Chapter 4

NMDA Receptors in Astroglia: Chronology, Controversies, and Contradictions from a Complex Molecule

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Abstract

The neurocentric theory dismissed for decades the role of glia in information handling within the central nervous system (CNS). Nevertheless, almost 3 decades ago, this started to change and today astrocytes are considered relevant players for this function. Astrocytes "listen" to neuronal communication, regulate it, and respond at the cellular and synctitial level. Ionotropic glutamate NMDA receptor (NMDAR) is critical in CNS. It mediates synaptic neuronal communication and it is involved in different mechanisms. However, NMDAR is also expressed by astrocytes, but its functional role in these cells has not been deeply investigated and has been a matter of debate in the last decades. In this chapter, we briefly outline NMDAR intracellular transduction pathways initiated by Ca^{2+} flux. Then, we review chronologically NMDAR expression and function in astrocytes that have been a source of controversies and apparent contradictions. Finally, some insights are presented regarding NMDAR in astrocytes in the context of the tripartite synapse concept and the recently described Ca^{2+} flux–independent metabotropic-like NMDAR function in astrocytes. Given the complex molecular nature of NMDAR, its critical role, and the relevance of astrocytes, the study of astrocytic NMDAR promises to provide further understanding of CNS physiology and pathology.

Keywords: NMDAR, astrocyte, glutamate, flux-independent, signal transduction, tripartite synapse

1. Introduction

The glutamate (Glu) N-methyl D-aspartate receptor (NMDAR) plays a fundamental role in the central nervous system (CNS) mediating synaptic neuronal communication based on its

ionotropic function. This Glu receptor is sensitive to different coagonist such as glycine (Gly) or D-serine (D-Ser), ions (Mg^{2+}, Zn^{2+}, H^{+}), or other molecules such as polyamines. It is involved in different functions that include memory, synaptic plasticity, and long-term potentiation and depression (LTP and LTD) among others [1, 2]. This central role in CNS is given mainly by its location in the postsynaptic membrane, where it mediates neuronal communication enabling extracellular (EC) Ca^{2+} and Na^{+} entry into the postsynaptic neuron. Functionally, at resting membrane potential, neuronal NMDAR is inactive because its pore is blocked by an Mg^{2+} ion. This scenario is modified when the presynaptic neuron depolarizes and releases vesicular Glu into the synaptic cleft. This in turn activates AMPA and Kainate ionotropic receptors that depolarize the postsynaptic membrane, thus allowing Mg^{2+} removal from the NMDAR pore and therefore its opening with the consequent cationic flux in response to Glu and coagonist binding. For this reason, neuronal NMDAR is considered a coincidence detector that requires both membrane depolarization and ligand binding [1, 2].

NMDAR is a tetramer of homodimers or a heterotrimer conformed by two obligate subunits GluN1 coupled to GluN2 and/or GluN3 subunits. There is only one gene (*Grin1*) for GluN1 subunit that is present in all NMDAR described so far, since it plays a central role in NMDAR assembly in the endoplasmic reticulum (ER). There, GluN1 regulates NMDAR exit from this organelle due to ER retention signals that are masked after its assembly. In addition, there are four GluN2(A-D) genes (*Grin2a-d*) and two genes for GluN3 (A and B; *Grin3a-b*). It is well known that the mRNA of some of these genes undergoes posttranscriptional modification by alternative splicing, generating molecular variants that confer specific functional properties to NMDAR. This diversity of subunits also allows the assembly of different NMDAR according to subunits expressed by the cell, resulting in receptors with different features in terms of modulation, traffic, location, and biophysical properties, given in part by their interaction with different molecular partners. In addition, there are some posttranslational modifications of these subunits that also generate functional variants of NMDAR, such as phosphorylation, myristoylation, and proteolytic cleavage, among others [1, 2]. In the CNS, the expression of NMDAR subunits is regionalized, and notably, NMDAR is expressed by nonneuronal cells but also is widely expressed in cells from different mammal tissues including skin, testis, and pancreas, among many others [1–3].

All NMDAR subunits share a common structure with an EC region of ≈500 amino acids (aa); three transmembrane domains and two loops, the first one intracellular (IC) and the second EC; and an IC C-terminal domain that ranges from ≈200 aa in GluN1 and GluN3 subunits to ≈500 aa in GluN2 subunits (**Figure 1**). The EC region is comprised of two functional domains: the N-terminal domain (NTD) involved in subunit-subunit molecular interactions and the ligand binding domain (LBD) that in close interaction with the EC loop shapes the ligand binding site, Glu for GluN2 subunits and Gly or D-Ser for GluN1 and GluN3 subunits. The IC C-terminal domain mediates NMDAR molecular interactions that regulate its functional properties, for instance in the synapse with the postsynaptic density proteins that mediate the assembly of molecular clusters [1, 2].

NMDAR is a cationic channel with partial selectivity for Ca^{2+} conduction, explained at least in part by the DRPEER motif that binds this ion and is present near the second transmembrane domain of GluN1 that directly contacts the pore wall [2]. The entry of Ca^{2+} through NMDAR

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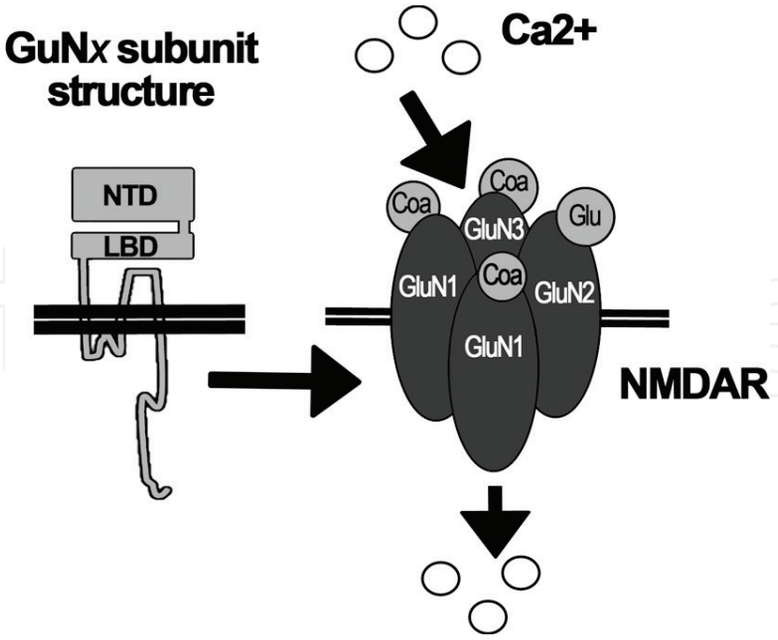

Figure 1. NMDAR subunit and channel structure (see text for details).

activates different IC signaling pathways that seem to depend upon its synaptic or extrasynaptic location (**Figure 2** and see below). It is well known that these pathways are involved in neuronal survival, growth, and differentiation, among other functions. On the other hand, it is also known that the excessive and persistent activation of NMDAR may result in the mechanism known as excitotoxicity, elicited by the excess of Ca^{2+} entry and the activation of IC pathways that lead to neuronal death (see below) [4, 5]. These Ca^{2+}-dependent IC pathways activated by NMDAR have also been referred to as Ca^{2+} flux–dependent metabotropic signaling [6].

Despite its wide expression and distribution in cells and tissues of mammals [3], NMDAR has been studied mainly in the neuronal context as a cationic channel [1, 2]. Nevertheless, now we know that NMDAR is expressed not only by other cells of the CNS such as astrocytes or oligodendroglia but also in non-CNS cells such as endothelium, platelets, and lymphocytes, among others [3, 7–9]. Moreover, there are some reports that have demonstrated NMDAR Ca^{2+} flux–independent functions and signaling [10–15], but only few of them have explored the molecular mechanisms that underpin this function.

Within the CNS, the role of astrocytes has transformed in almost a century from being considered supporting and metabolic cells to that of starring players in synaptic transmission among other functions. Indeed, since 1999, the concept of the tripartite synapse was coined by Araque et al. [16]. According to this idea, astrocytes play a relevant role beyond that of supporting cells, given their neurotransmitter receptor expression, neurotransmitter secretion, and IC Ca^{2+} waves elicited by neurotransmitters together with the early observed position of astrocytes that contact synapses and blood vessels. In this context, nowadays, a large amount of information supports the role of these cells in different CNS functions including synaptic transmission, synapse formation, development, LTP, and complex functions such as memory, sleep, and respiration, among many others [17] .

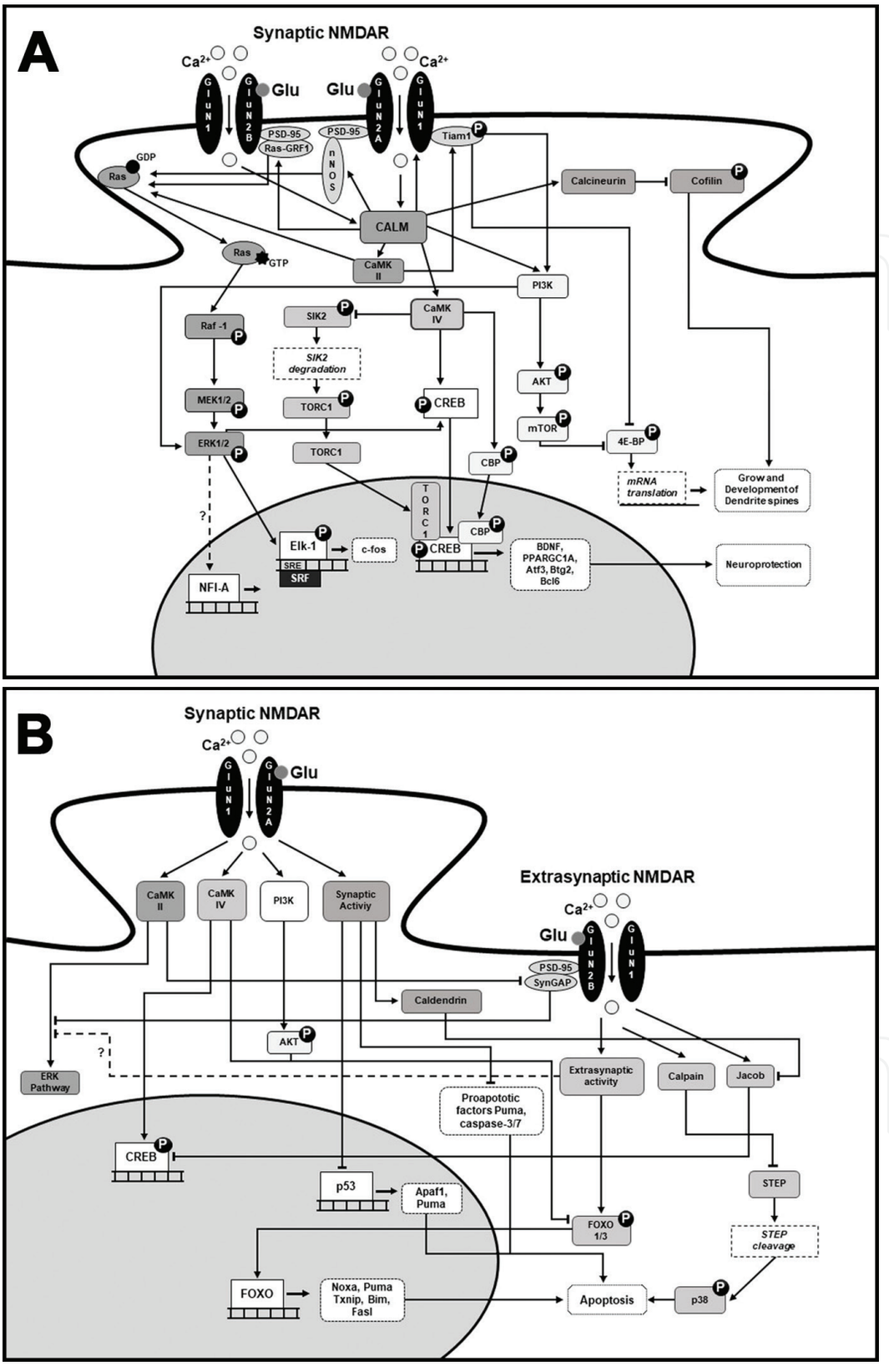

Figure 2. Ca^{2+} flux–dependent metabotropic signaling pathways in NMDAR. (A) Prosurvival, antiapoptotic pathways. (B) Prodeath, proapoptotic pathways (see text for details).

