background staining became more obvious. In contrast, in WT aromatase expression was particularly found around the cellbody as well as in cellular protrusions. In addition, cell alignment is disordered in *reeler*, however in WT cells are arranged in a distinct cellular band.

Quantitative analysis of aromatase expression was performed by ImageJ, followed by statistical analysis by SPSS. To analyze differences between group means an analysis of variance (ANOVA) was conducted, followed by a *post-hoc* test (Bonferroni). With WT male serving as reference a significantly increased aromatase expression was found in male and female *reeler*. In addition, the difference between female WT und female *reeler* was even highly significant. Results of immunohistochemistry are summarized in figure 3.18.

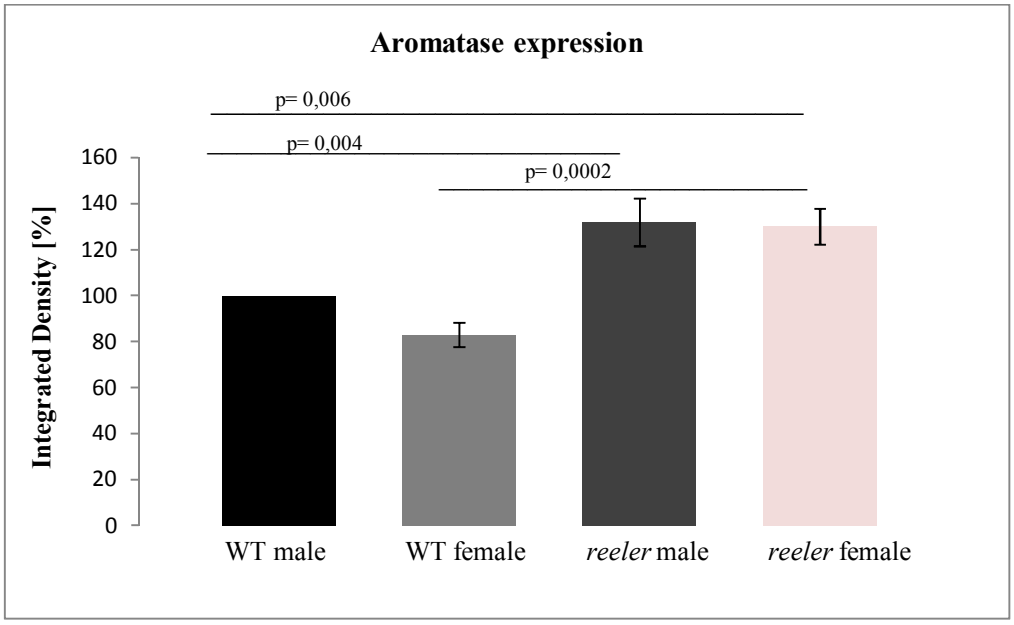
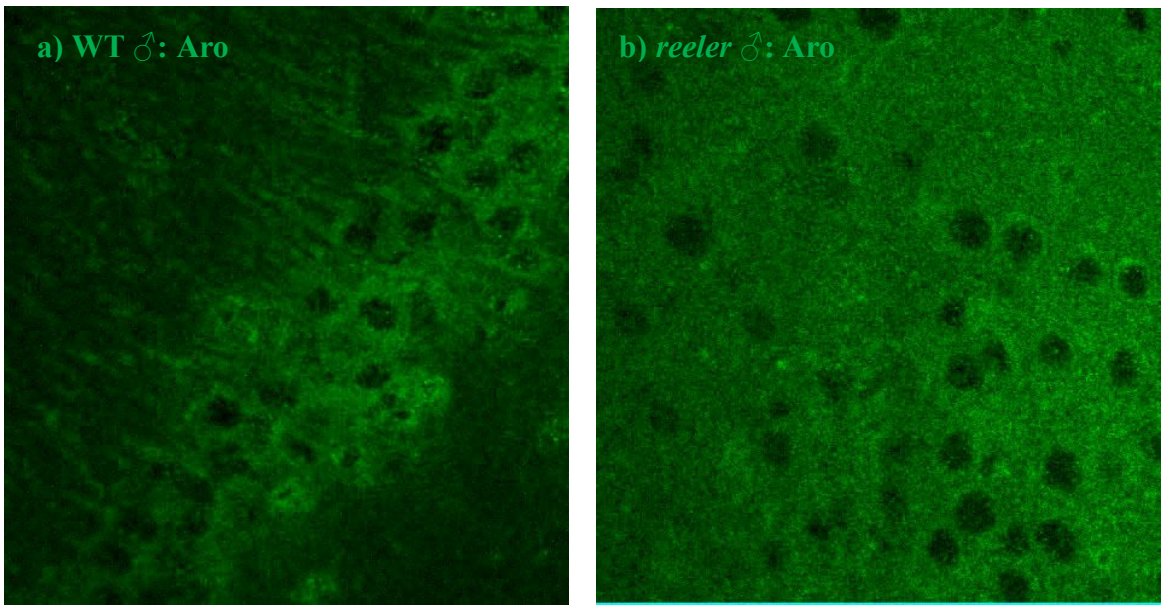


Figure 3.18: Quantification of aromatase immunoreactivity in the CA1 region of hippocampal slice cultures
Aromatase was immunohistochemically stained in acute slices of hippocampi originating from juvenile *reeler* and WT (5-6 weeks old). Compared to WT male (control, 100%) a significant increase of aromatase expression was found in *reeler* male and *reeler* female. In addition, *reeler* female aromatase expression was significantly higher compared to female WT. Illustrated is Integrated Density in % ±SEM, n=36 (WT male); n=33 (WT female); n=34 (*reeler* male); n=35 (*reeler* female). (Mean differences: WT male - WT female 0,0327; 95%-Confidence Interval (CI) [-0,0624; 0,1279]; WT male - *reeler* male: -0,1234, CI [-0,2178;-0,0289]; WT male – *reeler* female -0,1174, CI [-0,2112; -0,0237]; *reeler* male – *reeler* female: 0,0059, CI [-0,0891; 0,1009]; WT female – *reeler* female: -0,1502, CI [-0,2460; -0,0544]. Statistical analysis: ANOVA, followed by post-hoc test (Bonferroni).

Keeping in mind that aromatase expression might be gender specific I focused again on differences between WT and *reeler* in males and females separately. Statistical analysis showed in both genders a significant increase of aromatase expression when comparing *reeler* to WT. Results are shown in figure 3.19. and 3.20..

