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The discovery of GluA3-dependent synaptic plasticity

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Chapter 1:

General Introduction

Memories allow us to know how to ride a bike, what it feels like when we fall off it, and where we parked it the last time we used it. These are very different types of memories and they depend on different regions of the brain. Overall, memories determine who we are; they shape our lives. The information is encoded and stored in neuronal networks. When this neuronal system is altered memories can be lost, as it is the case of Alzheimer's disease, where people's lives are dramatically changed.

In my thesis entitled "The discovery of GluA3-dependent synaptic plasticity" I start by introducing relevant concepts as synaptic plasticity, learning and memory, describing the brain structures of interest for this work, namely the hippocampus and cerebellum, and presenting AMPA receptors and Alzheimer's disease. Finally, I introduce the experimental chapters that form the core of this thesis.

1.1. Learning and memory

Learning is the process by which we acquire information about the world, and memories are the stored knowledge. Even the simplest animals have the ability to learn from the environment, and this skill is highly developed in humans. What types of learning and memories have been described and studied? Which brain structures partake in memory formation? It is well accepted to classify memories into declarative (explicit) and non-declarative (implicit) ones.

Declarative memories code information about autobiographical events (episodic memory) as well as knowledge of facts (semantic memory), and they can be willingly evoked. Declarative memories depend on structures of the temporal lobe: extensive experimental work (Squire and Zola-Morgan, 1991) allowed to identify the involvement of the hippocampus and entorhinal, parahippocampal and perirhinal cortices. The amygdala, a structure belonging to the limbic system, can exert a modulatory effect on explicit memories. In rodents, widely accepted models of explicit memories include object recognition and spatial learning tasks like the Morris water maze. On the other hand, implicit memories are characterized by being automatic and reflexive. Their formation and their recall are not conscious processes. Classically, this type of memory is gradually formed and improved. Implicit memories are classified into procedural memory (of habits and skills), priming, non-associative learning and classical conditioning.

Fear conditioning is a form of Pavlovian conditioning that is often used in research. In this task, the animal learns to associate a neutral stimulus (conditioned stimulus, CS), with an aversive unconditioned stimulus (US), so that re-exposure to the CS will lead to a conditioned behavioral response. In the case of contextual fear conditioning (Figure 1), the electric foot shock (US) is given in a certain context (CS) that becomes threatening. The conditioned response of the animal is to freeze, meaning a complete lack of movement except for breathing. A fear conditioning memory has components of both explicit and implicit learning: learning the context of the fearful situation involves explicit memory and depends on the hippocampus, since lesions in this brain area impair the memory (Kim and Fanselow, 1992). The association of the context with fear is implicitly generated and requires the amygdala (Phillips and LeDoux, 1992).

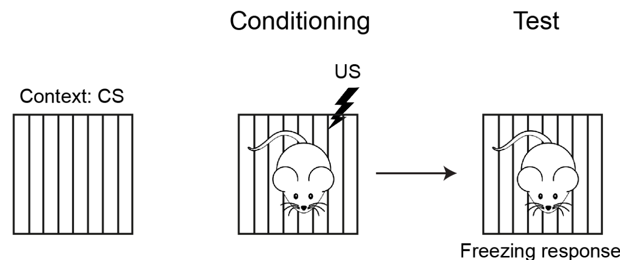

Figure 1: Schematic representation of contextual fear conditioning. During the conditioning session, the mouse learns to associate the conditioned stimulus (CS, the context) with the unconditioned stimulus (US, electric foot shock). When tested, the mouse will freeze as a conditioned response to the CS.

1.2. Synaptic plasticity and learning and memory

1.2.1. Historical overview

William James (1890) was one of the first to question the physiological bases of the formation of associations. According to his "law of habit", the condition allowing associations to be made is the coactivity of elementary brain processes (James, 1890). He was hypothesizing on the concept of neural plasticity. In 1893, Eugenio Tanzi proposed that changes provoked by experience were located in neural junctions (Tanzi, 1893) that would in 1897 be named synapses by Charles Scott Sherrington (Sherrington, 1897). Santiago Ramon y Cajal (1909) suggested that the nervous system was not a *sinctium* but an ensemble of separated neurons that were

only connected in specialized synaptic sites. He also proposed that learning was the result of new ramifications in the existing neurons rather than of neuron proliferation (Ramon y Cajal, 1894, 1909). These morphological changes would allow the strengthening of connections, rendering the transfer of information more efficient. Donald Hebb, in his book "The organization of behavior: a neurophysiological theory" (1949), brought forward the hypothesis about the neural processes underlying memory formation. He suggested that cell assemblies worked together in order to represent information, and these assemblies were distributed along large cortical areas. He postulated that "when an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased" (Hebb, 1949). In more modern terms, when presynaptic glutamate release coincides with postsynaptic depolarization, synaptic connections are strengthened, modifying synaptic ensembles. This concept was later supported by the work of Bliss and Lomo, who reported that in the hippocampus repetitive stimulation of cell ensembles resulted in a persistent increase in synaptic strength, evidenced by an increase in the extracellularly recorded excitatory postsynaptic field potentials (EPSPs) (Bliss and Lømo, 1973). They were to first to record a form of synaptic plasticity, which came to be known as long-term potentiation (LTP) (Douglas and Goddard, 1975). The discovery of Bliss and Lomo provided an experimental analogue to the learning-induced changes in synaptic connectivity postulated by Hebb, and it was the stepping-stone to numerous lines of research into synaptic plasticity and learning.

