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Glycine release from glutamatergic presynaptic terminals in CA1 region of mice hippocampus

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# Glycine release from glutamatergic presynaptic terminals in CA1 region of mice hippocampus

By

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# Abstract

Glycine plays two roles in neurotransmission. It acts as the main inhibitory neurotransmitter in the spinal cord and brainstem and is a co-agonist of the *N*-methyl-D-aspartate receptor (NMDAR) throughout the central nervous system. Changes in extracellular levels of glycine modulate NMDAR functions and consequently, are critical for excitatory synaptic transmission. In the hippocampus, the extracellular levels of glycine at excitatory synapses are regulated by the presence of glycine transporters (type 1; GlyT1) located both on glia surrounding the synaptic cleft and on presynaptic terminals. Although the mechanism of glycine reuptake is well established, the mechanism of glycine release is still unknown. Here, we used immunocytochemical, confocal and electrophysiological techniques to determine the mechanism underlying glycine release in the *stratum radiatum* of the CA1 region of mouse hippocampus. Quantitative analysis of triple immunolabelings combining astrocytic, neuronal, glycinergic and glutamatergic markers showed that glycine was preferentially accumulated in glutamatergic presynaptic terminals. In addition, we found that the vast majority of the glutamatergic presynaptic terminals contained glycine and faced postsynaptic NMDARs. Moreover, using electrophysiological recordings we found that glycine released following electrical stimulation activated few glycine receptors on the postsynaptic neurons but instead, strongly modulated the amplitude of NMDAR currents. Overall, our data indicate that glycine is released from glutamatergic presynaptic terminals upon neuronal activity to modulate NMDAR function.

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# Abbreviations

**ACSF** artificial cerebrospinal fluid

**AMPAR** α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor

**CdCl** cadmium chloride

**CGP** 3-[[(3,4-dichlorophenyl)methyl]amino]propyl]diethoxymethyl)- phosphinic acid

**CHO** chinese hamster ovary

**CNS** central nervous system

**CSF** cerebrospinal fluid

**D-AP5** D(-)-2-Amino-5-phosphonopentanoic acid

**EGTA** ethylene glycol tetraacetic acid

**GABA** γ-aminobutyric acid

**$GABA_AR$** $GABA_A$ receptors

**GAD** glutamic acid decarboxylase

**GCS** glycine cleavage system

**GFAP** glial fibrillary protein

**GlyRs** glycine receptors

**GlyT** glycine transporter

**GMS** glycine modulatory site

**HEPES** *N*-2-hydroxy-ethylpiperazine-*N*_-2-ethanesulphonic acid

**IR** immunoreactivity

**KAR** kainite receptor

**LTP** long-term potentiation

**NBQX** 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo- [*f*] quinoxaline-7-sulphonamide

**NFPS** N[3-(4-fluorophenil)-3-(4'-phenilphenoxy)]propylsarcosine

**NMDAR** N-methyl-D-aspartate receptor

**QX-314** lidocaine N-ethyl bromide

**SHMT** serine hydroxymethyltransferase

**SNARE** soluble N-ethylmaleimide-sensitive-factor attachment protein receptor

**SR** stratum radiatum

**TTX** tetrodotoxin

**VGluT** vesicular glutamate transporter

**VIAAT** vesicular inhibitory amino acid transporter

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# Publications

1. Chen J., Ghazawi F., **Bakkar W.**, and Li Q. **(2006)** Valproic acid and butyrate induce apoptosis in human cancer cells through inhibition of gene expression of Akt/protein kinase B. Molecular Cancer, 5: 71

2. Muller E., **Bakkar W.**, Legendre P., Martina M., and Bergeron R. **(2007)** Glycine release from glutamatergic presynaptic terminals in mice hippocampus. (Submitted)

# Abstracts

1. Emilie Muller, **W. Bakkar**, P. Legendre, M. Martina and R. Bergeron (2007) Glycine release from glutamatergic presynaptic terminals in CA1 region of mouse hippocampus. Society for Neuroscience.

# Chapter 1: INTRODUCTION

***Preface***

Neurons communicate at synaptic contacts *via* chemical and electrical synapses in a process known as synaptic transmission. The fundamental process that triggers synaptic transmission is the action potential, a propagating electrical signal that is generated by exploiting the electrically excitable membrane of the neuron. Upon action potential arrival, neurotransmitters are released by the presynaptic signal-emitting neuron and bind to specific receptors expressed on the postsynaptic signal-receiving neuron. Excitatory neurotransmission in the central nervous system (CNS) is mainly mediated by the neurotransmitter glutamate, while inhibitory neurons, often referred to as interneurons, inhibit their target by releasing either GABA and/or glycine. The balance between the excitatory and inhibitory action of different neurotransmitters is essential for proper brain function; if this balance is severed it can consequently lead to severe neuropathophysiologies.

## A) Glycine's dual role: inhibition and excitation

### *1) Glycine and inhibitory neurotransmission*

#### *a) Glycine as an inhibitory neurotransmitter*

Glycine (**Fig. 1**) and γ-aminobutyric acid (GABA) are the main inhibitory neurotransmitters in the nervous system [1]. While fast inhibitory transmission is mainly GABAergic in the forebrain, it is mostly mediated by glycine in more caudal areas, notably in the brainstem and spinal cord, where it contributes to both motor and sensory

functions [2]. Nevertheless, Jonas and colleagues demonstrated that in rat spinal cord, glycine and GABA could be co-released from the same presynaptic terminal to co-activate corresponding glycine receptors (GlyRs) and $GABA_A$ receptors ($GABA_ARs$) co-expressed at postsynaptic densities of motoneurons [3]. Such mixed inhibitory glycinergic-GABAergic synapses have, since then, been morphologically and functionally described in several distinct brainstem areas [4;5], as well as in the cerebellum [6], the abducens nucleus [7] and in the lateral superior olive nucleus [8]. Their existence has also recently been suggested in forebrain areas such as the hippocampus [9]. While pure glycinergic or GABAergic synapses do exist, these studies show that glycine and GABA are not necessarily segregated, and strongly suggest a wider distribution of mixed inhibitory synapses in the nervous system than what was previously thought.

The role of glycine as one of the most important inhibitory neurotransmitter in the nervous system has been demonstrated more than 35 years ago. While glycine is a universal cellular component which enters in the composition of proteins, peptides or nucleotides, it is also present in glycinergic interneurons which control motor rhythm generation underlying locomotor behaviour and are implicated in the coordination of spinal reflex activity [2]. In the CNS, glycine is highly concentrated in caudal areas like the brainstem, and its detection decreases towards more rostral areas [10]. At the glycinergic synapse, glycine release into the synaptic cleft is triggered by a $Ca^{2+}$-dependent fusion of glycine containing vesicles with the presynaptic plasma membrane and results in the activation of postsynaptic GlyRs. Glycine ultimately decreases neuronal activity by hyperpolarizing the membrane potential via $Cl^-$ influx through strychnine sensitive GlyRs. The plant alkaloid strychnine is a highly selective and extremely potent

competitive antagonist of GlyRs; and strychnine sensitivity is currently the most definitive means of discriminating glycinergic from GABAergic synaptic currents [11].

GlyRs (**Fig. 2**) are pentameric receptors composed of α (4 isoforms: α1, α2, α3, α4) and β subunits, which can be either homomeric $(\alpha)_5$ or heteromeric $(\alpha)_2(\beta)_3$ [2;12]. The β subunit is required for the postsynaptic clustering of GlyRs. Their kinetic properties depend on their subunit composition and their stoechiometry [2;13;14]. The α1β heteromeric receptor is the predominant form of GlyRs expressed in the adult nervous system, and is mainly involved in fast inhibitory glycinergic transmission [2]. In contrast, the α2 homomeric receptor, known as the neonatal form of GlyRs, displays a large conductance state (~100pS) and slower response kinetics reported to be incompatible with synaptic function [14]. Glycinergic inhibitory transmission plays a crucial role in motor coordination, thus the physiological significance behind the developmental speeding of synaptic currents due to α2 to α1 subunit switch is to enhance motor skill reflex [15].

*b) Glycine metabolism, presynaptic release machinery and vesicular uptake at glycinergic synapse*

Little is known regarding glycine metabolism in the mammalian brain; however, two enzymes implicated in glycine metabolism have been characterized: the glycine cleavage system (GCS) and the enzymatic complex serine hydroxymethyltransferase (SHMT) [16]. Both enzymes have been localized in the mammalian brain where SHMT is a mitochondrial enzyme, expressed in neurons and astrocytes [17] while GCS is specifically expressed in the mitochondria of astrocytes [18]. Both function in a reversible manner to produce glycine from L-serine and L-serine from glycine. However, glycine in

glycinergic presynaptic terminals arises mainly from its re-uptake from the extracellular space *via* specific glycine transporters [19] before it is stored in presynaptic vesicles. Vesicular transporters, located on the membrane of neurotransmitter storage vesicles, are responsible for maintaining vesicular neurotransmitter content and allowing exocytotic neurotransmitter release [20].

Glycine and GABA share and compete for the same vesicular transporter known as the Vesicular Inhibitory Amino Acid Transporter or VIAAT [21;22]. The designation of VIAAT as a GABA/glycine transporter is based on evidence from morphological studies showing the presence of VIAAT in both GABAergic and glycinergic synaptic terminals [23], from biochemical studies on neurotransmitter uptake [24] and from electrophysiological data revealing co-release of GABA and glycine from single vesicles in spinal cord neurons [3]. However, VIAAT is reported to have a ~ 60-fold higher affinity for GABA than for glycine [24] and a recent biochemical study confirmed a physical association between VIAAT and GAD65 [25], one of the enzymes involved in GABA synthesis. This is in line with a study in which homozygous knock-out mice lacking VIAAT display a reduction of GAD65 protein levels [26]. Thus, despite multiple studies indicating VIAAT as the carrier for both GABA and glycine, based on the observations above which seem to favour GABA transport, one cannot rule out the possibility of an additional vesicular transporter for glycine.

*c) Glycine transporters: localization, structure and function*

Neurotransmission at chemical synapses proceeds with remarkable speed. Indeed, at individual synaptic sites, the post-synaptic response to transmitter release lasts only for milliseconds. Such rapid kinetics require an effective clearance of neurotransmitter from

the synaptic cleft, achieved through either diffusion or enzymatic degradation but predominantly, through re-uptake by specific transporters located on neuronal and glial plasma membranes [27]. Specific high-affinity transporters have been characterized for several amino acid neurotransmitters and classified into two families: a) the 12-transmembrane domain, $Na^+/Cl^-$-dependent family that includes transporters for glycine, GABA, serotonine and dopamine; b) the glutamate transporter family [28].