The aim of the present chapter is to review the expression and function of NMDAR in astrocytes, cells in which this topic has been a matter of debate and apparent contradictions. These have emerged mainly because astrocytes do not elicit action potentials as neurons do and thus NMDAR activation paradigm cannot be achieved, but also because NMDAR in astrocytes presents singularities not observed in neuronal NMDAR. Nevertheless, now there is a good amount of experimental evidence that has demonstrated NMDAR function in these cells.

In the first section of this chapter, we outline the Ca^{2+} flux–dependent metabotropic IC transduction pathways started by NMDAR activation, studied in the context of synaptic function, to substantiate the diversity of IC signals activated by this receptor. We then account chronologically the findings regarding the expression and function of NMDAR in astroglia that have been matter of some controversies and apparent contradictions. Finally, some insights are presented regarding NMDAR expression by astrocytes, with special attention to the tripartite synapse concept, and the Ca^{2+} flux–independent metabotropic-like NMDAR function recently reported. Given the complex molecular nature of NMDAR, its critical role in the CNS, both studied mainly in the neuronal context, and the relevance of astrocytes in the CNS evidenced in the last decades, the reexamination of NMDAR functions in astroglia may provide the basis to further gain insight into brain physiology and pathology.

2. NMDAR Ca^{2+} flux–dependent IC signaling

Until today, the canonical function of NMDAR is that of a cationic channel with partial selectivity for Ca^{2+}. This ion has been described in multiple signaling pathways ranging from bacteria to mammals and probably possesses the greatest universality and versatility as a second messenger [18]. Several IC signaling pathways are activated depending upon Ca^{2+} entry through NMDAR, and some mediate signaling to cell nucleus where gene expression is regulated (**Figure 2**) [5, 18, 19]. These Ca^{2+} dependent IC pathways triggered after NMDAR activation have been also referred as NMDAR metabotropic signaling [6]. The diversity of these IC pathways is influenced by NMDAR location and organism age among other variables [1, 20, 21]. Importantly, Ca^{2+}-dependent IC signaling mediates NMDAR function in LTP, memory, learning, neuronal plasticity, and survival, among other mechanisms but also mediates Glu excitotoxicity that leads to neuronal death [5].

Notably, there is a signaling dichotomy of NMDAR that has long been acknowledged [22]. A good amount of evidences supports divergent signaling between synaptic (prosurvival, antiapoptotic) and extrasynaptic (prodeath, proapoptotic) NMDAR [5], and even their genomic programs have been analyzed [23]. Interestingly, synaptic NMDAR is mainly assembled by GluN1/GluN2A subunits, whereas extrasynaptic NMDAR is mainly assembled by GluN1/GluN2B subunits [5, 24]. However, this dichotomy has presented inconsistencies in some tested models [25]. In the following subsections, we outline the IC signaling evoked by NMDAR that for convenience is separated into prosurvival, antiapoptotic signaling and prodeath, proapoptotic signaling. Since the review of these pathways is not the aim of this work, we invite the reader to please refer to the cited reviews and references therein for further

information. Although some of these pathways have also been investigated in astrocytes, it must be considered that their involvement must be contextual since NMDAR in astrocytes contains different assembled subunits and these cells perform distinct functions.

On the other hand, as mentioned above, some reports have found that NMDAR also activates IC pathways independently of Ca^{2+} flux in neurons and astrocytes, also termed Ca^{2+} flux–independent metabotropic-like signaling, although its molecular mechanisms have been poorly studied. This kind of signaling is described in the section of NMDAR in astrocytes since recent work has demonstrated its occurrence in these cells, and then it is analyzed in the following section.

2.1. Prosurvival, antiapoptotic signaling

2.1.1. Calmodulin (CALM) pathway

One of the central pathways, if not the central one, associated with NMDAR Ca^{2+} flux is that of calmodulin (CALM). This small protein (148 aa) is a Ca^{2+} sensor that in turn activates the function of different enzymes, ion channels, aquaporins, and other proteins [26]. Consequently, CALM activation is a critical, necessary step for the activation of several downstream pathways associated with NMDAR. Malenka et al. demonstrated that CALM and CaMKII activation is necessary to induce LTP in rodent hippocampal slices. In their experiments, IC injection into CA1 pyramidal cells of calmidazolium (CALM antagonist) or a CALM-binding peptide blocked LTP [27]. Later, it was found that c-fos NMDAR-dependent activation required CALM activity in cultured rat hippocampal neurons, thus suggesting that CALM mediates signaling to cell nucleus [28]. In addition, CALM itself was found to modulate NMDAR function through its interaction with GluN1 subunit [29]. For a review of CALM functions in NMDAR signaling, please refer to the work by Xia and Storm [26].

2.1.2. Ca^{2+}/calmodulin-dependent protein kinase (CaMK) pathway

Consistent with the central role of CALM in NMDAR signaling, one of the most studied pathways is that of Ca^{2+}/calmodulin-dependent protein kinase (CaMK), of which two isoforms are active in NMDAR signaling. The role of these serine/threonine kinases was demonstrated by Bading et al. [28], who found in cultured rat hippocampal neurons that NMDAR triggered activation of CaMK (CaMKII, because of the inhibitors tested), since the addition of DL-2-Amino-5—phosphopentanoic acid (APV or AP-5), a competitive NMDAR antagonist, decreased CaMK activity. Furthermore, also in cultured rat hippocampal neurons, Ca^{2+}/CALM induced binding of CaMKII to the cytoplasmic domain of GluN2B, resulting in the translocation of the kinase to the synapse, allowing the persistent CaMKII activity and facilitated response to extracellular Ca^{2+}, mechanisms that regulate and potentiate synaptic strength [30]. CaMKII is involved in multiple neuronal functions such as LTP [31], dendritic spine density [32], and neuronal survival [20, 33], among others. These functions are achieved through activation of several downstream signaling pathways such as Ras/mitogen-activated protein kinase (MAPK) pathway and neuronal nitric oxide synthase (nNOS), among others (see below). For a review on the role of CaMKII in the synapse, please refer to the recent reviews [31, 34].

On the other hand, CaMKIV has also been involved in NMDAR-mediated functions. One of the earliest reports on NMDAR-dependent CaMKIV activation was that by Impey et al. [35]. Their results in cultured rat primary hippocampal neurons suggested that activation of CaMKIV is dependent upon NMDA, since APV treatment blocked its downstream signaling. In addition, NMDAR-mediated transcription was attenuated by treatment with CaMKIV inhibitors or in CaMKIV dominant-negative models [35]. CaMKIV has been extensively studied in downstream pathways, such as that of cAMP response element-binding protein (CREB; see below).

2.1.3. Ras/mitogen-activated protein kinase (MAPK) pathway

One of the most important and studied IC pathways activated by CaMKII is the Ras/MAPK pathway. Ras GTPase is a key molecule since when it is bound to GTP it activates different effectors. Raf-1 kinase is one of the effectors that in turn phosphorylates MEK1/2 (mitogen-activated protein kinase kinase) that phosphorylates and activates extracellular-regulated kinase (ERK1/2 or mitogen-activated protein kinase (MAPK)). ERK1/2 activation results in the regulation of different genes in the cell nucleus [36].

Bading and Greenberg [37] demonstrated that Glu treatment of cultured rat hippocampal cells resulted in the rapid and transient tyrosine phosphorylation of a 39-kilodalton protein suggested to be microtubule-associated protein kinase-2 (or ERK 2). NMDAR was responsible since APV blocked this effect that required Ca^{2+} influx. Later, Yun et al. [38] demonstrated that nitric oxide (NO), synthesized by nNOS, is a key player in NMDAR-mediated Ras activation. nNOS is a well-known target of CALM and it is activated after NMDAR mediated Ca^{2+} flux [39]. Moreover, NMDAR stimulation failed to activate Ras in $nNOS^{-/-}$ cultured cortical neurons, and ERK activation through NO and Ras is Ca^{2+} flux–dependent through NMDAR [38]. Later, it was found that Ca^{2+}/CALM-dependent Ras guanine nucleotide release factor (RasGRF1) directly interacts with GluN2B subunit, and this interaction mediates MAPK pathway activation [40]. For further details on this pathway, please refer to previous reviews [18, 41].

However, not always NMDAR activity leads to ERK activation. It has been shown that under certain circumstances ERK can be inhibited by NMDAR generating apoptotic signals (see below).

2.1.4. Phosphoinositide 3-kinase (PI3K) pathway

Phosphoinositide 3-kinase (PI3K) is a target of CALM [42]. Chandler et al. [43] demonstrated that PI3K-mediated NMDAR induced activation of ERK in cultured cortical neurons, since wortmannin (PI3K inhibitor) decreased phospho-ERK2 levels. This observation, together with PI3K catalytic subunit binding to phosphorylated GluN2 previously reported, let the authors suggest that NMDAR activation could directly mediate PI3K activation. This was later confirmed when it was found that exposure to NMDA significantly enhanced phospho-Akt levels in cultured rat cortical neurons [44]. This increase was blocked by MK-801, a pore blocker of NMDAR, or APV, but also by wortmannin and KN-93 (CaMKII inhibitor), indicating that PI3K and CaMKII play a role in NMDA activation of Akt/ERK signaling. The Akt pathway modulates actin dynamics and promotes the synthesis of proteins through

the kinase mammalian target of rapamycin (mTOR) that in turn phosphorylates the factor 4E-binding protein (4E-BP) that controls mRNA translation [45].