1.2.2. Synaptic plasticity: LTP and its link to learning

In general, synaptic plasticity can occur at the presynaptic and/or the postsynaptic site. Presynaptically, it occurs generally by a change in the neurotransmitter release probability. In the case of postsynaptic modifications, the amount of receptors, as well as their properties, can change. In addition, the type of receptors that are expressed can be altered. Other synaptic changes that do not directly involve alterations in synaptic strength can also occur, such as structural plasticity of spines. All of these mechanisms of synaptic plasticity could be participating together in the process of memory storage, potentially with other neuronal changes as adult neurogenesis and alterations in neuronal excitability.

Long-term potentiation (LTP; Figure 2) is the type of synaptic plasticity most thoroughly studied in its relation to learning and memory, and it is widely proposed to

be the cellular correlate of these processes. LTP occurs in a very short period of time, generates strengthening of synaptic transmission and can last for several hours or longer. Over the last decades, extensive work using pharmacological as well as genetic approaches has shed light into the molecular mechanisms and involvement of LTP in learning and memory.

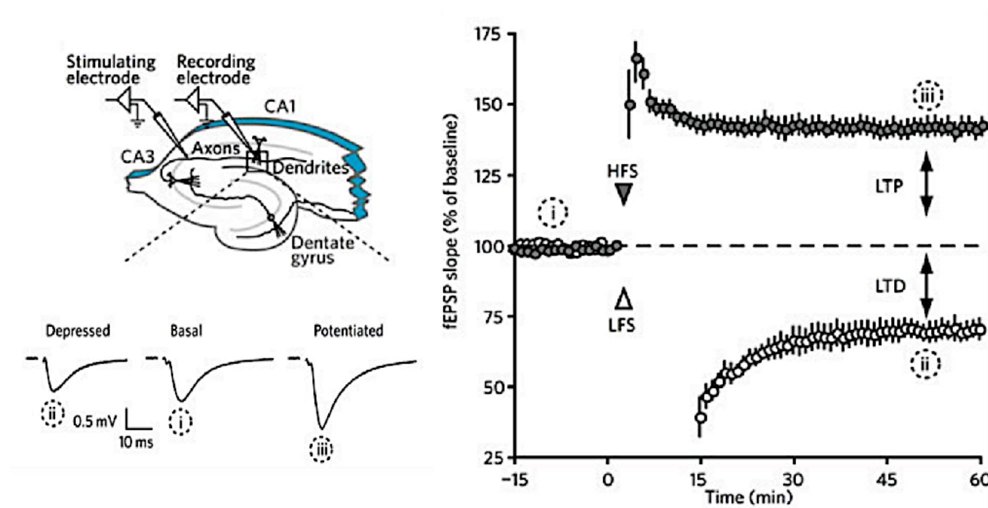

Figure 2: Representative figure showing LTP and LTD. Scheme of a hippocampal brain slice and the location of the stimulation and recording electrodes (top left). Examples of field excitatory postsynaptic potentials (fEPSPs) measured from synapses under (i) basal, (ii) depressed or (iii) potentiated conditions (bottom left). LTP (closed circles) and LTD (open circles) induced following high frequency stimulation (HFS) or low frequency stimulation (LFS) respectively (right). Adapted from Fleming and England, 2010.

The mechanism that establishes LTP has been extensively investigated and different hypothesis have led to strong debates. Over the years, conflicting evidence pointed either to a pre- or to a post- synaptic origin. In short, the evidence of a presynaptic origin was based on the fact that after LTP there was a decrease in synaptic failures, which was an observation that at the time could only be explained by the failure to release neurotransmitter presynaptically. Eventually, this was settled with the discovery of postsynaptically silent synapses (Liao et al., 1995), containing NMDA receptors but not AMPA receptors. Upon LTP induction these synapses start expressing AMPA receptors as well, thereby becoming unsilenced, increasing synaptic strength and decreasing the number of synaptic failures.

The most studied form of LTP in the hippocampus is NMDA receptor-dependent. NMDA receptors in the postsynaptic membrane are activated when presynaptic activation, leading to neurotransmitter release, coincides with postsynaptic

depolarization. Calcium influx through these receptors triggers molecular pathways such as the activation of calmodulin-dependent kinase II (CaMKII), which phosphorylate postsynaptic proteins including the carboxy-terminal region of the AMPA receptor subunit GluA1, facilitating the trafficking of GluA1-containing AMPA receptors into the postsynaptic density (PSD). This results in a rapid and stable increase in synaptic strength.

Whereas one focus of LTP-related research is to unravel its detailed mechanisms, another central field is the one that studies the relation between this form of synaptic potentiation and learning and memory. Several lines of work provide evidence to consider LTP as a fundamental process for memory formation. Fear conditioning produces LTP (Fedulov et al., 2007; Rogan et al., 1997; Whitlock et al., 2006). The most compelling causal link between learning and LTP was recently strengthened showing that LTP induction *in vivo* can reactivate a previously inactive memory (Nabavi et al., 2014).

Long-term synaptic plasticity involves not only strengthening of synaptic connections by means of LTP but also synaptic weakening, which occurs through long-term depression (LTD; Figure 2). The weakening of synapses in LTD can occur through a postsynaptic mechanism via internalization of AMPA receptors or presynaptically as a result of a decrease in neurotransmitter release. Over the years, in the field of study of NMDA receptor-dependent LTD it was established that low frequency stimulation activates NMDA receptors allowing a calcium influx that is lower than in the case of LTP, leading to the activation of calcineurin and internalization of GluA2-containing AMPA receptors into clathrin coated vesicles (Carroll et al., 1999; Lüscher et al., 1999; Man et al., 2000). However, this classic view was challenged since it has recently been shown that calcium influx is not necessary for LTD: NMDA receptors have a metabotropic function that is sufficient to trigger a signaling cascade that may release CaMKII from the synapse and will cause synaptic depression (Aow et al., 2015; Dore et al., 2015; Nabavi et al., 2013) and spine shrinkage (Stein et al., 2015).

