Received: 28 August 2020 | Revised: 10 November 2020 | Accepted: 3 December 2020

DOI: 10.1111/bph.15349

REVIEW ARTICLE

# Presynaptic release-regulating NMDA receptors in isolated nerve terminals: A narrative review

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The existence of presynaptic, release-regulating NMDA receptors in the CNS has been long matter of discussion. Most of the reviews dedicated to support this conclusion have preferentially focussed on the results from electrophysiological studies, paying little or no attention to the data obtained with purified synaptosomes, even though this experimental approach has been recognized as providing reliable information concerning the presence and the role of presynaptic release-regulating receptors in the CNS. To fill the gap, this review is dedicated to summarising the results from studies with synaptosomes published during the last 40 years, which support the existence of auto and hetero NMDA receptors controlling the release of transmitters such as glutamate, GABA, dopamine, noradrenaline, 5-HT, acetylcholine and peptides, in the CNS of mammals. The review also deals with the results from immunochemical studies in isolated nerve endings that confirm the functional observations.

**KEYWORDS**
GluN subunit, glutamate, glycine, presynaptic NMDA receptor, synaptosomes, transmitter release

## 1 | INTRODUCTION

The amount of transmitter released from nerve terminals does not exclusively depend on the efficiency of the mechanism of exocytosis but also on the activation by ambient ligands of receptors located presynaptically on nerve terminals (Langer, 1997, 2008; Raiteri, 2006). When focussing on the role of glutamate as a presynaptic modulator of transmitter release, it emerges that both metabotropic (mGlu) and ionotropic glutamate receptors mediate this function. However, although the presynaptic, release-regulating mGlu receptors have been largely described in term of functions and pharmacological profiles, the existence and the role of presynaptic ionotropic glutamate receptors, particularly the NMDA receptor subtypes, still remains matter of debate. First proposed as almost pure postsynaptic receptors, evidence was then provided supporting their presence also at the presynaptic level.

The first report suggesting the existence of presynaptic release-regulating NMDA receptors appeared in the late '70s, when Spencer (1976) showed that ligands of these receptors can modify transmitter outflow. More than 40 years later, the knowledge of these receptors has greatly increased, and there is, nowadays, a consensus on their existence and role in synaptic communication.

**Abbreviations:** [$^3$H]DA, [$^3$H]dopamine; 5,7-Cl-Kyna, 5-7-dichloro kynurenic acid; 7-Cl-Kyna, 7-chloro kynurenic acid; CCK-LI, cholecystokinin; CP-101,606, (1*S*,2*S*)-1-(4-hydroxy-phenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol; CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; D-AP5, D-2-amino-5-phosphonovaleric acid; D-AP7, D-(−)-2-amino-7-phosphonoheptanoic acid; DL-tBOA, DL-threo-*b*-Benzyloxyaspartate; GluN, glutamate NMDA; GlyT1, glycine transporter type 1; GS91755, cis-4-[phosphomethyl]-piperidine-2-carboxylic acid; HA-966, (±)3-amino-1-hydroxy-pyrrolidin-2-one; mGlu receptor, metabotropic glutamate receptor; NAc, nucleus accumbens; NFPS, (*N*-[3-(4¢-fluorophenyl)-3-(4-phenylphenoxy)propyl]) sarcosine hydrochloride; PKC, protein kinase C; RO25-6981, (α*R*,β*S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol maleate; SRIF-LI, somatostatin; TTx, tetrodotoxin.

In recent years, several reviews were dedicated to the NMDA receptors located presynaptically and to their role as release-regulating receptors, but almost all of them focussed on the electrophysiological observations supporting this conclusion, paying few attentions, if ever, to the data from isolated nerve endings (synaptosomes; Bouvier et al., 2018; MacDermott et al., 1999; Rodríguez-Moreno et al., 2010). To fill the gap, this review emphasizes the functional observations obtained with this tissue preparation that, starting from the late '80s, support the existence of release-regulating presynaptic NMDA receptors in the CNS. I apologize for omissions in the coverage of the existing literature.

## 2 | EXPERIMENTAL APPROACHES TO DETECT PRESYNAPTIC RELEASE-REGULATING NMDA RECEPTORS

As already mentioned, most of the articles seeking to prove the existence of the presynaptic NMDA receptors deal with electrophysiological studies measuring the effects of NMDA antagonists on the spontaneous and/or the miniature excitatory/inhibitory postsynaptic currents in slices (Banerjee et al., 2016; Corlew et al., 2008; Duguid, 2013; Pinheiro & Mulle, 2008). These studies provided evidence of the presence of presynaptic release-regulating NMDA receptors at defined subsets of synapses, mainly located in the cortex and the hippocampus (Buchanan et al., 2012; Crozier et al., 2007; Froemke et al., 2005; Larsen et al., 2014; McGuinness et al., 2010). The main shortcoming of this experimental approach, however, is that the NMDA-mediated responses also could involve (as related to presynaptic events) the signalling of postsynaptic NMDA receptors. The results are therefore correctly interpretable only if the postsynaptic receptors are blocked in advance (Berretta & Jones, 1996; Corlew et al., 2007; Brasier & Feldman, 2008; Woodhall et al., 2001; but see Rodríguez-Moreno & Paulsen, 2008).

Apart from slice preparations, preparations of purified nerve endings (synaptosomes), isolated from selected regions of the CNS were also used to confirm the existence of presynaptic NMDA receptors controlling transmitter exocytosis. The first findings from these experiments date from the late 80's/early 90's (Desce et al., 1992; Fink et al., 1989; Pittaluga & Raiteri, 1990). In general, in these studies, dynamic conditions (e.g., the up-down superfusion of a thin layer of synaptosomes; Raiteri et al., 1974) were applied to monitor the transmitter release from synaptosomes in the absence or in the presence of receptor ligands. These experimental conditions largely avoid the effects of the extracellular biophase, decreasing some of the disadvantages that mainly affect electrophysiological recordings (see for further technical information Raiteri & Raiteri, 2000; Pittaluga, 2016, 2019; Figure 1). This approach also permits the differentiation of spontaneous from depolarization-evoked, release of transmitters, a discrimination that cannot be easily achieved with other approaches.

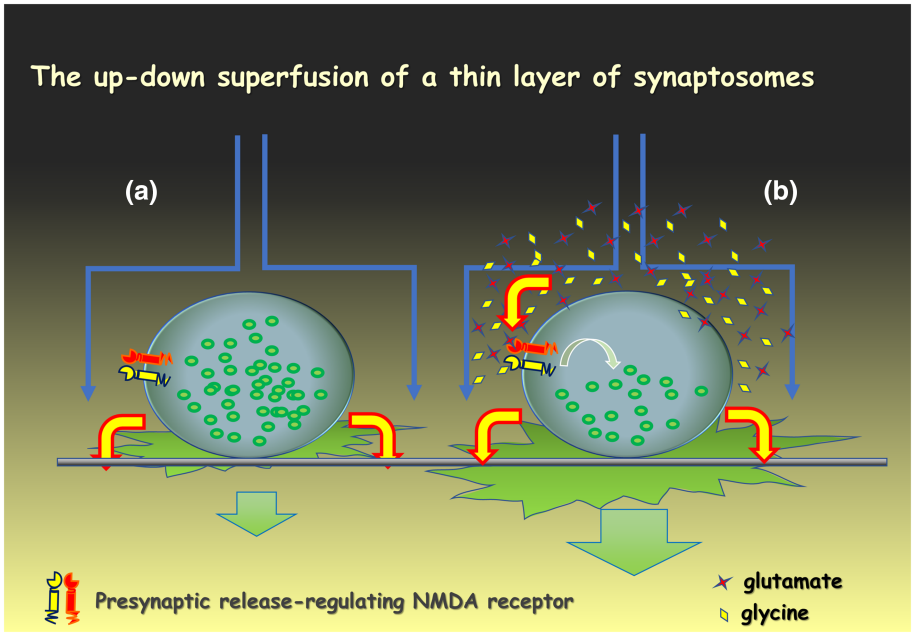


**FIGURE 1** The up-down superfusion of a thin layer of synaptosomes as first proposed by Maurizio Raiteri and colleagues in 1974. (a) The continuous flowing of the superfusion medium assures the quick removal of any endogenous substance released by the superfused particles, then minimizing the shortcomings due to the presence of the biophase (which has major effects on electrophysiological recordings in slices). (b) In this dynamic condition, presynaptic release-regulating receptors are activated by receptor ligands exogenously added to the superfusion medium. By acting at the respective binding sites (e.g., on the respective GluN subunits), the orthosteric agonists added to the superfusion medium (e.g., glutamate and glycine for the NMDA receptors) influence the molecular events controlling vesicular exocytosis, then causing significant changes to transmitter overflow that could be quantified and correlated with the activation of the presynaptic receptors. Notably, the technique also permits distinguishing between the spontaneous and the depolarization-evoked release of a selected transmitter, a discrimination that cannot be easily achieved in electrophysiological studies (see for technical information Raiteri & Raiteri, 2000)