(A)



(B)

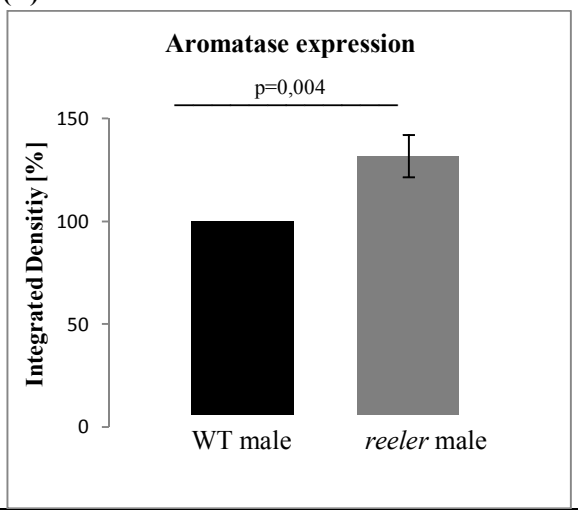
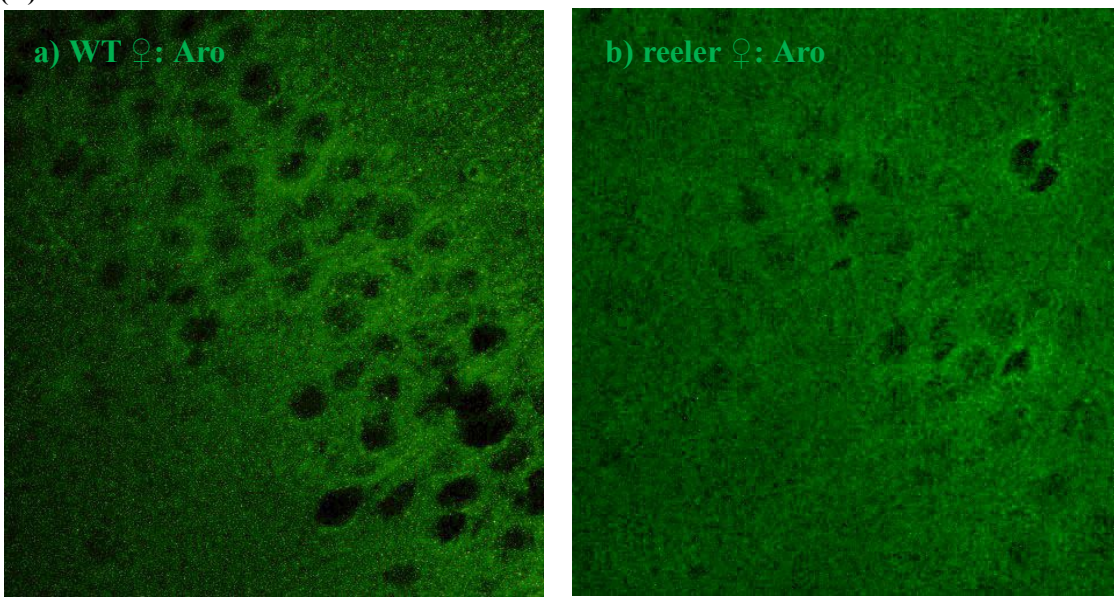


Figure 3.19: Quantification of aromatase immunoreactivity in CA1 region of hippocampal slice cultures in males
A) Hippocampal slices of 5-6 weeks old male WT and *reeler* were immunohistochemically stained to detect aromatase expression in CA1. As it can be seen staining of aromatase was far less specific in male *reeler* (a) compared to male WT (b).
B) A significant increase by 32% of aromatase expression was found in male *reeler* compared to male WT. Shown are mean values as a percentage [Integrated Density in %] ±SEM, n=36 (WT male), n=34 (*reeler* male), t-test.

(A)



(B)

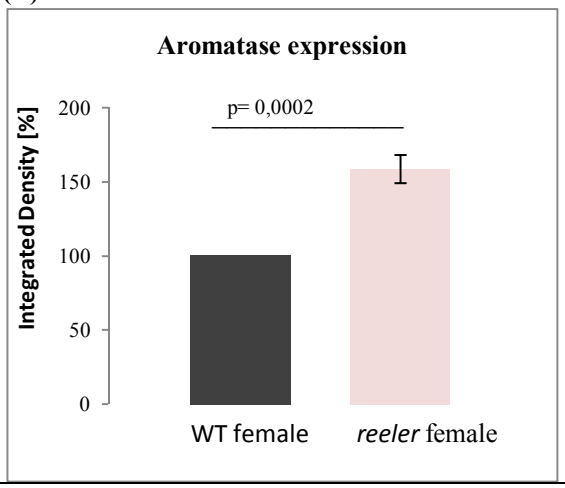


Figure 3.20: Quantification of aromatase immunoreactivity in CA1 region of hippocampal slice cultures in females
A) Hippocampal slices of 5-6 weeks old female WT and *reeler* were immunohistochemically stained to detect aromatase expression in CA1.
B) In females aromatase expression was highly significant increased by almost 60% in *reeler* compared to WT. Shown are mean values as a percentage [Integrated Density in %] ±SEM, n=33 (WT female), n=35 (*reeler* female); t-test.

4. Discussion

The brain is subject to continuous remodeling. Generally speaking, the central nervous system gathers, processes and generates information. However, what seems simple is beyond all imagination. What happens exactly during processing, which factors are involved? On cellular level information is transduced from one synapse to another and due to changes in use, behavior, neuronal processes and a lot more synaptic connectivity can be modified. This is summarized by the umbrella term `synaptic plasticity´.
Certain factors influence synaptic plasticity; many of them are not fully understood yet but we know that hormones, i.e. estrogens like 17ß-estradiol, play an important role regarding morphological and physiological conditions of a synapse, hence synaptic plasticity, especially in the hippocampus (Fester et al. 2011; Fester and Rune 2014; Spencer et al. 2008).

4.1 Estrogen-induced spine synapse formation?

Aromatase is the final enzyme in estrogen synthesis and has been shown to be essential for the maintenance of spine synapses in the hippocampus (Kretz et al., 2004; Zhou et al., 2007). After aromatase inhibition spine synapse density is reduced in female WT; however, this effect can be rescued by estrogen. Without previous aromatase inhibition there is no estrogen-induced upregulation of hippocampal spine synapses. This might be a ceiling effect due to product inhibition. A simplistic theory is visualized in figure 4.1.

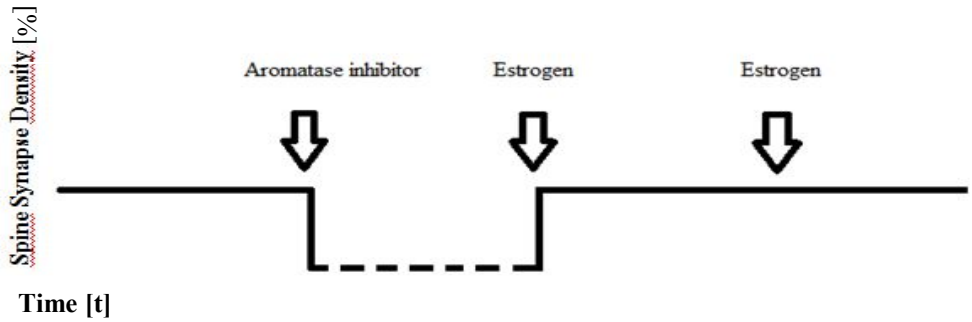


Figure 4.1: No increase in spine synapses is seen after application of estrogen without previous treatment with letrozole
Shown is spine synapse density after estrogen treatment with and without previous letrozole treatment. Additional application of estrogen without preceding letrozole treatment does not increase spine synapse density. This might be considered as product inhibition.

4.2 Disturbed synaptogenesis in *reeler*

Together with findings of decreased aromatase activity in *reeler* (Meseke et al. 2017, in press) and reduced spine synapse density after aromatase inhibition we hypothesized that a reduction in spine synapses in *reeler* might be rescued by estrogen treatment.
Two theories both ending up in reduced spine synapse density in *reeler* were scrutinized. Assuming an interaction between Reelin and estrogen, enabling synergistically synaptogenesis, it seems possible that due to Reelin deficiency estrogen, in turn cannot

exert its influence on synaptogenesis. Put differently, if the counterpart of estrogen is missing, which is the case in *reeler*, synaptogenesis might be disturbed.
Alternatively, the migratory defect of GnRH-neurons (Cariboni et al. 2005) and its impact on aromatase activity as well as on downstream events could explain disordered synaptogenesis. Both theories are outlined in figure 4.2.