Events experienced by the individual are believed, at the cellular level, to modify synapses, thereby shaping neuronal pathways. One very important feature of synaptic plasticity related to learning and memory is its specificity. When a new memory is formed, only a subset of neurons is considered to be modified. The combination of neurons whose synapses are activated represent the memory engram, a term coined over a century ago (Semon, 1904). The study of the location of the memory engram was initiated with lesion experiments (Lashley, 1950). It is this

specific combination of synapses on a subset of neurons that encodes a memory (Hayashi-Takagi et al., 2015; Rumpel et al., 2005), and its artificial activation leads to the recall of the memory (Liu et al., 2012; Ramirez et al., 2013), whereas inactivation of the engram cells inhibits memory retrieval (Denny et al., 2014; Tanaka et al., 2014). This sparse coding of memories allows the brain to store an enormous amount of information. If instead the majority of synapses on a large proportion of neurons would go through synaptic modifications during learning, this would unlikely lead to specific memory formation, or if it did then the number of distinct memories stored in the brain would be very limited.

Most of the studies mentioned above on the mechanism of LTP as well as its relation with memory, and many other types of learning and memory research, are performed in the hippocampus. This brain structure has been of great interest along the years and it is described in more detail below.

1.3. The hippocampal formation

Since the case of patient H.M. was described in 1957 (Scoville et al., 1957), in which the removal of the hippocampus and surrounding medial temporal lobe structures caused anterograde amnesia, the hippocampal formation has been strongly linked to the formation of declarative memories. Even though this is historically the most famous case, before this discovery it had already been proposed that the hippocampal formation was amongst the ones important for memory processes (von Bechterew, 1900; Glees and Griffith, 1952; Grünthal, 1947).

The hippocampal formation (HF; Figure 3), c-shaped in rodents, is one of the principal structures of the medial temporal lobe that has a role in memory formation. The HF is divided in cytoarchitectonically distinct regions: the subiculum, the dentate gyrus (DG) and the hippocampus proper (HP). The latter is in turn subdivided into three areas, named after their similarity with a ram’s horn: *Cornu Ammonis* 1-3 (CA1, CA2 and CA3).

The principal input to the HF arrives from the entorhinal cortex (EC) through the perforant path to the granule cells of the DG, although projections will arrive to all subdivisions of the HF. The EC in turn receives information from various cortical regions. The granule cells in the DG, through so-called mossy fibers, project to CA3. CA3 projects locally to itself and sends Schaffer collaterals to CA1. CA1 pyramidal

neurons in turn project to the subiculum and deep layers of the EC. Finally, the output of the subiculum is mainly the EC. The HF also receives input and sends projections to a number of other brain regions, rendering it a highly connected structure. The neuronal structure of the HP is laminar through its different sub regions. The cell type that prevails in the HP is the pyramidal neuron, whose cell body is located in the *stratum pyramidale*, where somas are densely packed forming an easily identifiable layer. Pyramidal neurons have an apical dendritic tree and a basal one.

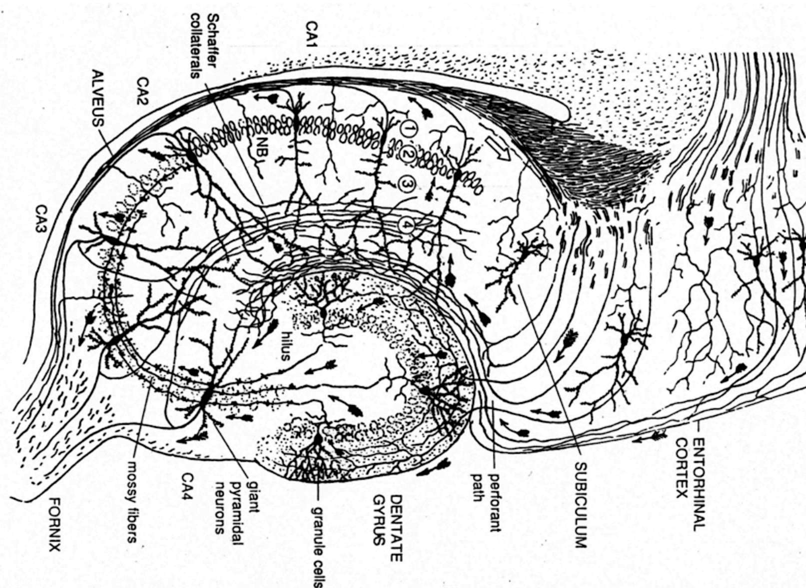

Figure 3: Components of the hippocampal formation. The subiculum, part of the EC, the DG and CA1-CA3 regions amongst others are indicated. The hippocampus proper is divided in *stratum oriens* (1)*, stratum pyramidale* (2)*, stratum radiatum* (3) *and stratum lacunosum-moleculare* (4). Interneurons are not present in the illustration (adapted from Ramon y Cajal 1911).