However, synaptosomes also suffer from some technical limitations mainly due to the heterogeneity of the preparation. The synaptosomal suspension isolated from a selected CNS region consists of particles isolated from synaptic processes of different neuronal subpopulations that innervate the structure. Although monitoring the release of a selected transmitter allows the isolation, from a functional point of view, of a specific synaptosomal subpopulation, this heterogeneity limits (e.g., when the synaptosomal subpopulation represents a low percentage of the entire population) the use of immunochemical approaches to confirm the presence of the receptor proteins in a defined synaptosomal subpopulation. Actually, as for electrophysiological studies, the functional results from superfused synaptosomes should be accompanied by immunochemical results to confirm the presence of the receptor proteins in the superfused particles. Unfortunately, this is sometimes difficult, if even impossible, not only when monitoring the release of transmitter from a synaptosomal subpopulation poorly expressed within the entire synaptosomal suspension (as above introduced, but see Paragraph 4.2) but also when trying to purify synaptosomes from certain physically small-sized, subregions of the CNs, such as the L3/L4 layer of the somatosensory motor cortex).

## 3 | PRESYNAPTIC NMDA HETERORECEPTORS CONTROLLING DOPAMINE RELEASE

### 3.1 | Making short a long story: An historical perspective

The existence of presynaptic NMDA receptors controlling **dopamine** release emerged while dissecting the cortical glutamatergic projections to the dopaminergic striatal neurons. The iontophoretic application of glutamate to striatal neurons caused an excitatory response that was suppressed by L-glutamate diethylester, a compound with generalized antagonistic activity towards ionotropic glutamate receptors (Spencer, 1976). Soon later, Glowinski and collaborators (Giorguieff et al., 1977) and Roberts and Sharif (1978) demonstrated that glutamate stimulated [$^3$H]dopamine release in rat striatal slices through a **tetrodotoxin** (TTx)-resistant mechanism, suggesting that the cortico-striatal afferent terminals impinge directly onto the dopaminergic ones. Comparable results were obtained in slices containing dopaminergic nerve terminals (Cheramy et al., 1986; Marien et al., 1983; Snell & Johnson, 1986) or in dendritic processes of cultured cells (e.g., those isolated from the substantia nigra; Cheramy et al., 1981; Marien et al., 1983). Again, the releasing activity was TTx insensitive, indicating that voltage-sensitive **$Na^+$** channels were not involved. As a whole, the data were interpreted as indicating the existence of release-regulating glutamatergic receptors, although they did not allow these receptors to be located definitely on nerve terminals or on dendritic processes.

Trying to gain information on the pharmacological profile of the glutamate receptor(s), Roberts and Anderson (1979) (but see also Marien et al., 1983) found that NMDA mimicked glutamate in controlling the release of preloaded [$^3$H]dopamine in striatal slices. This observation led to propose the existence of NMDA heteroreceptors, presynaptically located on striatal dopaminergic processes. A few years later, this hypothesis was definitively proved by Glowinski's group (Desce et al., 1992; Krebs et al., 1991). The authors demonstrated that the release of newly synthetized [$^3$H]dopamine from striatal synaptosomes superfused with a medium lacking **$Mg^{2+}$** ions (to favour activation of NMDA receptors) was significantly augmented when synaptosomes were exposed to micromolar NMDA in the presence of saturating glycine. **Glycine** potentiated the releasing activity in a **strychnine**-insensitive manner, and the releasing activity was TTx resistant but prevented by **dizocilpine** or physiological amounts of magnesium ions in the biophase ($[Mg^{2+}]_{out}$). Comparable conclusions were described by Johnson and Jeng (1991) and by Wang (1991), confirming that striatal dopaminergic nerve endings are endowed with $Mg^{2+}$-sensitive, NMDA heteroreceptors controlling dopamine exocytosis (Tables 1 and 3). These studies also demonstrated the relevance of the sensitivity to the $Mg^{2+}$ ions as a pharmacological tool, to associate functional responses to the activation of NMDA receptors. Indeed, sensitivity to this divalent cation and to **dizocilpine (MK-801)** represents a major discriminating feature of the pharmacological properties of the NMDA receptors, first introduced by Meguro et al. (1992) and Mori et al. (1992).

### 3.2 | Distribution in the CNS

Presynaptic NMDA heteroreceptors controlling dopamine release are not limited to striatal synaptosomal preparations (Chéramy,

**TABLE 1** Effects of NMDA, glycine or NMDA+glycine on the spontaneous and the depolarization-evoked transmitter release

| | Spontaneous release | | | Depolarization-evoked release | | |
|---|---|---|---|---|---|---|
| | NMDA | Glycine | NMDA/glycine | NMDA | Glycine | NMDA/glycine |
| Dopamine | ↑ | n.e. | ↑↑ | n.d. | n.d. | n.d. |
| Noradrenaline | ↑ | n.e. | ↑↑ | n.d. | n.d. | n.d. |
| Acetylcholine | n.e. | n.e. | n.e. | ↑ | n.e. | n.d. |
| GABA | n.d. | n.d. | ↑ | n.d. | n.d. | n.d. |
| CCK-LI/SRIF-LI | n.e. | n.e. | n.e. | ↑ | ↑ | ↑; n.a. |
| Glutamate | ↑ | n.e. | ↑↑ | n.d. | n.d. | n.d. |

*Note.* ↑/↑↑, increased transmitter release; n.d., not determined; n.e., no effect; n.a., not additive.

Godeheu, L'Hirondel, & Glowinski, 1996; Desce et al., 1994; Keita et al., 1997; L'hirondel et al., 1999; Livingstone et al., 2010; Mennini et al., 1997; Pittaluga et al., 2001; Risso et al., 2004; Salamone et al., 2014; Whittaker et al., 2008), but also can be found in other CNS regions. In the hippocampus, a significant increase of the spontaneous release of [$^3$H]dopamine was observed when hippocampal synaptosomes were exposed to NMDA/glycine in the absence of $Mg^{2+}$ ions (Malva et al., 1994). Again, the marked sensitivity to dizocilpine suggested the involvement of presynaptic release-regulating NMDA receptors. Similarly, NMDA concentration dependently released [$^3$H]dopamine from synaptosomes isolated from the prefrontal cortex of adult rats, in a $Mg^{2+}$ and dizocilpine-dependent fashion, proving the involvement of the NMDA receptor subtype (Grilli et al., 2009). Notably, the existence of presynaptic NMDA receptors on dopaminergic striatal synaptosomes was also confirmed with non-functional approaches. In 2014, Salamone and colleagues described the immunocytochemical co-localization of the dopamine transporter (**DAT**; used as a selective marker of dopaminergic terminals), of the **GluN1** receptor subunit (to highlight the presence of NMDA receptors) and of synaptophysin (used as marker of presynaptic particles) in synaptosomes isolated from the nucleus accumbens (NAc) of adult rats.

### 3.3 | The pharmacological profile

Over the years, selective NMDA receptor agonists/antagonists acting at the different GluN subunits became available and were used to define the subunit composition of the NMDA receptors. These compounds allowed the characterization of the presynaptic NMDA receptors controlling dopamine release, and also allowed the association of particular GluN subunits with the presynaptic receptors.