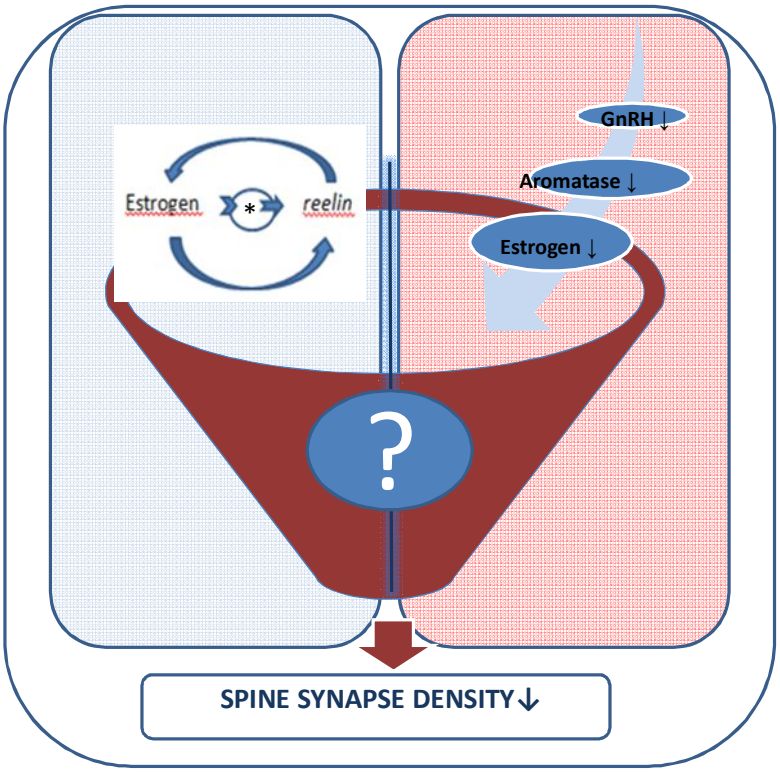


Figure 4.2: Possible explanations for reduced spine synapse density in *reeler*
In *reeler* synaptogenesis is disturbed; different theories are trying to explain the underlying mechanism. In this figure two mechanisms I examined are represented, although highly simplified.
Possibly estrogen and Reelin influence each other directly, that is estrogen binds via estrogen receptor α (ER α) on Cajal-Retzius cells (see *), whereby Reelin expression is induced. In which way Reelin influences estrogen needs further examination. However, Reelin and estrogen are both involved in synaptogenesis, and in *reeler* where Reelin expression is reduced synaptogenesis is negatively affected. Alternatively, due to disordered migration of GnRH-neurons and possibly subsequent reduced aromatase expression in *reeler,* spine synapse density might be decreased.

Aspects favoring the first idea are findings by Bender and colleagues. In 2010 they found in organotypic slice cultures of early postnatal hippocampus an estrogen dependent Reelin expression in Cajal-Retzius cells (CR) (Bender et al. 2010). These cells express and release Reelin (Förster et al. 2006) but, interestingly, they express estrogen receptor α (ERα) as well. Immunohistochemical experiments showed that in WT, external application of estrogen increases expression of Reelin in CR cells while blockade of ERα or inhibition of aromatase decreases it. Accordingly Reelin might be responsive to estrogen (Bender et al. 2010)
Referring to the second concept I investigated the theory whether spine synapses are reduced as a consequence of migratory defects of GnRH neurons. Similar to gonadal aromatase, hippocampal aromatase is potentially under influence of GnRH (Prange-Kiel et al. 2008). In *reeler*, however, GnRH neurons show migratory defects possibly leading to

reduced numbers of these cells and disordered aromatase regulation which, in turn could cause reduced concentration of estrogen (Cariboni et al. 2005).
I approached the two theories by determining spine synapse density and by quantifying expression of aromatase in *reeler*. Apical dendrites from CA1 pyramidal neurons project to the stratum lacunosum moleculare (SLM) of the hippocampus. Cajal-Retzius cells secrete their product, the extracellular enzyme Reelin, to this layer (Frotscher 1998). Indeed, synaptogenesis is as a function of estrogen treatment most prominent in the CA1 region (Spencer et al. 2008), therefore I focused my evaluation on this part of the hippocampus.

4.3 Quantification of spine synapses

Inspection of the hippocampal slices by electron microscopy did not show any qualitative differences between various groups after incubation. In both genotypes the neuronal ultrastructure and characteristic pyramidal cell layers were well preserved and did not differ from the *in vivo* situation. Of each mouse I analyzed 48 neuropil fields, randomly selected from the whole CA1 region. Morphological differences between certain areas within this region can be neglected due to the coincidental procedure and the high number of samples.
I determined the number of spine synapses in hippocampal slice cultures of WT and *reeler* mice using unbiased stereological methods. Stereology uses random systematic sampling to provide unbiased and quantitative data. Stereology can be used not only for one- and two-dimensional probes but it can analyze three-dimensional samples as well (West 1999). This is a profound benefit concerning synapse counting.
Synapse counting was performed using the disector technique (Sterio 1984). Only those synapses were counted being visible on one of the two neighboring slices. If synapses were present on both slices they were ignored. This excludes the risk of counting a synapse twice.
As a restriction, one must note that even if all criteria defining a synapse (see section Material and Methods) were respected and the counts repeated several times there is still a risk of subjectivity.

4.3.1 Spines and spine synapses: a small but subtle difference

I hypothesized that loss of dendritic spines in *reeler*, shown by Niu and co-workers (Niu et al. 2008) is due to an altered neurosteroid synthesis within the hippocampus, at least in females. Niu et alii determined the number of spines along apical dendrites of fluorescently labeled CA1 pyramidal neurons *in vivo* by confocal microscopy. YFP-labeled dendrites from WT, heterozygous and homozygous *reeler* mice, disregarding the gender, were analyzed in brain sections from transgenic mice perfused on P21 and P32, respectively. From experimental and control samples 25-35 dendritic segments of selected neurons from multiple sections or slices were analyzed. Based on this experimental method they demonstrated a reduction in dendritic spines in heterozygous and homozygous *reeler* mice. In their quantitative analysis they found in second- to fourth-order branches values of 0.94±0,02 spines/µm in WT, about the half in heterozygous and about a quarter in homozygous mice. The same tendency was found when they analyzed terminal apical branches. To verify results and investigate the role of Reelin regarding spine formation they also quantified spines in hippocampal slice cultures after eleven days *in vitro*, obtained from postnatal mice P4. Again, spines are significantly reduced, both in heterozygous and homozygous *reeler* mice. Interestingly, when they subsequently