High affinity uptake of glycine from the extracellular space is mediated by GlyT1 and GlyT2 (**Fig. 3**) transporters believed to be found on astrocytes and neurons, respectively. Both of these transporters are expressed under several isoforms: alternative splicing generates five GlyT1 isoforms (a, b, c, d and e) differing in only their N-terminal sequence [29] while alternative promotor usage generates three GlyT2 isoforms (a, b, and c) [30]. Glycine uptake is energetically coupled with the transmembrane sodium gradient maintained by the $Na^+/K^+$- ATPase [30;31]. The stoechiometry of substrate/ion co-transport is 3 $Na^+/Cl^-$/glycine for GlyT2 and 2 $Na^+/Cl^-$/glycine for GlyT1 [32]. This difference in ionic coupling implies that, under physiological conditions, the driving force available for glycine uptake in neurons is much higher than for glial cells. In addition, GlyT2 has a severe limitation for reverse uptake, which suggests an essential role of GlyT2 in maintaining a high intracellular glycine pool, thereby facilitating refilling of synaptic vesicles [33]. In contrast, the lower $Na^+$ transport stoechiometry has been suggested to allow a reverse function of GlyT1 transporter upon changes in ionic concentration gradients or membrane potential [32].

Although they share approximately 50% amino acid sequence identity, GlyT1 and GlyT2 transporters have distinct pharmacological properties and display a complementary

rather than an overlapping distribution pattern suggesting different functions. GlyT1 transporters display a more widespread expression throughout the nervous system. While they are highly expressed in regions enriched in glycine, and are mostly detected on astrocytes [34], they are also expressed in more rostral areas devoid of glycinergic transmission, like in the hippocampus [35]. In this area, their expression has been detected both on astrocytic and neuronal compartments [34]. In contrast, the distribution pattern of GlyT2 transporters is similar to that of glycine-immunoreactivity (-IR). GlyT2 transporters are predominantly expressed in the spinal cord, brainstem and cerebellum [36;37]and are concentrated at the plasma membrane of presynaptic terminals facing GlyRs [37;38]. Thus, GlyT2 transporters are reliable markers of glycinergic presynaptic terminals.

Recent studies have demonstrated that at glycinergic synapses, neuronal presynaptic GlyT2 transporters are crucial for efficient neurotransmitter loading of synaptic vesicles, while astrocytic GlyT1 transporters would be preferentially involved in quickly clearing the synaptic cleft [19]. In contrast, GlyT1 transporters expressed in brain areas devoid of glycinergic transmission have recently been shown to be involved in excitatory neurotransmission [39].

*2) Glycine: modulator of excitatory neurotransmission*

*a) NMDA receptors*

The amino acid glutamate is the major excitatory neurotransmitter in the CNS [40]. It acts by binding to several distinct glutamate receptors classified as ionotropic receptors, involved in fast excitatory neurotransmission, or metabotropic receptors, associated to G-proteins and intracellular signaling pathways [41]. Based on their distinct pharmacological

and biophysical properties, three subtypes of glutamate-gated ionotropic receptor channels have thus far been identified: N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPARs) and kainate receptors (KARs).

NMDARs (**Fig. 4**) are subject to a voltage-dependent block by physiological $Mg^{2+}$ concentrations. They are highly permeable to $Ca^{2+}$ ions and act as a coincidence detector, allowing ion flow only when both pre- and postsynaptic cells are excited [42]. More importantly, NMDAR function requires not only the binding of glutamate but also of a co-agonist glycine or D-serine. To date, seven NMDAR subunits have been characterized: one NR1, four NR2 (A-D), and two NR3 (A, B) subunits. NMDARs assemble as heterotetramers in the endoplasmic reticulum (ER) to form functional channels composed of two NR1 subunits containing the glycine-binding site and two NR2 subunits containing the glutamate-binding site [42;43]. In contrast, NMDARs containing NR3 subunits are only sensitive to glycine. Based on their composition in NR2 subunit subtype, NMDARs will differ in their biophysical and pharmacological properties, including their sensitivity to $Mg^{2+}$, open probability, deactivation time, single channel conductance and interactions with intracellular signalling molecules [44].

*b) Glycine and glutamatergic synapses*

Johnson and Ascher demonstrated in 1987, that NMDAR activation required the binding of both glutamate and the co-agonist glycine [45]. However following immunohistochemical studies revealing a similar distribution of D-serine amino acid and NMDARs notably in the forebrain [46-48], it has been shown that D-serine displays a ~3

fold higher affinity for NMDARs than does glycine [49]. D-serine, released from astrocytes upon glutamate receptor activation through calcium- and SNARE-dependent exocytotic pathways, modulates NMDARs function [50]. Thus both glycine and D-serine can act as co-agonist of NMDAR activation and have been reported to enhance NMDAR-mediated responses [51]. Moreover, stimulation of the glycine site with either amino acid primes the receptors for clathrin-dependent endocytosis [52].

Whether the glycine modulatory site (GMS) of NMDARs is saturated or not *in vivo* was a matter of debate for a few decades. Indeed, the glycine concentration in the cerebrospinal fluid (CSF) bathing the synaptic cleft is in the micromolar range [53], which is sufficient to saturate the glycine site. However, several recent *in vitro* studies performed in acute hippocampal or brainstem slices reported a potentiation of NMDAR function upon administration of glycine or the potent and selective GlyT1 antagonists which increase the extracellular concentration of glycine [54-56]. Likewise, heterozygous knockout mice for GlyT1 transporters exhibit an enhanced NMDAR-mediated excitatory transmission in the hippocampus compared with wild type littermates [39; 57; 58]. These data clearly indicate a glycine-mediated modulation of NMDAR function. Interestingly, a more recent study highlighted a close relationship between GlyT1 transporters and glutamatergic synapses. Indeed in several forebrain areas such as the hippocampus, GlyT1 transporters have been detected not only on surrounding astrocytes [59] but also on pre- and postsynaptic elements of glutamatergic synapses [34]. Moreover, biochemical studies demonstrated that NMDARs and GlyT1 transporters are physically linked [34]. Collectively, these data strongly support the idea that the level of glycine in the synaptic

cleft tightly regulated by GlyT1 transporters, modulates NMDAR activity. This level is kept below the 'set point' of the NMDAR internalization by the presence of GlyT1 [39; 52].

Despite numerous evidence involving GlyT1 transporters in the modulation of NMDARs function, the source of glycine and the mechanism by which glycine is released at glutamatergic excitatory synapses in forebrain regions, notably the hippocampus, are presently unknown. It has been suggested however, that a reverse GlyT1 transporter function could account for glycine release at NMDARs-containing synapses under specific pathological conditions [32].

**B) Source of glycine at glutamatergic synapses in hippocampus**

*1) Glycine afference to the hippocampus*

*a) Functional glycinergic synapses*

Due to the strong glycine-immunoreactivity detected in the lower nervous system, glycinergic transmission was initially thought to be exclusively present in spinal cord and caudal areas of the CNS. Nevertheless, novel techniques provide clear evidence that GlyRs as well as glycine containing fibers and glycinergic cell bodies are more widely distributed within the CNS. These have been notably detected in the cochlear nuclei, the cerebellar cortex, deep cerebellar nuclei, the thalamus and hypothalamus, the area postrema and more recently, in some upper brainstem and forebrain areas [60; 2]. However, the mere presence of morphologically identified glycine-immunoreactive fibers and GlyRs, does not suggest a physiological role such as glycinergic transmission. In fact, electrophysiological studies indicated that fast glycinergic transmission is restricted to

spinal cord [61];[62], brainstem, Golgi cells of the cerebellum [63] and on retinal ganglion cells [64].

In the hippocampus, while GlyRs and GlyT2 transporters expression has been reported [9], no glycinergic afferences or fast glycinergic transmission has been detected thus far. In contrast, GlyRs are expressed at low density, mostly at extrasynaptic sites and seem to be mostly involved in tonic inhibition and attenuate pyramidal cells excitability. Moreover, fast inhibitory transmission in hippocampal slices is mainly mediated by GABA [65].

*2) Aim of study*

The goal of my research is to determine the source of glycine at the CA1 glutamatergic synapse in the mouse hippocampus and to gain insight into its release mechanism(s) at this synapse.

In the hippocampus (**Fig. 5**), glutamatergic Schaffer collaterals arising from the CA3 pyramidal cells innervate CA1 pyramidal cells in the *stratum radiatum* and constitute the main glutamatergic afferences. At these glutamatergic excitatory synapses, NMDAR function was shown to be modulated by the extracellular concentration of glycine, which in turn, is tightly regulated by GlyT1 transporters [39;66]. However, the cellular sources of glycine, as well as the mechanisms underlying its release in this region, are not known. Thus, I combined immunocytochemical, confocal microscopy and electrophysiological techniques to determine the site and mechanism of glycine release at glutamatergic excitatory synapses in the *stratum radiatum* of the CA1 region of mouse hippocampus.

Immunocytochemical techniques in combination with confocal microscopy were used to determine the specific distribution pattern of glycine within the CA1 region of the hippocampus. I used whole-cell patch-clamp technique and the 'sniffer-patch' technique **(Fig. 6)** to gain insight into the mechanisms involved in glycine release in the stratum radiatum of the CA1 hippocampus. This method which exploits the natural high affinity of ligand-gated ion channels for their native neurotransmitter to provide a sensitive detection system, dates back to 1983, when it was used for the first time to detect elicited and spontaneous release of ACh from developing growth cones [67;68]. For our purpose, the principle behind this technique is to form an outside-out patch by excising a small piece of membrane from CHO cells constitutively expressing $\alpha_2$ homomeric glycine receptors. The $\alpha_2$ subunit of GlyRs is chosen because of its high sensitivity to glycine and its ability to detect very minute amounts of this amino acid. This outside-out patch of membrane, rich in GlyRs, is then translocated to the *stratum radiatum* where it will serve as a sensor of glycine release upon various experimental conditions.

