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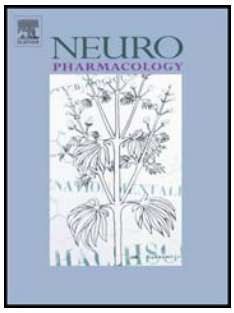

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Dysfunctional synapse in Alzheimer's disease – a focus on NMDA receptors

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Abstract

Alzheimer's disease (AD) is the most prevalent form of dementia in the elderly. Alterations capable of causing brain circuitry dysfunctions in AD may take several years to develop. Oligomeric amyloid-beta peptide (Aβ) plays a complex role in the molecular events that lead to progressive loss of function and eventually to neurodegeneration in this devastating disease. Moreover, *N*-methyl-D-aspartate (NMDA) receptors (NMDARs) activation has been recently implicated in AD-related synaptic dysfunction. Thus, in this review we focus on glutamatergic neurotransmission impairment and the changes in NMDAR regulation in AD, following the description on the role and location of NMDARs at pre- and post-synaptic sites under physiological conditions. In addition, considering that there is currently no effective ways to cure AD or stop its progression, we further discuss the relevance of NMDARs antagonists to prevent AD symptomatology. This review posits additional information on the role played by Aβ in AD and the importance of targeting the tripartite glutamatergic synapse in early asymptomatic and possible reversible stages of the disease through preventive and/or disease-modifying therapeutic strategies.

Keywords: Alzheimer's disease; amyloid-beta peptide; synaptic dysfunction; NMDA receptors; GluN2A and GluN2B subunits; tripartite synapse

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1. Introduction

Alzheimer's disease (AD) is the most common form of dementia and the most prevalent neurodegenerative disease in the elderly population, affecting almost 40 million people worldwide. AD progression has been associated with a gradual damage in function and structure in the hippocampus and neocortex, the vulnerable brain areas used for memory and cognition. AD is characterized by synaptic loss, abnormal amyloid-beta peptide (Aβ) processing of Aβ precursor protein (APP) and hyperphosphorylation of tau, a microtubule associated protein. High levels of intracellular Aβ and the accumulation of the secreted form are believed to be central causative factors for AD (reviewed by Ferreira et al., 2010). Tau was shown to interact with APP both *in vitro* and *in vivo* (Barbato et al., 2005) and $A\beta_{1-42}$ aggregates promote *in vitro* tau aggregation in a dose-dependent manner (Rank et al., 2002), suggesting a direct link between senile plaques and neurofibrillary tangles in AD.

AD has been associated with an impairment of cholinergic terminals, which appear largely vulnerable, followed by glutamatergic terminal dysfunction and finally by the lesion of the somewhat more resilient GABAergic terminals (Bell and Claudio, 2006). The fact that glutamate is the principal excitatory neurotransmitter in the brain areas mainly affected in AD is in accordance with the impairment in glutamate neurotransmission that occurs in this disease. Thus, the ionotropic glutamate receptor subtype *N*-methyl-D-aspartate (NMDA) (described in *section 3*) has been implicated in memory function and is believed to be involved in AD progression. In fact, recent findings posit that Aβ induces an increase in cytosolic calcium levels that may underlie mitochondrial calcium dyshomeostasis and ultimately damage the neurons, namely by activating NMDA receptors (NMDARs) (reviewed by Ferreira et al., 2010).

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2. Synaptic dysfunction in AD

In its most incipient clinical form, early symptoms of AD like confusion and loss of episodic and working memory can be postulated to be due to network disconnections produced by oligomeric forms of Aβ (reviewed by Selkoe, 2002). Concordantly, synaptic dysfunction was observed in Tg2576 mice presenting early increased Aβ levels (Calkins et al., 2011;Tamagnini et al., 2012).

Synapses are the fundamental units of information transfer and storage in the brain, composed of pre- and postsynaptic compartments. Synapse transmission, or neurotransmission, consists in the release of neurotransmitters, which in turn bind and activate receptors located at postsynaptic or presynaptic sites. The role of glial cells has been also recognized, giving rise to the concept of a tripartite synapse organization (**Figure 1**). In fact, astrocytes may respond to neuronal activity through an elevation of internal Ca^{2+} concentration, which further leads to the release of neurotransmitters able to cause feedback regulation of neuronal activity and synaptic efficacy (Araque et al., 1999). Thus, neurotransmission implicates functional pre- and post-synaptic sites, as well as operational astrocytes.

Functional synapses require active mitochondria, which are mainly involved in the generation of energy (ATP and NAD^+), regulation of cell signaling and calcium homeostasis. It was reported that synaptic mitochondria are more susceptible to Ca^{2+} overload than nonsynaptic mitochondria (Brown et al., 2006). Accordingly, Du and colleagues (2010) identified differences in synaptic *versus* nonsynaptic mitochondrial properties and function of mitochondrial populations isolated from AD transgenic mice brain overexpressing the human mutant form of APP and Aβ (Du et al., 2010). In this study, synaptic mitochondria from young transgenic mice showed an increase in Aβ accumulation, increased mitochondrial permeability transition, a decline in both respiratory function and activity of cytochrome c oxidase, as well as increased

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mitochondrial oxidative stress. In AD patients, oxidative stress markers were demonstrated to correlate with Mini-Mental Status Examination scores; importantly, oxidative stress was more localized to the synapses, with levels increasing in a disease-dependent manner (Ansari and Scheff, 2010). However, recent findings showed that intrinsic bioenergetic capacities, including respiration, calcium handling, and transmembrane potentials were maintained in presynaptic nerve terminals isolated from different symptomatic AD mouse models (J20, Tg2576, and APP/PS), when compared with age-matched controls (Choi et al., 2012).

2.1. Aβ at presynaptic level and glial cells

Recent studies link the defects in function of presynaptic boutons associated with presynaptic protein dysfunction to the etiology of several neurodevelopment and neurodegenerative diseases, including AD (reviewed by Waites and Garner, 2011). On the other hand, Aβ may exert a physiological function at the presynaptic terminal, as the peptide may be essential for neurotransmitter release (Puzzo et al., 2011). Nevertheless, a brief exposure to a very low concentration of Aβ resulted in impairment of long term potentiation (LTP) produced by pre-synaptic defects (Russell et al., 2012). Morphological and biochemical synaptic changes associated with aging may contribute to exacerbate the damaging effects of Aβ, particularly at presynaptic level (Quiroz-Baez et al., 2013), suggesting that Aβ oligomers can cause presynaptic dysfunction. According to these authors, synaptic terminals obtained from aged rats were shown to be more sensitive to Aβ toxicity, evidencing an age-related decline in mitochondrial activity, reduced antioxidant contents and increased oxidative stress markers in both resting and depolarized conditions. In addition, ultrastructural changes including increased mitochondrial size and a significant reduction of synaptic vesicles contents

were also observed in presynaptic nerve endings from rat hippocampus exposed to Aβ at different ages (Quiroz-Baez et al., 2013).

Aβ was also shown to inhibit presynaptic P/Q Ca^{2+} channels, suppressing spontaneous synaptic activity (Mezler et al., 2012;Nimmrich et al., 2008), along with the activation of presynaptic α7-nicotinic acetylcholine (ACh) receptors (Dougherty et al., 2003). More recently, Aβ was reported to directly modulate recombinant P/Q-type and also N-type Ca^{2+} channels in HEK293 cells and blockade of presynaptic Ca^{2+} channels reversed Aβ-induced functional deficits in synaptic transmission (Hermann et al., 2013). Moreover, synthetic Aβ peptide species were also shown to potentiate K^{+}-induced glutamate release from normal rodent hippocampus (Kabogo et al., 2010).