2.1.5. cAMP response element-binding protein (CREB) pathway

One of the most studied pathways associated to NMDAR/CaMKIV is that of cAMP response element-binding protein (CREB), which plays a critical role in synaptic NMDAR-mediated neuroprotection. Impey et al. [35] showed that NMDAR activates transcription of CREB-responsive genes in cultured rat hippocampal neurons. CaMKIV was responsible for phosphorylating CREB-binding protein (CBP), a CREB coactivator. Moreover, mutation of CBP Ser301 impaired NMDAR and CaMKIV-stimulated transcription, whereas dominant-negative CaMKIV inhibited NMDAR-mediated activation of CREB-CBP. These authors showed that CaMKIV mediated a transient early phase of NMDAR-dependent CREB phosphorylation, while MAPK mediates a later persistent phase, suggesting the convergence between MAPK and CaMKIV pathways. In addition, the transducer of regulated CREB activity 1 (TORC1) modulates NMDAR-dependent CREB activation. Although the role of TORC1 in LTP had been previously demonstrated [46], Sasaki et al. [47] found NMDAR-dependent TORC1 transcription activation mediated by CaMKIV and salt-inducible kinase 2 (SIK2) degradation after oxygen-glucose deprivation (OGD).

Multiple genes related to cell survival are upregulated by NMDAR-dependent CREB activation. Nonetheless, it has been found that NMDAR activity-dependent shut off of genes is also an important process for neuronal survival. Zhang et al. [48] found in cultured mouse hippocampal neurons that NMDAR-mediated CREB regulation of Aft3 required nuclear Ca^{2+} transients and CaMKIV activity. Aft3 acts as a transcriptional repressor and protects neurons from apoptosis and extrasynaptic NMDAR-induced cell death triggered by NMDA or OGD. For a review, please refer to the work by Hardingham [5].

2.1.6. T-cell lymphoma invasion and metastasis 1 (Tiam1)

Tolias et al. [49] identified Rac-1 guanine nucleotide exchange factor (GEF) Tiam1 as a molecule involved in NMDAR downstream signaling. Tiam1 interacts and colocalizes with NMDAR that after activation allows Tiam1 phosphorylation, depending upon Ca^{2+} flux. It is suggested that such phosphorylation is mediated by CaMKII since it phosphorylates Tiam1 and increases Rac1 GTP exchange [50]. Using Tiam1 RNAi-infected neurons, this GEF was found to be necessary for NMDAR-dependent activation of Akt and phosphorylation of 4E-BP that mediates NMDAR-dependent mRNA translation [49].

2.1.7. Cofilin/calcineurin pathway

Calcineurin is a Ca^{2+}/CALM-dependent serine/threonine protein phosphatase that in neurons was found to downregulate NMDAR itself after its activation by Ca^{2+} entry through NMDAR [51]. In this work with acutely isolated adult rat neurons, the duration of NMDA channel openings is prolonged when calcineurin is inhibited by okadaic acid (phosphatase inhibitor) or its specific inhibitor FK506. On the other hand, it was found that NMDAR activation enhanced F-actin content in dendritic spines, associated to LTP, and such increase was

mediated by cofilin deactivation that stimulates actin filament turnover and therefore growth and development of dendritic spines [52].

2.1.8. Serum response factor (SRF) and ETS domain containing protein (Elk-1)

The transcription factors serum response factor (SRF) and ETS domain containing protein (Elk-1) were found to mediate NMDAR Ca^{2+}-dependent transcriptional response in cultured rat hippocampal neurons [53]. In this work, it was determined that c-fos gene transcription through the serum response element (SRE) present in its promoter could be achieved by two different pathways: one by SRF independent of Elk-1 and the second dependent upon Elk-1 phosphorylation, mediated by ERK.

2.1.9. p53 and p53 upregulated mediator of apoptosis (PUMA) suppression

NMDAR has also been shown to inhibit proapoptotic factors. Lau and Bading [54] demonstrated in cultured mouse hippocampal neurons that synaptic NMDAR suppressed p53 expression. This effect was blocked by MK-801 and included the repression of proapoptotic p53 target genes apoptosis protease activating factor 1 (*apaf1*) and p53 upregulated modulator of apoptosis (PUMA). Léveillé et al. [55] demonstrated that in cultured mouse cortical neurons, blockage of NMDAR with MK-801 induced the activation of caspase-3/7 following an apoptotic insult. They also confirmed that synaptic NMDAR protected neurons by suppressing the expression of proapoptotic PUMA that through Bcl2 associated X protein (Bax) promotes mitochondrial release of apoptotic factors, such as cytochrome C, and facilitates the action of proapoptotic members of the Bax subfamily. Released cytochrome C binds to APAF-1, which oligomerizes and recruits procaspase-9 that then becomes activated, forming the apoptosome [56].

2.2. Prodeath, proapoptotic signaling

2.2.1. ERK pathway inhibition

Chandler et al. [43] found an ambivalent ERK regulation by NMDAR in rat neuronal cultures. High concentration of NMDA decreased phospho-ERK2 levels in 1 mM extracellular Ca^{2+}, but activated it in 100 μM extracellular Ca^{2+}. The inhibition was accompanied by a decrease of phospho-CREB. Later, Ivanov et al. [57] demonstrated in cultured rat hippocampal neurons that ERK activation or inhibition depended upon the pool of NMDAR activated. Synaptic NMDAR activated ERK, whereas extrasynaptic NMDAR inhibited it. However, more recently, it was reported that ERK pathway activation in cultured rat cortical neurons depended on the days *in vitro* (DIV) of the culture and NMDA concentration. However, effects were sensitive to ifenprodil (GluN2B receptor antagonists), thus suggesting that GluN2B containing NMDAR may activate or inhibit ERK pathway [21].

2.2.2. Synaptic Ras GTPase-activating protein (SynGAP)

Synaptic Ras GTPase-activating protein 1 (SynGAP1) has been shown to interact with PDZ domain containing proteins such as postsynaptic density protein 95 (PSD-95), in the PSD, where it exists as a macromolecular complex together with the NMDAR subunit GluN2B [58].

SynGAP is inhibited by its phosphorylation by CaMKII that in turn stops inactivation of Ras that leads to MAPK pathway activation [59]. In cultured rat hippocampal neurons, they used RNAi to specifically suppress SynGAP expression that resulted in sustained ERK activation following NMDAR stimulation instead of transient activity [58].

2.2.3. Forkhead box protein O (FOX O)

Forkhead box protein O (FOXO) class of transcription factors can promote neuronal death [60]. FOXO targets proapoptotic genes including Bcl2-interacting mediator of cell death (Bim), PUMA, and Fas ligand (FasL), among others. It has been demonstrated in cultured rat cortical neurons that synaptic NMDAR prevents FOXO nuclear export and suppression of FOXO1 gene through PI3K/Akt activation [61]. Similarly, Dick and Bading [62] found that in cultured rat hippocampal neurons, synaptic activity inhibits nuclear translocation of FOXO3 and that dominant negative CaMKIV(K75E) expression blocked protective activity. Contrarily, extrasynaptic NMDAR activity induced FOXO3 nuclear import resulting in apoptosis.

2.2.4. Juxtasynaptic attractor of caldendrin on dendritic boutons (Jacob)

A binding partner of the neuronal Ca^{2+}-binding protein caldendrin critical for extrasynaptic NMDAR signaling was identified named juxtasynaptic attractor of caldendrin on dendritic boutons protein (Jacob) [63]. These authors found that Jacob knockdown prevented CREB shut off after extrasynaptic NMDAR activation. When imported into the nucleus, Jacob causes CREB dephosphorylation and promotes apoptosis, effects prevented by Caldendrin, which binds Jacob in a Ca^{2+}-dependent manner elicited by synaptic NMDAR activity. Contrarily, extrasynaptic NMDAR activity promoted nuclear accumulation of Jacob and neuronal death [5, 63].

2.2.5. Calpain/p38 MAP pathway

The calpain pathway is another important pathway that is related to the extrasynaptic activity of NMDARs. Calpain is a Ca^{2+}-dependent protease that has been widely implicated in neurotoxicity [64]. Xu et al. [65] demonstrated that in cultured rat cortical neurons the activation of extrasynaptic NMDARs strongly activates calpains, whereas synaptic NMDAR fails to activate this protease. Striatal-enriched protein tyrosine phosphatase 61 (STEP61) is a substrate for calpain and is rapidly cleaved after strong glutamatergic stimulation such as during excitotoxic or ischemic insult [66, 67]. Extrasynaptic stimulation of NMDARs evoked calpain-mediated proteolysis of STEP61 and activation of p38 MAPK. p38 contributes to cell death induced by chronic NMDA or Glu exposure [65]. Previously, Kawasaki et al. [68] observed p38 activation after Glu treatment in mature cerebellar granule cells, mediated by NMDAR.

3. Chronological track of NMDAR expression and function in astroglia

When considering the role of NMDAR in astrocytes, it is convenient to first acknowledge the hypothesis made by nineteenth century scientists such as Fridtjof Nansen, William Lloyd

Adriezen, or Carl Ludwig Schleich who suggested that neuroglial cells could play a more relevant role than that of gluing the CNS, after the name neuroglia was coined by Virchow in 1856 [17]. After almost a century of studying CNS from the perspective of the neurocentric theory, this conception was found to be correct. Previous recent reviews have examined the expression of NMDAR in neuroglial cells [69, 70]. Here, we present a chronological comprehensive review about how the expression and function of NMDAR in astrocytes have been studied in the last 40 years. Throughout these decades, different controversies and apparent contradictions have been encountered, but today, NMDAR expression and function in astrocytes are well documented and accepted. Moreover, NMDAR in astrocytes could play a relevant role in CNS pathologies and therefore offer a window to develop new therapeutic strategies.

In this review, we focus in NMDAR expression and function of brain astrocytes, although we recapitulate some early approaches in other neuroglial cells. It must be noted however that different laboratories documented the expression and function of NMDAR in Muller, Bergman, and radial glial cells or spinal astrocytes mainly in the 1990s. Please refer to the reviews by Dzamba et al. [70] or Verkhratsky and Kirchhoff [69] who examined the work on these cells.

3.1. Is it there? The early years: 1960s, 1970s, and 1980s

In 1967, it was observed that cortical glial cells *in situ* did not respond to Glu [71]. Later, Hösli et al. [72] found that in spinal organotypic cultures, astrocytes depolarized after treatment with Glu (100 μM). However, it was postulated that such depolarization was due to K^+ released by neurons, since 4-aminopyridine (blocker of Kv1 receptors) inhibited such effect and it was observed only in astrocytes adjacent to neurons but not in isolated astrocytes. Remarkably, no change in membrane resistance was observed in these cells, but neither in astrocytes from the olfactory cortex that also responded to Glu [72, 73]. However, few years later, it was demonstrated that neuron-free cultured astrocytes from newborn rat hemispheres responded to Glu treatment (100 μM-1 mM) in a Na^+-dependent manner. These groups also demonstrated that NMDAR was not involved in Glu response of astrocytes, since NMDA treatment alone (100 μM-1 mM) did not generate membrane depolarization [74, 75]. Instead, Glu was found to open Na^+/K^+ channels in cultured rat astrocytes that shared many properties with neuronal kainate/quisqualate receptor [76]. This finding was confirmed by Pearce et al. [77] that measured Ca^{2+} efflux and breakdown of inositol phospholipids in cultured rat cortical astrocytes and found no response with 100 μM NMDA. Usowicz et al. [78] showed that type-2 cerebellar astrocytes also depolarized in response to Glu, and consistently with previous findings, these cells did not respond to NMDA application (30–100 μM). Consistently, Backus et al. [79] observed that cultured rat cerebral astrocytes had no change in membrane potential with NMDA, even with the coagonist Gly or in Mg^{2+}-free solution, conditions that would favor neuronal NMDAR response. These studies, most of them electrophysiological, the traditional approach to study neuronal NMDAR, set the basis to establish that astrocytes do not express NMDA-type Glu receptors.