# Chapter 2: MATERIALS AND METHODS

## Immunocytochemistry

Swiss wild-type mice (8- to 10-weeks-old) were deeply anesthetized with an overdose of pentobarbital (60 mg/kg) and intracardiacally perfused with 4% (v/v) paraformaldehyde (PFA) in a phosphate-buffered saline (PBS) solution in agreement with the guidelines of the Canadian Council of Animal Care. After a 30 min post-fixation at 4°C, the brain was removed and frozen in liquid nitrogen. The brain was then sliced with a cryostat (Leica CM 3050 S) to obtain hippocampal coronal slices of 12 µm. For triple labeling including glycine receptor (GlyR; 4a) detection (glycine-VGluT1-VGluT2-VGluT3-4a), slices were fixed for 10 min in methanol-acetic (95/5) at -20°C. All slices were then quenched with 50 mM $NH_4Cl$ in PBS for 30 min and permeabilized with 0.2% (w/v) Triton and 0.25% (w/v) fish gelatin in PBS for 30 min to enhance the penetration of primary antibodies. The slices were then incubated with primary antibodies in 0.25 % (w/v) gelatin in PBS overnight at 4°C and subsequently with secondary fluorescent antibodies in 0.25% (w/v) gelatin in PBS for 2 hours at room temperature. Slices were rinsed, mounted with Vectashield (Vector, Burlingame, CA, USA). Control slices, in which one or all the primary antibody were omitted in triple labeling experiments, were inspected for possible cross-reactivity between different immunoreactions or false labeling provided by the secondary antibodies.

## Antibodies

The presence of the glycine amino-acid was detected using a rat anti-glycine antibody (1:500; Immunosolution;). Presynaptic terminals were identified using a

monoclonal antibody raised against synaptophysin, a protein involved in the presynaptic exocytosis machinery (1:1000; Sigma, Canada). The astrocytic compartments were identified using a rabbit anti-GFAP (Glial Fibrillary Protein) antibody (1:500; Chemicon, USA). Glutamate and glycine presynaptic transporters were identified using three guinea-pig antibodies (1:500 each; Chemicon, USA) raised against vesicular glutamate transporters type 1, 2 and 3 (VGluT1, VGluT2 and VGluT3), and a guinea-pig antibody raised against the glycine transporter type 2 (GlyT2; 1:1000; Chemicon, USA), respectively. Glycinergic vesicular transporters were detected using a rabbit anti-(Vesicular Inhibitory Amino Acid Transporter) VIAAT antibody (1:1000; gift from B. Gasnier, Institut de Biologie physicochimique, France). The VIAAT antibody recognizes the common presynaptic vesicular transporter of glycine and GABA [21; 23]. GABAergic terminals were detected using a combination of mouse anti-synaptophysin-IR (see above) and an antibody which recognizes the two isoforms (65 and 67) of the enzyme Glutamic Acid Decarboxylase (GAD) which synthesize GABA (rabbit anti-GAD65/67, 1:250; Chemicon, USA). GlyRs were detected using a monoclonal antibody (4a) which recognizes all GlyR subtypes (1:1000; Synaptic System, Germany), while the NMDARs were identified using a rabbit anti-NR1 subunit antibody (1:500; Chemicon, USA). Multiple labelings were performed using compatible triplets or quintuplets of primary antibodies raised in different species.

To allow fluorescent detection of primary antibody raised against glycine, we used a Cy3-conjugated donkey anti-rat IgG (1:500; Jackson ImmunoResearch, USA). For fluorescent detection of primary antibodies raised in mice against synaptophysin or GlyRs, we used a Cy5-conjugated goat anti-mouse IgG (1:500; Jackson

ImmunoResearch). For fluorescent detection of primary antibodies raised in rabbit, we used either a FITC-conjugated goat anti-rabbit IgG (1:500; Jackson ImmunoResearch, USA) for GFAP detection or a Cy5-conjugated goat anti-rabbit IgG (1:500; Jackson ImmunoResearch, USA) for GAD65/67, VIAAT and NR1 detection. Primary antibodies raised in guinea pig (VGluT1, VGluT2, VGluT3 and GlyT2) were detected using a FITC-conjugated goat anti-guinea pig IgG (1:500; Jackson ImmunoResearch, USA).

**Immunofluorescence, confocal microscopy and quantification**

Fluorescence images of hippocampal slices were acquired by sequential scanning using an LSM 510 confocal laser-scanning microscope (Zeiss, Germany) with a 63x oil-immersion objective. Typically, stacks of 10-20 sections (1024*1204 pixels) with an interval of ~ 0.3 µm from the *stratum radiatum* of the CA1 hippocampus were scanned twice to optimize the signal-to-noise ratio. All quantifications were done manually. A cluster was characterized by a sharp increase of fluorescence intensity, as previously defined [69]. Cy3, FITC and Cy5 channel images were merged using Adobe Photoshop 7.0.1. The criterion to define a co-localization was a superimposition of at least five pixels of each labelling. Isolated clusters were defined as the ones that were not co-localized with other clusters. Single densities, as well as the densities of co-clusters (double or triple) were determined to calculate percentage of co-localization. For each labeling, 10 sections from two different animals were computed. Statistical significance was determined with paired *t*-tests (two-tailed). A $P < 0.01$ was considered statistically significant. Values are means ± SEM.

## Electrophysiology

***Preparation of hippocampal slices.*** Coronal brain slices containing the hippocampus were obtained from WT Swiss mice (8- to 10-weeks old). Prior to decapitation, the animals were anaesthetized using an isofluorane vaporizer (Stoelting, Wood Dale, IL, USA), in agreement with the guidelines of the Canadian Council of Animal Care. The concentration of isofluorane was 2-5%, the $O_2$ rate was $1 L.min^{-1}$.

The brain was removed and placed in an oxygenated (95% $O_2$ – 5% $CO_2$) artificial cerebrospinal fluid (ACSF) solution at 4 °C containing (mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$ and 10 glucose. The osmolarity of the ACSF was adjusted to 300 $mosmol.l^{-1}$ and the pH to 7.2 using HCl (1M). Hippocampal slices (300 μm) were cut with a vibrating microtome (Leica VT 1000S, Germany) and incubated for 1h in an oxygenated chamber at room temperature before they were used for experiments.

***Cell culture.*** Chinese hamster ovary (CHO; a gift from Dr. Pascal Legendre, UMR 7102 Dynamique de la Synapse, France) cells were maintained in a 95% air- 5% $CO_2$ humidified incubator at 37°C in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 30% glucose, fetal bovine serum (Invitrogen), streptomycin and zeocin (Invitrogen). Cells were passed every 5-6 days. For electrophysiological recordings, confluent cells were detached using a trypsine solution to provide a medium full of cells. Cells (50μl drops) from this medium were seeded onto glass coverslips previously coated with poly-ornithine (Sigma) and subsequently incubated for 2 hours prior to experiment. The medium was renewed every second day.

***Data recording and analysis.*** In voltage clamp experiments, whole cell patch-clamp recordings were performed with an intracellular solution containing (mM): 130 Cs-methanesulfonate, 10 *N*-2-hydroxy-ethylpiperazine-*N*_-2-ethanesulphonic acid (HEPES), 10 KCl, 2ATP, 0.2 GTP-tris (hydroxyl-methyl) aminomethane (GTP), 5 lidocaine N-ethyl bromide (QX-314) and 10 cesium-BAPTA. When indicated in the text, the synthetic peptide derived from the proline-rich domain of dynamin I ($100\mu g.ml^{-1}$, QVPSRPNRAP, dynaminPRD) [70];[71] was added to the intracellular solution.

Whole-cell patch-clamp recordings to evaluate glycine release were performed using an intracellular solution containing (mM): 140 $CsCl_2$, to further maximize the driving force of chloride ions, 2 $MgCl_2$, 10 Hepes, 2 ATP, 0.2 GTP, 5 QX-314 and 10 EGTA. The pH was adjusted to 7.2 using CsOH (1M) and the osmolarity to 280 $mosmol.l^{-1}$. The pipettes had a resistance of 3-5 MΩ when filled with these solutions.

In the outside-out patch configuration experiments, carried-out to study the mechanism of glycine release, an intracellular solution containing (mM): 130 cesium chloride, 4 $MgCl_2$, 10 Hepes, 10 EGTA, 2 ATP, was used to form a gigaseal with the membrane of the CHO cells. The pH was adjusted to 7.2 and the osmolarity to 280 $mosmol.l^{-1}$. The patch was done in an ACSF containing (mM): 126 NaCl, 2.5 KCl, 1 $MgCl_2$, 1.25 $NaH_2PO_4$, 10 glucose, 26 $NaHCO_3$, and 2 $CaCl_2$. When indicated, we added to the base ACSF solution: 35mM $K^+$ to mimic depolarization, tetrodotoxin (TTX; 1μM) to block inward $Na^+$ currents thereby preventing generation of action potentials and cadmium chloride (CdCl; 50 μM) to block all calcium channels. The pH was adjusted to 7.2 and the osmolarity to 300 $mosmol.l^{-1}$.

Voltage-clamp and single channel recordings were obtained with a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA, USA) under visual control using differential interference contrast and infrared video microscopy (IR_DIC; Leica DMLFSA, Germany). The recordings were performed at room temperature from individual pyramidal cells of the CA1 region of the hippocampus voltage-clamped at -65mV.

Post-synaptic currents were evoked by electrical stimulation of the Schaffer collaterals with a bipolar microelectrode positioned in the *stratum radiatum.* The stimulation intensity consisted of 100 $\mu$s current pulses (10–200 μA) and was adjusted to evoke an EPSC amplitude in the range of 40–80 pA at a membrane potential ($V$m) of −65 mV. Bridge balance was monitored during the recordings. Recordings with series resistance higher than 25 MΩ were discarded. Compensation was 70-80%.

To isolate the NMDAR-mediated component of evoked responses, I used ACSF containing a low concentration of MgCl2 (0.1mM) with osmolarity maintained by $CaCl_2$, and the $\alpha$-amino-3-hydroxy-5- methylisoxazole-4-propionic acid receptor (AMPAR) antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo- [*f*] quinoxaline-7-sulphonamide (NBQX, 20 μm), the $GABA_A$ receptor antagonist picrotoxin (50 μm), the $GABA_B$ receptor antagonist 3-[[(3,4-dichlorophenyl)methyl]amino]propyl]diethoxymethyl)-phosphinic acid (CGP 52432, 10 μm) and the glycine receptor antagonist strychnine (1 μM).

To evoke glycinergic currents, a 500 ms train of 100 μs current pulses at 33Hz was applied. To isolate the glycinergic currents, we used ACSF containing NMDAR

antagonist D(-)-2-Amino-5-phosphonopentanoic acid (D-AP5; 50 μM), AMPAR antagonist NBQX (20 μM), the $GABA_A$ receptor antagonist gabazine (2 μM) and the $GABA_B$ receptor antagonist CGP 52432, (10 μM). Glycinergic currents were blocked using strychnine (1μM).