Deleterious effects of Aβ oligomers were shown to be present on multiple steps of synaptic vesicle trafficking (Park et al., 2013). Synaptic vesicle pool is composed by recycling and resting pools, the former including a readily-releasable pool and reserve pool; indeed, the size of the recycling pool and its regeneration kinetics are important factors for the efficacy of synaptic function. Endogenous Aβ peptides appear to have a crucial role in activity-dependent regulation of synaptic vesicle release, pointing out for the primary pathological events that lead to compensatory synapse loss in AD (Abramov et al., 2009). Accordingly, acute treatment of cultured rat hippocampal neurons with Aβ oligomers was very recently shown to reduce the recycling pool, increase the resting pool of synaptic vesicles, decrease vesicle endocytosis and regeneration, and to increase the release probability of the readily-releasable pool, while its recovery was shown to be delayed, leading to a weaken synaptic transmission (Park et al., 2013). Interestingly, these effects were dependent on Aβ, since they were prevented by an antibody against Aβ. Moreover, reduction of the pool size was prevented by calpain or CDK5 inhibitors, while the defects in endocytosis were averted following overexpression of phosphatidylinositol-4-phosphate-5-kinase type I-γ, indicating that these two downstream pathways are involved in Aβ oligomers-induced

presynaptic dysfunction (Park et al., 2013). In addition, it was also demonstrated that Aβ reduced the magnitude of exocytosis and that the remaining synaptic vesicles displayed a much slower speed of endocytosis, thus inhibiting presynaptic function (Parodi et al., 2010). By using electron microscopy, these authors also reported that Aβ-treated neurons displayed reduced number of synaptic vesicles, especially those near the presynaptic active zones and a reduction in several presynaptic proteins (Parodi et al., 2010). Accordingly, presynaptic proteins such as SNAP-25, synaptophysin, and synaptotagmin were reduced in brains of patients with AD (Reddy et al., 2005) and in the hippocampus of Tg2576 mice 1 month after injection of Aβ into the third ventricle (Chauhan and Siegel, 2002). Recent findings by Russel and co-authors (2012) evidenced a time-dependent interaction of Aβ with synaptophysin in presynaptic terminals of hippocampal neurons. Furthermore, Aβ disrupted the complex formed by synaptophysin and another vesicle associated protein, VAMP2, increasing the amount of primed vesicles and exocytosis; electrophysiology recordings in hippocampal brain slices confirmed that Aβ affects baseline neurotransmission (Russell et al., 2012). Additionally, Aβ oligomers can alter dynamin-1, a neuron-specific GTPase that pinches off synaptic vesicles, allowing them to re-enter the synaptic vesicle pool (Kelly et al., 2005;Kelly and Ferreira, 2006).

In the synaptic cleft, clearance of glutamate occurs by glutamate transporters localized in both pre-synaptic terminals and also in astrocytes (**Figure 1**), precluding in this way the deleterious effects exerted by glutamate, namely excitotoxicity. Aβ can disrupt astrocytic calcium signaling and gliotransmitter release, which are vital processes for astrocyte-neuron communication (reviewed by Vincent et al., 2010). The sporadic form of AD seems also to be due to dysfunctional glutamate clearance. In this context $A\beta_{1-42}$ can downregulate the astrocytic glutamate uptake capacity (Matos et al., 2008), promoting glutamate receptor activation (**Figure 1**). More recently, these authors demonstrated that Aβ decreased GLAST and GLT-I expression in astrocytes from wild

type, but not from A(2A)R (adenosine 2A receptor) knockout mice, known to modulate astrocytic glutamate uptake. This impact of Aβ on glutamate transporters and uptake was also confirmed in an *ex vivo* astrocyte preparation (gliosomes) from rats intracerebroventricularly injected with $A\beta_{1-42}$ (Matos et al., 2012).

Upon exposure to Aβ, astrocytes and microglia become activated, extending their hypertrophic processes to physically separate the neurons from Aβ fibrils, thus playing a neuroprotective role. Despite some controversy, there are also evidences that astrocytes can bind and take up Aβ in processes involving different internalization pathways, including scavenger receptors (reviewed by Mohamed and Posse de, 2011), suggesting a role in Aβ accumulation and clearance. Accordingly, it was previously shown that microglia facilitates the conversion of soluble and oligomeric Aβ to the fibrillar form within invaginations in the surface of the plasma membrane; this highlights the potential benefit of blocking the initial intracellular accumulation of Aβ in neurons and astrocytes, and of inhibiting microglia-mediated assembly of fibrillar Aβ, which is particularly resistant to degradation in AD brain (Nagele et al., 2004). In a very recent study, temporal cortex of AD patients showed a high number of $GFAP^+$ astrocytes and $MHC2^+$ microglia, compared with non-demented subjects; however, similar numbers of total astrocytes and microglia were observed and remained constant over the clinical course of the disease, suggesting that phenotypic change of existing glial cells, rather than a marked proliferation of glial precursors, accounts for by the majority of glial responses observed in the AD brain (Serrano-Pozo et al., 2013).

3. NMDA receptors

3.1. GluN2A and GluN2B expression and regulation

NMDARs are cationic channels gated by the neurotransmitter glutamate, having critical roles in excitatory synaptic transmission, plasticity as well as in excitotoxicity in the central nervous system (CNS). NMDAR subunits are encoded by three families of

genes coding for GluN1, GluN2 and GluN3 subunits (Cull-Candy et al., 2001). Functional NMDARs are heterotetramers composed of two glycine or D-serine-binding GluN1 subunits and two glutamate-binding GluN2 (GluN2A-D) subunits or, in some cases, glycine-binding GluN3 (GluN3A/B) subunits (Kohr, 2006). Activation of NMDARs leads to cytosolic free intracellular calcium (Ca^{2+}_{i}) increase (MacDermott et al., 1986) required for LTP and long-term depression (LTD) (Muller et al., 2009;Fetterolf and Foster, 2011) and, more generally, for synaptic plasticity (MacDonald et al., 2006;Lau et al., 2009). The most widely expressed NMDARs contain the obligatory subunit GluN1 plus either GluN2B or GluN2A or a mixture of the two; therefore, in next sections we will focus on these two subunits.

All NMDARs subunits share a common membrane topology, consisting of three transmembrane segments and a re-entrant pore-loop. Despite structural similarities, there are pharmacological differences between NMDAR subunits. Endogenous Mg^{2+} and MK-801 are channel blockers and D-APV is a competitive antagonist that inhibits NMDARs non-selectively, whereas the GluN2A subunit is antagonized by the competitive antagonist NVP-AAM077, which was further demonstrated to have a better selectivity for the GluN2D subunit; the GluN2B subunit is also selectively blocked by the non-competitive antagonists ifenprodil and Ro 25-6981, among others (reviewed by Ogden and Traynelis, 2011). Recently, TCN201 was described as a potent GluN2A antagonist but dependent on the GluN1 co-agonist concentration (Edman et al., 2012).

Expression of different NMDAR subunits differs along different brain areas and during development. GluN1 mRNA expression represents 67-88% of the total subunit gene expression in the brain (Goebel and Poosch, 1999). In the rat fetal brain, GluN1 as well as GluN2A subunits are mildly expressed in restricted areas such as the temporal region of the cerebral cortex and the hippocampus, and become widely expressed throughout the whole brain in neonates (Takai et al., 2003). On the other hand, GluN2B subunit, which is mildly expressed in hippocampus and temporal cortex in fetal brain,

becomes hardly expressed in the neonatal brain after 7 days of life, being absent from the brain stem (Takai et al., 2003). In humans, GluN1 levels are low in embryonic prefrontal cortex and increases after birth (Henson et al., 2008), remaining constant with age in whole brain (Law et al., 2003). Moreover, expression of GluN2B mRNA is higher in the neonate than in older brains, whereas GluN2A mRNA remains constant after birth, leading to an age-related increase in GluN2A/2B transcript ratio (Law et al., 2003). It is believed that the pre- and postnatal progressive changes in subunit expression could contribute to the variation in NMDARs-mediated synaptic plasticity during development. Interestingly, GluN2B subunit levels are reduced in old mouse frontal cortex, suggesting alterations in memory processes during aging (Kuehl-Kovarik et al., 2000).