The involvement of the GluN1 subunit was first proposed based on the high sensitivity of the receptor to ambient glycine. Low nanomolar glycine, unable by itself to elicit dopamine release, was required to permit the response mediated by NMDA, while increasing its concentration to micromolar level almost doubled the NMDA-induced signal (Krebs et al., 1991; Pittaluga et al., 2001). The allosteric activity of glycine was mimicked, although less efficiently, by **D-serine** (Table 2), it was insensitive to **strychnine** but prevented by selective antagonist(s) at the strychnine-insensitive, glycine-sensitive allosteric binding site of the NMDA receptors (Table 3), including 7-chlorokynurenic acid (7-Cl-Kyna), 5,7-dichlorothiokynurenic acid (5,7-Cl-Kyna) and (±)3-amino-1-hydroxy-pyrrolidin-2-one (**HA-966**; Krebs et al., 1991; Mennini et al., 1997; Pittaluga et al., 2001). Notably, 7-Cl-Kyna also prevented the release elicited by NMDA

**TABLE 2** Effects of glutamatergic and glycinergic agonists on the presynaptic release-regulating NMDA receptors

| | NMDA | Quinolinic acid | Glycine | D-serine |
|---|---|---|---|---|
| Dopamine | Full agonist | n.e. | Co-agonist | Co-agonist |
| Noradrenaline | Full agonist | Full agonist | Co-agonist | Co-agonist |
| Acetylcholine | Full agonist | n.d. | n.e. | n.d. |
| GABA | Full agonist | n.d. | Co-agonist | n.d. |
| CCK-LI/SRIF-LI | Full agonist | n.d. | Full agonist | Full agonist |
| Glutamate | Full agonist | Full agonist | Co-agonist | Partial agonist |

*Note*. n.d., not determined; n.e., no effect.

**TABLE 3** Effects of ions, pH and receptor antagonists on the NMDA auto and heteroreceptors

| | $Mg^{2+}$ ions | Dizocilpine | D-AP5CGS19755 | 7-Cl-Kyna | $Zn^{2+}$ ions | (*RS*)-CPP | Ifenprodil | pH 6.0 | pH 8.0 |
|---|---|---|---|---|---|---|---|---|---|
| NMDA heteroreceptors | | | | | | | | | |
| Dopamine (striatum) | ↓ | ↓ | ↓ | ↓. | n.e. | n.d. | ↓ | n.e. | n.e. |
| Dopamine (NAc) | ↓ | ↓ | ↓ | ↓. | n.e. | n.e. | ↓ | n.e. | n.e. |
| Noradrenaline | ↓ | ↓ | ↓ | ↓. | n.e. | n.d. | ↓ | ↓ | ↑ |
| Acetylcholine | n.e. | ↓ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| GABA | ↓ | ↓ | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| CCK-LI/SRIF-LI | n.e. | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| NMDA autoreceptors | | | | | | | | | |
| Hippocampus | n.e. | ↓ | ↓ | ↓ | ↓ | n.d. | ↓ | ↓ | ↑ |
| Nucleus accumbens | ↓ | ↓ | n.d. | ↓ | n.d. | ↓ | n.e. | n.d. | n.d. |
| Cortex | n.e. | ↓ | | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Spinal cord | ↓ | ↓ | n.d. | n.d. | ↓ | n.d. | ↓ | ↓ | n.d. |

*Note*. ↓, decreased transmitter release; n.d., not determined; n.e., no effect.

alone (in the absence of exogenously added glycine, Krebs et al., 1991), stressing a major role for ambient, contaminating glycine in disclosing the NMDA-mediated control of dopamine release.

GluN1 subunits exist in eight different splice variants, depending on the presence or the absence of the extracellular N1 cassette and of the intraterminal C1 and C2 sequences (Zukin & Bennett, 1995). The N1 sequence controls the pH sensitivity of the NMDA receptor. It blocks the pH sensor, limiting the sensitivity of the receptor to ambient protons. Studies dedicated to monitor the pH sensitivity of the NMDA receptors controlling dopamine exocytosis in striatal synaptosomes showed that the releasing activity was unmodified when the pH of the external milieu was either reduced to 6.6 or augmented to 8.0 (Pittaluga et al., 2001; Table 3). The observations seemed most consistent with the involvement of a GluN1 subunit containing the N1 sequence. As far as the C1 and C2 sequences are concerned, these cassettes contain **protein kinase C (PKC)**-sensitive phosphorylation sites. Unfortunately, selective ligands for these sites are not available, and PKC inhibitors cannot be used to predict their presence, because PKC-dependent phosphorylation also occurs in the **GluN2** subunits. It was therefore generically proposed that N1-containing GluN1 subunits (the $GluN1_{+xx}$ one; see, for the GluN1 subunit classification, Zukin & Bennett, 1995) were involved in the expression of these receptors, at least in striatal nerve endings (Pittaluga et al., 2001).

Besides the GluN1 subunits, NMDA heteroreceptors on dopaminergic nerve endings also consist of GluN2 subunits, as suggested by the finding that NMDA is pivotal to trigger the releasing activity. Accordingly, broad spectrum GluN2 antagonists (Table 3) including D-2-amino-5-phosphonovaleric acid (**D-AP5**) and *cis*-4-[phosphomethyl]-piperidine-2-carboxylic acid (**CGS19755**, selfotel) prevented release (Chéramy et al., 1996; Mennini et al., 1997; Pittaluga et al., 2001). The NMDA receptors controlling dopamine release are insensitive to quinolinic acid and only slightly affected by kynurenic acid (Pittaluga et al., 2001). Furthermore, they were unaffected by nanomolar zinc ions (Paoletti & Neyton, 2007) or (*RS*)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid [(*RS*)-CPP], two **GluN2A**-preferring NMDA antagonists, but they were sensitive to ligands acting at the **Glu2B** subunits (viz., **infenprodil**, (1*S*,2*S*)-1-(4-hydroxy-phenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol; **CP-101,606**, traxoprodil) and (α*R*,β*S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol maleate, Ro 25-6981; Mennini et al., 1997; Pittaluga et al., 2001; Salamone et al., 2014; Table 3). As for the **GluN2C** and **GluN2D** subunits, the lack of selective ligands for these two subunits limited at that time the study of their participation in the NMDA receptor assembly. The involvement of the GluN2C, however, seems unlike considering its almost negligible expression in several CNS regions, including the striatum (Harney et al., 2008; Misra et al., 2000). However, the GluN2D subunit is developmentally regulated in the striatum, becoming almost undetectable at 49 days postnatally. Furthermore, if present, it preferentially locates extrasynaptically (in axonal and dendritic processes, far from the synaptic active zone), being absent from synapses in striatal neurons (Dunah et al., 1996), indirectly ruling out its participation in the presynaptic NMDA heteroreceptor.

Whether the NMDA heteroreceptors controlling dopamine release also involve the **GluN3** subunit was not investigated, but the available data allow some speculations. Our knowledge of the GluN3 subunit has greatly increased in recent years and, today, we know that this subunit also exists presynaptically (Larsen et al., 2011; Musante et al., 2011; Olivero, Vergassola, Cisani, Usai, & Pittaluga, 2019). The GluN3-containing NMDA receptors were proposed to preferentially consist of GluN1/GluN3 and of GluN1/GluN2/ GluN3 assemblies. The former assembly is activated by glycine alone, whereas the latter also requires glutamate to exert its function and it is potentiated by HA-966, a mimic of glycine (Al-Hallaq et al., 2002; Perez-Otano et al., 2001; Pérez-Otaño et al., 2016; Smothers & Woodward, 2007). Taking into consideration that NMDA receptor-evoked release of dopamine involves a glutamate-sensitive receptor whose releasing activity is antagonized by HA-966 (Mennini et al., 1997), it seems reasonable to exclude the participation of the GluN3 subunit in the NMDA heteroreceptors.

### 3.4 | The sensitivity to $Mg^{2+}$ ions

The NMDA receptors on dopaminergic terminals are highly sensitive to the presence of $Mg^{2+}$ in the external medium, because physiological concentrations of the divalent cation blocks their releasing activity (Krebs et al., 1991; Risso et al., 2004). The question therefore arises whether and how these receptors could modulate dopaminergic transmission in physiological conditions, for example, in the presence of high $[Mg^{2+}]_{out}$. It is known that the concomitant application of a depolarizing stimulus, such as that elicited by co-localized non-NMDA receptors (Desce et al., 1992) as well as other non-glutamatergic ionotropic receptors (viz., nicotinic receptors; Risso et al., 2004; Marchi & Grilli, 2010), allows the ionic block to be overcome, restoring the releasing activity. Several reviews already focused on these aspects will be not further discussed in this review (see Marchi & Grilli, 2010; Marchi et al., 2015).