Data were collected using pCLAMP 9 software (Axon Instrument, Foster City, CA). Analysis was performed *off*-line with the software Clampfit 9.0 (Axon Instrument, Foster City, CA). Statistical significance of the results was determined with paired *t*-tests (Two-tailed). A $P< 0.01$ was considered statistically significant. All values are expressed as means ± SEM.

Drugs: D-AP5, picrotoxin, gabazine, strychnine, N[3-(4-fluorophenil)-3-(4'-phenilphenoxy)]propylsarcosine (NFPS), TTX and CdCl were obtained from Sigma. NBQX, and CGP 52432 were obtained from Tocris (Bristol, UK). DynaminPRD (QVPSRPNRAP) were synthesized by SIGMS genosys (Cambridgeshire, UK).

# Chapter 3: RESULTS

## Glycine is present in the glutamatergic presynaptic terminals in the *stratum radiatum* of the CA1 of mouse hippocampus

Until recently, antibodies raised against glycine required glutaraldehyde fixative conditions, which resulted in strong tissue autofluorescence and prevented a clear establishment of glycine distribution pattern. Recently, a new antibody requiring paraformaldehyde fixative conditions has been produced. Paraformaldehyde fixative conditions provide weaker autofluorescence, and clearer establishment of glycine distribution [72]. We took advantage of this new antibody to investigate the distribution of glycine in the *stratum radiatum* of the CA1 region of mouse hippocampus.

### *Glycine accumulates in presynaptic terminals*

To determine the astrocytic *vs.* neuronal cellular distribution of glycine in the *stratum radiatum* of the CA1 region of the mouse hippocampus, we used an anti-glycine primary antibody requiring classical paraformaldehyde fixative conditions (see above) and performed a glycine-Glial Fibrillary Acidic Protein (GFAP)-synaptophysin triple immunolabeling (**Fig. 7**). GFAP is a protein specifically expressed in astrocytes while synaptophysin is a protein involved in the exocytosis machinery of presynaptic terminals, consequently their immunoreactivity (IR) will indicate the presence of astrocytic compartments and presynaptic terminals respectively.

We found that glycine-IR was uniform throughout the *stratum radiatum* of mouse hippocampus (**Fig. 7A**) and formed bright fluorescent clusters (**Fig. 7D**), while GFAP-

staining displayed only few labelled astrocytic processes (**Fig. 7E**). This poor GFAP distribution was likely due to the thin confocal sectioning of astrocytic processes. Indeed entire GFAP-positive astrocytes could easily be reconstructed from projected images acquired along the z-axis (**Fig. 7S**). Moreover, in the *stratum lacunosum moleculare,* where astrocytes are present in high density [73], GFAP-staining indicated a high density of glial cells (**Fig. 7B**). Synaptophysin-positive presynaptic terminals provided a high density of clusters (**Fig. 7F**). Although Soluble *N*-ethylmaleimide-sensitive-factor Attachment Protein Receptor (SNARE) proteins such as synaptophysin are also detected in astrocytes [74], only 14.6 ± 1.3 % of synaptophysin clusters co-localized with GFAP-IR (**Fig. 7I**), suggesting that synaptophysin clusters typically represented presynaptic terminals.

Glycine- and synaptophysin-IR provided similar staining patterns (**Fig. 7D, F**) which greatly overlapped (**Fig. 7G**). In contrast, glycine clusters were rarely apposed to GFAP-IR (**Fig. 7D-E**). We calculated that 58.2 ± 1.9 % of glycine clusters co-localized with synaptophysin clusters (**Fig. 7H**) while only 16.0 ± 1.2 % of glycine clusters co-localized with GFAP-IR (**Fig. 7H**). These data suggest that glycine is preferentially accumulated in presynaptic terminals rather than in GFAP-positive astrocytes. Glycine clusters which were neither co-localized with synaptophysin- nor with GFAP-IR (**Fig. 7H**) could represent glycine involved in metabolic processes or localized in other cellular subtypes.

Next, I estimated the glycine distribution over the population of synaptophysin-positive presynaptic terminals, and found that 62.6 ± 2.2 % of synaptophysin clusters co-localized with glycine clusters (**Fig. 7I**), indicating that ~2/3 of presynaptic terminals in

the *stratum radiatum* of the CA1 region of the mouse hippocampus potentially contained glycine.

*Co-existence of glycine and glutamate in the same presynaptic terminal*

Since glycine acts as a co-agonist of NMDAR [45], and since the extracellular level of glycine modulates NMDAR function at excitatory synapses [39], we have investigated whether presynaptic terminals which contain glycine could also contain glutamate. To do so, we performed a quintuple immunolabeling for glycine, vesicular glutamate transporters (VGluTs) and synaptophysin (**Fig. 8**). In the hippocampus, three types of VGluTs (VGluT1, VGluT2 and VGluT3) specifically load glutamate into presynaptic vesicles of glutamatergic terminals [75; 76; 77; 78]. To detect the whole population of vesicular glutamate transporters, we used three primary antibodies (recognizing VGluT1, VGluT2 and VGluT3) raised in the same specie and detect them with a single secondary antibody. From this point on, we will refer to the 3 types of VGluTs together as "VGluTs" and to the glycine-VGluT1-VGluT2-VGluT3-synaptophysin as "glycine-VGluTs-synaptophysin triple immunolabeling". We considered the concomitant detection of synaptophysin- and VGluTs clusters as a representation of glutamatergic presynaptic terminals.

Glycine-, VGluTs- and synaptophysin-IR clusters displayed similar distribution patterns (**Fig. 8A-C**) and numerous triple co-localizations, corresponding to presynaptic terminals containing both glycine and glutamate, were detected (**Fig. 8D**). Indeed, 76.5 ± 3.7 % of glycine-VGluTs co-clusters co-localized with synaptophysin clusters (**Fig. 8E**),

suggesting that glycine-VGluTs co-clusters mostly corresponded to presynaptic terminals containing both glycine and glutamate.

Next, I calculated that 74.2 ± 3.5 %, of synaptophysin-VGluTs co-clusters co-localized with glycine clusters (**Fig. 8F**) suggesting that the vast majority of glutamatergic presynaptic terminals contained glycine. Conversely, 60.5 ± 3.3 % of synaptophysin-glycine co-clusters co-localized with VGluTs clusters (**Fig. 8F**). These data indicate that ~ 60 % of presynaptic terminals containing glycine also contain glutamate, while ~ 40 % of presynaptic terminals contain only glycine or a neurotransmitter different from glutamate.

Overall, these results suggest that glycine could be distributed in different subtypes of presynaptic terminals, however it was preferentially found in glutamatergic presynaptic terminals.

*Glycine in mixed inhibitory glycinergic-GABAergic presynaptic terminals*

Considering that ~ 40% of presynaptic terminals containing glycine did not contain glutamate (see above), I then investigated if these contained glycine only.

Since recent studies have shown the co-localization of glycinergic and GABAergic markers in rat hippocampus [9; 79], I investigated the presence of GABA in presynaptic terminals containing glycine. To do so, I performed a glycine-glutamate acid decarboxylase 65 and 67 (GAD65/67)-synaptophysin triple immunolabeling (**Fig. 9**). To detect the presence of GABA, I used an antibody against both isoforms (65 and 67) of GAD, the enzyme that synthesizes GABA [80]. I chose to use both isoforms of GAD because although the GAD67 isoform is widely distributed in somatodendritic and axonal

compartments, it can also contribute to GABA synthesis in presynaptic terminals with GAD65 [81].

In agreement with studies showing the co-localization of glycinergic and GABAergic synaptic markers in the hippocampus [9; 79], we found that glycine- and GAD65/67-IR partially overlapped (**Fig. 9D**). In fact, 64.2 ± 4.1 % of synaptophysin-GAD65/67 co-clusters co-localized with glycine clusters (**Fig. 9E**), suggesting that numerous GABAergic presynaptic terminals may contain glycine, while only 27.2 ± 2.9 % of synaptophysin-glycine co-clusters, co-localized with GAD65/67 clusters (**Fig. 9E**).

Furthermore, by combining the quantifications obtained from glycine-VGluTs-synaptophysin and glycine-GAD65/67-synaptophysin triple immunolabelings, I found that the presynaptic accumulation of glycine occurs preferentially in glutamatergic (~60%) rather than in GABAergic (~27%) presynaptic terminals (**Fig. 9F**). The small proportion (~13%) of isolated synaptophysin-glycine co-clusters could represent presynaptic terminals which contain glycine only (**Fig. 9F**).

Overall, these results suggest that in the *stratum radiatum* of the CA1 region of mice hippocampus, glycine preferentially accumulates in glutamatergic presynaptic terminals.

*Glycine uptake and storage in glutamatergic presynaptic terminals*

I then focused my attention on presynaptic glutamatergic terminals containing glycine to determine the mechanism of glycine uptake. The presence of glycine in glutamatergic presynaptic terminals could be due to the presence of specific transporters which uptake glycine from the extracellular space. It has been reported that, in the CA1

hippocampus, glycine transporters type 1 (GlyT1s) are expressed on presynaptic neuronal elements and strongly co-localized with VGluT1 transporters [34]. This suggests that GlyT1 could be the transporter responsible for glycine re-uptake into glutamatergic presynaptic terminals. However, in the spinal cord and brainstem, glycine transporters type II (GlyT2s) specifically re-uptake glycine into presynaptic glycinergic terminals [82]. GlyT2s are also expressed in the CA1 hippocampus, although at a lower density than in the spinal cord [9]. To determine whether GlyT2s were presynaptically localized and whether they could contribute to glycine re-uptake in presynaptic terminals in the *stratum radiatum* of the CA1 mouse hippocampus, I performed a GlyT2-GFAP-synaptophysin triple immunolabeling (**Fig. 10**).

Surprisingly, GlyT2s co-localized equally with either synaptophysin clusters alone or GFAP-IR (**Fig. 10D**). On one hand, 47.5 ± 2.8 % of GlyT2 clusters co-localized with synaptophysin clusters alone (**Fig. 10E**). On the other hand, ~ 44 % of GlyT2 clusters co-localized with GFAP-positive astrocytes: 19 ± 2.7 % with GFAP-IR only and 25.1 ± 2.4 % with both synaptophysin clusters and GFAP-IR (triple co-localization) (**Fig. 10E**). It is noteworthy that the density of GlyT2 clusters was ~4.5 fold lower than that of glycine clusters (data not shown). Moreover, it is estimated that half of GlyT2 clusters were localized in presynaptic terminals. These data suggest that few presynaptic terminals containing glycine were likely to express GlyT2s. Interestingly, the density of GlyT2-synaptophysin co-clusters was not significantly different from the estimated density of pure glycinergic presynaptic terminals shown in **Fig. 9F** (data not shown). These data suggest that GlyT2s are unlikely to be present at glutamatergic presynaptic terminals, and that they could be used as markers of pure glycinergic presynaptic terminals in the

hippocampus. Consequently, the presence of glycine in glutamatergic presynaptic terminals could mainly arise from GlyT1s as suggested the data of Cubelos and co-workers [34].