For simplicity, throughout the remaining of this thesis we will refer to the hippocampal formation as hippocampus. Historically, different functions have been attributed to the hippocampus, such as olfaction and behavioral inhibition. During several decades, the hippocampus has been repeatedly implicated in spatial coding and different types of memory formation. During the 1970's, important new evidence supported these roles of the hippocampus. It was discovered that pyramidal neurons of the rat could code for spatial information. Due to its prominent role in memory formation and its unique organization, the hippocampus can be accounted as the most frequently used brain structure in the study of synaptic plasticity and memory encoding. In particular, the Schaffer collateral to CA1 synapse remains probably the most studied synapse in the brain.

Although synaptic plasticity was described and extensively studied in the hippocampus, the process occurs widely throughout different structures of the brain, including the cerebellum (Ito and Kano, 1982; Figure 4).

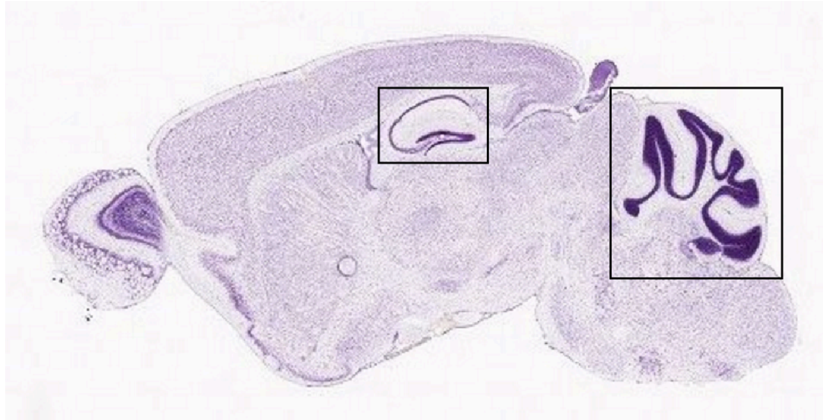

Figure 4: Location of the hippocampus and the cerebellum: Nissl staining of a sagittal mouse brain section. The hippocampus (left) and cerebellum (right) are marked by bliack squares. Image extracted from the Allan Brain Atlas (http://www.brain-map.org/).

1.4. The cerebellum

The cerebellum is part of the hindbrain, which also contains the medulla oblongata and pons. Composed by two hemispheres, it is located dorsally to the pons and posterior to the superior colliculus. The basic design is the same for the cerebellum and the cerebrum: an outer cortex with high neuronal density forming the grey matter, white matter and deep gangliar structures, in this case deep cerebellar and vestibular nuclei. The cerebellar cortex is divided in 10 lobules, each consisting of a tightly folded layer of cortical grey matter.

The first overview of the cerebellar circuitry was provided by Santiago Ramon y Cajal. Two of the main inputs to the cerebellum are climbing fibers, coming from the inferior olive, and mossy fibers. Purkinje cells (PC) were first described in 1837. They have a very characteristic morphology, with a bi-dimensional dendritic tree extremely ramified. Each PC receives input from one single climbing fiber (coming from the inferior olive) as well as multiple parallel fibers, which are the axons of granule cells, which in turn received input from mossy fibers. PCs are inhibitory cells, releasing GABA from their presynaptic sites, and they are the only output of the cerebellar cortex, projecting to cerebellar and vestibular nuclei neurons. PCs generate two types of spikes, namely simple and complex ones. The production of simple spikes is

intrinsic to the PC and their probability of occurrence is modulated by the activity of the parallel fibers. Complex spikes are caused by glutamate discharges from climbing fibers, since one climbing fiber contacts the PC in numerous synaptic sites. Granule cells, the smallest neurons in the brain, are contained in the granular layer. They have a very conserved morphology and their axons form the parallel fibers, which can connect with several PCs, although always making a single synaptic contact with each PC.

The cerebellum has important functions in motor behavior. Amongst them, in the aim towards a correct and stable vision, is the generation of optokinetic responses and vestibulo-ocular reflexes. The former occurs when the eyes rapidly move following a target in movement, while the latter is the ability to counterbalance a head movement to prevent the destabilization of the visual focus. The cerebellum is also a structure of foremost importance for the fine tuning and encoding of procedural memory, which is a type of implicit memory.

1.5. Glutamate receptors

Glutamate is the principal excitatory neurotransmitter in the central nervous system. Upon fusion of glutamate-containing presynaptic vesicles with the presynaptic membrane, the neurotransmitter is released into the synaptic cleft, where it can bind to different transmembrane receptors at the postsynaptic side. Glutamate receptors can be ionotropic, meaning that they are ligand-gated ion channels, or metabotropic, meaning G-protein coupled receptors acting through a signaling cascade that involves a secondary messenger. These receptors mediate glutamate-induced excitation of neurons.

There are three classes of ionotropic glutamate receptors (Figure 5): α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainate receptors, named after their respective artificial selective agonists. In this thesis, the main type of glutamate receptor under study is the AMPA receptor.

Figure 5: Classification of ionotropic glutamate receptors.

AMPA receptors (AMPARs) are ligand-activated cation channels. When activated, K^+ and Na^+ flow through their pore. As a result, postsynaptic currents having a reversal potential close to 0 mV are generated.

AMPARs are composed of four subunits by a dimer of two identical homo- or heterodimers that together form the tetramer. There are four types of subunits, namely GluA1, GluA2, GluA3 and GluA4, and their sequences are highly homologous, approximately 70%. Each subunit contains one ligand-binding site (Mayer, 2005). Upon binding of the ligand, the subunit changes its conformation. Agonist binding to at least 2 of the 4 subunits is necessary to allow the channel to open (Clements et al., 1998).