3.2. Rethinking the idea: the 1990s

In the seminal study by Cornell-Bell et al. [80], who described for the first time Ca^{2+} waves in cultured rat astrocytes in response to Glu, establishing the possibility that this kind of signaling could be relevant for CNS function, small inconsistencies regarding the expression of

NMDAR in astrocytes were observed. Using the IC Ca^{2+} (iCa^{2+}) probe Fluo-3, they found that cultured astrocytes did not respond to NMDA (100 μM) and Gly, although a small decrease of iCa^{2+} is observable in their published recordings. Nevertheless, these authors noted that APV "attenuated" the Glu response, because peak frequency and the amount of iCa^{2+} between peak responses were reduced, although maximal amplitude was maintained. Indeed, APV depleted a Glu-dependent gradual increase of iCa^{2+} masked between peak responses. Despite these findings suggesting that NMDAR could somehow be functional in these cells, it was concluded that astrocytes did not express NMDAR, consistently with previous reports. Simultaneously, Jensen and Chiu [81] also reported a lack of iCa^{2+} response measured with fura-2 in cultured rat cortical astrocytes to NMDA with or without Mg^{2+}. The same year, the Cornell-Bell group reported in a different work that Glu induced the formation of filopodia in astrocytes of mixed hippocampal cultures; however, this effect was not achieved with NMDA alone (100 μM) [82].

After Ca^{2+} waves' discovery in cultured astrocytes, Dani et al. [83] demonstrated the existence of Ca^{2+} waves in fluo-3 labeled astrocytes from organotypically cultured slices of rat hippocampus. These waves were elicited by neuronal electrical stimulation or bath application of NMDA (20 μM). However, since these waves were secondary to Ca^{2+} rise in neurons, these authors inferred that they were indirectly elicited, resulting from neurotransmitter released from NMDA-stimulated neurons and not from a direct stimulation of NMDAR in astrocytes.

Later, Holzwarth et al. [84] also found no iCa^{2+} response measured with Fura-2 to NMDA in cultured rat cortical astrocytes, whereas Seifert and Steinhauser [85] did the same observation in acute isolated mice hippocampal astroglial cells by patch clamp. These results reinforced the notion that astrocytes lacked functional NMDAR. However, in parallel and contradiction with this conception, two different groups suggested astrocyte activity evoked by NMDAR. Steinhauser et al. [86] reported that in postnatal (9–12 days) mice hippocampal brain slices, in a small group of glial cells termed passive (34% of them glial fibrillary acidic protein + [GFAP+] and characterized by time-independent currents) NMDA (1 or 5 mM) elicited currents detected by patch clamp. However, these responses varied: 41% presented an inward current, 32% a small outward current, and 27% did not respond. Nevertheless, these authors did not unequivocally identify astrocytic NMDAR as the responsible receptor. The second work by Porter and McCarthy [87] was made in rat hippocampal slices from young animals (9–13 days) labeled with Fura Red AM or Calcium Green-1 and recorded with confocal microscopy. In this work, it was reported that 75% of recorded astrocytes, identified by GFAP, presented iCa^{2+} rise in response to NMDA (50 μM), blocked by APV, although only 45% of these responses persisted in the presence of tetrodotoxin (TTX) that blocks neuronal activity. These observations suggested that astrocytes have functional NMDAR, although authors considered that Glu release mediated by presynaptic NMDAR, even with TTX, offered an alternative explanation for their observations. On the other hand, the other 55% of cells confirmed that neuronal activity could also elicit astrocyte responses, as inferred by Dani et al. [83].

In the meanwhile, different groups looked for the expression of NMDAR with histological techniques. Conti et al. [88] initially suggested that in the cerebral cortex of adult rats, virtually all (95.7%) GFAP+ cells did not express the mRNA for GluN1 (NMDAR1 or NR1) as

evidenced by *in situ* hybridization and electron microscopy (EM). Nevertheless, Gracy and Pickel [89] found by immunohistochemistry (IHC) combined with EM that in the basolateral amygdala 20% of staining with anti-GluN1 antibodies (Abs) corresponded to distal tips of astrocytic processes. Moreover, in a second work from Conti [90], it was found by IHC-EM, that in the cortex of adult rats, some distal processes and rare cell bodies of astrocytes were positive for GluN1 and GluN2A/B, although this was not evident by light microscopy. Similarly, Petralia et al. [91] found by IHC-EM and light microscopy in the dorsal cochlear nucleus some glial processes and wrappings of the synapses, and therefore possibly astrocytes, labeled with Abs against GluN1 and GluN2A/B. Likewise, Bockstaele and Colago [92] found in the nucleus coeruleus by IHC-EM the presence of GluN1 in astrocytic processes. Also, Farb et al. [93] reported that in the basal nuclei of the amygdala, glial processes were labeled by GluN1 Abs, detected by IHC-EM. Therefore, despite initial observations that supported the notion of astrocytes lacking NMDAR, several studies found evidences supporting its expression in tissue astrocytes of different brain regions.

Later, Pasti et al. [94] obtained further evidence with brain slices indicating that iCa^{2+} increase in cortical or hippocampal astrocytes in response to NMDA (100 μM) or neuronal electrical stimulation was a secondary effect of neuronal activity, not a direct action on putative astrocytic NMDAR. Consistently, Cai and Kimelberg [95] found no response to NMDA (100 μM) in GFAP+ acute isolated astrocytes from rat hippocampus. Notably, Gottlieb and Matute [96] in the same year found by IHC the expression of GluN2A and GluN2B subunits in rat hippocampus reactive astrocytes after transient ischemia. The expression of these subunits was maximal after 28 days of ischemia. This study constituted the first evidence suggesting that astrocytic NMDAR could play a role in the development of reactive astrocytes and therefore in pathology.

Two years after, Nishizaki et al. [97] detected currents by patch clamp elicited by NMDA (1 mM) in human cultured astrocytes obtained from the white matter surrounding a tumor. These currents were potentiated by Gly, sensitive to Mg^{2+}, independent of Glu transporters, but curiously were not sensitive to APV. Instead, they were partially sensitive to a G-protein inhibitor and increased when iCa^{2+} was depleted by inhibiting the sarcoendoplasmic reticulum Ca^{2+}/ATPase (SERCA), suggesting that currents were the result of store-operated Ca^{2+} entry (SOCE). In addition, it was observed with fura-2/AM that iCa^{2+} increased in response to NMDA, but this response was only partially inhibited by extracellular Ca^{2+} depletion and was also APV insensitive. With these findings, the authors suggested that NMDA elicited a response through a receptor distinct from NMDAR, perhaps through the activation of G-protein–coupled receptors (GPCR) that released Ca^{2+} from IC stores. However, Shelton and McCarthy [98] found no "clear" iCa^{2+} response to NMDA (100–400 μM) in rat astrocytes labeled with Calcium Green-1 AM from hippocampal slices obtained from 30 to 38 day animals, in Mg^{2+}-free solution with Gly and TTX. This contrasted with their previous observation in younger animals (9–13 days) [87] and led the authors to suggest that NMDAR expression could change during development. Simultaneously, Conti, et al. [99] reported that in human cortex, distal astrocyte projections had GluN1, GluN2A, and GluN2B labeling detected by IHC-EM, whereas astrocyte cell bodies were only occasionally labeled. Some of the labeled distal projections of astrocytes surrounded the axon terminal but others were present in areas not related with synapses.

3.3. It is there and it works: the 2000s

In 2001, Schipke et al. [100] identified functional NMDAR in cortical astrocytes of wild-type mice and transgenic mice with enhanced green fluorescent protein (EGFP) under control of GFAP promoter (EGFP-GFAP mouse). These authors isolated EGFP+ cells to obtain mRNA, perform RT-PCR, and test NMDAR subunit expression. In these experiments, GluN1, GluN2B, and GluN2C were expressed in astrocytes, whereas GluN2A, GluN2D, and GluN3 were not found. In addition, efforts were made to identify NMDAR subunits by western blot, but they were unsuccessful due to low cell yields. Patch clamp experiments in brain slices from 7 to 28 day animals showed that NMDA (100 μM) elicited currents in most (72%) of EGFP+ cells, but also in astrocytes from wild-type animals. Although the main component of these currents was found to be indirectly mediated by neuronal activity as previously identified, there was also a component related solely to direct NMDAR function in astrocytes. This response was blocked by MK-801 and Mg^{2+} suggesting that NMDAR was similar to neuronal NMDAR. Consistently, using Ca^{2+} indicators, it was found that NMDA increased iCa^{2+} observed mainly in distal projections, but also in the cell soma, consistently with previous ultrastructural observations that identified NMDAR mainly in distal projections.

This same year, Kondoh et al. [101] studied again NMDA (1 mM) responses by patch clamp in human astrocytes from the white matter surrounding a tumor. Essentially, the results reported were the same and consistent with their previous findings [97], but they also discarded that the cultured cells were from tumoral origin. Oddly, NMDA-elicited currents were potentiated by kynurenic acid (KYNA), a nonselective ionotropic Glu receptor antagonist. Also, the receptor mediating the response to NMDA was found to be less permeable to Ca^{2+}. With these observations, the authors suggested that astrocytes express a novel form of NMDAR, regulated by GPCR, as suggested by their sensitivity to a G-protein inhibitor, perhaps through the assembly of particular nondescribed subunits expressed in these cells.

Then, the group by Krebs et al. [102] reported that in rat hippocampus, NMDAR subunits GluN1 and GluN2B were observed in GFAP+ cells by IHC 3 days after transient ischemia, peaking at 28 days, and declining by 56 days, but were not detected in cells from intact animals. These observations were consistent with those made by Gottlieb and Matute [96]. In addition, GluN1 and GluN2B subunit expression was confirmed in astrocytes from postnatal (2–4 days) hippocampal neuron-glia cocultures subject to anoxia, where no neurons survived by day 3. In contrast, GluN2B was not found in pure cultured hippocampal astrocytes after anoxia. Functionally, NMDA (0.5–1 mM) elicited iCa^{2+} responses in astrocytes obtained from neuron-glia cocultures subject to anoxia or astrocytes acutely isolated from ischemic hippocampi. These responses were APV sensitive, and in acute isolated astrocytes, they were partially blocked by ifenprodil. These authors suggested that NMDAR function in astrocytes could provide the basis for new therapies to ameliorate the effects of stroke.