incubated organotypic slices obtained from *reeler* with recombinant Reelin a rescue effect regarding spine reduction was seen. This finding made them conclude that a reduction of spines in *reeler* is directly caused by Reelin deficiency.
In contrast to the findings by Niu (Niu et al. 2008) I did not find any difference between postnatal WT and homozygous *reeler* when I determined the number of spine synapses in hippocampal slice cultures. However, in my experimental set-up I focused on the entire spine synapse in hippocampal CA1 pyramidal neurons, rather than on the postsynaptic element only. Quantitative analysis indicated no difference with respect to spine synapse density in postnatal WT and homozygous *reeler* mice (Figure 3.1., section Results). To explore the idea whether estrogen could rescue a potential spine synapse reduction slices obtained from *reeler* were incubated with estrogen to see if spine synapse formation will be induced. However, no increase in spine synapses was found.
As we know by now there is a sexual dimorphism regarding synaptic plasticity in response to sexual steroids (Brandt et al. 2013; Fester et al. 2016). Therefore results were analyzed again with respect to the gender. However, in both sexes and both genotypes no increase in spine synapse number was found (Figure 3.6., section Results). Thus, it appears that neuronal connectivity might not be affected in postnatal *reeler* mice, even if the number of spines is reduced. Methodological reasons could account for this discrepancy between my findings and those of Niu et alii. They determined numbers of spines using the Golgi-impregnation technique, whereas I determined numbers of spine synapses per volume in various areas of the hippocampus. The Golgi-technique is biased since not all neurons are stained but only a randomly selected number of neurons. Usually, neurons are selected according to the degree of staining. In contrast, determination of spine synapses in randomly chosen various areas may be more representative. Furthermore, the discrepancy could also suggest that spines do not necessarily have a presynaptic partner. Spines are extremely dynamic and can form within minutes (Engert and Bonhoeffer 1999).
In addition it might be possible that a lack of Reelin becomes apparent only later during development, i.e. it is rather necessary for the maintenance of synapses instead of their formation during pre- and postnatal development. I therefore performed further investigations to determine spine synapse density in mature *reeler* mice. In addition I determined spine synapse density in young adult mice (about 4 weeks) to get an idea what happens during development.
Interestingly, while in adult *reeler* no reduction of spine synapses was seen, in 4 week old animals the number of spine synapses is reduced significantly. Keeping the sexual dimorphism in mind results were re-assessed with the focus on males and females separately. Strikingly, both females and males showed a significant reduction at the age of 4 weeks but not in adults in *reeler* (Figure 3.10. and 3.14., section Results).
In fact, results from spine synapse determination cast a direct reciprocal interaction between Reelin and estrogen, enabling synaptogenensis, into doubt. Assuming estrogen stimulates Reelin in order to perpetuate synaptogenensis spine synapse density should be significantly reduced in *reeler* since homozygous mice do not express Reelin at all. This holds true in developing animals, however, data from adults show that synapse formation is accomplished regardless of the existence of Reelin since both genotypes have about the same spine synapse density. Possibly different mechanisms are already established in adults to provide intact synaptic connectivity. In addition, it could be possible that synaptogenesis is disturbed indirectly due to down-regulated aromatase expression. In the next section aromatase as a potential key factor will be addressed.

4.4 Is it all about aromatase?

It has been frequently shown that hippocampal neurons express aromatase, the final enzyme in estrogen biosynthesis (For review see Pelletier, 2010; Shibuya et al., 2003); moreover, hippocampal neurons are capable of synthesizing estrogen de novo (Hojo et al., 2004; Prange-Kiel et al. 2003). Next to mechanisms like gene transcription and substrate availability (Fester et al. 2011) gonadotropins are likely to regulate hippocampal estrogen synthesis. In gonads, an estrogen-regulated feedback mechanism is established operating via the hypothalamus-pituitary-gonadal axis to induce a GnRH-mediated, dose dependent release of estrogen. As in gonads, GnRH-receptor protein and GnRH-receptor mRNA have been detected in rat central nervous system (Jennes et al. 1997; Prange-Kiel et al. 2008). Interestingly, treatment of hippocampal slice cultures from rats with GnRH affected the release of estradiol in a specific dose-dependent manner (Prange-Kiel et al. 2008). In addition, GnRH-induced estradiol synthesis in hippocampal neurons can be inhibited by simultaneous treatment with GnRH and the aromatase inhibitor letrozole, indicating that aromatase is possibly inter alia influenced by GnRH. Furthermore, recent data from *in vitro* experiments point to a GnRH-mediated modulation of spine synapse density. In fact, spine synapse density was increased in response to GnRH treatment while GnRH together with letrozol had no effect (for review see Brandt et al., 2013; Fester et al., 2011). These findings suggest spine synapse density might be associated with pulsatile GnRH-release carried out by estrogen synthesis.
As it is known *reeler* show a migratory defect for GnRH-neurons (Cariboni et al. 2005; Rakic and Caviness 1995), so estrogen synthesis by aromatase, as a consequence, might be irregular. In short, based on the assumption that aromatase is via LH and FSH influenced at least to some extent by GnRH it seems possible that aromatase expression is affected in *reeler* due to migratory defects of GnRH-neurons resulting in a disturbed hypothalamic-pituitary-gonadal axis. However, with aromatase being down-regulated like it might be the case in *reeler* one could assume that Reelin itself influences estrogen synthesis by modulating aromatase expression. To address the question how aromatase is expressed when Reelin is missing like in the mutant I examined aromatase expression in young adult *reeler* compared to WT.

4.4.1 Immunoblot assay to determine aromatase expression

Detection of aromatase by immunoblotting showed stronger expression in WT than in *reeler*, both in females and males. The strongest expression was found in female WT. However, it is important to note that I used whole hippocampus tissue but not only the CA1 region like I did with electron microscopy. However, this applies for all four different groups of animals. I repeated the blotting three times with lysates of the same animals. Due to time limitations, these results are only preliminary considering the small sample number. Next to the influence of the menstrual cycle (see below) methodical reasons need to be considered. In fact, analyzing the blots with ImageJ was complicated as immunoblots often show protein bands not being well defined, i.e. aromatase signal differentiation was frequently ambiguous. This phenomenon appeared in both experiments; however, reasons for double-layered protein signal remains to be unraveled. Later on immunoblots will be discussed and put in context as certain disregarded factors of the experimental set-up might be of particular importance.

4.4.2 Visualization of aromatase expression by immunohistochemistry

Unlike immunoblotting where the whole hippocampus was examined, immunohistochemical experiments were evaluated with the focus on aromatase expression in CA1 only. The expression was stronger in *reeler* compared to WT and the strongest in *reeler* male. However, an essential morphological difference was found between the two genotypes. While in WT the CA1 pyramidal neurons are organized in a proper line there is no cellular organization in *reeler* at all. The cells are distributed all over the whole area making it difficult to identify certain pyramidal neurons. Pictures of a specific size were taken with the confocal microscope, covering as much neurons as possible. However, due to the fact that in *reeler* cells are widely distributed it was hard to get as much cells on a picture as in WT (see figure 4.3.). To eliminate this bias aromatase expression was calculated per cell by dividing whole staining intensity by the number of cells per picture.