Regulation of NMDARs function is a complex process involving numerous proteins in the cell, particularly a variety of protein kinases. Phosphorylation of GluN2B Tyr1472 enhances NMDARs activity by increasing its number at the synaptic membrane (Goebel et al., 2005;Goebel-Goody et al., 2009). Tyrosine kinase Src, as well as Fyn kinase, are involved in the upregulation of GluN2B-containing NMDARs at the surface of the plasma membrane by phosphorylation of Tyr1472 (Sinai et al., 2010;Xu et al., 2006) (**Figure 2**). On the contrary, the tyrosine phosphatase striatal enriched protein ($STEP_{61}$) leads to decrease of GluN1/GluN2B receptor complexes from the neuronal surface by dephosphorylating the Tyr1472 residue (Kurup et al., 2010). In the same way, GluN2B phosphorylation of Ser1480 by casein kinase 2 (CK2) disrupts the interaction of GluN2B with the scaffold protein post-synaptic density 95 (PSD-95) and synaptic-associated protein 102 (SAP102), two proteins involved in the anchorage of NMDAR to the synaptic membrane, and decreases its surface expression in neurons (Chung et al., 2004). On the other hand, decreased synaptic GluN2B leads to an increase in synaptic GluN2A expression (Sanz-Clemente et al., 2010). Moreover, other post-translational modifications have been implicated in synaptic NMDAR activity

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regulation; *e.g.* S-nitrosylation of NMDARs leads to a decrease in channel opening, modulating NMDARs activity (Lipton et al., 1998;Kim et al., 1999).

Importantly, there are other types of NMDAR regulation not involving post-translational modifications. The calcium-dependent protease calpain downregulates NMDARs function through degradation of GluN2A and GluN2B subunits (Wu et al., 2005). The Wnt pathway can also be involved in the regulation of NMDARs function in later stages of development; in fact, Wnt ligands are necessary to maintain basal levels of NMDARs synaptic transmission and Wnt5 specifically up-regulates synaptic NMDAR currents in rat hippocampal slices (Cerpa et al., 2011). More recently, it has been demonstrated that the glutamate metabotropic receptor mGluR7 reduces the association of NMDARs with PSD-95, and the consequent surface level of NMDARs, in an actin-dependent manner (Gu et al., 2012). Evidences for cytoskeletal and plasma membrane involvement in NMDARs regulation are numerous and affect NMDAR presence at the synapse. The membrane phospholipid phosphatidylinositol bisphosphate (PIP_2) is important for the maintenance of NMDARs at the cell surface (Mandal and Yan, 2009). Blocking PIP_2 reduces NMDAR-mediated currents, whereas application of PIP_2 enhances these currents (Mandal and Yan, 2009). Moreover, cofilin, an actin depolymerizing factor which links actin and PIP_2 is required for NMDARs regulation, suggesting that a decrease in PIP_2 leads to cofilin release and actin depolymerization, which in turn promotes NMDARs internalization (Mandal and Yan, 2009). Active myosin light chain kinase enhances NMDARs-mediated whole-cell and synaptic currents, increasing actin-myosin contractility, which leads to increased membrane tension on NMDARs or to altered physical relationships between NMDAR anchored proteins, such as PSD-95 (Kornau et al., 1995) and cytoskeleton (Lei et al., 2001). PSD-95 is linked indirectly to cortactin, a protein that promotes actin polymerization, by a succession of linker proteins (**Figure 2**). Thus, NMDARs are indirectly linked to actin cytoskeleton indicating that cytoskeleton alterations may affect NMDARs surface availability. Indeed, binding of reelin, a secreted glycoprotein involved in synaptic

plasticity, to its receptors ApoER2 (apolipoprotein E receptor 2) or VLDLR (very low density lipoprotein receptor) triggers Dab1 phosphorylation and activation, which further leads to actin polymerization (Suetsugu et al., 2004) (**Figure 2**). Moreover, Dab1 activation induces Src phosphorylation (Ballif et al., 2003;Bock and Herz, 2003) and increases NMDARs activity (Chen et al., 2005) (**Figure 2**). Therefore, reelin inhibition was shown to decrease GluN2B subunit availability at the synapse (Groc et al., 2007).

3.2. Synaptic and extrasynaptic localization and activation of NMDARs

NMDARs subunits differ not only in temporal expression, as described above, but also in cellular localization. In fact, NMDARs can be synaptic or extrasynaptic (**Figure 1**). Synaptic NMDARs composition changes quickly after synapse formation. Thus, synapses containing predominantly GluN1/GluN2B represent immature sites, whereas mature sites are more predominantly composed by NMDARs composed of GluN1/GluN2A subunits (Tovar and Westbrook, 1999;Lopez de and Sah, 2003). Moreover, extrasynaptic NMDARs are usually concentrated at points of contact containing adhesion factors with adjacent processes such as axons, axon terminals or glia (Petralia et al., 2010) and are largely composed by GluN1/GluN2B heteromers (Tovar and Westbrook, 1999;Petralia, 2012) (**Figure 1**). Functionally, synaptic and extrasynaptic NMDARs are gated by different co-agonists (Papouin et al., 2012), respectively, D-serine released by astrocytes (Kang et al., 2013) and glycine released by both astrocytes and neurons (Holopainen and Kontro, 1989), as observed in both the hippocampus and in cerebellar granule cell cultures (**Figure 1**). Papouin and colleagues demonstrated that the availability of the co-agonists matches the preferential affinity of each subunit for its own co-agonist and that glycine and D-serine inhibit NMDAR surface trafficking in a subunit-dependent manner, influencing also

NMDARs subcellular localization in the hippocampus (Papouin et al., 2012). Moreover, as described before in this review, phosphorylation of GluN2B at Tyr1472 enhances NMDARs activity, increasing its number at the synaptic membrane (Goebel et al., 2005;Goebel-Goody et al., 2009), whereas phosphorylation of Tyr1336 is associated with enrichment of extrasynaptic NMDARs (Goebel-Goody et al., 2009).

It was initially believed that only synaptic NMDARs were implicated in the synaptic transmission process; however, there is growing evidence regarding the involvement of extrasynaptic NMDARs in the transmission of information from the presynaptic terminal. Harris and colleagues demonstrated that short stimulations with low frequencies on CA1 hippocampal slice pyramidal neurons engaged extrasynaptic NMDARs, while higher frequencies engaged both synaptic and extrasynaptic NMDARs receptors, suggesting that extrasynaptic receptors participate in physiological synaptic transmission (Harris and Pettit, 2008). On the other hand, it seems that LTP is mediated only by synaptic NMDARs, whereas LTD requires both synaptic and extrasynaptic receptors (Papouin et al., 2012).