## 4 | PRESYNAPTIC NMDA HETERORECEPTORS CONTROLLING NORADRENALINE RELEASE

### 4.1 | A non-synaptic release-regulating NMDA receptor

By the end of the 1980s, two groups provided functional evidence of the existence of presynaptic NMDA heteroreceptors controlling **noradrenaline** release from hippocampal nerve terminals (Fink et al., 1989; Pittaluga & Raiteri, 1990). These results were soon later confirmed by other groups (Malva et al., 1994; Wang et al., 1992), which also extended the study to other CNS regions, including the olfactory bulb (Wang et al., 1992), the spinal cord (Klarica et al., 1996; Nankai et al., 1998; Sundström et al., 1998 and the cortex of rodents (mice and rats; Fink et al., 1989; Wang et al., 1992; Pittaluga

et al., 1996; Gemignani et al., 2004; Longordo et al., 2006) and humans (Pittaluga & Raiteri, 1994). Because noradrenergic processes from the locus coeruleus do not make synaptic contact in the regions they innervate, these presynaptic NMDA receptors would be better defined as extrasynaptic receptors (Langer, 2008) that sense glutamate reaching the noradrenergic nerve endings and varicosities by the mechanisms of 'volume diffusion' (Pittaluga, 2016; Vizi et al., 2004) to modulate noradrenaline exocytosis.

### 4.2 | The subunit composition and the pharmacological profile

At a first glance, the pharmacological profile of the NMDA receptors controlling noradrenaline release largely resembles that of the NMDA receptors on dopaminergic terminals (see Table 1–3). In both cases,

i. the releasing activity is detectable in basal condition by omitting external $Mg^{2+}$ ions (Fink et al., 1989; Pittaluga et al., 2000; Pittaluga & Raiteri, 1990, 1992a);
ii. unavoidable contamination of glycine in the experimental solutions allows receptor activation (low nM; Longordo et al., 2006; Pittaluga & Raiteri, 1990; Raiteri et al., 1992, 1993, 2000), whereas higher, micromolar, concentrations reinforce the NMDA-mediated releasing activity (Pittaluga et al., 1993, 1996; Pittaluga & Raiteri, 1992b);
iii. channel blockers including dizocilpine and memantine prevent, although to a different extent, the releasing activity (Pittaluga et al., 1996; Pittaluga & Raiteri, 1994);
iv. the analysis of the subunit composition suggested the involvement of both the GluN1 and GluN2 receptor subunits (Malva et al., 1994; Pittaluga et al., 2001).

The NMDA receptors on noradrenergic terminals, however, differ from those in dopaminergic nerve endings in their GluN1 composition. 7-Cl-Kyna and HA-966 behaved as receptor antagonists, whereas D-serine and **D-cycloserine** potentiated, although less efficiently than glycine, the NMDA-induced effect. (Longordo et al., 2006; Pittaluga & Raiteri, 1990, 1992b; Table 2). The marked sensitivity to glycine led Wang and colleagues to hypothesize that the presynaptic NMDA receptors on noradrenergic terminals could consist of an oligomeric complex of GluN1 subunits (Wang et al., 1992; Wang & Thukral, 1996). This hypothesis, however, contrasted with the agonist-like activity of glutamate and glutamate agonist(s) reported by other groups (Fink et al., 1989; Malva et al., 1994; Pittaluga & Raiteri, 1990). Conflicting results were also obtained when studying the proton sensitivity of these NMDA receptors. Wang provided evidence suggesting a low sensitivity to external protons (Wang et al., 1992). However, we found a marked dependency on the external pH, the releasing activity of these heteroreceptors being reduced at pH ≅ 6.6, but significantly potentiated at pH ≅ 8.0 (Pittaluga et al., 2001), most consistent with the presence of GluN1 subunits bearing the N1 cassette (the $GluN1_{-xx}$, Zukin & Bennett, 1995), which makes the receptors in noradrenergic terminals different from that controlling dopamine exocytosis (Table 3).

As far as the GluN2 subunit is concerned, the participation of this subunit was postulated because either NMDA or quinolinic acid elicits a dizocilpine-sensitive noradrenaline release that was prevented by broad spectrum GluN2 antagonists (e.g., **kynurenic acid**, D-AP5 and CGS19755). To note, selective GluN2B antagonists (e.g., ifenprodil and CP-101,606) mimicked D-AP5, whereas nanomolar **$Zn^{2+}$** ions, which selectively block GluN2A subunits, did not, suggesting that GluN2B rather than the GluN2A subunits are most likely to be involved in the receptor composition (Table 3; Malva et al., 1994; Pittaluga & Raiteri, 1992b; Pittaluga et al., 2001). The proposed composition in GluN subunits, however, cannot be confirmed with immunochemical studies because of the low percentage (less than the 1%) of the noradrenergic terminals in the hippocampal synaptosomal preparation. Furthermore, there are no published data concerning the possible participation of GluN2C and D subunits.

An unexpected finding was that the potency and the efficiency of GluN2B antagonists and channel blockers as well, mainly depend on the mode of activation of the NMDA heteroreceptors. As already discussed for the NMDA receptors on dopaminergic terminals (paragraph 3.4), the $Mg^{2+}$ blockade of the NMDA receptors can be overcome by applying a concomitant depolarizing stimulus that permit the opening of the voltage-dependent ion channel associated with the NMDA receptors. The local depolarization could be achieved by activating AMPA receptors co-localized with NMDA receptors. Such AMPA-induced activation of NMDA receptors, however, triggers conformational changes to the NMDA receptors in noradrenergic terminals that are expressed as increased affinity of GluN2 antagonists (e.g., D-AP5) and of dizocilpine at the receptors (Luccini, Musante, Neri, Brambilla Bas et al., 2007; Pittaluga & Raiteri, 1992a, 1992b; Raiteri et al., 1992). These pharmacodynamic adaptations were not observed in dopaminergic synaptosomes, further strengthening the conclusion that the presynaptic NMDA receptors in noradrenergic and dopaminergic terminals have different pharmacological and functional profiles. The detailed mechanism(s) underlying these conformational changes remains so far unexplored, although modifications in the GluN compositions as well as in the phosphorylation of the GluN subunits could play a role (Dorville et al., 1992; Zuo et al., 1993).

## 5 | PRESYNAPTIC NMDA HETERORECEPTORS CONTROLLING 5-HT RELEASE

The existence of presynaptic release-regulating NMDA receptors in 5-hydroxytryptaminergic nerve endings has been poorly investigated. In 1995, Manfred Gothert and colleagues demonstrated that in cortical slices pre-incubated with [$^3$H]**5-HT** and superfused with a solution from which $Mg^{2+}$ was removed, NMDA elicited the release of radioactive tracer in a concentration-dependent fashion (Fink et al., 1995). The NMDA-mediated releasing activity was prevented by the (*E*)-2-amino-4-methyl-5-phosphonopent-3-enoic acid (**CGP37894**), an antagonist

acting at the glutamate GluN2 subunit, by 5,7-Cl-Kyna and by dizocilpine. Furthermore, it was largely insensitive to TTx, suggesting that it was located in nerve endings. Finally, omission of external $Ca^{2+}$ ions almost totally prevented the releasing activity, compatible with the conclusion that the NMDA releasing activity relies on the influx of external $Ca^{2+}$ ions (the authors speculated through a voltage-dependent associated ionic channel located near the glutamate receptor). These results led the authors to propose the existence of presynaptic release-regulating NMDA receptors in cortical 5-hydroxytryptaminergic nerve endings. Because the NMDA-induced releasing activity was largely prevented by ifenprodil and eliprodil, the authors also hypothesized that GluN1/GluN2B receptor assemblies mainly account for these NMDA receptors (Fink et al., 1995, 1996). To the best of my knowledge, however, other results supporting the existence of NMDA controlling 5-HT release from synaptosomes have not been published.

## 6 | PRESYNAPTIC NMDA HETERORECEPTORS CONTROLLING GABA RELEASE

The existence of presynaptic release-regulating NMDA receptors in GABAergic axon terminals was first inferred with electrophysiological studies. Bath application of NMDA increased the frequency of spontaneous inhibitory synaptic currents (Farrant & Cull-Candy, 1991; Llano et al., 1991) and this effect was prevented by D-AP5, but it was resistant to TTx (Glitsch & Marty, 1999). The conclusion was also supported by results obtained in cultured cells from the retina and the cortex, showing that NMDA elicites a $Na^+$-dependent, $Ca^{2+}$-insensitive release of [$^3$H]GABA release from a cytosolic intracellular pool (Belhage et al., 1993; Duarte et al., 1993). The releasing activity was proposed to rely on $Na^+$ ions flowing through the ionic channel associated with the NMDA receptor, which might have caused a local depolarization of plasma membranes, thus favouring the reversal of the GABA transporters and the release of GABA from the cytosolic pool (Levi & Raiteri, 1993).