Glycine re-uptaken into glutamatergic presynaptic terminals through GlyT1s could either be freely diffusing and used in metabolic processes or stored in presynaptic vesicles for further exocytosis. To investigate this question, I performed a glycine-VGluTs-Vesicular Inhibitory Amino Acid Transporter (VIAAT) triple immunolabeling (**Fig. 11**). VIAAT is the vesicular transporter for both glycine and GABA [21]. Quantifications from this triple immunolabeling showed that 53 ± 2.1 % of glycine-VGluTs co-clusters co-localized with VIAAT clusters (**Fig. 11E**). Since we have assumed that glycine-VGluTs co-clusters represent the presynaptic terminals containing glycine and glutamate (see above) and since no other vesicular transporter other than VIAAT has been reported for glycine thus far, these data imply that approximately half of glutamatergic presynaptic terminals contain glycine which is loaded into vesicles.

**Frequency-dependent release of glycine from presynaptic terminals**

Since the immunocytochemical analysis of the glycine distribution in the *stratum radiatum* of the CA1 region of mouse hippocampus indicated that glycine was mainly distributed in presynaptic glutamatergic terminals, I then investigated whether glycine could be released from these terminals. Recordings of glycinergic currents were carried out on CA1 pyramidal cells voltage-clamped at -65mV, with a solution in the recording pipette that kept the $Cl^-$ equilibrium close to 0mV. Since few GlyRs are expressed at postsynaptic densities in rat hippocampus [9], the $Cl^-$ equilibrium close to zero provides at -

65 mV a driving force for chloride ions that maximize the glycinergic responses. Glycinergic currents were evoked every 10s through the stimulation of glutamatergic Schaffer collaterals and isolated in the presence of AP5 (50μm), NBQX (20μm) and gabazine (2μm). These inward currents displayed an amplitude of 3.25 ± 0.71 pA (N=10) and were abolished under bath application of strychnine, (1μM; **Fig. 12A**), a highly specific GlyR antagonist. We further induced glycine release by applying a train at 33 Hz (duration of 500 ms) (**Fig. 12B**). The trains induced glycinergic currents whose amplitude slowly increased to reach a plateau at the end of the 500 ms train (**Fig. 12B**), suggesting a frequency-dependent release of glycine from presynaptic terminals. The amplitude of strychnine-sensitive glycinergic currents at the plateau was 5.30 ± 0.07 pA (N=10; **Fig. 12C**), which was slightly but not significantly higher (unpaired *t*-test, $P<0.05$) than what we observed under single stimulation. To increase the extracellular level of glycine we blocked the GlyT1s with the high affinity GlyT1s antagonist N[3-(4-fluorophenil)-3-(4'-phenilphenoxy)]propylsarcosine (NFPS). Bath application of NFPS (300 nM) did not significantly increase the glycinergic currents evoked by single stimulation (4 ± 1.30 pA in NFPS, N=5 *vs.* 3.25 ± 0.71 pA in control; N=10; $P>0.05$), but significantly increased (unpaired *t*-test, $P<0.01$) the amplitude of the glycinergic currents evoked by the trains (8.40 ± 0.40 pA, N = 10 in NFPS *vs.* 5.30 ± 0.07 pA; N=10, in control **Fig. 12B and C**).

Overall, these data showed that postsynaptic GlyRs were activated by a frequency-dependent glycine release from presynaptic terminals.

The small amplitude of strychnine-sensitive glycinergic currents recorded was likely due to the low density of postsynaptic GlyRs facing presynaptic terminals

containing glycine. To test this proposition, I performed a GlyRs-VGluTs-glycine triple immunolabeling (**Fig. 13**), using an antibody which recognizes all GlyR subtypes.

Results indicate that the GlyR-IR was weak and diffused and provided few GlyR clusters (**Fig. 13C**). More so, only 27.4 ± 2.3 % of GlyR clusters faced the glutamatergic presynaptic terminals containing glycine recognized by the glycine-VGluTs co-clusters (**Fig. 13E**). Conversely, only 18.7 ± 1.6 % of glycine-VGluTs co-clusters were apposed to GlyR clusters (**Fig. 13E**). These data illustrate that postsynaptic GlyRs and glutamatergic presynaptic terminals containing glycine were rarely facing one another which could explain the small amplitude of strychnine-sensitive glycinergic currents recorded following the stimulation of Schaffer collaterals (See **Fig. 12**). Moreover, these results also imply that glycine released from presynaptic terminals could have another role other than activating GlyRs.

*Glycine release depends on network activity*

To further evaluate the mechanisms of glycine release, we adapted the sniffer technique (**Fig. 6**) to CHO cells expressing homomeric $\alpha_2$ GlyRs. In brief, this technique involves pulling the membrane of CHO cells until an outside-out patch is formed. This bulb of membrane, which contains GlyRs, is then translocated to the *stratum radiatum* of hippocampal slices where it will serve as a sensor of glycine release upon various experimental conditions. It has been reported that high potassium can mimic depolarization, and facilitate glycine release from presynaptic terminals of rat spinal dorsal horn neurons [83]. Applying high potassium (35mM $K^+$) to our slice resulted in a massive increase in glycine release (19.74 ± 7.06pA/sec, n=10), that was significant

(paired *t*-test, $P<0.05$, **Fig. 16**) compared with control (0.89 ± 0.33 pA/sec, n=10) (**Fig. 14**). This suggests that glycine release depends on network activity as a whole.

Glycine release partially relies on synaptic activity and on vesicular mechanisms

Next, I wanted to assess whether this potassium-induced glycine release relied more specifically on synaptic activity. To do so, I adapted the sniffer technique in presence of high extracellular concentrations of potassium (35mM $K^+$) and tetrodotoxin (TTX; 1μM), a potent neurotoxin which blocks action potentials in nerves by binding to the pores of the voltage-gated, fast $Na^+$ channels in nerve cell membranes. TTX abolished the potassium-induced glycine release (2.35 ± 0.84 pA/sec, n=3) (**Fig. 15**) and was not significantly (unpaired *t*-test, $P>0.05$) different compared to control conditions (**Fig. 16**). These results suggest that synaptic activity does play a role in the release of glycine neurotransmitter.

I have shown that half of the glutamatergic presynaptic terminals contain glycine which is loaded into vesicles (**Fig. 11E**); suggesting perhaps a calcium-mediated release. To investigate whether glycine release occurs as a result of a calcium-dependent vesicular mechanism, I used the sniffer technique in presence of high extracellular concentrations of potassium (35mM $K^+$), TTX (1μM) and cadmium chloride (CdCl; 50 μM) to block calcium channels which provide calcium required for vesicular release. The potassium-induced release of glycine was further reduced in the presence of cadmium chloride (0.93 ± 0.52 pA/sec, n=4) (**Fig. 15**) and was not significantly different from control conditions (unpaired *t*-test, $P>0.05$; **Fig. 16**) suggesting a dependence of external calcium and implying a vesicular mechanism in the release of glycine.

Glycine released from glutamatergic presynaptic terminals modulates NMDAR function

It is known that glycine acts both as an inhibitory neurotransmitter at glycinergic synapses and as a co-agonist of NMDAR activation at excitatory synapses [45]. In addition, we have previously reported that glycine potentiates NMDAR-mediated synaptic transmission in the CA1 mouse hippocampus [39]. In light of these reports and according to our results, we hypothesized that the physiological role of glycine released from glutamatergic presynaptic terminals could be to modulate the postsynaptic NMDAR function rather than to activate GlyRs.

To test this hypothesis, I investigated whether NMDARs were facing glutamatergic presynaptic terminals containing glycine, by performing a glycine-VGluTs-NR1 triple immunolabeling (**Fig. 17**). We identified NMDARs using an antibody against the NR1 subunit (this subunit is a necessary component for the function of the NMDAR complex), while glutamatergic presynaptic terminals containing glycine were identified by the detection of glycine-VGluTs co-clusters (see **Fig. 8**). Glycine, VGluTs and NR1 clusters showed a similar distribution pattern (**Fig. 17A-C**). Indeed, 64.51 ± 3.7 % of NR1 clusters were apposed to glycine-VGluTs co-clusters (**Fig. 17E**), indicating that most of the NMDARs were facing glutamatergic presynaptic terminals containing glycine. Importantly, almost all glycine-VGluTs co-clusters were apposed to NR1 clusters (84.31 ± 2.8 %; **Fig. 17E**). Overall, these data indicated that glutamatergic presynaptic terminals containing glycine were frequently facing postsynaptic NMDARs and strongly suggested that glycine-glutamate presynaptic terminals play a role in NMDAR function.

In light of these data, I investigated whether the activity-dependent glycine release could affect NMDAR function. I evoked NMDAR-mediated currents in CA1 pyramidal cells with two different intervals of stimulation (6 and 30 s; **Fig. 18A**), in absence and presence of NFPS (300 nM). The NMDAR-mediated component of the post-synaptic current was pharmacologically isolated in a low $Mg^{2+}$ ACSF containing NBQX, picrotoxin, CGP 52432 and strychnine (see Methods). We observed that, depending on the interval of stimulation, the application of NFPS (300 nM) changed the amplitude of NMDAR-mediated evoked currents (stable over 5 min in absence of NFPS). We observed that, when the interval of stimulation was 30s, the NMDAR current amplitude slowly increased over a 15 min period (**Fig. 18A**). On the other hand, when the interval of stimulating was 6s, the NMDAR current amplitude decreased (**Fig. 18A**). Since the NMDAR currents seem to work as a sensor of extracellular glycine level, these results strongly suggested that the amount of glycine released was different depending on the interval of stimulation. More importantly, these data showed that GlyT1 transporters were not responsible for glycine release.

The decrease in the amplitude of NMDAR currents observed during the 6s-stimulation, could be due to the induction of NMDAR glycine-clathrin-dependent internalization [52]. To test this hypothesis, I repeated the same experiment with a DynaminPRD peptide, known to block clathrin-dependent endocytosis [70; 84; 71], in the intracellular solution. As expected, I observed a large increase of the amplitude of NMDAR-mediated evoked currents (from 22.94 ± 2.28 pA in control to 37.88 ± 6.30 pA, $N = 8$; $P < 0.05$) with the DynaminPRD peptide (**Fig. 18B**). These results suggest that

the extracellular level of glycine reached high concentrations when stimulations was applied every 6s, and was sufficient to reach the internalization threshold for NMDARs.