The same year, Zhang et al. [103] published a study reporting NMDAR participation in iCa^{2+} responses to Glu in cultured rat cortical astrocytes (87% of cells as described in their following publication) using APV. This is to our knowledge the first report that found response to NMDA in nonhypoxic cultured rat cortical astrocytes. A year after, the same group published

these results but also found that NMDA (50–100 μM) elicited iCa^{2+} response that was sensitive to APV in a large proportion (72%) of cultured rat cortical astrocytes [104]. Interestingly, APV inhibition was reverted only after a 30-min incubation in APV-free solution.

Two years later, strong evidence was provided by the group of Verkhratsky indicating that NMDAR mediated currents in acute isolated and tissue mouse cortical astrocytes (17–22 days) [105]. Profiting the advantages of the EGFP-GFAP mouse model for the identification of astrocytes, these authors found three different components of currents elicited by Glu application in acute isolated astrocytes using AMPA/kainate receptor, NMDAR, and Glu transporter inhibitors (NBQX, APV, and L-TBOA, respectively). In particular, APV blocked the sustained component of the Glu-induced current. They also demonstrated that NMDA elicited robust currents potentiated by Gly in 87% of acute isolated astrocytes at −40 or −80 mV membrane potential, indicating their insensitivity to Mg^{2+}, in contrast with NMDAR-mediated currents in neurons and previous work with hippocampal astrocytes [100]. Robust currents required 10–100 μM NMDA but were observable with 30 nM NMDA, they were blocked by MK-801, and ifenprodil only partially reduced currents in 43% of cells. Importantly, electrical stimulation of neurons in brain slices from 17 to 22 day animals also evoked Mg^{2+}-insensitive cortical astrocyte currents of which the fast component was blocked by MK-801, suggesting NMDAR involvement. Glu transporters also mediated these currents, but in contrast with observations in isolated astrocytes, AMPA/kainate receptors were poorly involved, although preventing their desensitization increased current amplitude. Interestingly, miniature spontaneous currents were also observed in tissue astrocytes that were independent of TTX, partially blocked by APV (32%) but fully blocked by APV and NBQX combination. All in all, this work demonstrated that astrocytes express functional NMDAR that mediate neuron-astrocyte communication.

Contrary to these findings, the same year different groups reported that NMDAR is not involved in iCa^{2+} response of astrocytes. Wang et al. [106] investigated astrocyte Ca^{2+} activity in the barrel cortex of adult mouse (6–8 weeks) in response to whisker stimulation. In this work, it was found that iCa^{2+} activity in astrocytes was consistently triggered by whisker stimulation. However, iontophoretic application of APV did not modify astrocytic iCa^{2+} activity although postsynaptic currents were suppressed, suggesting that astrocytic NMDAR is not involved in their response. In addition, Serrano et al. [107] studied glial involvement in heterosynaptic depression in rat (14–21 days) hippocampal slices. Although these authors found that NMDA elicited iCa^{2+} responses in astrocytes that were blocked by APV, these responses were delayed minutes after NMDA application. Indeed, TTX revealed that these glial responses were indirect and mediated by neuronal activity, as previous works had reported. Likewise, Kato, et al. [108] found in cultured cortical astrocytes from newborn mouse that NMDAR antagonists MK-801, ifenprodil, or Ro25-6981 did not block iCa^{2+} rise in response to Glu, as it was observed in cultured neurons. Notably, Glu induced astrocytes' activation (measured by morphological changes and GFAP expression) in neuron/astrocyte cocultures or cultured astrocytes, but this effect was blocked by MK-801 or ifenprodil only in neuron/astrocyte cocultures. This supported that neuronal NMDAR mediated neuron-glia signaling mediated astrocyte activation, resembling previous observations made in tissue astrocytes. In addition, Edling et al. [109] found that c-fos induction by Glu in newborn rat cultured astrocytes was not blocked by MK-801.

In 2008, a transcriptome database for three cell types of the CNS (neuron, astrocyte, and oligodendrocyte) from mouse forebrain was published, describing developmental changes between postnatal days 1 and 30 [110]. In this work, astrocytes were isolated by fluorescence-activated cell sorter (FACS) from a transgenic mouse expressing EGFP under control of S100β promoter, an astrocyte marker. Results showed that *in vivo* but also cultured astrocytes express NMDAR subunits GluN1, GluN2C, and GluN3A, the subunits included in the arrays. In particular, GluN2C was enriched in mature astrocytes. Importantly, after expression profile analysis, the authors observed that cultured astrocytes expressed many of the genes expressed by *in vivo* astrocytes and did not express the enriched genes in neurons or oligodendrocytes. However, they concluded that cultured astrocytes do not represent the same cell type as *in vivo* astrocytes, but instead an immature stage of the astrocyte lineage or a reactive astrocyte phenotype.

Simultaneously, Serrano et al. [111] found evidence suggesting astrocytic NMDAR function in the hippocampus, in contrast to their previous findings. These authors first distinguished two populations of cells in hippocampal slices from the EGFAP-GFAP mouse (10–18 day). These cells had different current/voltage curves, GFP levels, and cell-coupling and therefore were designated linear and outward rectifying glial cells (probably NG^{2+} cells). NMDA (25 μM) depolarized both types of cells, but in linear glial cells, TTX blocked partially (43%) the depolarization, while in outward rectifying cells, TTX had no effect. These observations suggested the direct involvement of astrocytic NMDAR beyond an indirect role mediated by neuronal activity as suggested by their own work and other previous reports. These authors further suggested that diversity of glial cells in the hippocampus could underpin distinct observations regarding NMDAR function in hippocampal astrocytes.

3.4. NMDAR composition, peculiar function, and therapy window: the 2010s

Palygin et al. [112] reported again that acute isolated astrocytes from EGFP-GFAP mouse (3 months) elicited currents in response to NMDA (30 μM). These currents presented slow desensitization kinetics, were blocked by APV, and were insensitive to Mg^{2+} as previously reported by the same group [105]. In addition, NMDA (30 μM) also elicited iCa^{2+} responses in acute isolated astrocytes measured with Fura-2 that were 43% of that elicited with Glu. iCa^{2+} responses were also evoked in astrocytes from cortical slices by neuronal electrical stimulation and were proportional to stimulus intensity. The iCa^{2+} response was blocked by TTX (100%) or decreased by APV (34%) or UBP141 (29%; antagonist of GluN2C/D containing NMDAR). With these results, the authors confirmed that astrocytic NMDAR participated in neuron-glia signaling and suggested that they should be assembled with GluN2C/D subunits and GluN3 that could provide lack of Mg^{2+} block and low Ca^{2+} permeability.

The same year, Lee et al. [113] reported that human astrocytes also expressed functional NMDAR. In this work, all seven NMDAR subunits were detected by RT-PCR in cultured astrocytes from fetal brains or adult brains and by immunofluorescence in human cultured fetal astrocytes. They found iCa^{2+} responses measured with Fura-2 to Glu (20 μM- 2.5 mM) or quinolinic acid (QUIN; agonist of NMDAR; 40 nM- 5 μM) that were blocked by MK-801 or memantine (NMDAR antagonist). However, three aspects were atypical in these experiments:

(a) responses to Glu and QUIN were not transient but sustained even after 30 s of agonist removal; (b) disparate concentrations of Glu (500 μM) and QUIN (1 μM) were required to elicit a similar iCa^{2+} response; and (c) these experiments were made with a fluorometer despite employing live microscopy. On the other hand, cytotoxicity levels induced by Glu or QUIN were prevented by MK-801 or memantine, but again Glu and QUIN concentrations to achieve the same response were disparate (500 μM vs. 500 nM, respectively).

After, Jiang et al. [114] reported that in cultured rat cortical astrocytes the NO donor sodium nitroprusside (SNP) or NMDA (10 μM for 18 hrs) induced Carboxyl-terminal PDZ ligand of nNOS (CAPON) translocation form cytoplasm to cell nucleus. The authors assumed NMDAR function since SNP effect was prevented by MK-801. Extraordinarily, they observed that SNP induced GluN2B localization in the cell nucleus, supporting previous findings suggesting putative NMDAR nuclear translocation [115, 116]. On the other hand, Zhou et al. [117] characterized NMDAR subunit expression in mouse cultured astrocytes at different times and in a model of ischemia. They found GluN1, GluN2A, and GluN2B expression by RT-PCR and immunofluorescence (only GluN1 and GluN2B). The expression of GluN1 decreased with time (4 weeks), GluN2A was increased, and GluN2B was slightly increased. Ischemia, actually an incubation in medium without serum, glucose, and equilibrated with 85% N2 and 0% O2, caused a bell-shaped response in GluN1, GluN2A, and GluN2B gene expression.

The following year, a study by Palygin et al. [118] characterized pharmacologically the NMDAR in acute isolated cortical astrocytes from the EGFP-GFAP mouse (4–8 weeks). In these cells, NMDA (50 μM) evoked inward currents in all astrocytes tested that were inhibited by UBP141 (62%) and were Mg^{2+} independent, whereas in neurons, only a slight inhibition was observed (9%) and they were Mg^{2+} dependent. Ifenprodil did not block astrocyte responses (3%), whereas it had a marked effect on neurons (58%). Memantine at low concentration blocked mainly astrocyte responses (39%) but not neuronal responses (7%), whereas at high concentration, it blocked both responses by 72% in astrocytes and 46% in neurons. In addition, using cortical brain slices, astrocyte and neuronal synaptic responses to NMDA or afferent stimulation were recorded. Astrocyte currents were dependent (TTX blocked them) and proportional to neuronal activity, therefore termed glial synaptic currents (GSC), but had slower rise and decay times compared with neuronal responses. In these experiments, similar results were observed to those observed in acute isolated cells with UBP141, ifenprodil, and memantine. When MK-801 was applied to astrocytes intracellularly, their response was decreased and no further effect was achieved with UBP141 or APV, although Glu transporter inhibitors almost fully abolished astrocytic response. In addition, measuring simultaneously iCa^{2+} with Fluo-3 and currents in astrocytes, it was found that NMDAR contributes importantly (89%) to current response, whereas it mediated approximately half of iCa^{2+} response (50–55%). The Ca^{2+} permeability (Pa_{Ca}/P_{Na}) of astrocytic NMDAR was calculated and found lower than that of neurons (3.4 astrocytes vs. 7.5 neurons), as suggested previously. This value is similar to that of NMDAR containing GluN3 subunits and together with Mg^{2+} independence suggested that GluN3 subunits are assembled into NMDAR of astrocytes. These authors also obtained evidences suggesting that diheteromeric (GluN1/GluN3) NMDAR is present in astrocytes. Together, these findings indicated that NMDAR in cortical astrocytes is assembled with GluN1, GluN2C or D, and GluN3 subunits.

Later the same year, the same group reported the participation of NMDAR, AMPA, and P2X receptors and Glu transporters in astrocyte responses to synaptic activity using the same model [119]. Their results showed that the participation of these molecules change in time. In particular, NMDAR increases its participation in both evoked and spontaneous GSC from the young (1 month) to adult (6 months) animals, declining in old animals (18–21 months). The same behavior was observed for membrane current density and iCa^{2+}.