Importantly, NMDARs location influences its coupling to pro-death or pro-survival. The extracellular signal-regulated kinases (ERK), which promote a signaling cascade important for neuronal plasticity and survival, are regulated, in part, through NMDARs activation. Thus, the synaptic pool of NMDARs activates ERK, promoting cell survival (Ivanov et al., 2006;Leveille et al., 2008), whereas the extrasynaptic pool of NMDARs triggers mitochondrial membrane potential breakdown, as well as cell body and dendritic damage (Leveille et al., 2008), inducing a signaling pathway that inactivates ERK (Ivanov et al., 2006). Interestingly, the simultaneous activation of synaptic and extrasynaptic NMDARs induces ERK activation, weaker than those mediated by synaptic NMDARs alone (Ivanov et al., 2006). Moreover, activation of synaptic NMDARs also leads to activation of the cAMP response element binding protein (CREB), a transcription factor also related to cell survival pathways (Kaufman et al., 2012;Zhou and Sheng, 2013) and brain-derived neurotrophic factor (BDNF) gene

expression (Hardingham et al., 2002), whereas activation of extrasynaptic NMDARs activated a general and dominant CREB shut-off pathway (Kaufman et al., 2012;Hardingham et al., 2002); this effect could be prevented by the use of memantine (Kaufman et al., 2012), commonly used as AD treatment, and which preferentially blocks extrasynaptic NMDARs at therapeutic concentration (Leveille et al., 2008;Xia et al., 2010). These data suggest that extrasynaptic NMDARs activation contributes to excitotoxicity. Conversely, other authors showed that activation of extrasynaptic NMDARs alone did not trigger cell death, but activation of both extrasynaptic and synaptic NMDARs induced cell death program, being this excitotoxic effect dependent on the magnitude and duration of co-activation (reviewed by Zhou and Sheng, 2013). A a recent study further suggested that NMDA-induced neurotoxicity is mediated only by synaptic NMDARs (Papouin et al., 2012). However, since NMDA is not an endogenous NMDAR agonist, this result can be discussed regarding to its relevance for similar *in vivo* conditions. By implicating synaptic NMDARs in excitotoxicity and evidencing the involvement of extrasynaptic NMDARs in synaptic transmission, this study calls into question whether cell survival or cell death depend on the activation of synaptic or extrasynaptic receptors or may rather depend upon NMDARs composition at the membrane surface and the interaction with selective proteins.

4. NMDA receptors in AD

4.1. NMDARs-related post-synaptic dysfunction in AD: influence of Aβ peptide

Overactivation of NMDARs was initially hypothesized to occur at early stages of AD; indeed, recent reports indicate that activation of NMDARs by Aβ accumulation may occur at early stages of the disease (reviewed by Parameshwaran et al., 2008), and that Aβ oligomeric species evoke an immediately Ca^{2+}_{i} rise through activation of GluN2B-containing NMDARs in cultured cortical neurons (Ferreira et al., 2012), although the mechanisms by which Aβ causes synaptic deficits involving NMDARs

remains to be clarified. Despite this, memantine, an uncompetitive open channel blocker of NMDARs, has been mostly prescribed as a memory-preserving drug for moderate- to late-stage AD patients (Reisberg et al., 2003) (see detailed effects of memantine in section 5).

The mRNA and protein levels of NMDAR subunits have been largely studied in different models of AD and in AD brains. A first study in transgenic mice expressing the C-terminal of APP demonstrated that NMDAR protein levels were unchanged compared to control mice (Sandhu et al., 1993). Conversely, recent studies showed that presenilins knock-out leads to an early increase in GluN2A subunit expression at post-synaptic densities with a concomitant reduction at non-synaptic sites before synaptic loss (Aoki et al., 2009). In *in vitro* studies, the effect of Aβ on NMDARs has been also demonstrated. Ronicke and colleagues (2011) demonstrated that early neuronal dysfunction induced by Aβ is mediated by an activation of GluN2B subunits in primary neuronal cultures and hippocampal slices from rat and mouse. Moreover, treatment of rat organotypic slices containing pyramidal neurons with Aβ oligomers decreased dendritic spine density and reduced NMDAR-mediated Ca^{2+} influx (Shankar et al., 2007). In humans, Jacob and colleagues reported a downregulation of GluN1 subunit in brains of AD patients in various stages of the disease (Jacob et al., 2007). GluN1 mRNA levels were significantly lower in AD, compared with control brains (Hynd et al., 2001) and the GluN1 isoform containing an N-terminal splice cassette appeared drastically decreased in the disease, suggesting that this isoform may increase cell vulnerability in AD (Hynd et al., 2004b). Moreover, levels of GluN2B mRNA and protein, as well as GluN2A levels were decreased in susceptible regions of postmortem human AD brain, such as the hippocampus and the cortex (Hynd et al., 2004a;Bi and Sze, 2002;Mishizen-Eberz et al., 2004). In comparison to AD susceptible brain areas, there were no alterations in NMDARs subunit expression in cerebellum of AD patients (Bi and Sze, 2002). Conversely, other studies evidenced that mRNA levels of GluN1 (Bi

and Sze, 2002) and GluN2A subunits (Mishizen-Eberz et al., 2004) were unchanged in AD patient's brains.

Additionally to a decrease in mRNA levels, the decrease in GluN2B and GluN2A subunits could be due also to a decrease in reelin levels, a protein that mediates NMDAR activity, and which is depleted in AD brains (Herring et al., 2012). On the other hand, the decrease in NMDAR subunits may also be due to an increase in $STEP_{61}$, which contributes to the endocytosis of GluN1/GluN2B and GluN1/GluN2A receptors (Snyder et al., 2005;Kurup et al., 2010). Reducing $STEP_{61}$ activity by genetic manipulations in different AD mice models reversed cognitive and cellular impairment (Zhang et al., 2010), supporting a fundamental role for NMDARs activation in AD. Importantly, co-expression of mutated APP and NMDAR subunits in embryonic kidney cells decreased receptor internalization and thus increased surface levels of GluN1/GluN2B and GluN1/GluN2A, linked to enhanced NMDARs currents (Cousins et al., 2009). NMDARs, more particularly those containing GluN2B subunits, appear in numerous studies as mediators of Aβ-induced neurotoxicity. Indeed, Aβ oligomers induce $Ca^{2+}{}_{i}$ dysregulation and neuronal death through activation of NMDARs (Alberdi et al., 2010) and inhibit LTP (Chen et al., 2000;reviewed by Rowan et al., 2003;Li et al., 2011). Also, we previously demonstrated that GluN2B subunit activation is involved in Aβ-induced $Ca^{2+}{}_{i}$ homeostasis deregulation (Ferreira et al., 2012). In this work we also showed that GluN2A-NMDARs antagonism potentiates $Ca^{2+}{}_{i}$ rise induced by a high concentration of Aβ, suggesting that GluN2A and GluN2B subunits have opposite roles in regulating $Ca^{2+}{}_{i}$ homeostasis. Moreover, Aβ modulated NMDA-induced responses and *vice versa*; indeed, pre-exposure to Aβ decreased NMDA-evoked $Ca^{2+}{}_{I}$ rise and pre-exposure to NMDA decreased Aβ response. In addition, simultaneous exposure to Aβ plus NMDA synergistically increased $Ca^{2+}{}_{i}$ levels, an effect mediated by GluN2B-containing NMDARs (Ferreira et al., 2012). Previously, we also demonstrated that Aβ induced ER stress and NADPH oxidase-mediated superoxide production, which was prevented by ifenprodil, suggesting an important role of NMDAR GluN2B subunits

(Costa et al., 2012). Moreover, we showed that Aβ induced DNA fragmentation and microtubule depolymerization, as well as neurite retraction in a NMDAR-dependent manner, particularly through GluN2B-containing NMDARs (Mota et al., 2012). Interestingly, in primary neuronal cultures, overexpression of human tau caused cell death, which was prevented by treatment with ifenprodil, a GluN2B selective antagonist, suggesting that GluN2B subunit also mediates tau-induced neurotoxicity (Amadoro et al., 2006). Importantly, using antibodies against the N-terminal component of GluN1 or GluN2B subunits, our data supports that Aβ oligomers are able to bind NMDARs extracellularly (Costa et al., 2012). On the other hand, NMDARs activation (Lesne et al., 2005), and more particularly extrasynaptic activation (Bordji et al., 2010), triggers increased production and secretion of Aβ, which is preceded by a shift from APP695 to Kunitz protease inhibitory domain (KPI) containing APPs, isoforms exhibiting a high amyloidogenic potential, followed by a shift from α-secretase to β-secretase-mediated APP processing, largely suggesting a circuit in which Aβ facilitates NMDARs activation, which in turn promotes Aβ production.