Soon after, it was proposed that presynaptic NMDA receptors can enhance GABA release at cerebellar-interneurons Purkinje cells synapses (Duguid & Smart, 2004). The activation of these presynaptic NMDA receptors elicited a long-lasting increase in GABA release from stellate neurons that enhanced the frequency of spontaneous inhibitory postsynaptic currents through a mechanism involving the $Ca^{2+}$-induced $Ca^{2+}$- release from intra-terminal stores (Rossi et al., 2012; see Moreau & Kullmann, 2013). NMDA receptors were also proposed to have a role in the facilitation of GABA release between multipolar interneurons and pyramidal cells (De-May & Ali, 2013).

As far as the results with synaptosomes are concerned, the existence and the role of the NMDA receptors controlling GABA release is still a matter of debate. In 1993, we could not observe an NMDA-mediated releasing activity in cortical synaptosomes (Bonanno et al., 1993) which, on the contrary, was described by Breukel and colleagues in synaptosomes isolated from the hippocampus (Breukel et al., 1998). Exposure of hippocampal synaptosomes in superfusion to NMDA in the presence of saturating glycine but in the absence of external $Mg^{2+}$ significantly increased the spontaneous release of endogenous GABA in a dizocilpine-sensitive manner (Tables 1–3). The releasing activity was independent on external $Ca^{2+}$ ions but dependent on the reversal of the amino acid transporter. Further studies are needed to investigate the cellular events accounting for the release of the inhibitory amino acid elicited by the NMDA receptors.

## 7 | PRESYNAPTIC NMDA HETERORECEPTORS CONTROLLING ACETYLCHOLINE RELEASE

The existence of presynaptic release-regulating NMDA heteroreceptors controlling acetylcholine release first originated from studies in slices (Lehmann et al., 1983; Lehmann & Scatton, 1982; Ransom & Deschenes, 1989; Scatton & Lehmann, 1982). Evidence was then provided showing that presynaptic glutamate receptors belonging to the NMDA receptor subtype are present at the developing neuromuscular synapses of *Xenopus*, where they promote the release of acetylcholine (Fu et al., 1995). Soon after these findings, Morari et al. (1998) proved the existence of presynaptic NMDARs controlling the release of [$^3$H]acetylcholine from striatal synaptosomes. The authors demonstrated that NMDA did not affect the basal release of [$^3$H]acetylcholine but significantly potentiated the overflow of ACh elicited by a mild depolarizing stimulus (Table 1). Such NMDA-induced effects occurred in a concentration-dependent manner, and they were blocked by low micromolar concentrations of dizocilpine, despite the presence of $Mg^{2+}$ ions (Table 3). The NMDA-mediated facilitation of [$^3$H]acetylcholine exocytosis was controlled by co-localized metabotropic $mGlu_{2/3}$ receptors (Mela et al., 2006; Marti et al., 1999; Marti et al., 2003), further supporting the proposed presynaptic location of the NMDA receptors under study and the exocytotic nature of the transmitter release.

As to the subunit composition of these NMDA receptors, the available data do not allow any conclusion. Morari et al. (1998) demonstrated that GluN1 proteins exist in striatal synaptosomes and that GluN1 co-immuno-precipitate with GluN2B subunits. However, the acetylcholine exocytosis evoked by low micromolar NMDA was unaffected by exogenous glycine (Table 2) posing the question of the functional role of the GluN1 protein, if present, in dictating the function of the presynaptic NMDA receptors (Morari et al., 1998). Furthermore, data concerning the effects of GluN2-selective ligands are so far lacking.

## 8 | PRESYNAPTIC NMDA HETERORECEPTORS CONTROLLING THE RELEASE OF PEPTIDES: A NON-CONVENTIONAL PRESYNAPTIC NMDA HETERORECEPTOR

The available data in the literature suggests the existence of presynaptic NMDA receptors controlling the release of cholecystokinin

(CCK-LI) and somatostatin (SRIF-LI) in cortical synaptosomes (Gemignani et al., 2000; Paudice et al., 1998) having comparable pharmacological profiles.

NMDA as well as glycine failed to modify, by themselves, the spontaneous release of both peptides but significantly potentiated their exocytosis elicited by a mild depolarizing stimulus. These observations were best interpreted by assuming that both the somatostatinergic and the cholecystokinergic terminals express presynaptic NMDA receptors whose activation increases the availability of $Ca^{2+}$ ions in the cytosol to a level insufficient, per se, to trigger peptide overflows, but sufficient to reinforce the depolarization-evoked, $Ca^{2+}$-dependent exocytosis. NMDA or glycine-mediated effects occurred in a concentration-dependent manner but were not additive (Tables 1 and 2).

The NMDA- or glycine-induced facilitation of peptides exocytosis was significantly reduced by antagonists acting at either the glutamate binding site (D-AP5 or CGS19755) or the glycine binding site (7-Cl-Kyna), respectively, which could suggest the presence of a classic GluN1/GluN2 heteromeric receptor assembly. Furthermore, the agonist-evoked facilitation of peptides exocytosis was prevented by ifenprodil and $Zn^{2+}$ ions, compatible with the participation of both GluN2A and GluN2B subunits to the NMDA complex (Table 3). Data on the effects of selective GluN2C or GluN2D antagonists are not available, but the low expression of these subunits in the cortex of adult mice would limit their involvement in the NMDA receptor assemblies.

These NMDA heteroreceptors were insensitive to the presence of $Mg^{2+}$ ions but were inhibited by non-competitive antagonists acting at the associated voltage-dependent ionic channel (e.g., (*RS*)-CPP, dizocilpine, memantine). D-serine mimicked glycine in potentiating the exocytosis of CCK-LI and SRIF-LI from cortical synaptosomes in an ifenprodil and $Zn^{2+}$ ions-sensitive manner (Table 2). Finally, lowering the external pH to 6.4 significantly reduced the NMDA-evoked facilitation of peptide exocytosis. Unexpectedly, the external alkalinisation at pH = 8.0 also reduced the facilitation elicited by NMDA or by D-serine (Table 3), leaving unsolved the question on the involvement of GluN1 subunits lacking the N1 cassette (e.g., the GluN1-1a subunit for instance) in the NMDA receptor assembly (Gemignani et al., 2004). Based on these observations, it was proposed that cortical nerve terminals releasing SRIF-LI and CCK-LI possess 'non-conventional' NMDA receptors typified by a tri-heteromeric GluN1/GluN2A/GluN2B assembly that can be operated by glycinergic agonists in the apparent absence of the glutamate co-agonist, but in the presence of physiological amounts of $Mg^{2+}$ ions (Paudice et al., 1998).

At that time, the involvement of the GluN3 subunits in the expression of NMDARs was not taken in consideration, but the available data, particularly the fact that glycine acts as a full agonist, allow some speculation on this regard. As already mentioned (see Paragraph 3.3), the GluN3-containing NMDA receptors are roughly subdivided in GluN1-GluN2-GluN3 subunits assembly, (the so-called GluN3-NMDA receptors) and in GluN1-GluN3 subunit complexes (Al-Hallaq et al., 2002; Pachernegg et al., 2012; Perez-Otano et al., 2001, 2016; Smothers & Woodward, 2007). As far as the stoichiometry of the GluN3-NMDA receptor subtypes is concerned, it is generally accepted that it can vary depending on the neuronal subpopulations and the CNS regions involved (Paoletti, 2011; Pilli & Kumar, 2012; Stroebel et al., 2017). In general, however, these receptors are typified by low $Ca^{2+}$ permeability and are relatively insensitive to external $Mg^{2+}$ ions and glycine, and some glycine agonists act as full agonists at the receptor in a 5,7-Cl-Kyna-sensitive manner. However, the GluN1-GluN3 complexes are not activated by NMDA or glutamate, they are insensitive to D-AP5 and to the channel blockers ($Mg^{2+}$, dizocilpine, memantine, (*RS*)-CPP), and they behave as glycine-gated receptors, glycine acting as a full agonist and D-serine as a partial agonist (Chatterton et al., 2002; Henson et al., 2012; Pachernegg et al., 2012). Finally, protons potentiate the glycine-mediated responses at the GluN1/GluN3 complexes but inhibit the GluN3-NMDA receptors (Cummings & Popescu, 2015).