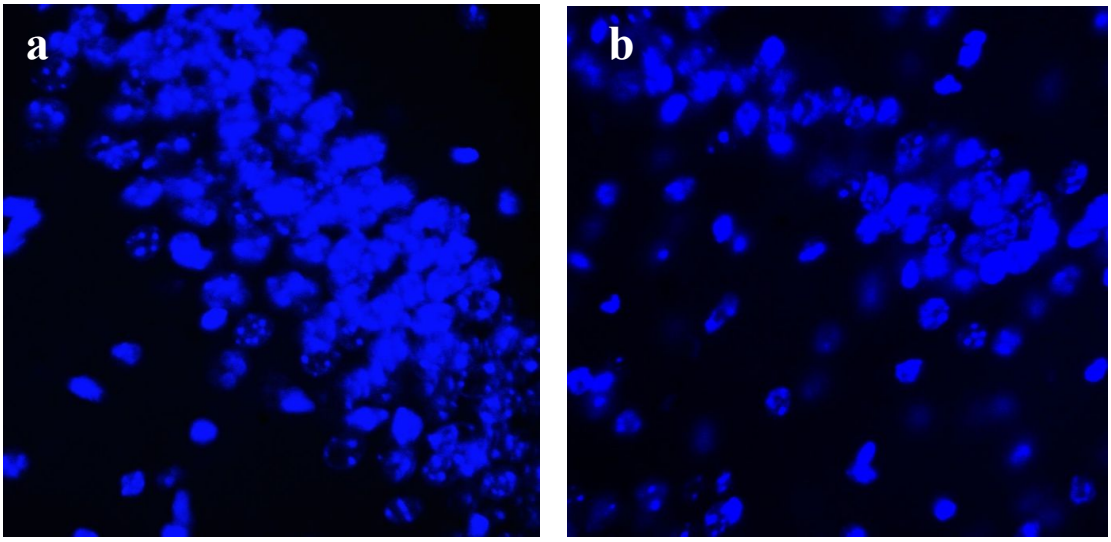


Figure 4.3: Pyramidal cells of stratum pyramidale in WT and *reeler*
Represented are details of laser scanning micrographs with DAPI-stained cell bodies of mainly pyramidal cells originating from WT and *reeler* CA1- region. In WT (**a**) cells are arranged in a distinct cell layer whereas in *reeler* (**b**) cells are placed irregularly. In *reeler* it is hard to differentiate pyramidal cells from other cell types and because of far-scattered positioning of cells in *reeler* possibly less pyramidal cells were included compared to WT. However, total staining was related to amount of cells.

In addition background staining was a big problem in general but especially in *reeler*. As it is commonly known aromatase expression is predominantly found around the cells as well as in cellular protrusions (Lephart, 1996; Roselli, 1995). As it can be seen in figure 3.17 (see section Results) in WT staining was found indeed predominantly around cell nuclei; however, in *reeler* pictures were stained rather homogenous. Although background staining was taken into account and reduced while analyzing the pictures with ImageJ it cannot be eliminated completely. As every sample was treated identically factors like temperature differences, antibody quality or microscopic adjustments can be excluded.

4.5 Impact of cyclicity

It has been previously shown that the amount of estrogen in the hippocampal tissue varies with the estrus cycle, i.e. it is for example higher in proestrus than in estrus animals (Fester et al. 2012; Kato et al. 2013). The impact of varying estrogen levels on changes in spine density was already shown in 1990 by Woolley and colleagues (Woolley et al. 1990).

Furthermore, McEwen et al. claimed in 2002 that the cyclic turnover of synapses in the hippocampus is a function of gonadal estrogen levels in the serum (McEwen 2002). However, it is commonly known by now that neurons, in particular hippocampal neurons are equipped with enzymes of steroidogenesis enabling them to synthesize estrogen *de novo* (Kretz et al. 2004; Pelletier 2010; Prange-Kiel et al. 2003; Shibuya et al. 2003). Comparable to gonadal estrogen synthesis hippocampal *de novo* synthesis is possibly influenced by pulsatile release of gonadotropins, namely GnRH. Taken together, the cyclic stage of an animal exerts a dominating influence on the expression and activity of aromatase and estrogen levels and possibly even on spine synapse density. To apply these findings to the results obtained from quantification of aromatase expression and determination of spine synapse density one has to admit that results should be viewed critically since animals were not staged initially. At the age of 5-6 weeks animals are pubescent, i.e. pulsatile GnRH release is usually established. Vaginal opening which is used as an external index of puberty onset in mice occurs around day 26 (Nelson et al. 1990). Since we cannot certainly tell at what stage of development *reeler* mice reach puberty we speculate that it must be around the same developmental stage. In addition, we know that *reeler* mice do have an estrous cycle, however, no strict cyclicity. As it was shown in a previous doctoral thesis of our laboratory *reeler* mice have prolonged cycle stages and certain stages were not distinguishable from each other. In addition the cycle itself was often incomplete (Schmahl 2012).
Reflecting data from immunoblot, consequences of cyclicity becomes obvious. Strikingly, in all repetitions one protein band of one specific female *reeler* was more prominent than others. Interestingly the corresponding ovary gave rise to a strong signal of the same band as well (see figure 3.15. in section Results). In fact, this female *reeler* is possibly shortly before ovulation with highest aromatase expression. When repeating analysis but without that specific animal results were rather different; in that case aromatase expression is the least in female *reeler*.
Unpublished data from our institution showed a significant reduction of aromatase in adult *reeler*. The abundance of aromatase in 12-week old mice all being in the same phase of estrous cycle were determined. There it has been shown that at this age aromatase is reduced. However, these experiments were carried out with animals being staged prior to immunoblot. To verify and correlate the two findings it would be necessary to validate the cycle stage of younger animals as well.

4.5.1 Steroid- and synaptogenesis: a gender-based issue?

Considering gender-specific differences the contention of cyclic variations in males need to be subject of critical analysis. Comparable to gonadal estrogen synthesis hippocampal *de novo* synthesis is apparently up to pulsatile release of GnRH (Prange-Kiel et al. 2008); furthermore, hippocampal and ovarian estrous cycle seem to be paralleled by GnRH (Brandt et al. 2013) as high levels of serum estrogen during follicular phase triggers an increased hypothalamic release of GnRH which in turn stimulates ovarian estrogen synthesis via LH but also induces hippocampal estrogen synthesis directly (Brandt et al. 2013; Prange-Kiel et al. 2008). In contrast, experiments with male rats show a tonic and acyclic release of LH in response to GnRH providing a steady state of testosterone (Gillies and McArthur 2010). As testosterone exerts a negative feedback on the release of GnRH its concentration should be rather constant.
It can be reasonably assumed that aromatase protein content is up to the secretion of GnRH. However, while GnRH triggers the activity of aromatase in females, in males GnRH stimulation does not increase estrogen concentration; it rather seems as if in males

aromatase activity is suppressed but conversion of testosterone to dihydrotestosterone (DHT), a non-aromatizable androgen is stimulated (own unpublished observations). Furthermore, synaptic plasticity is especially affected in females by inhibition of aromatase and to a lower extent in males (Fester et al. 2012; Leranth et al. 2004; Vierk et al. 2012). Indeed, estrogen has no such effect on synaptogenesis in males as in females (Leranth et al. 2004). Interestingly, our further experiments showed that correspondent to the effect of estrogen on synaptic plasticity in females, in males it is controlled by DHT (Unpublished data). In fact, estrogen might be lowered in males due to a stimulating effect of GnRH on 5-alpha-reductase, the enzyme converting testosterone to dihydrotestosterone (DHT). As Leranth et al. already demonstrated in 2003 (Leranth et al. 2003) maintenance of spine synapse density in males seems to be controlled by DHT. After gonadectomy an increase of spines was found following treatment with estrogen in females but not in males. Vice versa, treatment with DHT rescued the reduction of spine synapses in castrated males.