The structure of each subunit can be divided into an extracellular domain, a ligand binding domain, a transmembrane domain forming the channel pore and a cytoplasmic carboxy-terminal domain. It is in the latter that more differences are found between subunits. While GluA1 and GluA4 (and GluA2L, a splice variant of GluA2) have long carboxy-terminal domains, these are short in GluA2 and GluA3 (and GluA4short, a splice variant of GluA4) subunits. The carboxy-terminal domains allow the subunits to interact with specific cytoplasmic proteins. In addition, each subunit has different and specifically regulated phosphorylation sites. Glutamatergic synaptic plasticity acts through modulation of AMPAR-mediated synaptic transmission, making AMPARs very interesting to study synaptic plasticity (Kessels and Malinow, 2009).

AMPAR subunits are differentially expressed throughout the brain. They play distinct roles in synaptic plasticity, learning and memory, diseases and overall neuronal function, and some subunits have to date been more thoroughly characterized than others. There are no known pharmacological tools to selectively block or activate one

particular subunit. A genetic approach is rather used to unravel the role of the subunits. For example, transgenic mice or viral introduction of unaltered or modified subunits are commonly used tools.

Certain subunit compositions can render AMPARs inwardly rectifying, meaning that ion flow in the outward direction becomes minimal. This is caused by polyamines, which at a depolarized holding potential (+40 mV) will block the channel pore. The rectification index is thus measured as the ratio of the AMPAR current observed at -60 mV to that at +40 mV. GluA2 is the determinant subunit for this characteristic: if it is part of the AMPAR, rectification at positive potentials does not occur. Therefore, this property allows studying the composition of AMPARs that contribute to synaptic transmission. AMPARs lacking the GluA2 subunit conduct Ca^{2+} in addition to K^{+} and Na^{+}.

1.5.1. GluA1

GluA1 is the most studied AMPAR subunit in the context of synaptic plasticity and learning and memory. It mainly forms heterodimers with GluA2 (GluA1/2-AMPARs) and it can also form homomers (GluA1/1-AMPARs).

Different genetic tools have been used to study the role of GluA1. Knocking out its gene allowed assessing in which processes GluA1 is a crucial player. Overexpressing GluA1 will tend to form homomeric GluA1/1-AMPARs rather than heteromers with GluA2, rendering AMPARs inwardly rectifying, and this acts as a so-called electrophysiological tag. Other tools include expressing mutated GluA1 subunits to determine the role of its regions and specific amino acids, expressing a pore-dead version of the receptor that does not conduct current, or expressing the cytoplasmic tail that interacts with GluA1-specific binding proteins competing with endogenous GluA1. It is currently not possible to pharmacologically block or activate selectively one type of AMPAR.

GluA1 is responsible for the majority of AMPAR-mediated currents in the synapse as well as extra-synaptically (Andrásfalvy et al., 2003; Lu et al., 2009). As mentioned previously, GluA1 is delivered to the synapse upon LTP (Hayashi et al., 2000; Kakegawa et al., 2004; Shi et al., 2001, 1999) and it has been shown to be crucial for LTP in different regions of the brain such as the hippocampus, amygdala and cortex (Mack et al., 2001; Rumpel et al., 2005; Zamanillo et al., 1999). The trafficking of this subunit in and out of the synapse is an activity-dependent process, stimulated by NMDAR activation.

GluA1 has four known phosphorylation sites in its intracellular carboxy-terminal domain: Serine 818 (Boehm et al., 2006), 831 (Barria et al., 1997; Roche et al., 1996) and 845 (Roche et al., 1996) and Threonine 840 (Lee et al., 2007). It is phosphorylated at S831 by protein kinase C (PKC) and CaMKII (increasing single channel conductance), at S845 by cAMP-dependent protein kinase (PKA) (increasing mean open probability), and at S818 and T840 by PKC. These phosphorylations in the carboxy-terminal domain of GluA1 modify the properties of the subunit (Lee et al., 2010), for example by increasing surface levels in the postsynaptic density, facilitating the process of LTP. However, it has been recently suggested that these phosphorylations hardly take place *in vivo* (Hosokawa et al., 2015).

Interestingly, some GluA1 properties can change depending on the experimental model. When expressed in organotypic hippocampal slices GluA1 is restricted from the synapses and only traffics to the synapse upon LTP induction or CaMKII activation (Hayashi et al., 2000; Kakegawa et al., 2004; Shi et al., 1999). Recombinant GluA1 expressed in dissociated cultured neurons can travel to the synapse in the absence of LTP induction (Lissin et al., 1998; Passafaro et al., 2001). It is well possible that dissociated neuronal cultures have a higher level of intrinsic neuronal activity that promotes the insertion of GluA1 into synapses. One study found that also in hippocampal slices GluA1 is delivered to the synapse without the need of activity (Granger et al., 2013), and proposed that it is the GFP tag that prevents trafficking to synapses, making the GFP-tagged GluA1 model less relevant. However, when trying to unify these views in the same experimental setup (Nabavi et al., 2014) activity-independent entry of GluA1 into synapses was not observed either in the presence or absence of a GFP tag. Importantly, when expressed *in vivo* GluA1 can be found in dendritic spines (Mack et al., 2001), but only traffics into synapses upon behavioral experience. For instance, fear learning drives recombinant GluA1 to the synapse in the amygdala (Rumpel et al., 2005). In addition, whisker-experience involves the insertion of GluA1-containing AMPARs into synapses of the barrel cortex, and clipping whiskers prevents GluA1 from trafficking into these synapses (Makino and Malinow, 2011; Takahashi et al., 2003; Zhang et al., 2015). In conclusion, the majority of studies are in line with the model that GluA1-containing AMPARs only go into synapses upon LTP-like activity.