It has been suggested that enhancement of GluN2A activity and/or the reduction of GluN2B activity may be used in order to halt the early Aβ-mediated synaptic dysfunction (Liu et al., 2010). Taking into account the importance of ERK for cell survival (described previously in this review), a negative regulation by extrasynaptic NMDARs, mainly composed of GluN2B subunits (Tovar and Westbrook, 1999;Petralia, 2012), may be one of the early signaling events determining brain injury in AD (Ivanov et al., 2006). Moreover, in a recent work oligomeric Aβ caused selective loss of synaptic GluN2B responses, promoting a switch in subunit composition from GluN2B to GluN2A, a process normally observed during development (Kessels et al., 2013). Since GluN2A subunits have been implicated in protective pathways, whereas GluN2B subunits appear to increase neuronal vulnerability (Liu et al., 2007), the early increase in GluN2A and decrease in GluN2B subunit-composed NMDARs activity may be an attempt to reduce Aβ-induced neuronal dysfunction.

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4.2. NMDARs in the presynapse and astrocytes – influence in AD?

NMDARs are mainly located at postsynaptic densities of excitatory synapses; however, their localization on presynaptic axon terminals and astrocytes has been evaluated in the past two decades. Thus, we may hypothesize that changes in receptor composition and/or activation also take place in synaptic sites during AD progression, although there is scarce information regarding this topic.

Several evidences suggest a role for presynaptic NMDAR localization in reshaping synaptic transmission by regulating presynaptic glutamate release (for review see Corlew et al., 2008;Huang et al., 2011). Very recently, the presence of GluN1, -2B, -3B and -2D, but not GluN-2A or -2C, was established at presynaptic sites in nerve terminal membranes and in mossy fiber axons by using postembedding electron microscopy immunogold cytochemistry (Berg et al., 2013). The presynaptic localization of NMDARs is in accordance with the idea that these receptors can act as autoreceptors (reviewed by Duguid, 2013). The first suggestion for the autoreceptor role of NMDARs appeared in 1991, when Martin and colleagues showed that NMDAR antagonists reduced K^+-evoked glutamate release form CA1 hippocampal neurons (Martin et al., 1991). Moreover, in rat striatum, it has been suggested that activation of NMDAR by endogenous glutamate enhances glutamate release, evidencing that this regulation is, in part, linked to presynaptic NMDARs activation (Bustos et al., 1992). In addition, blockade of NMDARs in rat entorhinal cortex reduced the frequency but not the amplitude of glutamate-mediated spontaneous excitatory postsynaptic currents, which persisted even when postsynaptic NMDARs were blocked (Berretta and Jones, 1996), once again suggesting that presynaptic NMDARs may act as autoreceptors.

Presynaptic NMDARs can be involved in synaptic plasticity such as certain types of LTP (Berg et al., 2013) and also in timing-dependent LTD in the visual cortex (Sjostrom et al., 2003). In the rat spinal cord, presynaptic NMDARs inhibited glutamate release

from primary sensory neurons (Bardoni et al., 2004), suggesting a positive or a negative control of presynaptic glutamate release exerted by presynaptic NMDARs activation. Importantly, during cortical development, the loss of presynaptic NMDARs function was shown to correlate with depletion of presynaptic receptors, contributing to a switch between pre- and postsynaptic NMDARs (Corlew et al., 2007). Accordingly to these authors, presynaptic receptors are involved in LTD induction during development, whereas in older mice LTD induction requires the activation of postsynaptic receptors (Corlew et al., 2007), pointing out for a dynamic localization of NMDARs at the synapse during development. Despite the general limited information on presynaptic NMDARs, data suggest that autoreceptor regulation of synaptic transmission is one of the key factors determining information processing in the CNS.

In a study performed by Bell and colleagues (2007), the density of glutamatergic presynaptic boutons and the abundance of dystrophic neurites were quantified in midfrontal gyrus brain tissue from subjects with no cognitive impairment, mild cognitive impairment, or mild- to severe-stage AD. These authors concluded that subjects with mild cognitive impairment displayed a paradoxical elevation in glutamatergic presynaptic bouton density, similar to that observed in the cholinergic system, which then depletes and drops with disease progression (Bell et al., 2007). These results pointed out that dystrophic neurite generation and reduced presynaptic bouton densities detrimentally influence neurotransmission and cognitive function in later stages of AD.

Astroglial NMDARs, shown to be present in the cortex and the spinal cord, are characterized by weak Mg^{2+} block and moderate Ca^{2+} permeability (reviewed by Parpura et al., 2012)). Moreover, there are evidences for Ca^{2+}-dependent glutamate release from astrocytes in response to the Ca^{2+} ionophore used as a secretagogue. In addition, Ca^{2+} ionophore-stimulated astrocytes can also release D-serine, a co-agonist of the glycine-binding site of the NMDAR (reviewed by Parpura et al., 2012). Evidence

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for the existence of functional NMDAR expression in human primary astrocytes was also described by Lee and colleagues (2010). These authors showed that all seven currently known NMDAR subunits (Glu-N1, -2A, -2B, -2C, -2D, -3A and -3B) are expressed in astrocytes, although at different levels; notably, astrocytic glutamatergic system has been also implicated in several neuropathological conditions, including in AD (Lee et al., 2010).

5. NMDARs as targets for therapeutic intervention in AD

The several studies reviewed above demonstrate the importance of NMDARs in AD progression. Furthermore, NMDARs activation, particularly extrasynaptic NMDARs (Bordji et al., 2010), promote neuronal Aβ secretion (Lesne et al., 2005). Thus, the use of treatments targeting NMDARs seem to be a promising therapeutic option to counteract AD progression.

NMDARs are fundamental for normal synaptic function, which implicates that a full inhibition of these receptors triggers important secondary effects; thus, to reduce the possibility of side effects, the maximum dose tolerated may be not therapeutically effective (reviewed by Hardingham and Bading, 2010). Many compounds can target and block NMDARs, namely MK-801 (reviewed by Woodruff et al., 1987), 1-benzyl-1,2,3,4-tetrahydro-β-carboline, which acts inside the ion channel as the MK-801 (Espinoza-Moraga et al., 2012), and huperzine A, which acts as a non-competitive inhibitor interacting at the polyamine binding site (Zhang and Hu, 2001). However, as referred before, considering the involvement of NMDARs in synaptic function, a complete blockade of NMDARs is associated with important secondary effects, such as severe memory impairment. Selective antagonism of NMDARs subunits involved in excitotoxic events, using low concentrations of a pharmacological compound, appears to be a good strategy in order to avoid secondary effects; moreover, the refinement of a

drug able to selectively inhibit pathologically activated NMDARs, without interfering with normal receptor activation, represents an important challenge for AD therapy (reviewed by Lipton, 2004;Lipton, 2007).

Memantine is an open channel blocker with low affinity, which preferentially antagonizes NMDARs excessively activated; moreover, due to its relatively fast off-rate memantine does not substantially accumulate in the channel to interfere with synaptic transmission (reviewed by Lipton, 2004). Several *in vitro* and *in vivo* studies evidenced the neuroprotective effect of memantine on Aβ toxic actions (reviewed in Danysz and Parsons, 2012). Thus, *inter alia,* in mature hippocampal neurons memantine was shown to prevent oligomeric Aβ-induced oxidative stress (De Felice et al., 2007) as well as the disruption of axonal transport trafficking (Decker et al., 2010), DNA fragmentation, microtubule deregulation and neurite retraction (Mota et al., 2012) . Interestingly, memantine leads to a significant decrease in secreted APP and Aβ peptide levels in human neuroblastoma cells (Ray et al., 2010) and in cortical levels of $A\beta_{1-42}$ in APP/PS1 transgenic mice after 8 days of treatment (Alley et al., 2010), suggesting a role for memantine in the regulation of APP processing. *In vivo*, memantine protects against neuronal degeneration induced by $A\beta_{1-40}$ intracranial injections (Miguel-Hidalgo et al., 2002) and prevents cognitive impairment in the same animals (Miguel-Hidalgo et al., 2012). Moreover, in 3xTg-AD mice, memantine improved cognition and reduced levels of both insoluble Aβ and hyperphosphorylated tau (Martinez-Coria et al., 2010).