4.5.1.1 Estrogen and generation of LTP

In addition to experiments aimed to determine spine synapse density we also analyzed the effect of letrozole on Long-Term-Potentiation (LTP), an electrophysiological parameter of memory which is known to induce spine formation (Yuste and Bonhoeffer 2001). It has been frequently shown that estrogen influences synaptic plasticity. Next to the influence of estrogen on the formation of spines and spine synapses in the hippocampus it possibly increases Long-Term-Potentiation (LTP) at hippocampal CA3-CA1 synapses (Vierk et al. 2012). In contrast, with estrogen synthesis inhibited, generation of LTP is impaired and spine synapses disappear. Vierk et alii compared ovariectomized females, intact females and males regarding generation of LTP and spine synapse loss in response to letrozole treatment (Vierk et al. 2012). Indeed, inhibition of aromatase leads to impairment of LTP, however, it can be rescued by estrogen (Brandt et al. 2013; Vierk et al. 2012). After one day of treatment with letrozole LTP was significantly reduced but spine synapse loss was only seen after 2 days. Apparently, LTP precedes spine synapse loss. Altogether, aromatase inhibition impairs LTP followed by spine synapse loss. Eventually this might explain cognitive deficits as it has been shown several times in pilot studies indicating that aromatase inhibitors used for the therapy of hormone responsive breast cancer have an effect on cognition and memory in women (Shilling et al. 2003b; Zhou et al. 2010).
As noted above, experiments were performed with males and females separately since recent findings point onto sex-specific differences. While in females LTP was reduced by about 60% after 1 day and 95% after 7 days, respectively, such effect was not found in males. In males a reduction by 20% was detected after 1 day, remaining at this level for up to 7 days (Brandt et al. 2013; Vierk et al. 2012). These findings construe the effects of spine synapses being gender-specific as well. In females spine synapses were reduced by 26% after 7 days of treatment. However, in males no spine synapse loss was found after the same time of treatment.

4.6 Expression and activation of steroid enzymes

To be in line with the outlined paradigm of sex-specific differences the following, theoretical conditions should be met:

1. In male WT GnRH levels are in steady state → aromatase is rather inactive but 5-alpha-reductase is active → synapse density is high
2. In female WT GnRH levels are high → aromatse is activated → synpapse density is high

→ No difference regarding synapse density in males and females
→ Aromatase is reduced in males but increased in females

3. In male *reeler* GnRH-levels are depressed → aromatase is not inhibited and 5-alpha-reductase is possibly not activated → synapse density is low
4. In female *reeler* GnRH levels are depressed → aromatase is not activated and 5-alpha-reductase is possibly increased → synapse density is low
   → No difference regarding synapse density in males and females
   → Aromatase is reduced in females, but increased in males

Table 4.1: Protein expression, hormone levels and spine synapse density as a function of genotype and gender

	GnRH	Aromatase	5-alpha-reductase	Estrogen	DHT	Spine synapse density
WT male	↔	↓	↑	↓	↑	↑
WT female	↑	↑	↓	↑	↓	↑
reeler male	↓	↑	↓	↑	↓	↓
reeler female	↓	↓	↑	↓	↑	↓

In the present paper, results are consistent with this concept in the majority of cases, except data from immunohistochemistry. Theoretical explanations for the latter were already discussed earlier.
This paradigm, however, is a rather simplistic and extremely theoretical construct. Various factors involved in the circuitry were not considered or even not known and understood. In addition, the interaction between GnRH and 5-alpha-reductase and associated mechanisms were no issue in this paper, regulations are certainly rather complex and not inferable from interactions between GnRH and aromatase. An antipodal activity of aromatase and 5-alpha-reductase, for instance, appear quite oversimplified; furthermore, the function of 5-alpha-reductase in females was disregarded.
Moreover, recent data question whether aromatase expression can be equated with aromatase activity. But, what are the mechanisms to activate aromatase? As we know, next to slow mechanisms like gene transcription and enhanced protein synthesis regulating aromatase activity there are also rapid mechanisms (Balthazart et al. 2003; Fester et al. 2016). Rapid de-activation of aromatase, however, depends on protein phosphorylation. In fact, Ca^{2+}-dependent protein phosphorylation decreases the activity of aromatase within 10-15 minutes while in the absence of calcium it is dephosphorylated and thus enhanced (Balthazart et al. 2003). Interestingly, it has been shown frequently that estrogen induces Ca^{2+} influx into neurons via L-type channels and also calcium release from internal stores (Fester et al. 2016; Meethal et al. 2009; Zhao et al. 2005). Subsequently, aromatase becomes phosphorylated and its activity is downregulated; however, protein expression is apparently increased (Fester et al. 2016). These findings imply an autocrine regulation of estrogen synthesis in hippocampal neurons. Referring to the regulation of steroid synthesis in the hippocampus with release of GnRH being subject to estrogen concentrations one can imagine the complexity of a fine-tuned circuitry enabling steroidogenesis and, possibly

even synaptogenesis. Moreover, the significance of sex specific mechanisms to activate steroid enzymes was not even taken into account in this concept.

4.7 Questions and issues to be focused on in future

Information originating from or going to the central nervous system (CNS) or the periphery sometimes has to travel long distances before it finally reaches its destination. Along the way, several synapses are passed and information is processed which requires a fine-tuned transmittance. Communication between synapses depends on neurotransmitters and its receptors. It is well known that excitatory synaptic transmission is mainly mediated by glutamate which binds on either ionotropic or metabotropic receptors (Vyklicky et al. 2014). The N-methyl-D-aspartate receptor (NMDAR) belongs to the ionotropic receptors and it has been subject of many discussions due to its role in synaptic plasticity. Long-term-potentiation (LTP), being the neuronal correlate for learning, can be generated only in the presence of NMDAR. Interestingly, Reelin has an indirect effect on NMDAR via intracellular processes (Fatemi 2004). As there are already certain experiments focusing on the interaction of NMDAR and Reelin (Beffert et al. 2005; Chen et al. 2005) it will be challenging to understand the expression pattern and functionality of NMDAR in *reeler*.
To understand sex-specific differences in sexual steroid-induced synpatogenesis steroid enzymes deserve closer attention. In this present paper I focused on aromatase only; other steroid enzymes, for example 5-alpha-reductase were rather disregarded. If there is a sex-dependent expression and activation of steroid enzymes as described earlier, what are the effects of 5-alpha-reductase and DHT in females? Does 5-alpha-reductase need to be activated? Furthermore, is there a harmonized mechanism between aromatase and 5-alpha-reductase and their products to enable steroidogenesis and possibly even synaptogenesis sex-dependently? In addition, effects of 5-alpha-reductase and DHT in *reeler* were only shortly addressed; however, thorough research focusing on expression pattern and activity of that enzyme might help to get a closer insight into phenotypic differences. In fact, many questions remain to be clarified; we are just at the beginning to understand the complexity of neuroendocrinological processes. Further research needs to be done with the focus on the role of steroid enzymes and their interaction with Reelin, in general and particularly regarding sex-specific differences.

5. Summary

During the last decades a *de novo* synthesis of steroids in different parts of the brain has been repeatedly demonstrated. Furthermore, the function of especially estrogen has been in focus of interest of neuroendocrinological examinations. By now we know that processes like establishing neuronal connections or synaptic plasticity is critically influenced by neurosteroids. It has been previously shown that also the extracellular matrix protein Reelin is affected by the neurosteroid estrogen. Treatment of hippocampal slice cultures with estrogen induces an increased Reelin expression; in addition, its expression is reduced after inhibition of aromatase, the final enzyme in estrogen synthesis, by letrozole. Furthermore, aromatase and its product estrogen are essential for formation and maintenance of spine synapses, especially in the hippocampus. Interestingly, recent data indicate that in murine granulose cells aromatase expression can be upregulated by Reelin. In fact, Reelin and estrogen possibly cooperate reciprocal enabling certain processes like i.e. synaptogenesis in the brain or well-ordered reproduction.