# Chapter 4: Discussion

In the present study, I have investigated glycine distribution in the CA1 *stratum radiatum* of mouse hippocampus. My data indicates that glycine is mainly localized to presynaptic terminals rather than in astrocytes. Interestingly, the majority of presynaptic terminals containing glycine also contained glutamate and were apposed to NMDARs. Furthermore, glycine could be released from presynaptic terminals in a frequency-dependent manner to modulate NMDAR function. Finally, my results rule out the involvement of GlyT1 in the release of glycine and suggest that perhaps glycine is released *via* vesicular mechanisms in at least a subpopulation of glutamatergic neurons.

*Glutamatergic presynatpic terminals contain glycine*

Glycine concentration in the cerebrospinal fluid is approximately 10 μM, and some have suggested this could be a major source of glycine at excitatory synapses [85]. However, the glycine concentration in the vicinity of NMDARs might be reduced to below 1 μM due to high affinity glycine transporters (GlyTs) [86; 87; 88; 54; 89]. Knowing that a reciprocal communication exists between neurons and glia [90; 91], some have proposed that glycine could be released from astrocytes in the vicinity of glutamatergic synapses [85]. This hypothesis is partially based on the fact that in the hippocampus, a strong expression of GlyT1 is found on glial cells [29]. However, it has been shown that GlyT1s are also expressed on pre- and postsynaptic neuronal elements [34]. Interestingly, my immunocytochemical data demonstrate that glycine is highly present in synaptophysin-positive presynaptic terminals, while only low levels of glycine were detectable in GFAP-positive astrocytes.

Recently, Song and colleagues [79] have shown that, in rat hippocampus, glycine is mainly concentrated in interneurons. This study supports our data regarding glycine accumulation in neuronal cells; however, it contrasts with our results because we did not detect many interneurons showing a strong glycine staining. This apparent discrepancy could be explained by differences in species (rat/ mouse) or by different techniques in intracardiac perfusion. Indeed Song and colleagues [79] sequentially perfused ACSF and paraformaldehyde fixative solutions. The former ACSF perfusion could wash small amino acids like glycine and increase the detection threshold of small glycine clusters. We avoided the ACSF flush to prevent this inconvenience and used a 10-fold lower concentration of primary antibody to specifically detect glycine-IR.

Vesicular glutamate transporters (VGLUTs) are essential to glutamatergic synapses and determine the glutamatergic phenotype of neurons [92]. Thus to assess the nature of these presynaptic terminals which contain glycine, a quintuple labelling with glycine-VGluT1-VGluT2-VGluT3-synaptophysin was performed and revealed that glycine clusters frequently co-localized with VGluTs-synaptophysin co-clusters (**Fig. 8F**) which suggests the accumulation of glycine in presynaptic glutamatergic terminals. Interestingly, the immunocytochemical co-detection of glycine and glutamate neurotransmitters in the same presynaptic terminals coincides with the fact that glycine acts as a co-agonist of NMDAR activation [93]. In support of our hypothesis, a strong overlap of GlyT1 and VGluT1 transporters expression has recently been reported in the rat hippocampus [34]. Moreover, the co-existence of glycine and glutamate in presynaptic terminals in the cerebral cortex was suggested by the presynaptic co-localization of high-affinity uptake systems specific for both neurotransmitters [94].

*Vesicular release of glycine*

Using electrophysiological recording, we have found that glycinergic currents increased following electrical stimulation of glutamatergic Schaffer collaterals, strengthening our hypothesis of the co-existence of glycine and glutamate in the same presynaptic elements. We also found that the amount of glycine released from presynaptic terminals was different whether we used a 6s or a 30s interval of stimulation **(Fig. 18A),** indicating that glycine release was dependent of neuronal activity. This theory is also supported by previous reports showing glycine release from primary cultures of cerebellar granule cells [95] and spinal dorsal horn inhibitory synapses [83] following perfusion with solutions containing high concentrations of potassium which are known to mimic presynaptic depolarization [96]. Similarly, I adapted and performed the sniffer technique from CHO cells expressing homomeric-$\alpha_2$ GlyRs and noticed a massive, potassium-dependent, glycine release **(Fig. 14)**. However, because potassium is applied liberally to the extracelluar solution, one can only conclude that release depends on network activity as a whole; but cannot exclude that release could occur as a result of synaptic activity alone.

The potassium-induced depolarization might cause an influx of $Na^+$ into presynaptic nerve terminals through voltage-dependent $Na^+$ channels, which may promote release of glycine [83]. Thus, I investigated the effects of TTX, a $Na^+$ channel inhibitor, on the potassium-induced increase in glycine release. The application of TTX in the presence of high concentrations of potassium resulted in glycine release that was not significantly different from control **(Fig. 15-16)**. Overall, these results suggest that synaptic activity is partially responsible for glycine release which coincides with our

functional data, where stimulation of Schaffer collaterals induces release of glycine (**Fig. 12**) to activate the few postsynaptic GlyRs.

Several studies suggest that reversal of glycine uptake might play a major role in increasing the concentration of glycine next to the synaptic cleft [85; 33], implying that glycine could be released by presynaptic GlyT1s. However, my electrophysiological data refutes this theory because glycine is still released despite the blockade of GlyT1 transporter (**Fig. 18A**). Instead, glycine could be released *via* a classical vesicular mechanism, as was demonstrated for D-serine which is released from astrocytes upon glutamate receptor activation [50]. In fact, elevating external potassium concentrations can lead to an influx of $Ca^{2+}$ through voltage-gated calcium channels located on presynaptic terminals which could then promote glycine release through a vesicular mechanism [83] and explain the residual glycine observed in the presence of TTX (**Fig 15**). Therefore, I applied cadmium chloride, a calcium channel blocker, in the presence of high potassium concentrations and TTX to determine if release depends on extracellular entry of $Ca^{2+}$ ions (**Fig. 15**). In these conditions, glycine release was not significantly different from control (**Fig. 16**), correlating with my immunocytochemical results, which indicate that a high proportion of glutamatergic presynaptic terminals containing glycine also co-localize with VIAAT, the vesicular glycine transporter (**Fig. 11**). In addition, according to a recent study, the co-expression of GlyT1 with VIAAT is sufficient to induce glycine vesicular release [22]. Altogether, the above findings point to a vesicular calcium-dependent mechanism implicated in the release of glycine. However, we cannot completely rule out the possibility that GlyT1 reverse activity could contribute to glycine release in physiological conditions. It is possible that glycine is released through both

vesicular mechanisms and reverse activity of its transporter, as it has been shown for GABA neurotransmitter in mouse spinal cord synaptosomes [97]. In support of this, glycine release from rat hippocampal synaptosomes has been reported to be calcium and sodium-dependent [96], suggesting a calcium-dependent vesicular release and sodium-dependent GlyT1 reverse function [33].

*Glycine released from presynaptic glutamatergic terminals modulates NMDAR function*

It is well established that in the brainstem and spinal cord, inhibitory synapses are GABAergic and/or glycinergic [3], while in the hippocampus, most if not all inhibitory synapses are thought to be GABAergic [98]. GlyRs have been localized to extrasynaptic sites rather than at postsynaptic elements of hippocampal synapses [9], where they could be important mediators of tonic inhibition [99; 100; 79] similarly to the function of extrasynaptic $GABA_ARs$ [101; 102]. Likewise, the small amplitude of glycinergic currents we observed upon activation of postsynaptic GlyRs by evoked glycine release (**Fig. 12**), and the low percentage of GlyRs apposed to presynaptic glutamatergic terminals containing glycine (**Fig. 13**), suggests that glycine, released from presynaptic terminals in the hippocampus, has another physiological role other than GlyR activation. In fact, GlyRs can be activated by taurine, an endogenous GlyR agonist [103] which is more efficient than glycine as a potential anticonvulsant in epileptic activity [104]. Accordingly, presynaptic release of glycine could activate postsynaptic NMDARs, which would coincide with the fact that glycine was shown to modulate NMDAR amplitude and NMDAR-dependent events such as long-term potentiation (LTP) in the hippocampus [39].

Recent studies suggest that in many brain regions, including hippocampus, D-serine rather than glycine may serve as the endogenous ligand for the glycine-binding site of NMDARs [48; 105; 106; 107]. However, our results indicate otherwise, as we found that the majority of glutamatergic terminals containing glycine were apposed to NMDARs (**Fig. 17E**), and that glycine released from glutamatergic presynaptic terminals modulates NMDAR-current amplitude **(Fig. 18A)**. Furthermore, D-serine is of astrocytic origin and has been shown not to be released from presynaptic terminals. Although both glycine and D-serine act as co-agonists of NMDARs, their individual localization and release mechanisms suggest perhaps a distinct role in NMDAR activation. For instance, glycine originating from presynaptic glutamatergic terminals and released into the synaptic cleft with glutamate could directly activate NMDARs while, astrocytic D-serine could modulate receptor activity *via* a longer pathway, involving metabotropic receptors. Altogether, these results indicate that glycine's primary role, at the CA1-glutamatergic synapse, is not to activate GlyRs; but rather to modulate excitatory neurotransmission by activating NMDARs.

## Future developments

The results presented in this study are innovative because they show, for the first time, the source of glycine and elucidate the possible mechanisms of its release at the CA1-glutamatergic synapse in the hippocampus. However, more work is needed to fully understand the molecular basis behind the release of glycine in the vicinity of NMDARs.

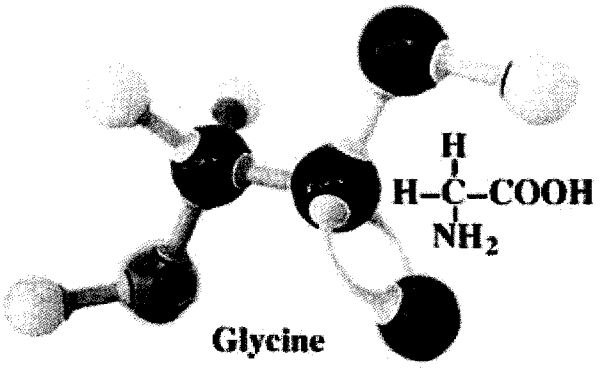
We showed that glycine release depends on calcium entry, however, calcium can also be mobilized from intraterminal stores; thus it would be interesting to determine if

mitochondrial and/or endoplasmic reticulum calcium plays a role in the release of this neurotransmitter. Furthermore, depolarization caused by high levels of external potassium could activate astrocytes, causing release of glutamate and potentially inducing glycine release indirectly through the activation of glutamate transporters found on presynaptic terminals.