Also in 2011, Martins de Souza et al. [120] published a proteome analysis of cultured astrocytes (cell line 1321 N1) treated with MK-801 or clozapine, performed with 2D gel electrophoresis followed by MALDI-TOF/TOF mass spectrometry. MK-801 treatment (8–72 h) regulated the expression of different proteins that belong to the energy pathway, cell communication, or cell growth, among others.

One year later, Gerard and Hansson [121] published a paper in which they described that rat cortical astrocytes cocultured (9–11 days) with endothelial cells presented iCa^{2+} responses to NMDA (100 nM-100 μM) measured with Fura-2. These responses were fully blocked by APV or ifenprodil, indicating that NMDAR with GluN2B subunit mediated these responses, subunit that was detected by immunofluorescence. Interestingly, the amplitude of these responses was blocked only partially (50%) in Ca^{2+}-free solution or with Cd^{2+}. Moreover, Ca^{2+} depletion from IC stores with caffeine and thapsigargin almost fully blocked (90%) response amplitude, whereas a combination of IC Ca^{2+} depletion and extracellular Ca^{2+}-free solution fully blocked the response. These results indicated that the source of Ca^{2+} was mainly the IC pools. Consistently, xestospongin C (XesC; inhibitor of inositol tris-phosphate [IP3] receptors) importantly (80%) diminished iCa^{2+} response amplitude and in combination with Ca^{2+}-free extracellular solution reached more than 90% inhibition. These authors also found that lipopolysaccharide (LPS) treatment increased NMDA iCa^{2+} response or IL-1β secretion, effects blocked by APV or ifenprodil. These results constituted the first suggestion that in cultured astrocytes the NMDAR could elicit a metabotropic-like flux-independent response beyond its ionotropic function, although these authors did not rule out the possibility that this response was actually a Ca^{2+}-induced Ca^{2+} release (CICR). Few groups had previously reported that some neuronal functions were mediated by a metabotropic-like, Ca^{2+} flux–independent NMDAR [12].

In 2013, a new expression profile analysis for young (2.5 months) and old (15–18 months) mouse astrocytes and microglia was published [122]. In this work, astrocytes were isolated from mouse brain through FACS using Glu transporter-1 (GLT-1) labeling. The mRNA of these cells was obtained, and cDNA was synthesized and then hybridized in an expression array. Consistent with the work by Cahoy et al. [110], astrocytes were enriched with GluN2C subunit expression in young and old astrocytes. Similar to the previous transcriptome analysis, these authors also found glutamate ionotropic receptor NMDA-type subunit-associated protein 1 (Grina) high expression. In addition, young astrocytes expressed higher levels of GluN3A in comparison to old astrocytes. Unfortunately, when this review was written, the list of genes in the arrays used were not publicly available and therefore it could not be confirmed which other subunits of the NMDAR were included in this array. Certainly, the expression of GluN2 and GluN3 subunits without GluN1 would not result in NMDAR assembly in the ER and transport to the plasma membrane, given the current paradigm and therefore GluN1

subunit expression is inferred. Rusnakova et al. [123] also published a study in which expression of NMDAR subunits was confirmed by single cell quantitative RT-PCR in acute isolated astrocytes from postnatal EGFP-GFAP mouse brains at days 10, 20, 30, and 50. In this work, NMDAR subunits GluN1, GluN2A, GluN2B, GluN2C, GluN2D, and GluN3A were expressed with different levels by these cells. In addition, this study also confirmed the expression of these NMDAR subunits at 3, 7, and 14 days after ischemia.

A new transcriptome database was published later by the Barres group based on RNA library sequencing [124]. This database included several brain cell types: neurons, astrocytes, myelinating, new and precursor oligodendrocytes, microglia, endothelium, and pericytes. In particular, cortical astrocytes were isolated from a transgenic mouse expressing EGFP under control of aldehyde dehydrogenase (Aldh 1 l1) promoter. In the open database at the Stanford University site, astrocytes are reported to express all NMDAR subunits with different levels (https://web.stanford.edu/group/barres_lab/brain_rnaseq.html). These expression levels in astrocytes are paired with expression levels in neurons for comparison and were reported in fragments per kilobase of transcript sequence per million mapped fragments (FPKM) as follows: GluN1 (*Grin1*) astrocytes ≈3 vs. >60 neurons; GluN2A (*Grin2a*) ≈ 0.1 vs. ≈ 0.8; GluN2B (*Grin2b*) ≈ 0.6 vs. ≈ 3; GluN2C (*Grin2c*) >25 vs. <1; GluN2D (*Grin2d*) <0.5 vs. ≈ 1.4; GluN3A (*Grin3a*) ≈ 5 vs. ≈ 6; GluN3B (*Grin3b*) ≈ 0.2 vs. ≈ 0.3. Thus, this study confirmed previous findings in other expression databases [110, 122] indicating GluN1, GluN2C, and GluN3A expression in tissue astrocytes but also found low expression levels of the other NMDAR subunits.

Later, Haustein et al. [125] studied spontaneous Ca^{2+} transients in mouse hippocampal astrocytes infected with an adenovirus containing the genetically encoded Ca^{2+} indicator (GECI) GCaMP. In this work, APV application did not significantly modify soma or projection spontaneous Ca^{2+} transient peak responses, amplitude, or kinetics. However, if traces of these experiments in the work by Haustein et al. [125] are conscientiously analyzed, some subtle differences are observed that perhaps could not be detected due to the statistical analysis or the population of peaks analyzed. However, if this could be true, the effect of APV was contrary to that described by Lalo et al. [105], because in this case, it appears as if NMDAR blockade increased spontaneous iCa^{2+} transients.

The following year, one of us published a work in which serendipitously a metabotropic-like Ca^{2+} flux–independent NMDAR was found in cultured rat cortical astrocytes [15]. In these cells, the expression of the seven NMDAR subunits was found at the mRNA and protein level by immunofluorescence and of GluN1 by WB. Interestingly, it was found that acid-NMDA (1 mM; pH 6.0) elicited iCa^{2+} responses in Fluo-4 labeled astrocytes that were not blocked by MK-801 nor by Ca^{2+}-free extracellular solution, but they were blocked by APV, KYNA, XesC, ryanodine (inhibitor of ryanodine receptors), or GluN1 knockdown by siRNA. Later, we found that iCa^{2+} response was elicited by the NMDAR but in response to acid pH that regulates NMDAR canonic function [2], rather than to NMDA itself. Also, acid-NMDA treatment depleted mitochondrial membrane potential ($m\Delta\psi$). These results strongly suggested that cultured astrocytes express an NMDAR than generates Ca^{2+} release from IC pools, mediated by IP3R and ryanodine receptors as suggested earlier by Gerard and Hansson [121]. However, in contrast to their work, we ruled out that CICR was involved since MK-801 and extracellular Ca^{2+}-free

solution did not block this response. These observations COULD help to explain the initial findings in cultured astrocytes that reported no electrophysiological response to NMDA or no iCa^{2+} response to low concentrations of NMDA. Nevertheless, the molecular mechanisms that enable this noncanonical function of the NMDAR still remain to be investigated.

The same year, Jimenez-Blasco et al. [126] reported that in cultured rat cortical astrocytes they observed iCa^{2+} rise measured with Fura-2 elicited by NMDA (1–100 μM). Curiously, NMDA effect was delayed by 500–1000 s after its application, presumably due to the low permeability of NMDAR in astrocytes. NMDA effect was partially sensitive to extracellular Ca^{2+}-free solution, suggesting NMDAR canonical ionotropic function, but it was fully blocked by Ca^{2+}-free solution in combination with SERCA inhibition by thapsigargin, evidencing also Ca^{2+} release from IC pools, although CICR was not ruled out. Moreover, iCa^{2+} rise was sensitive to U73122, inhibitor of phospholipase C (PLC), suggesting that IP3 synthesis could be involved in this effect, in agreement with our results. In addition, long-term NMDA treatment (20 μM, 8 h) promoted the activation of the PLC/protein kinase C (PKC)/p35/cyclin-dependent kinase (Cdk5) pathway that leads to nuclear factor erythroid 2-related factor 2 (Nrf2) activation and its nuclear accumulation, effect blocked by MK-801. Consistently, NMDA treatment activated the transcription of Nrf2-target genes.

Long-term effects of NMDA on astrocytes were reported also by Obara-Michlewska et al. [127], who tested the expression of inwardly rectifying K^+ channels (Kir4.1). In these experiments, the treatment of cultured rat astrocytes with NMDA (100 μM, 72 h) decreased the expression of Kir4.1 at the mRNA and protein level, effect reverted by MK-801 or APV. In addition, in a rat model of acute liver failure, memantine attenuated the decrease of Kir4.1 mRNA in the rat cortex.

On the other hand, Morquette et al. [128] described a role of astrocytes in the rat central pattern generator of the dorsal part of the trigeminal main sensory nucleus. In this study, it was found that astrocytes in this nucleus presented membrane currents or iCa^{2+} rise elicited by electric stimulation or NMDA treatment (1–2 mM), while neuronal activity turned from tonic to bursting and was also accompanied by iCa^{2+} rise. NMDA-elicited depolarizations in astrocytes were insensitive to TTX, whereas an inhibitory cocktail (CNQX, TTX, Cd^{2+}, and L-trans-pyrollidine-2,4-dicarboxylic acid [PCD] inhibitor of Glu uptake) blocked 53% of astrocyte response but almost fully blocked neuronal response, suggesting that astrocytic NMDAR was involved. Also, MK-801 diffused intracellularly in astrocytes blocked NMDA-elicited response in these cells. Together, these results strongly suggested that these astrocytes expressed functional NMDAR.

However, Otsu et al. [129] studied with the Ca^{2+} sensor Rhod-2 iCa^{2+} responses in glomerular astrocytes in the juvenile (14–21 day old) mouse olfactory bulb. In this work, it was found that electrical stimulation of odor sensory neurons elicited iCa^{2+} responses in glomerular astrocytes and neurons. Astrocyte responses were delayed and elicited only with high stimulus intensities in contrast to neuronal ones that were elicited even with single pulse stimulation. Astrocyte and neuronal responses were blocked by a combination of APV and CNQX, similar to the observations made by Lalo et al. [105] in cortical astrocytes. Nevertheless, considering the delay between both responses, these authors suggested that in astrocytes, responses were mediated by postsynaptic activity (dendrite Glu release), instead of a direct stimulation of

astrocytic NMDAR or AMPAR. It must be noted that these observations were made measuring somatic Ca^{2+} dynamics, but the NMDAR role was not tested when a GECI mouse model was used. This could be relevant because it has been shown that somatic and projection iCa^{2+} responses have different molecular players [125, 130, 131].