To be in line with this concept one can presume a disturbed synaptogenesis with Reelin missing like in the natural occurring mutant *reeler* due to altered expression of steroidogenic enzymes like aromatase. Indeed, preceding experiments from our own laboratory demonstrated a reduced aromatase expression in *reeler*. In addition, Niu and co-workers (Niu et al. 2008) showed a reduced number of spines in hippocampal CA1-neurons in *reeler;* furthermore, when they treated organotypic slice cultures originating from *reeler* with recombinant Reelin they were able to demonstrate a rescue effect regarding spine density. Based on this phenomenon they postulated a direct effect of Reelin on synaptic plasticity.

Given this background aromatase expression in pubescent *reeler* was determined by immunoblotting and immunohistochemistry to assess a reduction as a consequence of Reelin deficiency. In addition by the aid of electron microscopy hippocampal slice cultures of *reeler* of different ages were analyzed with the focus on the entire synapse, rather than on the postsynaptic element only. In fact, aromatase expression appeared to be affected, however, no significant reduction was found. Spine synapse density is significantly reduced in developing animals but in postnatal and adult *reeler* no reduction was seen. Aspects like an upregulation of aromatase during development, synaptic pruning and certainly methodical reasons might influence observations and could explain the discrepancy between the results and the data of Niu et al.. In addition, the influence of cyclicity was not taken into account; however this holds true for experiments of Niu as well. To validate results experiments should be repeated with animals being staged previously. In fact, the assumption of a direct effect of Reelin on synaptic plasticity as stated by Niu et al. cannot be supported by the present data.

6. Zusammenfassung

Durch zahlreiche Arbeiten der letzten Jahrzehnte konnte gezeigt werden, dass neben den Gonaden und anderen Organen auch im Gehirn Steroidhormone *de novo* synthetisiert werden. Darüber hinaus ist inzwischen die Funktion des Östrogen im Gehirn gut untersucht und seine Notwendigkeit zum Beispiel zur Etablierung neuronaler Verbindungen und der synaptischen Plastizität gut belegt. Es konnte in vorangehenden Arbeiten gezeigt werden, dass auch das extrazelluläre Matrixprotein Reelin beeinflusst wird durch das Neurosteroid Östrogen. Durch die Behandlung von hippocampalen Schnittkulturen mit Östrogen kann ein Anstieg der Reelinkonzentration beobachtet werden. Darüber hinaus führt die Hemmung von Aromatase, dem finalen Enzym der Steroidbiosynthese, zu einer reduzierten Konzentration von Reelin. Insbesondere im Hippocampus sind Aromatase und ihr Produkt, also Östrogen, relevant für die Formation und den Erhalt von Dornensynapsen. Interessanterweise konnte kürzlich gezeigt werden dass in murinen Granulosazellen durch die Behandlung mit Reelin ein Anstieg der Aromataseaktivität und -expression erzielt werden kann. Tatsächlich scheinen sich Reelin und Östrogen wechselseitig zu beeinflussen, um verschiedene Prozesse wie zum Beispiel die Synaptogenese im Gehirn oder einen regulierten Reproduktionsvorgang zu gewährleisten.
Anhand dieses Konzepts ist im Falle einer Reelindefizienz wie bei der natürlichen Mausmutante *reeler* von einer gestörten Synaptogenese auszugehen, aufgrund einer veränderten Enzymexpression. Tatsächlich konnte in unserem Institut eine reduzierte Aromataseexpression in der Reeler Maus nachgewiesen werden. Des Weiteren wurde durch die Arbeitsgruppe Niu et al. (Niu et al., 2008) eine reduzierte Dichte von Dornenfortsätzen an hippocampalen Pyramidenzellen gezeigt; weiterhin konnte durch die Behandlung der Kulturen mit rekombinantem Reelin ein *rescue effect* erreicht werden. Durch diese Beobachtungen wurde die Schlussfolgerung gezogen, dass Reelin einen direkten Effekt auf die synaptische Plastizität habe.
Vor diesem Hintergrund war das Ziel dieser Arbeit zum Einen eine reduzierte Aromataseexpression als Folge der Reelindefizienz in jungen Reeler Mäusen mittels Immunblot und Immunhistochemie nachzuweisen. Darüber hinaus wurden mittels Elektronenmikroskopie hippocampale Schnittkulturen untersucht von mutierten Mäusen unterschiedlichen Alters mit dem Fokus auf die gesamte Synapse und nicht auf lediglich das postsynaptische Element. Tatsächlich zeigte sich eine veränderte Enzymexpression, jedoch keine signifikante Reduktion. In pubertären Tieren ist die Dichte der Synapsen signifikant herabgesetzt, jedoch zeigt sich weder in postnatalen noch in adulten Tieren ein vergleichbarer Effekt. Aspekte wie eine Hochregulierung während der Entwicklung, Synapseneliminierung und selbstverständlich methodische Gründe könnten als Erklärung dienen und die Diskrepanz zu den Ergebnissen von Niu et alii erklären. Darüber hinaus wurde der Einfluss der Zyklizität in der Durchführung der Experimente nicht berücksichtigt, wobei dies auch bei den Experimenten von Niu nicht der Fall war. Anhand der vorliegenden Daten kann jedoch ein direkter Effekt von Reelin auf die synaptische Plastizität nicht bestätigt werden.

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8. Appendix

8.1 List of abbreviations

°C	Degree Celcius
µm³	cubic micrometer
AB	Antibody
AD	Alzheimers disease
ANOVA	Analysis of variance
APOE	Apolipoprotein E
APOER2	Apolipoprotein E Receptor 2
APS	Ammonium Persulfate
Aro	Aromatase
bp	base pair
BSA	Bovine Serum Albumin
CA	Cornu Ammonis; Hippocampus proper
CA2+	Calcium
CaCl2	Calcium chloride
CI	Confidence Interval
CNS	Central Nervous System
CO2	Carbon dioxide
CR	Cajal-Retzius
CREB	Cyclic AMP-response Element Binding Protein
CYP	Cytochrome P
d	Day
DAB1	Disabled-1
DAPI	4',6-Diamidino-2-Phenylindole
DG	Dentate Gyrus
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
dNTP	deoxyribose Nucleoside Triphosphate
DTT	Dithiothreitol
E2	Estradiol
ECL	Enhanced Chemiluminescence
EDTA	Ethylendiamintetraacetat
EGF	Epidermal Growth Factor
EM	Electron Microscopy
ER	Estrogen Receptor
ERα	Estrogen Receptor α
ERβ	Estrogen Receptor β
fESPS	field Excitatory Postsynaptic Potential
FSH	Follicle-stimulating Hormone
GA	Glutaraldehyde
GABA	Gamma-Aminobutyric Acid
GapDH	Glyceraldehyde 3-phosphate Dehydrogenase
GnRH	Gonadotropin-releasing Hormone
GnRH-R	Gonadotropin-releasing Hormone Receptor
GSK3β	Glycogen Synthase Kinase 3-β
h	hour
HBSS	Hanks Balanced Salt Solution
HCl	Hydrogen Chloride