The possibility that the NMDA receptors controlling SRI-LI and CCK-LI exocytosis could belong to the 'glycine-sensitive' GluN1/GluN3A NMDAR subtype seems unlikey because (i) it is operated by NMDA and (ii) the external acidification would be expected to potentiate instead of inhibit the receptor-mediated releasing activity, as indeed observed. Rather, the pharmacological profile of the NMDA receptors controlling SRIF-LI and CCK-LI exocytosis, particularly their sensitivity to glycine and the inhibitory effect exerted by protons, seems most consistent with the conclusion that these receptors belong to the GluN3- NMDA receptor subtypes. In line with this view, the GluN3A subunit expression is high in somatostatin-containing GABAergic interneurons in the cortex, to a level that makes the Grin3a mRNA an efficient marker, distinguishing these cells from other interneurons in this CNS region (Matsuda et al., 2003; Wee et al., 2008). Further studies are needed to verify the hypothesis.

## 9 | PRESYNAPTIC NMDA AUTORECEPTORS CONTROLLING THE RELEASE OF GLUTAMATE FROM NERVE ENDINGS

### 9.1 | Presynaptic NMDA autoreceptors in the hippocampus

The first data suggesting the existence of presynaptic release-regulating NMDA autoreceptors originated from studies in rat hippocampal CA1 slices (Chapman & Bowker, 1987; Connick & Stone, 1988; Crowder et al., 1987; Martin et al., 1991; Nadler et al., 1990) showing that NMDA antagonists reduce the exocytosis of glutamate/aspartate elicited by high-$K^+$, while NMDA did not evoke any measurable release under depolarized or basal conditions, despite the omission of $Mg^{2+}$ ions in the external milieu. It was proposed that the endogenous glutamate could have saturated the respective binding site at the NMDA receptors, disabling exogenous NMDA agonists to activate the autoreceptors, but permitting the competition of NMDA antagonists at the agonist binding site, indirectly showing the activity of the endogenous amino acid as enhancer of its own release through

NMDA autoreceptors. Starting from these findings, several electrophysiological recordings in hippocampal slices (Mameli et al., 2005; Madara & Levine, 2008; McGuinness et al., 2010; reviewed by Duguid, 2013; Pinheiro & Mulle, 2008) supported the existence of NMDA receptors in different hippocampal subregions (Dalby & Mody, 2003; Madara & Levine, 2008; Mameli et al., 2005; Martin et al., 1991; McGuinness et al., 2010; Nadler et al., 1990; see Duguid, 2013; Pinheiro & Mulle, 2008).

The presence of functional NMDA receptors in CA1 glutamatergic terminals was then confirmed by using synaptosomes (Table 1). The release of aspartate, but not glutamate, elicited by a mild depolarizing stimulus from these terminals was reinforced by NMDA in a D-AP5-sensitive manner (Zhou et al., 1995). Next, Breukel et al. (1998) provided evidence of a $Ca^{2+}$-independent NMDA-mediated release of both aspartate and glutamate from a non-vesicular, cytosolic pool. The authors proposed that the NMDA autoreceptors could be located near glutamate transporters and that the proximity of the two proteins would permit their functional crosstalk, leading to an NMDA-induced reversal of the carrier and to the $Ca^{2+}$-independent outflow of the amino acid from the cytosolic pools. The existence of the NMDA autoreceptors was then confirmed in both rat and mice hippocampal synaptosomes (Luccini, Musante, Neri, Raiteri, & Pittaluga, 2007; Musante et al., 2011; Olivero, Vergassola, Cisani, Usai, & Pittaluga, 2019; Sequeira et al., 2001). The results indicated that NMDA in the presence of saturating glycine potentiated the spontaneous release of glutamate in a concentration-dependent manner through different molecular events strictly dependent on the concentration of the agonist. Low micromolar NMDA caused a transporter-independent, $Ca^{2+}$- and $Na^{+}$-dependent overflow of glutamate, whereas higher concentrations led to the reversal of the glutamate transporter and to the carrier-mediated DL-threo-*b*-benzyloxyaspartate (**DL-tBOA**)-sensitive glutamate overflow (Luccini, Musante, Neri, Raiteri, & Pittaluga, 2007).

### 9.1.1 | The glutamate binding site

The involvement of the GluN2 subunit in the expression of the NMDA autoreceptors was shown by the results with selective GluN2 ligands. Quinolinic acid released glutamate, although less efficiently than NMDA, whereas broad-spectrum antagonists acting at the glutamate binding site (D-AP5, D-(−)-2-amino-7-phosphonoheptanoic acid, D-AP7) inhibited the NMDA-evoked glutamate exocytosis from hippocampal slices and synaptosomes (Tables 2 and 3; Chapman & Bowker, 1987; Connick & Stone, 1988; Crowder et al., 1987; Luccini, Musante, Neri, Raiteri, & Pittaluga, 2007). Furthermore, low (nM) concentration of $Zn^{2+}$ ions (which selectively block the GluN2A subunit) and ifenprodil (that specifically targets the GluN2B subunits) prevented the NMDA-evoked glutamate exocytosis almost to a comparable extent (Table 3; Luccini, Musante, Neri, Raiteri, & Pittaluga, 2007; Musante et al., 2011; Olivero, Vergassola, Cisani, Usai, & Pittaluga, 2019). Comparable effects were also observed when incubating synaptosomes with antibodies recognizing the outer sequence of the GluN2A and of the GluN2B receptor proteins (which mimic antagonists at the respective GluN subunit in an experimental approach called the 'immunopharmacological approach' (see Di Prisco et al., 2012; Olivero et al., 2017; Olivero, Vergassola, Cisani, Roggeri, & Pittaluga, 2019; Olivero, Vergassola, Cisani, Usai, & Pittaluga, 2019).

Western blot analysis and confocal microscopy confirmed the presence of the GluN2A and the GluN2B subunits in both rats and mice hippocampal synaptosomes (Henson et al., 2012; Musante et al., 2011; Olivero, Vergassola, Cisani, Usai, & Pittaluga, 2019), particularly in the presynaptic component of the synaptic active zone that was isolated by applying a technique of subfractioning of synaptosomal lysates (see Feligioni et al., 2006; Phillips et al., 2001). Notably, in this fraction, the GluN2A and the GluN2B subunits were shown to physically associate, consistent with their co-participation in the NMDA autoreceptor (Musante et al., 2011). The possible participation of the GluN2C and the GluN2D subunits was not investigated due to their low, almost undetectable, expression in the hippocampal synaptosomal lysates (Olivero, Vergassola, Cisani, Usai, & Pittaluga, 2019).

### 9.1.2 | The glycine binding site

The participation of the GluN1 subunit (e.g., the $GluN1_{1xx}$; Zukin & Bennett, 1995) in the expression of the NMDA autoreceptors in hippocampal synaptosomes is supported by the finding that (i) glycine reinforces the NMDA-evoked release of glutamate in a concentration-dependent fashion; (ii) D-serine mimics, although less efficiently, glycine; (iii) 7-Cl-Kyna totally prevented the NMDA-mediated releasing activity, independently of the concentration of the co-administered glycine; (iv) increasing the concentration of external protons hampers the NMDA-evoked release efficiency (Tables 2 and 3; Luccini, Musante, Neri, Raiteri, & Pittaluga, 2007; Musante et al., 2011; Olivero, Vergassola, Cisani, Usai, & Pittaluga, 2019). These observations, however, also questioned the possible involvement of GluN3 subunits in the expression of the NMDA autoreceptors. In particular, the dual effect exerted by D-serine at these receptors (Table 2, e.g.; it potentiated NMDA-induced exocytosis in the presence of low concentration of glycine but significantly reduced that in the presence of saturating glycine; Musante et al., 2011; Smothers & Woodward, 2007) seemed to indicate participation of this subunit in the receptor complex.