In this year also, Dzamba et al. [132] analyzed the expression of NMDAR subunits in cortical astrocytes from the EGFP-GFAP mouse before and after ischemia by single cell quantitative RT-PCR. In these experiments, all NMDAR subunits were expressed by astrocytes of uninjured mouse with the exception of GluN3B that presented in very low levels, consistently with their previous work [123]. The expression of these genes was increased 7 and 14 days after ischemia, with the exception of GluN2C that was the highest expressed subunit in control conditions and GluN3B that did not increase nor was detected. In contrast, immunofluorescence experiments in brain slices showed only GluN3A expression in control animals, but after ischemia, NMDAR subunits GluN1 and GluN2B-D were also observed. iCa^{2+} responses were elicited in tissue astrocytes by NMDA (4–100 mM), were not blocked by TTX, but were sensitive to APV and memantine. In contrast to previous findings, ischemia reduced iCa^{2+} responses to NMDA. Also, cortical cultured astrocyte iCa^{2+} responses were elicited, but with higher NMDA concentration (500 μM), these responses were also reduced in astrocytes isolated from ischemic mice.

An additional study [133] tested the effect of MK-801 in GFAP expression in tissue hippocampal and cultured astrocytes from rat. In these experiments, MK-801 (6 days) increased GFAP expression in the hippocampus as measured by immunofluorescence and WB. Consistently, MK-801 (24 h) increased GFAP, BDNF, TrkB, and p75 expression at the mRNA and protein level in hippocampal cultured astrocytes. These results suggested that NMDAR activity mediates these effects in astrocytes. Moreover, with these results, the authors proposed that hippocampal astrocytes may participate in NMDAR hypofunction associated to the pathophysiology of schizophrenia.

In 2016, one study by Pinacho et al. [134] showed that MK-801 upregulated the expression of glycogen phosphorylase (PYGM) and RAC-1 when administered in mice but also in cultured rat cortical astrocytes (72 h).

Recently, Mehina et al. [135] studied astrocytes in mouse cortical slices and found that neuronal theta burst stimulation generated transient iCa^{2+} increase in astrocyte soma and projections, followed by a long-lasting decrease of cytoplasmic Ca^{2+} basal levels, perhaps related with observations made by Cornell-Bell et al. [80] with NMDA. Although the transient iCa^{2+} response in astrocytes was blunted by APV, this was not statistically significant; however, it did block the long-lasting decrease of cytoplasmic Ca^{2+} basal levels. Importantly, MK-801 applied intracellularly by the patch pipette also blocked the long-lasting decrease of basal Ca^{2+}, indicating that it was mediated by astrocytic NMDAR. Consistent with the role of NMDAR, it was also found that a NOS inhibitor mediated the decrease of basal Ca^{2+}, probably through its regulation of SERCA. These authors also showed that basal Ca^{2+} levels in astrocytes regulate long-lasting vascular tone, effect blocked by APV.

All in all, these studies have substantiated that astrocytes do express NMDAR at the mRNA, protein, and functional level. However, the reach of this conclusion has not been easy because

apparent contradictory findings or controversies have been often reported. A critical issue through this achievement has been the distinction between neuronal and astrocytic NMDAR, considering their location at or near the synapse, but also presynaptic membranes, very close to the perisynaptic astrocyte projection (PAP), where the NMDAR may mediate astrocytic Ca^{2+} responses relevant for information handling in the brain. Nevertheless, other sites of NMDAR actions besides the PAP should not be discarded.

An additional source of controversy has been the selection of the experimental approach and model to test NMDAR in astrocytes. It seems clear now that canonical ionotropic NMDAR function is not conserved in cultured cells compared with tissue astrocytes. Classical electrophysiological experiments initially set the basis to conceive the lack of NMDAR in cultured astrocytes. However, the same approaches applied to tissue or acute isolated astrocytes suggested NMDAR function. In this regard, it is very interesting why and how the NMDAR becomes a different functional molecule when astrocytes are cultured and what may be the physiological relevance (if any) at the cellular and tissular level (see below).

An important source of controversy has been the *a priori* expectations that NMDAR in astrocytes should have similar biophysical and functional properties as its well-studied neuronal synaptic counterpart. Pharmacological studies have already demonstrated that NMDAR in astrocytes may be assembled by different subunits that confer particular functional properties. Its multisubunit nature, diversity of subunits, and multiple posttranslational and posttranscriptional modifications suggest that NMDAR function is more complex as it goes beyond synaptic function. Interestingly, in line with this conception, some reports have already documented a noncanonical metabotropic-like, flux-independent NMDAR function in astrocytes but also in neurons [10–12, 15]. Intriguingly, phosphatidylinositol metabolism was found associated with NMDAR-mediated Ca^{2+} flux in Muller and Bergman cells [136, 137]. As a matter of fact, metabotropic-like, flux-independent function is not necessarily new for ionotropic Glu receptors. It has been reviewed elsewhere that this kind of mechanisms occurs for kainate receptors, some of them described almost 2 decades ago [138]. However, the cellular and molecular mechanisms that make this possible are poorly studied and become very relevant considering NMDAR function in other cells and tissues and flux-independent function observed in neuronal-mediated mechanisms.

Some of the methodological and experimental sources of controversy that precluded the acknowledgment of NMDAR expression and function in astrocytes are shared with those that precluded the acceptance of astrocyte iCa^{2+} dynamic responses in CNS handling of information and function. Please refer to the review by Khakh and McCarthy [139] who deeply discussed these aspects.

4. Insights regarding astrocytic NMDAR

Given the relevance of NMDAR in CNS, the expression of NMDAR in astrocytes, and its role in information handling in CNS, the intuitive conclusion is that NMDAR in astrocytes is implicated in this function, as some electrophysiological works have already demonstrated, detecting iCa^{2+} signals in response to synaptic activity. However, electrical activity in astrocytes may have a secondary role in comparison to other cellular activities such as for instance synctitial

communication, in contrast with their neuronal counterparts. Therefore, it is convenient to take into account the diversity of Ca^{2+}-dependent metabotropic IC signals elicited by NMDAR activation, outlined above, that could shape astrocyte activities. Since GluN2C is preferentially assembled into astrocytic NMDAR, then it is possible that IC pathways differ from GluN2A/B NMDAR, as some initial works have demonstrated. This is even more relevant when it is considered that astrocytes are involved not only in information handling, but also in energy administration, immune response to infection or tissue damage, development, or synaptogenesis, among other functions [17, 140]. In this regard, the IC pathways activated by Ca^{2+} entry through the NMDAR must be critical for the long-term effects that could occur not only to the astrocyte but also to its syncytium. Indeed, as described above, some groups have already started to study these long-term effects in astrocytes after chronic exposure to NMDA, and as history has demonstrated, cell culture is a useful tool to study these aspects. In addition, NMDAR has been shown to mediate IC signaling in astrocytes, function critically involved in neuronal survival. Furthermore, since astrocytes are also involved in the immune response and some evidences point to enhanced NMDAR activity in these cells after ischemia, thus it is possible to conceive that NMDAR in astrocytes is involved in immune function of these cells, as has been observed previously in lymphocytes [8]. Also, recent work has suggested that NMDAR of astrocytes could be involved in the hypofunction of NMDAR that has been proposed as an alternative hypothesis to understand schizophrenia [133]. More work is needed to investigate how NMDAR in astrocytes is related to other pathologies that have been related to astrocyte functions [141].

On the other hand, Ca^{2+} flux–independent metabotropic signaling by NMDAR in cultured astrocytes provides the grounds for a new framework of NMDAR activity, although more research is required to further confirm its occurrence in tissue astrocytes. Nevertheless, it must be noted that multifunctionality of cell membrane molecules is now well documented, and most of these mechanisms were initially described in cultured cells ([136]; and references therein). Indeed, it is possible that the effect observed by Nishizaki et al. [97] and Kondoh et al. [101] in cultured human astrocytes with a G-protein inhibitor could be related to NMDAR signaling inhibition itself instead of NMDA activation of an unknown receptor that elicited G-protein activation, or NMDAR regulation by a GPCR, as it was suggested. If true, this report could be the first hinting Ca^{2+} flux–independent metabotropic signaling by NMDAR. Therefore, this kind of signaling should not be discarded *a priori*, also considering that this Ca^{2+} flux–independent NMDAR function has also been observed in neuronal-mediated responses, and the number of works documenting this function is increasing [12, 142]. Moreover, flux-independent metabotropic signaling by other ionotropic Glu receptors have been known for almost 2 decades [138].

The experimental evidence indicates that in cultured astrocytes Ca^{2+} flux–independent metabotropic NMDAR signaling is dominant over its ionotropic function, that could be exacerbated by culture conditions, such as Glu concentration or the extracellular proteins present in fetal bovine serum. However, it is not possible to rule out that in tissue astrocytes some NMDAR, probably distributed at specific cellular locations, may elicit this kind of signaling. The currents observed in acute isolated astrocytes, the conservation of some classical ionotropic NMDAR function in astrocytes cocultured with endothelial cells, and its absence in pure cultured astrocytes support this notion [15, 105, 121], because cellular interactions may be involved in the conservation of canonical NMDAR function. Moreover, these observations may reflect a gradual conversion of NMDAR function due to some unknown mechanism that

may be related to membrane dynamics. In addition, our observations suggest that there is some sort of segregation of Ca^{2+} flux–dependent and –independent functions of NMDAR in cultured astrocytes [143, 144]. Consistent with these putative Ca^{2+} flux–dependent and –independent functions of NMDAR, long-term effects in cultured astrocytes and even iCa^{2+} rise in human cultured astrocytes have been blocked by MK-801 or memantine, thus substantiating a canonical ionotropic function. This somehow contradicts the fact that most electrophysiological recordings in cultured astrocytes have never found NMDAR-mediated currents, with the exception of the work with human astrocytes from the white matter that intriguingly were not sensitive to APV. These apparent contradictions may have their source in the experimental settings employed, as described above. For instance, the cell model employed may cooperate in this variability given the diversity of astrocytes [145, 146]. Also, the intracellular crosstalk among Glu ionotropic and metabotropic receptors and their regulation in the experimental models employed may interplay and result in the apparent contradictions observed. In addition, it is of note that most experiments studying NMDAR in cultured astrocytes have used high agonist concentrations that are within the Glu concentration reached in the synaptic cleft after neurotransmission [147]. Also, it is interesting that different physiological solutions have been used with cultured astrocytes that could be related to the observations made. Notably, experiments with human astrocytes have shown unique behaviors of NMDAR suggesting perhaps that in human cells NMDAR may have specific molecular features that are still unknown.