# Chapter 5: Conclusion

In conclusion, this study shed light on the long-time unanswered question regarding the source of glycine and the mechanisms through which it is released at the glutamatergic-CA1 synapse of mouse hippocampus. More importantly, it revealed that glycines' primary role at this synapse is to activate and modulate NMDAR function. NMDAR hypofunction is the leading hypothesis behind the etiology of schizophrenia, a brain disorder characterized by hallucinations, withdrawal and serious thought and speech disturbances. Hence, determining glycine's source and mechanism of release could help us better understand NMDAR modulation and develop innovative drugs which could perhaps treat this disease.

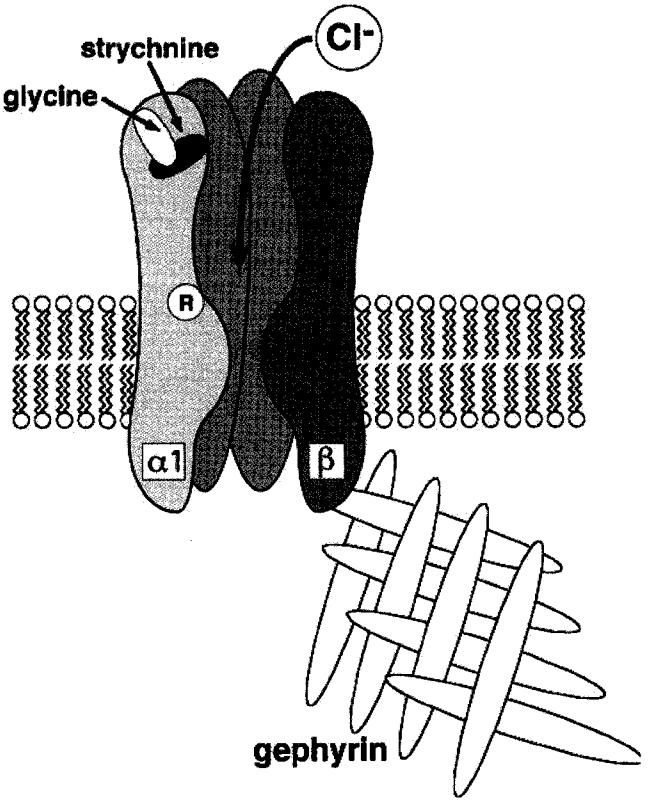
# Chapter 6: Figures




## Glycine structure

Glycine is the organic compound with the formula HO2CCH2NH2. It is one of the 20 amino acids commonly found in proteins. Its three letter code is gly, its one letter code is G, and its codons are GGU, GGC, GGA and GGG. Because of its structural simplicity, this compact amino acid tends to be evolutionarily conserved in, for example, cytochrome c, myoglobin, and hemoglobin. Glycine plays a dual role in the central nervous system: it is an inhibitory neurotransmitter, especially in the spinal cord, brainstem, and retina while it is a required co-agonist along with glutamate for NMDA receptors. [Online] URL: http://www.daviddarling.info/images/glycine.jpg

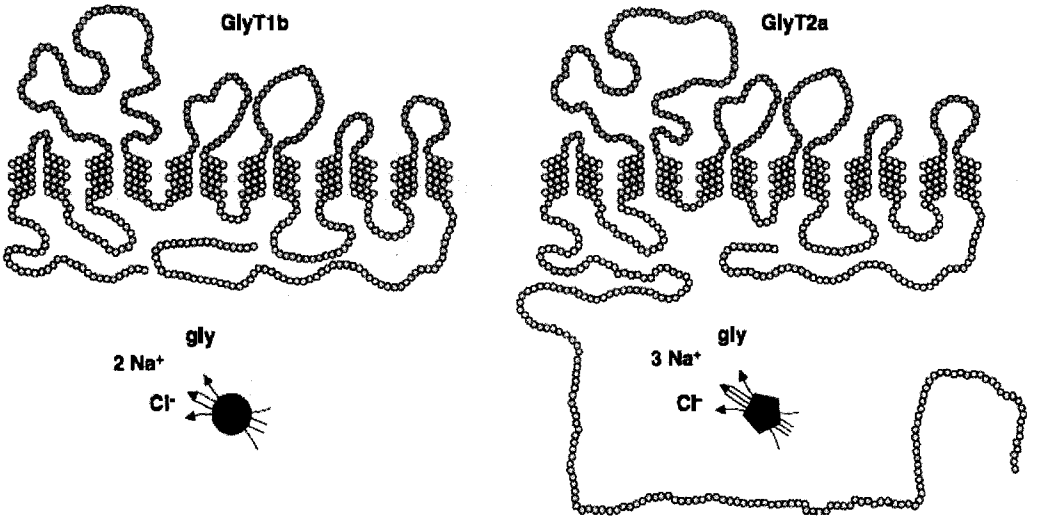
**Figure 1**




## Glycine receptor model

The glycine receptor (GlyR), a member of the family of ligand-gated ion channels, is one of the most widely distributed inhibitory receptors in the CNS. GlyRs are pentameric receptors composed of α (4 isoforms: α1, α2, α3, α4) and β subunits, which can be either homomeric (α)5 or heteromeric (α)2(β)3. The β subunit anchors the receptor to postsynaptic sites via its interaction with gephyrin. These receptors can be activated by a range of simple amino acids including glyince, and taurine, and are selectively blocked by the high-affinity competitive antagonist strychnine.
[Online] URL:http://www.biochem.uni-erlangen.de/MouseDB/imgs/GlyR.jpg

**Figure 2**

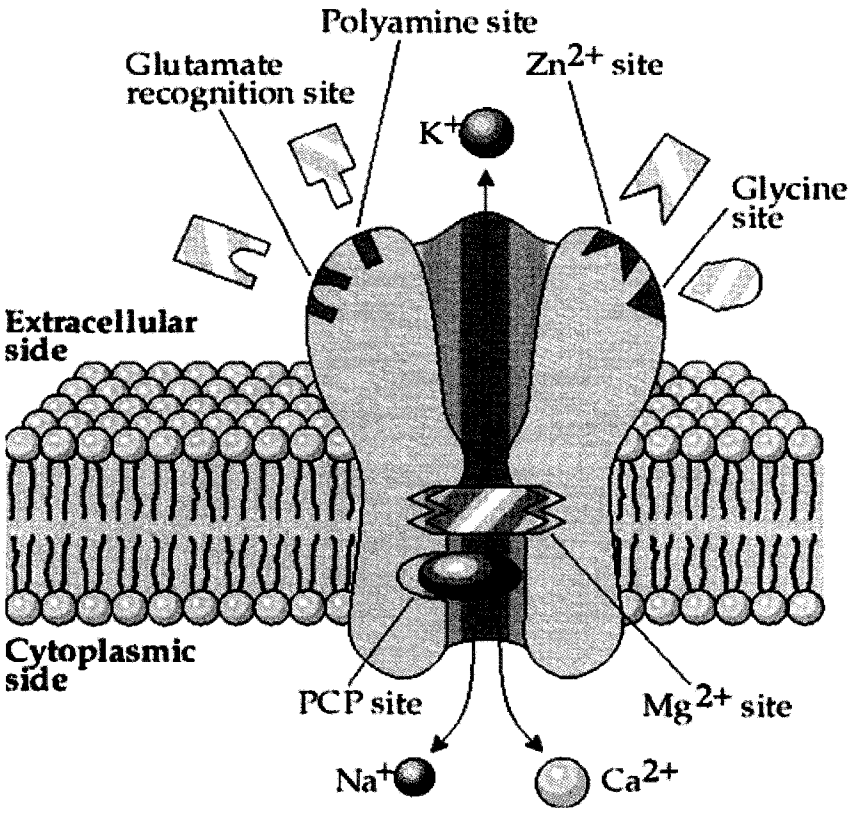



Supplisson & Roux, FEBS letters, 529 (1) 2002

## Glycine transporters: A model of GlyT1 & GlyT2

Glycinergic transmission is terminated via the action of two high affinity glycine transporters: astrocytic GlyT1 and neuronal GlyT2. Both transporters belong to the 12-transmembrane domain, sodium/chloride-dependent family of transporters and come in various isoforms. They differ in their distribution pattern, pharmacology, and stoechiometry.

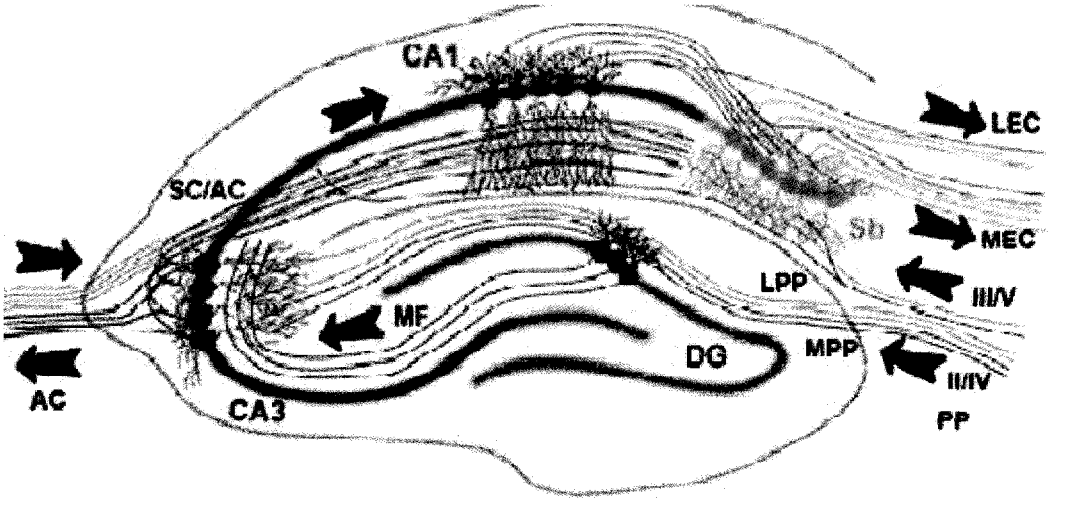
**Figure 3**




## NMDAR and its modulatory sites

Glutamate, the major excitatory neurotransmitter in the CNS, is known to bind to the NR2 subunit of the NMDAR. In addition, these receptors require the binding of co-agonist glycine to the strychnine insensitive, glycineB site found within the NR1 subunit of the receptor. NMDARs are voltage-dependent ligand-gated channels because at resting membrane potential, the receptors' channel is blocked by $Mg^{2+}$ and may also be blocked uncompetitively by PCP whose binding site resides close to that of $Mg^{2+}$. Once active, the receptor becomes permeant to $Ca^{2+}$, $Na^+$ and $K^+$ ions.
[Online] URL:http://www.securitylab.ru/_Article_Images/2005/10/nmda_receptor.gif