Memantine has been widely prescribed to provide symptomatic relief and enhance life quality in AD. In clinical trials, memantine has shown significant general benefits such as in aspects of language, memory, praxis, functional communication and in activities of daily living for AD patients (Wilkinson, 2012;Hellweg et al., 2012), even if studies revealed that it did not improve excessive agitation (Fox et al., 2012) or total brain or hippocampal atrophy after one year treatment (Wilkinson et al., 2012). Importantly,

memantine has shown to be well tolerated and the mechanism of action allows it to be safer than other non-selective NMDAR antagonists (Farlow et al., 2008). Interestingly, extrasynaptic NMDARs have been largely associated with NMDARs excitotoxicity in AD (Hardingham and Bading, 2010). In this respect, memantine was associated to a preferential blockade of extrasynaptic currents mediated by NMDARs, rather than synaptic currents in the same neuron (Xia et al., 2010), preferentially antagonizing overactivated receptores, which may explain the fact that memantine is well tolerated. Very recently, it was demonstrated that the use of a high dose of memantine (28 mg/day, almost three times the normal dose) is efficacious and still well tolerated and safe (Grossberg et al., 2013). However, memantine seems to increase the risk for somnolence, weight gain, confusion, hypertension, nervous system disorders and falling (reviewed by (Yang et al., 2013). Interestingly, Kotermanski and colleagues (2009) reported that Mg^{2+}, an endogenous blocker that binds near to the memantine binding site at physiological concentrations, decreases memantine inhibition of GluN2A- and GluN2B-containing receptors, while it has no effect on memantine inhibition of GluN2C- and GluN2D-containing receptors (Kotermanski and Johnson, 2009), suggesting that the hypothesized mechanism of action for memantine should be reviewed in order to reconsider the role of GluN2C/D subunits. However, taking into account that in the brain areas mainly affected in AD NMDARs are mainly composed by GluN2A and GluN2B subunits, this last observation may not be so relevant for the action of this compound in AD. Neramexane, an uncompetitive antagonist of NMDARs, has shown to be as efficient as memantine in enhancing long-term spatial memory in adult rats, but at lower doses (Zoladz et al., 2006), suggesting that this antagonist may represent an interesting alternative to memantine. Furthermore, taking into account the fact that extrasynaptic NMDARs have been associated with excitotoxicity in AD (Hardingham and Bading, 2010) and that extrasynaptic NMDARs are mainly composed by GluN2B-containing NMDARs (Tovar and Westbrook, 1999;Petralia, 2012), the use of a selective GluN2B subunit antagonists might be an interesting strategy to prevent

synaptic dysfunction in AD. We previously demonstrated that ifenprodil, a GluN2B antagonist, prevented Aβ-induced ER stress and hippocampal dysfunction (Costa et al., 2012) *and* Aβ-induced microtubule deregulation (Mota et al., 2012), as well as Aβ-induced Ca^{2+} rise (Ferreira et al., 2012) *in vitro.* Furthermore, in primary neuronal cell culture and hippocampal slices from rat and mouse, ifenprodil and Ro 25-6981, another GluN2B antagonist, prevented LTP impairment, baseline synaptic transmission reduction, neuronal spontaneous network activity decrease and retraction of synaptic contacts induced by Aβ oligomers (Ronicke et al., 2011). Moreover, in rats co-injected with Aβ and ifenprodil prevented Aβ-mediated inhibition of plasticity (Hu et al., 2009). Selective GluN2B antagonists were also demonstrated to be efficient at low doses by restoring Aβ oligomers-induced LTP impairment (Li et al., 2011;Rammes et al., 2011). These results suggest that preferentially targeting GluN2B subunit of NMDARs may be another way to prevent AD progression. However, there is a lack of clinical confirmation concerning the selective inhibition of GluN2B as a pharmacological therapy in AD. Interestingly, Rammes and colleagues (2011) also demonstrated that antagonism of metabotropic glutamate receptor 5 (mGluR5), which are mechanistically coupled to postsynaptic NMDARs, with low concentration of allosteric antagonist (MPEP, 2-methyl-6-(phenylethynyl)-pyridine) prevent Aβ oligomers-induced LTP impairment (Rammes et al., 2011), evidencing that the glutamatergic system may be considered as a target for the development of AD drugs. **Figure 3** shows the influence of NMDAR antagonists, namely selective GluN2B subunit antagonists and memantine, on rescuing Aβ-mediated pathophysiology by targeting the glutamatergic synapse and ultimately ameliorating AD cognitive deterioration.

In clinic, the concomitant use of memantine or their analogues together with drugs inhibiting ACh-esterases is frequent and seems to present positive results (reviewed in (Parsons et al., 2013). Thus, when patients receiving only ACh-esterase inhibitors are compared with patients receiving the same treatment plus memantine, the ability for

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independence, a factor that reflects cognitive capacity, is increased (Lopez et al., 2009). Regarding the effect of donepezil, one of the ACh-esterase inhibitors commonly used in the treatment of AD patients, Howard and colleagues have recently demonstrated that there are no evidences that the treatment of AD patients with both donepezil and memantine is beneficial compared with one of the drugs alone in late AD stages (Howard et al., 2012). However, this observation contrasts with the study by Tariot and colleagues (2004) and with a more recent one by Atri and colleagues (2013). Indeed, in moderate and severe AD patients the co-treatment significantly increased cognition, function and global status when compared to donepezil-treated patients and reduced rates of marked clinical worsening (Atri et al., 2013), and also improved measures of activities of daily living and behavior (Tariot et al., 2004). Moreover, the concomitant use of memantine plus donepezil is most efficient to reduce agitation than donepezil alone (Kano et al., 2013). Interestingly, galantamine, an ACh-esterase inhibitor used for AD treatment, not only potentiated nicotinic ACh receptors activity, but also improved NMDARs activity (Zhao et al., 2006); thus, concomitant use of memantine and galantamine prevented galantamine activation of extrasynaptic NMDARs (Zhao et al., 2006). A recent study has also demonstrated that memantine protects not only glutamatergic but also cholinergic septal neurons from Aβ-induced toxicity (Colom et al., 2013) which may explain, in part, the potentiation of the effect of ACh-esterase inhibitor by memantine, when used concomitantly in AD treatment. Importantly, co-administration of ACh-esterase inhibitors and memantine did not improve patient's life expectancy (Lopez et al., 2009).

6. Concluding remarks

Modified activity and regulation of postsynaptic NMDARs linked to Ca^{2+} dyshomeostasis have been assuming a great importance in AD pathogenesis. Indeed, changes in NMDARs appear to be involved in synaptic dysfunction in early stages of

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AD. In this perspective, and taking into account the tripartite synapse, both presynaptic and astrocytic NMDARs may also play a relevant role in some synapses, although they have been much less studied in the disease-context, compared to postsynaptic receptors. Moreover, differential regulation of synaptic and extrasynaptic NMDARs (namely by the co-agonists D-serine and glycine) and their differential composition (particularly in GluN2A and GluN2B subunits) seem to underlie distinct neuronal fates, either inducing cell survival or cell death. In this regard, interaction of oligomeric Aβ with extracellular NMDAR subunits (GluN1 and GluN2B) and the influence of the peptide on intracellular signaling pathways (e.g. reelin pathway linking Src-mediated activation of NMDAR) and/or with selective scaffold proteins seem to have a fundamental role in altering the integrity and function of the synapse in AD. Thus, selective inhibition of NMDARs-mediated excitotoxicity alone (with memantine or one of its analogues) or concomitantly with improvement of ACh receptor-mediated transmission may help to slow down the progression of synaptic disruption in AD. Unfortunately, these therapeutics do not trigger a complete cure or an improvement in life expectancy when applied in late stage AD and thus implementation of earlier therapeutic strategies targeting NMDARs and/or the intricate signaling pathways is needed.