However, the fact that cultured rat astrocytes present Ca^{2+} flux–independent metabotropic NMDAR signaling indicates at least the existence of a cellular and/or molecular mechanism that enables this function, although it could be overrepresented in cultured astrocytes due to culture conditions. This mechanism may be related to the expression of genes that let some authors conclude that cultured astrocytes represent an undifferentiated or activated phenotype of astrocytes. Nonetheless, since NMDAR is also expressed by endothelial cells, leukocytes, osteoblasts, platelets, or melanocytes, among other cells but also in a diversity of tissues including stomach, testis, thymus, ovaries, skin, pancreas, lung, kidney, or heart [3], it is likely that NMDAR function may encompass other mechanisms of action and regulation, beyond those described for synaptic or extrasynaptic NMDAR. For instance, leukocytes or endothelial cells are in contact with 40 times higher Glu (40 μM) in the blood than cells of CNS bathed by the cerebrospinal fluid (1 μM) [148]. Therefore, NMDAR in these cells must require particular cellular or molecular mechanisms to avoid its activation and the resulting entry of large quantities of Ca^{2} that could activate them or even be cytotoxic. Also, Mg^{2+} blockade should not work in these cells since Glu concentrations are constant, unless it is regulated differently to neuronal NMDAR. One alternative for this Ca^{2+} flux–independent metabotropic-like function is that in cell culture, the NMDAR associates with molecular partners that enable this function. Interestingly, it has been reported that NMDAR has been found to associate with metabotropic dopamine receptors [149]. There is also a possibility that Ca^{2+} flux–independent metabotropic-like NMDAR function could be the result of proteolytic cleavage, as that already reported for NMDAR subunit GluN1 [150], that occurs to other membrane molecules [151]. In addition, GluN2C subunit–mediated IC signaling, found highly expressed in most transcriptome analysis of astrocytes, but that has been poorly studied, could be involved.

Of particular interest is the fact that, according to our results, higher H^{+} concentration seems responsible to elicit NMDAR Ca^{2+} flux–independent metabotropic-like function. This is because

three main points are relevant for CNS: (a) It is well known that presynaptic Glu release causes an increase of H^+ concentration in the synaptic cleft [152]. (b) Under certain processes such as inflammation, hypoxia/ischemia, or hypercapnia, among others, very low pH levels may be reached in the brain [153]. (c) The NMDAR is well known to be regulated negatively by acidic pH, and the amino acid sequences that mediate this effect have been identified [2]. Then, Ca^{2+} flux–independent metabotropic-like function of NMDAR in astrocytes presents singularities, or even antagonic features when compared with canonical NMDAR that could be relevant for astrocyte function and therefore for the maintenance of brain homeostasis. Thus, the study of the molecular mechanisms that make possible Ca^{2+} flux–independent function of the NMDAR may open new possibilities to modulate NMDAR function in certain pathologies.

On the other hand, which advantage presents the Ca^{2+} flux–independent metabotropic-like NMDAR in astrocytes? Perhaps the easiest answer is that some sort of NMDAR signaling could be initiated without extracellular Ca^{2+} entry that could pose a menace for cellular homeostasis. This could be more advantageous if it is considered that in our experiments we found that Ca^{2+} flux–independent metabotropic-like NMDAR is dependent on H^+ levels above physiological normal levels [15]. However, the nature of the IC pathways initiated by this Ca^{2+} flux–independent metabotropic-like NMDAR still remains to be investigated, because the PLC/PKC/p35/Cdk5/Nrf2 pathway activation was sensitive to MK-801 similar to CAPON nuclear translocation that may lead to MAPK activation through NOS. Thus, it seems that activation of these pathways by long-term treatment with NMDA requires Ca^{2+} flux. To further analyze this question, it is then necessary to define whether this metabotropic-like function is mediated by a channel NMDAR with posttranslational modifications that is also capable to initiate signal transduction as has been observed in neuronal NMDAR [12, 142] or by a nonchannel NMDAR. In the first case then, some specific mechanism of channel blockade must act to avoid fast measurable Ca^{2+} flux through NMDAR. In the second case, perhaps other molecular mechanisms could result in a nonchannel NMDAR, as described above. However, this question requires further research to fully understand the mechanism that enables Ca^{2+} flux–independent metabotropic-like NMDAR in astrocytes and perhaps in neurons or other cell types.

All the above-described effects mediated by NMDAR in astrocytes must occur when it is located in (a) the astrocyte synaptic membrane (AsSm) of the PAP that directly contacts the synapse, analog to the postsynaptic membrane, and/or in (b) PAP extrasynaptic sites that could even include the soma. However, IHC-EM experiments have indicated that NMDAR in astrocytes is preferentially located in their projections. In addition, Lalo et al. [105] have already described the GSC, suggesting that Glu sensing takes place at the PAP or near it, thus somatic NMDAR function seems unlikely, although this needs to be tested. The PAP is a dynamic structure that surrounds the synaptic bouton (**Figure 3**) extending and retracting as a function of synaptic activity. The more neuronal activity, the more neurotransmitter and the more PAP coverage [125, 154, 155]. But, is the NMDAR present in the AsSm of PAPs? Or is NMDAR located in extrasynaptic sites in the PAP where it would only be activated by some Glu spillover? If it is located in AsSm, then NMDAR could be sensing tiny amounts of Glu, whereas if it is in extrasynaptic sites, then only high synaptic activity would lead to its activation. This is in fact a common feature of iCa^{2+} signaling in astrocytes, and it is observed with high synaptic activity, as described by different groups [105, 125]. However, this observation could be the result of the integration of synaptic activity carried out by astrocytes and thus

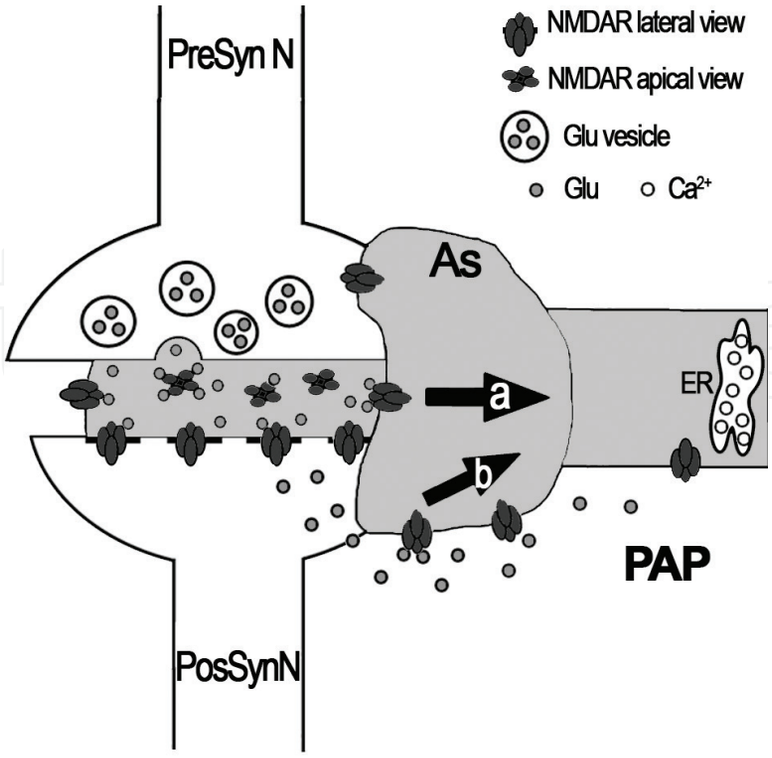

Figure 3. Astrocytic NMDAR putative locations. NMDAR may be located in the astrocyte synaptic membrane (AsSm) and/or astrocytic extrasynaptic membranes. IC signaling (a or b) in both places could be specific, b requiring Glu spillover. In a and/or b, the Ca^{2+} flux–independent metabotropic-like NMDAR could be involved, reaching IC Ca^{2+} pools such as the ER in the PAP. As, astrocyte; PreSynN, presynaptic neuron; PosSynN, postsynaptic neuron (see text for details).

does not necessarily rule out the presence of NMDAR in front of the synapse. Instead, it is possible that not enough receptors located at the AsSm are activated when low synaptic activity is elicited and thus the whole cell currents are not recorded. Therefore, at this point, it is not clear the exact location of astrocytic NMDAR; however, in this context, the density and organization of receptors at cell membrane in either location are relevant for the final result (**Figure 3**).

At this point, it is convenient to recall that Glu has been suggested to exert only metabotropic signaling (through mGluR, although the putative Ca^{2+} flux–independent function of NMDAR could participate) in extrasynaptic sites of the PAP, in view of the location of IC Ca^{2+} stores ≈1 μm far from the AsSm [156]. However, according to our biophysical modeling work, Ca^{2+} and IP3 diffusion coefficients (10X larger for IP3), together with mGluR location, support that synaptic astrocyte Glu metabotropic signaling may occur and reach IC Ca^{2+} stores within millisecond time scales depending upon PLC activity and number [157]. Moreover, PAP leaf-like morphology would optimize IP3 straightforward diffusion to IC Ca^{2+} stores. In contrast, although Glu has a ≈ 1.5 faster diffusion coefficient than IP3, the need to escape from the synaptic cleft and override the PAP enwrapping in the EC space and Glu multidirectional diffusion would perhaps make this alternative more difficult to occur, although not impossible when very high Glu secretion is reached (**Figure 3**).

Considering the ambivalent nature of NMDAR signaling described above (synaptic vs. extrasynaptic), an additional immediate question that comes up is which kind of signaling (prosurvival or prodeath) results from astrocytic NMDAR? Since astrocytes are resistant to

different insults including high levels of Glu, then intuitively the answer should be prosurvival signaling. In fact, this has been confirmed by different works, but with some differences in comparison with synaptic NMDAR signaling described above. Activation of PLC/PKC/p35/Cdk5/Nrf2, a prosurvival pathway, and CAPON nuclear translocation were described in cultured astrocytes in response to NMDAR [114, 126]. On the other hand, MK-801 treatment of cultured astrocytes upregulated glycogen phosphorylase and regulated the expression of growth and metabolic genes [134]. Together, these observations indicate that NMDAR in cultured astrocytes provide signaling that help to maintain homeostasis. In line with this idea, ischemia increases NMDAR subunits, perhaps providing better tools for cell survival.

5. Conclusions

Research regarding NMDAR expression and function in astrocytes has been full of apparent contradictions and controversies and some of them still remain puzzling. Nevertheless, it now seems clear that tissue astrocytes do express NMDAR subunits that are assembled into functional receptors that mediate membrane currents, but are different from the well-studied neuronal NMDAR. On the other hand, cultured astrocytes also express NMDAR subunits, but it is well documented that NMDAR ligands do not elicit recordable membrane currents. However, few groups have found that NMDAR in cultured astrocytes evoke Ca^{2+} flux–independent metabotropic-like signaling, with the exception of human cultured astrocytes in which currents present certain singularities. Intriguingly, some reports have documented that long-term effects of NMDAR agonists in cultured astrocytes are prevented by the pore blocker MK-801. Thus, more research is needed to elucidate NMDAR function and its consequences in cultured astrocytes. Although NMDAR function in cultured astrocytes may represent a rare phenomena that result from culture conditions, it cannot be discarded *a priori* that it may occur in tissue astrocytes. This possibility is supported by NMDAR wide expression in non-CNS cells that could also be relevant for NMDAR Ca^{2+} flux–independent effects also observed in neurons.

Moreover, the apparent contradictions and controversies found in the last decades in the study of NMDAR in astrocytes together with its wide expression in cells and tissues let us realize that the biology of NMDAR is much more complex than that described for synaptic NMDAR. This seems to be true even within the synapse, where presynaptic NMDAR has casted new complexities in its synaptic function. Also, the NMDAR subunit phylogenetic conservation reinforces this idea. However, the understanding of NMDAR function in astrocytes and other cells and tissues may lead to envision new therapeutic strategies as has been suggested previously.

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