HRS	Horse Radish Peroxidase
i.e.	id est
IHC	Immunohistochemistry
IntDen	Integrated Density
kDa	kilo Dalton
LCH	Lissencephaly
LH	Luteinizing hormone
LOG	Logarithm
LSD	Least Significant Difference
LTP	Long-Term-Potentiation
M	Mol
MD	Mean difference
MEM	Minimal Essential Medium
MgCl2	Magnesium chloride
ml	millilitre
mM	millimolar
Na2HPO4	Disodium Phosphate
NaH2PO4	Monosodium Phosphate
NaOH	Sodium Hydroxide
NMDA	N-Methyl-D-Aspartat
NMDAR	N-Methyl-D-Aspartat Receptors
OsO4	Osmium Tetroxide
P	Postnatal
PB	Phosphate Buffer
PBS	phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PSD-5	Postsynaptic Density Protein 95
rcf	relative centrifugal force
RELN	Gene encoding for Reelin
Rf	*reeler* female
RIPA	Radioimmunoprecipitation Assay
Rm	*reeler* male
rpm	revolutions per minute
RT	Room Temperature
s	second
SDS	Sodium Dodecylsulfate
SEM	Standard Error of the Mean
SFKs	Src Familiy Kinases
SLM	Stratum Lacunosum Molecular
SPSS	Statistical Package for the Social Sciences
Taq	Thermus aquaticus
TBE	Tris/Borate/EDTA
TBS	Tris-buffered Saline
TEMED	Tetramethylethylenediamine
TLE	Temporal Lobe Epilepsy
TRIS	Tris(hydroxymethyl)-aminomethan (THAM)
U	Enzyme unit
UKE	Universitätsklinikum Eppendorf
V	volt
VLDL	Very Low Density Lipoprotein

VLDLR	Very Low Density Lipoprotein Receptor
WB	Western Blot
WT	Wild Type
WTf	Wild Type female
WTm	Wild Type male
YFP	Yellow Fluorescent Protein
µ	mikro

8.2 Index of figures and tables

8.2.1 Figures

Figure 1.1: Anatomy of the hippocampus 7
Figure 1.2: Catalysis of estrogens from androgens, realized by the CYP450-protein aromatase. 8
Figure 1.3: Hypothalamo-Pituitary-Gonadal Axis 10
Figure 1.4: The rodent estrous cycle. 10
Figure 1.5: Regulation of steroid hormones by GnRH in WT. 12
Figure 1.6: Schematic diagram of the extracellular matrix protein Reelin. 13
Figure 1.7: Layering of the hippocampus by neuronal migration. 15
Figure 1.8: Cortical development in normal and *reeler*-like mice at different embryonic stages. 16
Figure 1.9: Reelin signalling cascade. 18
Figure 1.10: Consequences of migration defects of GnRH neurons in *reeler*. 19
Figure 1.11: Regulation of steroid hormones by GnRH in *reeler*. 19
Figure 3.1: Spine synapse 34
Figure 3.2: Two electron micrographs covering corresponding neuropil fields 35
Figure 3.3: Spine synapse density in hippocampal slice cultures of postnatal *reeler* 36
Figure 3.4: Spine synapse density in hippocampal slice cultures in postnatal WT after estrogen treatment 37
Figure 3.5: Spine synapse density in hippocampal slice cultures of postnatal reeler in response to estrogen treatment 37
Figure 3.6: Spine synapses in postnatal reeler in males and females separately in response to estrogen stimulation 38
Figure 3.7: Spine synapse density is significantly reduced in 4-week-old reeler 39
Figure 3.8: Differences between male pubescent reeler and WT 40
Figure 3.9: Differences between female juvenile reeler and WT 41
Figure 3.10: Spine synapse density is reduced in pubescent reeler 42
Figure 3.11: Spine synapse density in adult reeler 43
Figure 3.12: Spine synapse density in adult male *reeler* and WT 43
Figure 3.13: Spine synapse density in adult female reeler and WT 44
Figure 3.14: Summary of all data gathered from adult animals. Spine synapse density is not reduced in adult reeler 45
Figure 3.15: Western blot analysis of aromatase expression 46
Figure 3.16: Western blot analysis of aromatase expression in males and females separately 47
Figure 3.17: Aromatase immunoreactivity in the CA1 region of hippocampal slices 48
Figure 3.18: Quantification of aromatase immunoreactivity in the CA1 region of hippocampal slice cultures 49

Figure 3.19: Quantification of aromatase immunoreactivity in CA1 region of hippocampal slice cultures in males 50
Figure 3.20: Quantification of aromatase immunoreactivity in CA1 region of hippocampal slice cultures in females 50
Figure 4.1: No increase in spine synapses is seen after application of estrogen without previous treatment with letrozole.....51
Figure 4.2: Possible explanations for reduced spine synapse density in *reeler* 52
Figure 4.3: Pyramidal cells of stratum pyramidale in WT and *reeler* 56

8.2.2 Tables

Table 2.1: Animals used for Electron microscopy, Immunohistochemistry and Immunoblot 23
Table 2.2: Antibodies used for Immunohistochemistry and Immunoblot 26
Table 2.3: Primer used for amplification (1+2: WT; 1+3: *reeler*).....26
Table 2.4: Polymerase Chain Reaction, contents of Mastermix.....29
Table 2.5: PCR-Conditions 30
Table 2.6: Recipes for four gels for gelelectrophoresis 31
Table 4.1: Protein expression, hormone levels and spine synapse density as a function of genotype and gender 59

8.3 Danksagung

Von Herzen möchte ich mich bedanken bei all jenen Personen die mich durch Ihre Unterstützung zum Gelingen der Arbeit unterstützt haben.
Mein besonderer Dank gilt Frau Prof. Dr. Rune, zum Einen für die Möglichkeit in diesem interessanten Thema meine Arbeit erstellen zu können und dadurch unglaublich viel lernen zu dürfen. Besonders aber auch für die anregende Begeisterung für das Thema und die Wissenschaft sowie für die inspirierenden, ermutigenden Gespräche.
Dr. Lepu Zhou danke ich für die so wertvolle Betreuung. Für die stete Hilfsbereitschaft und Geduld sowie für die Unterhaltungen beim Tee. Maurice und Philipp, vielen Dank dass ihr so selbstlos wiederholt die Arbeit gelesen und korrigiert habt. Allen Mitarbeitern des Instituts für Neuroanatomie bin ich sehr dankbar für die freundliche und freundschaftliche Aufnahme im Institut und die Möglichkeit, jedem allzeit meine Fragen stellen zu können. Vielen Dank!

Ein besonderer Dank gilt meinen Eltern und meiner Familie für die unverzichtbare Unterstützung mich durch die emotionale Achterbahn zu begleiten. Ich bin euch sehr dankbar dafür, dass ihr mir ermöglicht habt meine Ziele zu erreichen.
Meinen Freunden danke ich dafür mir unermüdlich Mut zuzusprechen und mir bedingungslos zur Seite zu stehen.
Ein ganz besonderer Dank gebührt meinem Freund; danke dass Du mit mir in Hochphasen lachst und mich aus manch unüberwindbar scheinendem Tief herausholst.

8.4 Curriculum vitae

Entfällt aus datenschutzrechtlichen Gründen

Weitere wissenschaftliche Arbeiten:
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Publiziert am 13.06.2012 in The Journal of Neuroscience
http://www.ncbi.nlm.nih.gov/pubmed/22699893?dopt=Citation

8.5 Eidesstattliche Erklärung

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