In line with this hypothesis, incubation of hippocampal synaptosomes with an antibody recognizing the outer $NH_2$-terminal sequence of the GluN3A protein significantly reduced the NMDA-evoked release of glutamate, supporting evidence for the presence of this subunit in the receptor complex. Western blot analysis of the total hippocampal synaptosomal lysates and of the presynaptic synaptosomal fraction disclosed the presence of the GluN1 and GluN3A, but not of the GluN3B, immunoreactivities in both rats and mouse hippocampal preparations (Musante et al., 2011). Furthermore, the anti-GluN2B immune precipitate from the presynaptic component of the

hippocampal synaptosomes was immuno-positive for GluN1 and for GluN3A subunits, consistent with the physical interaction linking the GluN1 and the GluN3A proteins with the GluN2B one. Finally, confocal microscopy showed that mouse hippocampal synaptosomes immuno-positive for the **vesicular glutamate transporter 1 (VGLUT1)** and syntaxin-1A, also displayed immunolabelling for the GluN1 and the GluN3A proteins (Henson et al., 2012; Musante et al., 2011; Olivero, Vergassola, Cisani, Usai, & Pittaluga, 2019). Overall, these findings seemed best interpreted by assuming that the NMDA autoreceptors consist of GluN1-GluN2-GluN3 subunits. How GluN3A subunits fit into the NMDAR assembly remains, however, an unanswered question, although the possibility should be considered that GluN1 subunits associate with GluN2 subunits to form a tetra-heterodimeric complex (GluN1/GluN2A/GluN2B, Stroebel et al., 2017), which further evolve to a multimeric form including GluN3 subunits (Stys & Lipton, 2007).

### 9.1.3 | The sensitivity of the hippocampal NMDA autoreceptors to external cations

In 1998, Breukel and colleagues demonstrated that NMDA efficiently released glutamate and aspartate from CA1 synaptosomes despite the presence in the superfusion medium of physiological concentrations of $Mg^{2+}$ ions. The authors proposed that the voltage-dependent ionic channel associated with the NMDA receptors could have structural characteristics that permit the influx of cations having low mass (e.g., $Na^+$ ions), favouring a local depolarization of the plasma membranes and the consequent reversal of the glutamate carrier-mediated functions. More than a decade later, Musante et al. (2011) confirmed the low sensitivity of the NMDA autoreceptor to external $Mg^{2+}$ ions and its modulation by $Na^+$ and $Ca^{2+}$ ions in the external milieu (Breukel et al., 1998; Luccini, Musante, Neri, Raiteri, & Pittaluga, 2007; Musante et al., 2011). The authors however provided evidence that the $Na^+$ dependency of the releasing activity also relied on mechanisms of 'metamodulation' of the presynaptic NMDA receptors triggered by the activation of co-localized glycine transporter type 1 (**GlyT1**; Musante et al., 2011) that was permissive for the NMDA-mediated releasing activity.

In general, it is proposed that GlyT1s are located near NMDA receptors and modulate its function by maintaining the concentration of $[glycine]_{out}$ at sub-saturating levels (Aragón & López-Corcuera, 2005; Raiteri & Raiteri, 2010). GlyT1, however, is an electrogenic transporter, which provides a continuous influx of 2 $Na^+$ and 1 $Cl^-$ ions, while taking up glycine. Therefore, beyond controlling the concentration of external glycine, the transporter also depolarizes plasma membranes because of the net influx of positive charges that parallels its activity. We found that the NMDA-evoked releasing effect was abolished by the concomitant presence of the GlyT1 blocker *N*-[3-(4¢-fluorophenyl)-3-(4-phenylphenoxy)propyl] (**NFPS**, sarcosine hydrochloride), consistent with the view that GlyT1 'metamodulates' NMDA autoreceptors, favouring its releasing activity (Musante et al., 2011).

## 9.2 | Presynaptic release-regulating NMDA autoreceptors in other region of the CNS

Presynaptic release-regulating NMDA autoreceptors were reported to exist in several regions of the CNS (Banerjee et al., 2016; Bouvier et al., 2015; Bouvier et al., 2018; Corlew et al., 2008; Fan et al., 2014; Pinheiro & Mulle, 2008), and the studies involving synaptosomes support the existence of presynaptic NMDA autoreceptors in rat NAc (Zappettini et al., 2014), in mouse cortex (Marcelli et al., 2019; Nisticò et al., 2015) and spinal cord (Fariello et al., 2014).

As to the NMDA autoreceptors in glutamatergic nerve endings isolated from the NAc (Zappettini et al., 2014), these receptors co-localize with **nicotinic receptors** that are permissive to the NMDA-mediated releasing activity in physiological conditions (the presence of physiological $Mg^{2+}$ ions). In this case, the results from release studies were supported by data showing a significant increase of $Ca^{2+}$ ions in the synaptosomes exposed to NMDA and glycine, as well as by immunocytochemical data showing the co-localization of the GluN1 subunit protein and VGLUT1. In contrast to the NMDA receptors in hippocampal nerve endings, those in synaptosomes from the NAc were sensitive to (*RS*)-CPP and Ro256981, two GluN2A-preferring antagonists, but insensitive to ifenprodil (Zappettini et al., 2014), suggesting the existence of autoreceptors composed of the GluN1/GluN2A assembly in these terminals (Table 3).

However, the NMDA autoreceptors in spinal cord nerve endings consisted of a tri-heteromeric assembly of GluN1, GluN2A and the GluN2B subunits, as observed in the hippocampal terminals. Quite interestingly, in this case, lowering the external pH to 6.8 drastically reduced the releasing activity, while increasing it to 8.0 did not affected the receptor-mediated event. Whether these results could suggest the possible involvement of a GluN3 subunit remains to be investigated (Table 3; Fariello et al., 2014).

## 10 | CONCLUSION

In recent years, two reviews, the first from Pinheiro and Mulle (2008) and the second from Duguid (2013), suggested criteria that, if satisfied, could help to define the existence of presynaptic release-regulating NMDA receptors. Interestingly, some of these criteria can be successfully satisfied also by using the synaptosomal preparation, as briefly summarised below.

An important criterion to support the existence of presynaptic NMDA receptors is that NMDA agonist(s) can efficiently modulate the transmitter release probability as well as the $Ca^{2+}$ dynamics in nerve terminals (Table 1). Most of the functional data described in this review satisfy this criterion because it emerges that (i) the exposure of synaptosomes (which are pinched-off resealed nerve endings) to NMDA agonists affects transmitter release and (ii) the releasing activity is paralleled by $Ca^{2+}$ ions movements within the synaptosomal particles specialized for the release of the transmitter under study (e.g., glutamate; Zappettini et al., 2014; Nisticò et al., 2015).

Another criterion that is largely satisfied in release studies from superfused synaptosomes is that NMDA antagonists should prevent or reduce the agonist-induced effects. In almost all the reported studies (with the exception of the antagonist profile of the NMDA heteroreceptor controlling 5-HT and acetylcholine release), antagonists specific for selected subunits were found to modulate the NMDA/glycine-evoked transmitter release. Notably, as already discussed in the review, the results obtained with these ligands are attributed to their ability to target selected GluN subunits, and therefore, besides confirming the existence of presynaptic release-regulating NMDA receptors, they also aloow suggestions of the subunit composition of the receptor involved and the main feature of the associated ionic channel (Table 3).

Finally, another criterion to support the existence of presynaptic NMDA receptors is the presence of GluN subunit proteins at the -presynaptic structures. The review reports data from both immunocytochemical analysis and confocal microscopy demonstrating the presence of selected GluN subunits in synaptosomal particles specialized for the release of selected transmitters and in particular in the presynaptic fraction of the synaptosomal plasma membranes (e.g., the fraction corresponding to the presynaptic component of the synaptic active zone; Phillips et al., 2001). Furthermore, in some cases, the studies also show a clear correlation between the subunits and the transmitter specialization of the synaptosomal particles (Musante et al., 2011; Nisticò et al., 2015; Olivero, Vergassola, Cisani, Usai, & Pittaluga, 2019; Salamone et al., 2014; Zappettini et al., 2014).