The critical role for GluA1-trafficking in memory formation has been established using different model systems. Blocking GluA1-dependent LTP *in vivo* at different levels, such as knocking out GluA1 or introducing modified versions of GluA1 that disrupt

their trafficking, impairs learning or experience-dependent plasticity (Feyder et al., 2007; Rumpel et al., 2005; Takahashi et al., 2003). Blocking GluA1 traffic to the synapse in the hippocampus or amygdala impairs memory formation (Mitsushima et al., 2011; Rumpel et al., 2005). Mice lacking GluA1 have spatial working memory deficits (Reisel et al., 2002; Schmitt et al., 2003, 2004) and are impaired in fear conditioning (Humeau et al., 2007). However, in these animals other processes such as spatial reference memory remain unaltered (Reisel et al., 2002; Schmitt et al., 2003; Zamanillo et al., 1999), indicating that not all memory processes require GluA1-plasticity but likely depend on other types of plasticity.

Overall, GluA1 is considered the key subunit in several forms of synaptic plasticity and memory formation. This view was recently challenged in a study showing that for LTP to take place glutamate receptors are required independently of their subunit subtype. In this scheme, LTP only needs an adequate reserve pool of extrasynaptic glutamate receptors (Granger et al., 2013). However, the fact that any receptor can traffic does not imply that GluA1-lacking AMPARs do traffic upon LTP under normal circumstances, nor that GluA1 is not necessary for the normal regulation of LTP. GluA1 is an abundant subunit and phosphorylation of its C-tail facilitates trafficking to the PSD upon LTP. It is possible that there is a form of competition between AMPARs with different compositions: when LTP is induced GluA1-containing AMPARs would traffic more easily than those lacking GluA1. Only in the absence of GluA1 would other receptors may become more relevant for LTP.

1.5.2. GluA2

Virtually all the GluA2 subunits in the brain are present in their edited form: a single nucleotide substitution in the mRNA generates a protein where a neutral glutamine in the transmembrane domain is replaced by a positively charged arginine. This modification makes GluA2-containing AMPARs impermeable to calcium (Isaac et al., 2007). Furthermore, edited GluA2 cannot form homomers, likely because of electrostatic repulsion. As explained above, GluA2-lacking AMPARs are inwardly rectifying. This property is used to electrophysiologically differentiate subunit compositions of AMPARs. Additionally, overexpression of unedited GluA2 allows the formation of GluA2 homomers that are rectifying. Since GluA2 and GluA3 have short cytoplasmic tails, homomeric unedited GluA2/2 recombinant receptors have been used to assess the role of GluA1-lacking AMPARs, and their properties are often extrapolated to endogenous GluA2/3 heteromers. Interneurons contain a large proportion of GluA2-lacking AMPARs, which are calcium permeable, have fast

deactivation kinetics and enhanced single channel conductance. The majority of AMPARs in excitatory neurons contain GluA2.

The carboxy-terminal tail of GluA2 can be phosphorylated at S880, tyrosine 876 (both sites having a role in receptor trafficking), and at S863 (Lu and Roche, 2012). When phosphorylated at S880, the C-tail of GluA2 prevents its interaction with GRIP/ABP while the capacity to bind PICK1 is unaltered, modulating receptor trafficking (Chung et al., 2000; Matsuda et al., 1999, 2000). The phosphorylation at Y876 results in the same modifications in interactions with GRIP and PICK1, and disrupting the phosphorylation impairs endocytosis of GluA2 and therefore LTD (Chung et al., 2003).

LTP can be expressed in the absence of GluA2 (Jia et al., 1996). LTD in the hippocampus and cerebellum is a result of internalization of AMPARs. During this process, the carboxy-terminal domain of GluA2 interacts with proteins that trigger the endocytosis of the receptors, and LTD is absent in the cerebellum of GluA2 knock out mice (Chung et al., 2003).

1.5.3. GluA3

The main focus of this thesis will revolve around the subunit GluA3. Over the last decades of research, GluA3 has received considerably less attention than other AMPAR subunits like GluA1 or GluA2. Although GluA3 is present throughout the brain, there is conflicting evidence regarding its relative abundance. At the mRNA level, GluA3 is 10-fold less abundant than GluA1 and GluA2 in the hippocampus (Tsuzuki et al., 2001). However, there is evidence showing that at the protein level GluA1 and GluA3 are present in equivalent amounts in the CA1 region of the hippocampus (Kessels et al., 2009). Moreover, GluA3 total protein levels in the hippocampus are considerable (Schwenk et al., 2014).

It is then rather surprising to contrast this with the fact that in the absence of GluA1, when only GluA2/3-containing AMPARs are present, AMPAR currents in the hippocampus are low at synapses and virtually absent at extrasynaptic sites (Andrásfalvy et al., 2003; Lu et al., 2009). Correspondingly, when GluA3 is absent synaptic AMPAR currents remain unaltered or are only mildly reduced, and both LTP and LTD are still present (Lu et al., 2009; Meng et al., 2003).

GluA3-deficient mice show unaltered auditory and contextual fear conditioning memories (Humeau et al., 2007) and they perform normally in a variety of behavioral

tests. For all of the above-mentioned reasons GluA3 has, until this thesis, remained largely ignored.