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Figure legends:

Figure 1: *Tripartite glutamatergic synapse – a target for Aβ*. Upon presynaptic neuron stimulation achieved by Ca2+ entry through voltage-sensitive calcium channels or by presynaptic or perisynaptic receptor-operated calcium channels (e.g. namely NMDARs), released glutamate can activate NMDARs localized in the postsynaptic membrane (synaptic stimulation) leading to Ca2+ entry through the NMDARs and the propagation of the action potential. Glutamate can then be taken up by surrounding astrocytes through EAAT1/2 or by the presynaptic terminal through EAAT2/5, and then stored into vesicles (reviewed by (Corlew et al., 2008)), precluding excitotoxicity. In conditions of excessive glutamate release or impairment of clearance, namely due to the presence of Aβ, bulk extracellular glutamate concentration increases, leading to extrasynaptic NMDARs activation. The differential activation (synaptic *versus* extrasynaptic) can also be modulated by glycine released from neurons and/or astrocytes (Muller et al., 2013) or D-serine released by astrocytes (Kang et al., 2013). Note that both glycine and D-serine can also be taken up by the presynaptic terminal or astrocytes by their respective transporters. The figure shows potential intracellular and extracellular targets for Aβ.

Figure2: *NMDARs anchorage to the synaptic membrane*. NMDARs are indirectly linked to actin cytoskeleton through PSD-95, guanylate kinase-associated protein (GKAP), Shank and cortactin proteins. Reelin, involved in synaptic plasticity, links to its receptors ApoER2 or VLDLR and activates Dab1. ApoER2 associates with PSD- 95, thus coupling the reelin signaling complex to the NMDAR. Activation of Dab1 leads not only to Src activation, but also to glomerular-actin (G-actin) polymerization into filamentous-actin (F-actin).

Figure 3: *Aβ-mediated targets acting at the glutamatergic synapse in AD – influence of NMDAR antagonists*. Aβ directly interacts with NMDARs, increasing intracellular Ca^{2+}, which underlies ER and oxidative stress; NMDARs are also linked to microtubule depolymerization, contributing for decreased axonal transport and neurite retraction and eventually DNA fragmentation, a feature of apoptosis. Moreover, Aβ appears to mediate NMDAR subunit endocytosis, and evokes dynamin degradation through NMDAR activation. These processes lead to impairment in synaptic transmission and decreased LTP, which contribute to cognitive deterioration. Importantly, selective GluN2B subunit antagonists (e.g. ifenprodil and Ro 25-6981) and memantine have been shown to ameliorate AD-related cognitive deficits.