Starting from these considerations, the review describes a complex scenario that includes several NMDA receptors having different localization, different subunit composition and different conditions of activation. The complexity is consistent with a heterogenous pharmacological profile, heighted by using selective ligands for the glutamate and the glycine binding sites as well as compounds acting at the

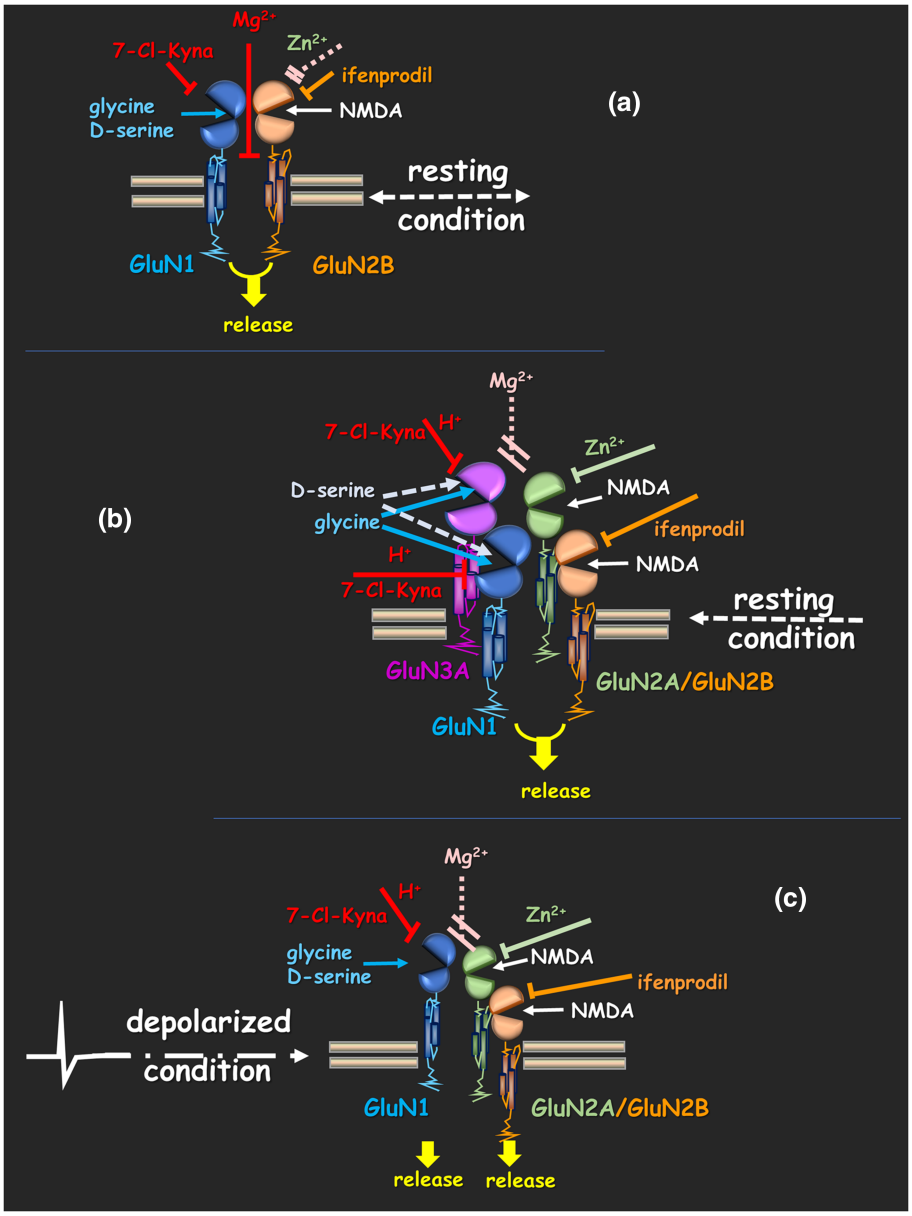


**FIGURE 2** Heterogeneity of the presynaptic release-regulating NMDA receptors. The pharmacological profile of the receptors was obtained using selective ligands for the GluN subunits, as well as compounds acting at the voltage dependent associated channel. The results are consistent with the presence of (a) dimeric GluN1/GluN2B assemblies in the dopaminergic and the noradrenergic nerve endings and of heteromeric GluN associations typified by a high level of complexity in (b) peptidergic and (c) glutamatergic terminals. The NMDA receptors also act differently on the transmitter outflow. (a and c) Presynaptic NMDA receptors controlling dopamine, noradrenaline and glutamate potentiate the spontaneous release of these transmitters, whereas (b) the presynaptic NMDA receptors controlling the release of CCK-LI and SRIF-LI significantly reinforce the exocytosis of the two peptides elicited by a mild depolarizing stimulus. Straight line with arrow: full agonist; dotted line with arrow: partial agonist; straight line with cap: antagonist/channel blockers; dotted line with breaks: antagonist/channel blockers inactive at the receptor

voltage dependent associated channel (see Figure 2 for an iconographic illustration).

In particular, analysis of the available data suggests the presence of dimeric GluN1/GluN2B assemblies in the dopaminergic and the noradrenergic nerve endings (Figure 2a) and of heteromeric GluN associations typified by a high level of complexity in peptidergic (Figure 2b) and glutamatergic (Figure 2c) terminals, possibly consisting of GluN3-containing GluN1/GluN2 NMDA heterocomplexes. There are however some NMDA receptors (e.g., those controlling acetylcholine and GABA release) whose subunit composition is poorly investigated, and others (e.g., the one controlling 5-HT release) whose existence depends on data from experimental approaches other than synaptosomes, but not confirmed in isolated nerve endings.

As already mentioned, the 'superfusion of synaptosomes' allows discrimination between basal and depolarized transmitter release and how these two events are controlled by autoheteroreceptors. By using this technique, it emerged that the presynaptic NMDA receptors affect differently the release of different transmitters. Presynaptic NMDA receptors controlling dopamine, noradrenaline and glutamate potentiate the spontaneous release of the transmitters (Table 1 and Figure 2) but display a different sensitivity towards the external $Mg^{2+}$ ions, which possibly reflects different subunit composition (including the participation of the GluN3 subunit to the receptor assembly; Table 3 and Figure 2). Other presynaptic NMDA receptors fail to affect the spontaneous release of transmitters but significantly augment their exocytosis elicited by a mild depolarizing stimulus (as in the case of the NMDA receptors controlling the release of acetylcholine and of peptides; Table 1 and Figure 2), and in most cases, the efficiency of the control of transmitter release slightly depends on the presence of $Mg^{2+}$ ions (e.g., the NMDA receptors controlling glutamate, peptides and acetylcholine release; Table 3 and Figure 2).

The NMDA-mediated modulation of transmitter release generally involves the influx of positive charges to the cytosolic compartments but also relies on mechanisms involving the reversal of co-localized transporters (e.g., the GLYT1; Musante et al., 2011) as well as the concomitant activation of co-localized receptors, for example, ionotropic (e.g., AMPA and nicotinic receptors; Pittaluga & Raiteri, 1992a, 1992b; Risso et al., 2004) and metabotropic (**$mGlu_1$ receptors**, **somatostatin type 5 receptors**, **CXCR4** chemokine receptors; Di Prisco et al., 2016) receptors that are permissive to the opening of the associated ionic channels.

Finally, the reciprocal role of glutamate and glycine at the presynaptic release-regulating receptors varies depending on the synaptosomal subpopulations and the transmitter involved (Table 2). For most of the presynaptic NMDA receptors described in this review, glutamate is essential for receptor activation, whereas glycine preferentially acts as a co-agonist, being a fine tuner of the releasing activity (it is permissive when added at nanomolar concentration, but allosteric at micromolar level, it is the case of the NMDA receptors controlling the release of catecholamines, glutamate and perhaps GABA). There are, however, NMDA receptors activated by glycine alone (e.g., the presynaptic release-regulating NMDA receptors controlling peptide exocytosis; Paudice et al., 1998) or receptors that are almost insensitive to the presence of glycine (e.g., those controlling the release of acetylcholine; Morari et al., 1998).

In summary, the data reviewed in this article manuscript substantiate the conclusion that presynaptic release-regulating NMDARs exist in selected regions of the CNS and control synaptic strength and efficiency, playing a major role in the neuronal network. Most of the functional properties of NMDA receptors relies on the subunit composition and on the co-localized receptors/transporters. Of relevance are the results concerning the effectsof agonists, antagonists and allosteric modulators on the presynaptic NMDA receptors because these results promise new opportunities to dissect the pre from the post NMDA-mediated component in synaptic transmission as well as to targeting therapeutically the presynaptic receptors.

## Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos et al., 2019; Alexander, Fabbro et al., 2019; Alexander, Kelly et al., 2019; Alexander, Mathie et al., 2019;).

### ACKNOWLEDGEMENTS

The review is dedicated to Professor Maurizio Raiteri and Professor Mario Marchi. Thanks for sharing the fascinating story of the presynaptic release-regulating NMDA receptors.

### CONFLICT OF INTEREST

The author declares no conflicts of interest.

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**How to cite this article:** Pittaluga A. Presynaptic release-regulating NMDA receptors in isolated nerve terminals: A narrative review. *Br J Pharmacol*. 2021;178:1001–1017. https://doi.org/10.1111/bph.15349