Globally GluA3-deficient mice do however show a number of altered phenotypes, including: increased sociability and aggressive behavior (Adamczyk et al., 2012), disturbed slow-wave brain oscillations and respiratory modulation, a tendency to suffer seizures (Steenland et al., 2008) and an increased alcohol consumption following deprivation (Sanchis-Segura et al., 2006).

1.5.4. GluA4

In pyramidal cells of the hippocampus GluA4 is highly expressed at the early stages of development. GluA4 trafficking properties have similarities with those of GluA1: they traffic to synapses in an activity dependent manner. In contrast to GluA1, spontaneous activity drives GluA4 trafficking in a manner independent of CaMKII (Zhu et al., 2000); phosphorylation of GluA4 at its C-tail by PKA is sufficient to trigger trafficking (Esteban et al., 2003). GluA4 levels decrease drastically during the first postnatal week in the hippocampus, where AMPARs will later consist of subunits GluA1, GluA2 and GluA3. In the barrel cortex, GluA4 delivery to the synapse triggered by whisker experience only occurs early in development (Miyazaki et al., 2012). Remarkably, GluA4 remains a significant component of AMPARs in other brain structures such as the cerebellum (Schwenk et al., 2014), where it is found in granule cells and in Bergman glia, in the latter forming calcium-permeable AMPARs together with GluA1 subunits (Matsui et al., 2005). GluA4 is present in several types of interneurons, which in general do not express GluA2 and consequently show a sharper decay in their AMPAR-mediated currents.

Compared to the vast knowledge on AMPARs in the hippocampus, the subunit composition and role of AMPARs in Purkinje cells of the cerebellum as well as their role in procedural cerebellar memory formation have not been extensively studied. There is sparse information about the roles of AMPAR subunit composition in plasticity of Purkinje cells (Bats et al., 2013; Douyard et al., 2007; Kakegawa and Yuzaki, 2005). Furthermore, although it is known that AMPAR-plasticity is involved in parallel fiber to Purkinje cell synapses during LTP and LTD (Chung et al., 2003; Kakegawa and Yuzaki, 2005; Steinberg et al., 2006), the full functional significance and the precise molecular pathways underlying this plasticity remain to be elucidated (Galliano and De Zeeuw, 2014; Gao et al., 2012).

1.6. Alzheimer’s disease

Neurodegenerative diseases are characterized by a loss of neural structure and function. Alzheimer’s disease (AD), a progressive age-related neurodegenerative disease, is the leading form of dementia in the elderly. It was fist described by Alois Alzheimer in 1906. The disease is characterized by the presence of two neuropathological elements: extracellular plaques that contain aggregates of the protein amyloid β, and neurofibrillary tangles composed of aggregates of the hyperphosphorylated protein tau. They are mainly formed in the cerebral cortex and subcortical regions, including the hippocampus. The clinical symptoms include impaired memory, personality changes, hallucinations and cognitive decline. The vast majority of patients present sporadic forms of AD, generally with a later onset in life. However, a fraction of the cases is linked with a genetic form of AD, termed familial AD, and the onset tends to be earlier in life. The early-onset AD is associated with mutations in the genes coding for the amyloid precursor protein (APP) and γ-secretase subunits presenilin 1 or 2 (PS1, PS2). Since all these mutations point towards an increased production of γ-secretase mediated cleavage products of APP, familial AD are believed to involve the increased production of amyloid β (Aβ). Late-onset and sporadic AD is associated with mutations in apolipoprotein E4 (ApoE4). Since ApoE4 influences the clearance of Aβ, one could speculate that late-onset and sporadic AD are usually associated with problems with Aβ clearance, while familial AD is linked with its high production.

Over the years, there has been extensive research aiming to understand what are the molecular mechanisms underlying Alzheimer's disease. Different hypothesis have been put forward.

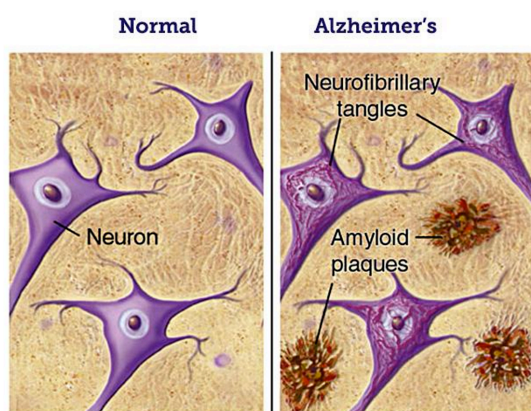

Figure 6: Schematic representation of neurons in a healthy and Alzheimer's brain. On the right, there are neurofibrillary tangles composed of tau and extracellular plaques composed of Aβ. Image from BrightFocus Foundation (http://www.brightfocus.org/).

The cholinergic hypothesis was the first one proposed to explain AD, in the 1970's. It was based on the fact that dysfunction and loss of cholinergic activity is a common early event in AD (Davies and Maloney, 1976). Furthermore, there is decreased activity and reduced levels of the components of the cholinergic system in AD patient's brains (Auld et al., 1998).

The tau hypothesis states that the main cause of AD is the abnormal phosphorylation of tau, a highly soluble microtubule-associated protein. This hyperphosphorylation of Tau leads to its aggregation and yields neurofibrillary tangles that accumulate inside the neurons, damaging cytoplasmic functions and interfering with axonal transport. In this frame, different proteins that phosphorylate tau and can cause AD have been identified: glycogen synthase kinase 3 and cyclin dependent kinase 5 (Noble et al., 2003; Spittaels et al., 2000).