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Figure 1

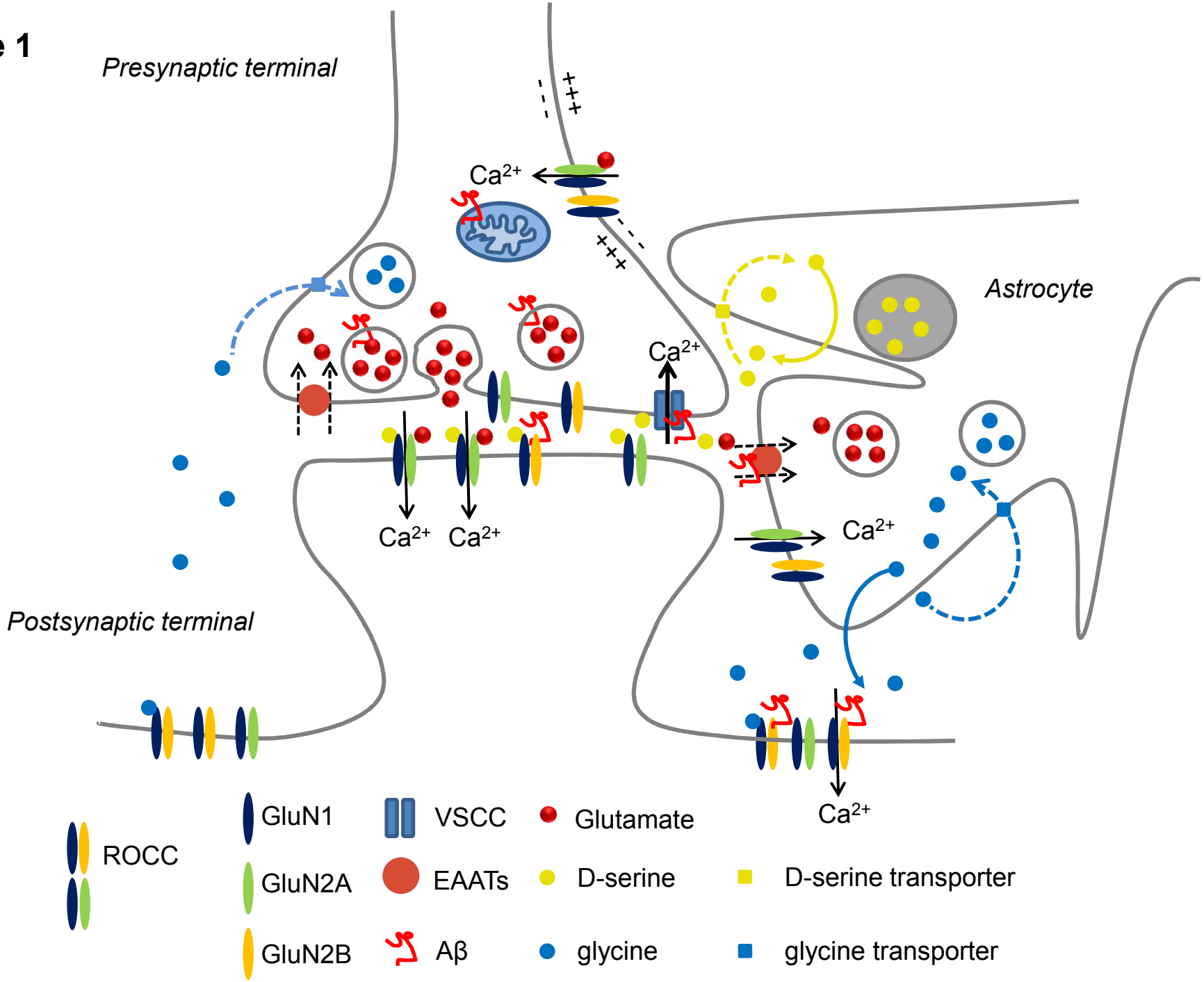


Figure 2

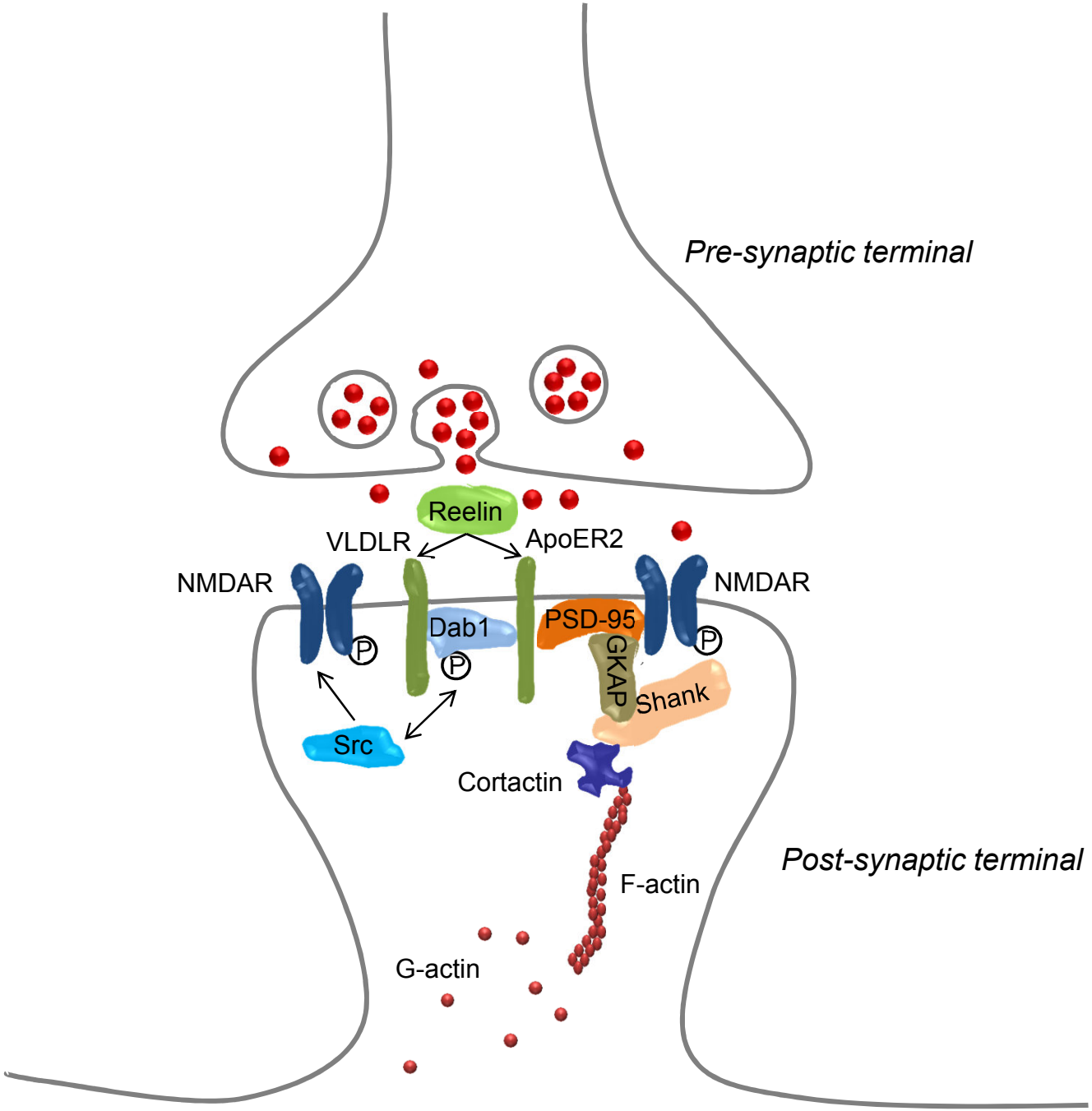
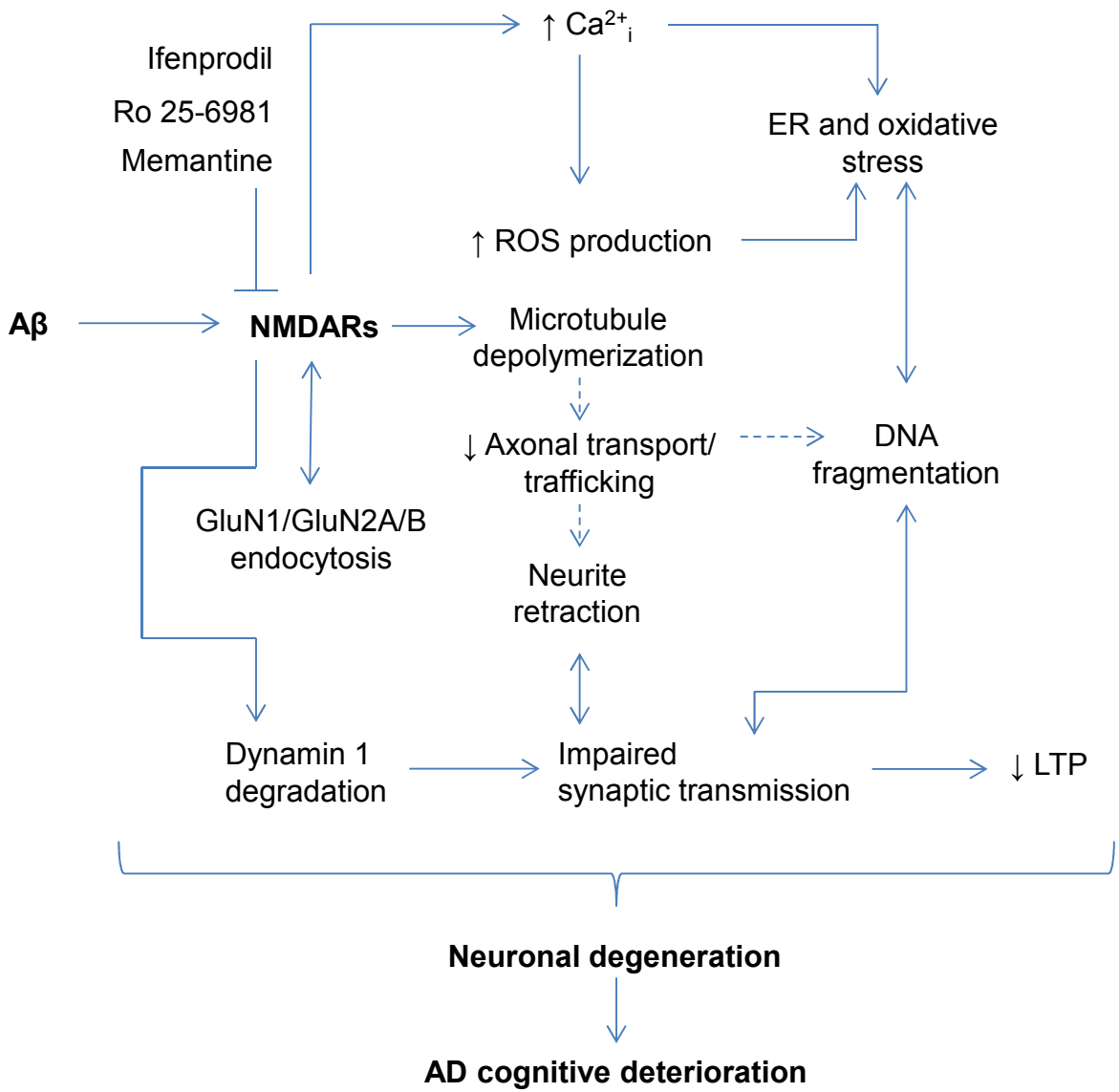

Pre-synaptic terminal
Reelin
VLDLR
ApoER2
NMDAR
NMDAR
Dab1
PSD-95
GKAP
Shank
P
P
P
Src
Cortactin
F-actin
G-actin
Post-synaptic terminal

Figure 3


↑ Ca2+i
Ifenprodil
Ro 25-6981
Memantine
ER and oxidative stress
↑ ROS production
Aβ
NMDARs
Microtubule depolymerization
↓ Axonal transport/ trafficking
DNA fragmentation
GluN1/GluN2A/B endocytosis
Neurite retraction
Dynamin 1 degradation
Impaired synaptic transmission
↓ LTP
Neuronal degeneration
AD cognitive deterioration

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Highlights

1. Aβ acts at the presynapse influencing synaptic vesicles and presynaptic proteins.
2. Postsynaptic dysfunction in AD is related with changes in NMDA receptor regulation.
3. AD may affect NMDA receptors located at the presynapse and in astrocytes.
4. NMDA receptors are relevant targets for therapeutic intervention in AD.

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