The third model is the amyloid β (Aβ) cascade hypothesis. The transmembrane amyloid precursor protein (APP) can be processed in two ways. In the non-amyloidogenic pathway, APP is firstly cleaved by α-secretase, whose site of action is inside the Aβ domain, and then cleaved by γ-secretase. Therefore, in this case the formation of the Aβ peptide is precluded. In the other option, known as the amyloidogenic pathway, APP is cleaved by β-secretase followed by the action of γ-secretase. One of the products of this processing is the Aβ peptide. The enzyme γ-secretase cleaves the protein in a variable site, producing peptides of different sizes: $A\beta_{40}$ and $A\beta_{42}$. Although both forms are found in aggregates, $A\beta_{42}$ is highly fibrillogenic, being thus the origin of large oligomers and plaques. An Aβ production-clearance unbalance leads to its accumulation in fibrils, originating neuropathological symptoms (Hardy and Selkoe, 2002).

The different hypotheses do not necessarily exclude each other. For instance, it is likely that tau is part of the Aβ cascade, since Aβ does not cause neurotoxicity in the absence of tau (Takashima et al., 1993).

The function and structure of neurons, but also of other cell types as microglia, astrocytes and muscle cells (endothelial and smooth) become altered by high levels of Aβ; the effects of Aβ on these other cell types can in turn indirectly further affect neurons (Mucke and Selkoe, 2012).

Originally, the amyloid cascade hypothesis pointed to Aβ insoluble plaques as the causative agent for AD (Hardy and Higgins, 1992). However, the discovery of soluble

oligomeric Aβ and its effects have provided evidence that this smaller form of aggregation of Aβ has a main role in the early synaptic failures seen in AD (Cheng et al., 2007; Lesné et al., 2006; Mucke et al., 2000; Shankar et al., 2008; Terry et al., 1991). The precise targets of Aβ and the mechanisms by which the peptide initiates the synaptic effects remain to be fully elucidated. Aβ has been found to interact with membrane receptors such as NMDARs, AMPARs, α7 nicotinic acetylcholine receptors and insulin receptors amongst others, although the physiological relevance (i.e. concentration) and the state of aggregation of the peptide have not always been fully controlled (Mucke and Selkoe, 2012). An increased production of Aβ results in a loss of spines, a reduced capacity for synaptic plasticity and synaptic depression through the endocytosis of AMPARs and NMDARs (Kamenetz et al., 2003; Lambert et al., 1998; Walsh et al., 2002). Synaptic depression mediated by amyloid β requires the activation of NMDAR function (Kamenetz et al., 2003; Kessels et al., 2013) in a manner independent of ion flux through the receptor channel (Kessels et al., 2013), which via a signaling cascade involving tau phosphorylation ultimately causes AMPAR internalization (Jin et al., 2011; Kamenetz et al., 2003; Mairet-Coello et al., 2013; Takashima et al., 1993). AMPAR endocytosis is crucial for depletion of NMDARs and spine loss to take place (Hsieh et al., 2006; Miyamoto et al., 2016). The metabotropic, ion-flux independent, function of NMDARs is not only required for Aβ-induced synaptic depression (Kessels et al., 2013), but also for spine shrinkage (Stein et al., 2015) and spine loss (Birnbaum et al., 2015). Research into the molecular mechanisms through which Aβ causes synaptic deficits is crucial for the future development of therapeutical strategies that effectively can inhibit or even prevent AD in the future.

1.7. Scope of this thesis

AMPA receptors are responsible for fast excitatory synaptic transmission in the brain. GluA1-containing AMPA receptors have been extensively studied and are known to play a key role in several forms of synaptic plasticity and memory formation. In contrast, GluA3-containing AMPA receptors have historically been ignored, because they have remained relatively invisible at the cell physiological level. This thesis aims to "put GluA3 on the map" by studying the role it plays in synaptic plasticity and behavior in the hippocampus and cerebellum, and establishing the relationship between GluA3 and amyloid β.

In **Chapter 2**, I describe our discovery that GluA3-containing AMPA receptors are present at synapses in large amounts, but they are invisible because they are closed/inactive/dormant under basal conditions and are hence electrically silent. However, when the intracellular cAMP level rises, GluA3 channels become functional, leading to a powerful synaptic potentiation. Fear, through an increase in norepinephrine levels, is a trigger for this newly found type of synaptic plasticity. We further show that this GluA3-plasticity plays no role in memory formation.

As I describe in **Chapter 3**, we find that GluA3-dependent synaptic plasticity is not a phenomenon restricted to the hippocampus: it also occurs in the cerebellum. In this brain structure, GluA3-containing AMPA receptors are responsible for long-term potentiation at the parallel fiber to Purkinje cell synapse and adaptation of the vestibulo-ocular reflex.

In **Chapter 4** I return to the hippocampus, turning the focus to Alzheimer's disease. Experiments in mouse models of Alzheimer's disease have shown that soluble oligomeric clusters of amyloid β trigger synaptic weakening, a loss of synapses and a reduction in synaptic plasticity. We find in this chapter that all these amyloid β-driven effects depend of AMPA receptor subunit GluA3 and that memory deficits in our mouse model of Alzheimer's disease are absent in a GluA3-deficient background, altogether suggesting that the expression of amyloid β-mediated synaptic and cognitive deficits require the presence of GluA3.

Finally, the main conclusions of this thesis are exposed and discussed throughout **Chapter 5**.

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