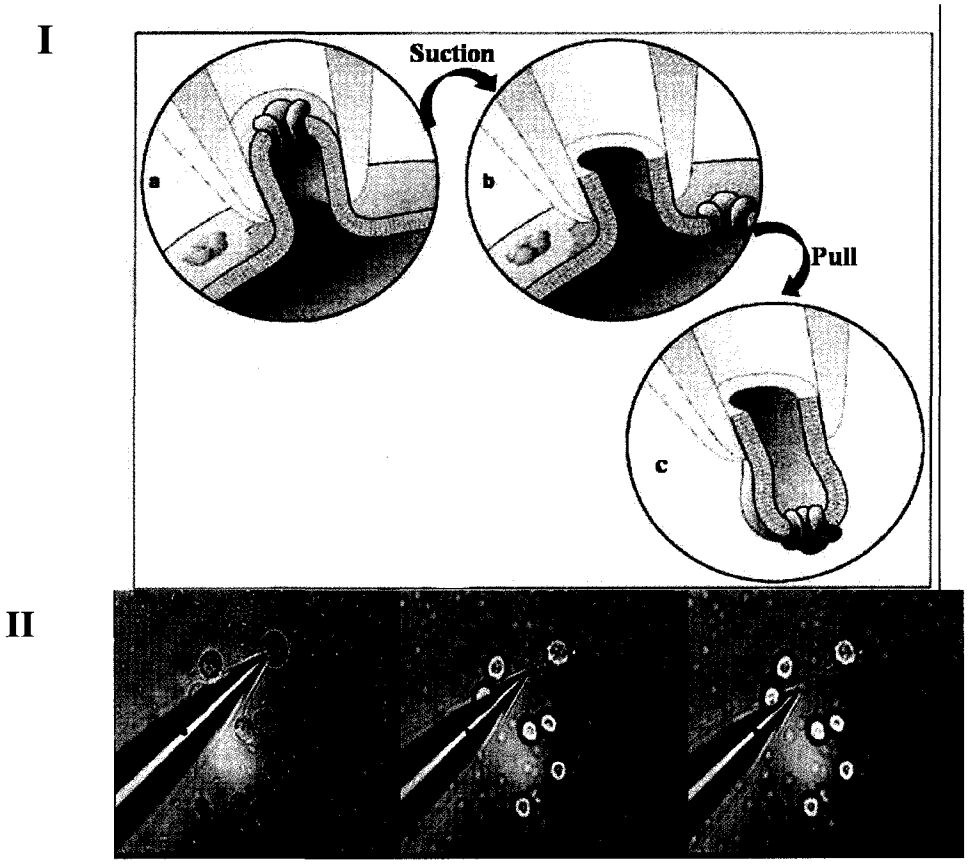
**Figure 4**




## The hippocampal network

The hippocampus forms a principally unidirectional network, with input form the entorhinal cortex (EC) that forms connections with the dentate gyrus (DG) and CA3 pyramidal neurons via the perforant path (PP). CA3 neurons also receive input from the DG via the mossy fibers (MF). They send axons to CA1 pyramidal cells via the glutamatergic Schaffer collaterals Pathway (SC), as well as to CA1 cells in the contralateral hippocampus via the Associational Commissural Pathway (AC). CA1 neurons also receive input directly from the PP and send axons to Subiculum (Sb). These neurons in turn send the main hippocampal output back to the EC, forming a loop. [Online]URL:http://www.bristol.ac.uk/Depts/Synaptic/info/pathway/figs/hippocampus.gif

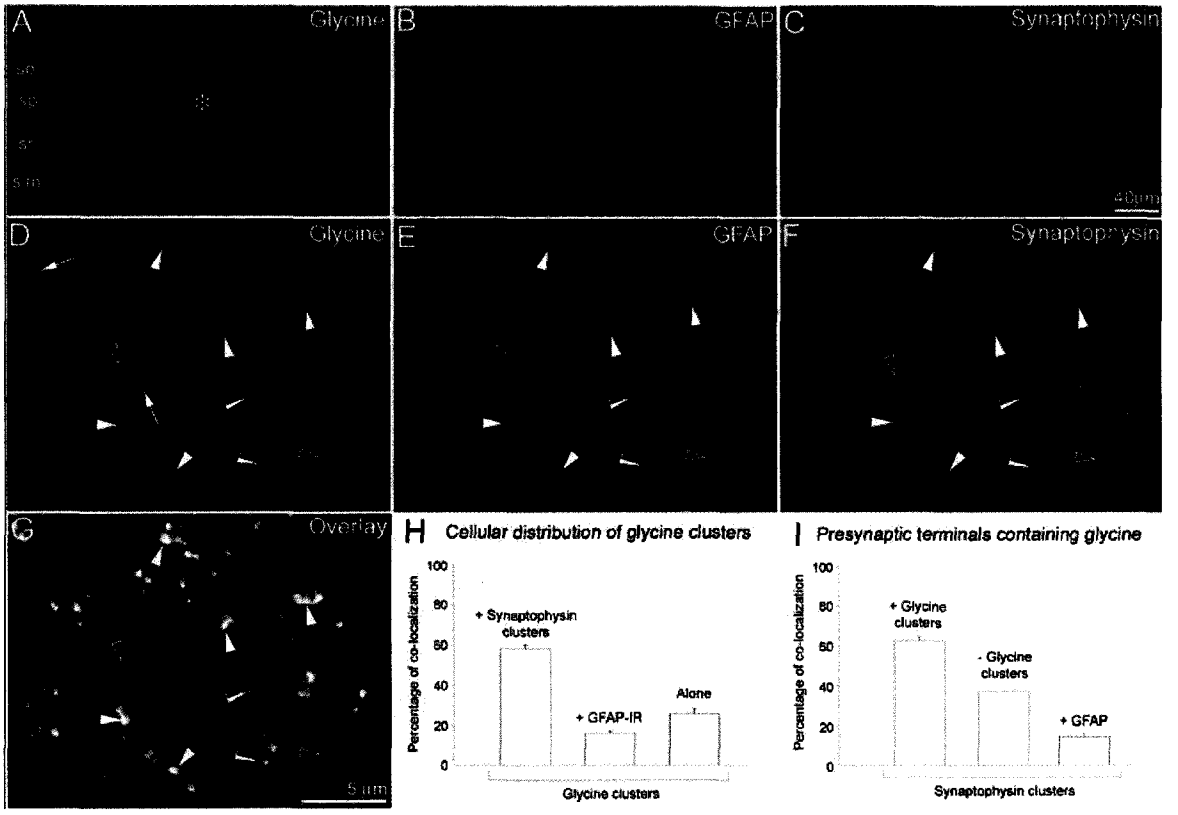
**Figure 5**




## The sniffer outside-out patch

The patch clamp technique in electrophysiology allows the study of individual ion channels in excitable cells such as neurons. (I) The outside-out patch is one of the variations of the basic technique which gives the experimenter the opportunity to study the properties of an ion channel when it is not in contact with its usual environment. In our experiments, we used a borosilicate pipette to perform an outside-out patch on CHO cells constitutively expressing homomeric α2 subunits of GlyRs. After gigaseal formation (a), suction is applied to attain the whole cell configuration (b). The electrode is then slowly pulled away from the cell allowing a bulb of membrane to bleb out from the cell (c). This piece of membrane which contains GlyRs, is then used in various conditions to detect glycine release in the SR of mouse hippocampus. (II) Real-time images showing step-by-step the formation of an outside-out patch.

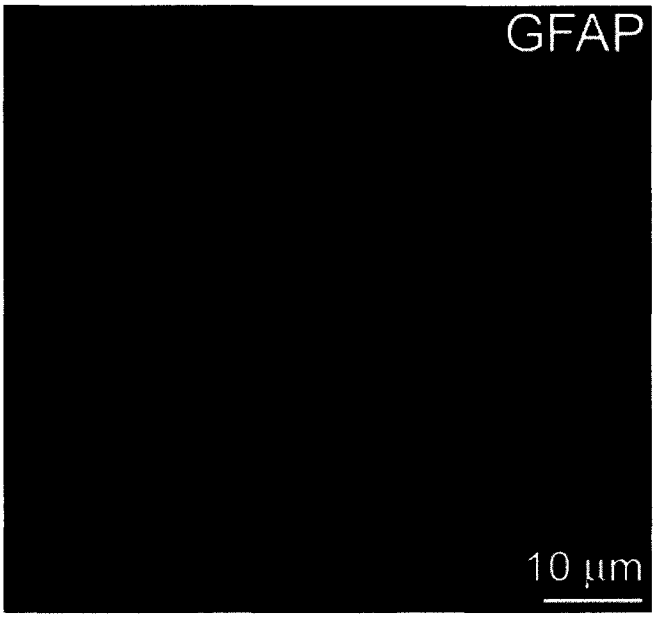
**Figure 6**




## Immunocytochemical detection of glycine in the *stratum radiatum* of the CA1 region of mouse hippocampus

(**A-C**) Immunofluorescent confocal sections of hippocampal slices triple labeled for glycine (**A**), GFAP (**B**) and synaptophysin (**C**). so, *stratum oriens*; sr, *stratum radiatum*; slm, *lacunosum moleculare*; sp; pyramidal layer. (**D-G**) Higher magnification of the *stratum radiatum* (asterisk) for glycine-IR (D), GFAP-IR (E), synaptophysin-IR (F) and merged images (G). Glycine-IR clusters (arrows). Glycine clusters co-localized with synaptophysin clusters (white arrowheads). Glycine clusters co-localized with GFAP-IR (half arrowheads) or isolated (black arrowheads). (**H**) Quantitative analysis of the percentage of glycine clusters co-localized with synaptophysin clusters, GFAP-IR or isolated. Note the high proportions of glycine-synaptophysin co-clusters. (**I**) Quantitative analysis of the percentage of synaptophysin clusters co-localized with glycine clusters or GFAP-IR. Data are from 10 confocal images from 2 different animals. Values are mean ± SEM.

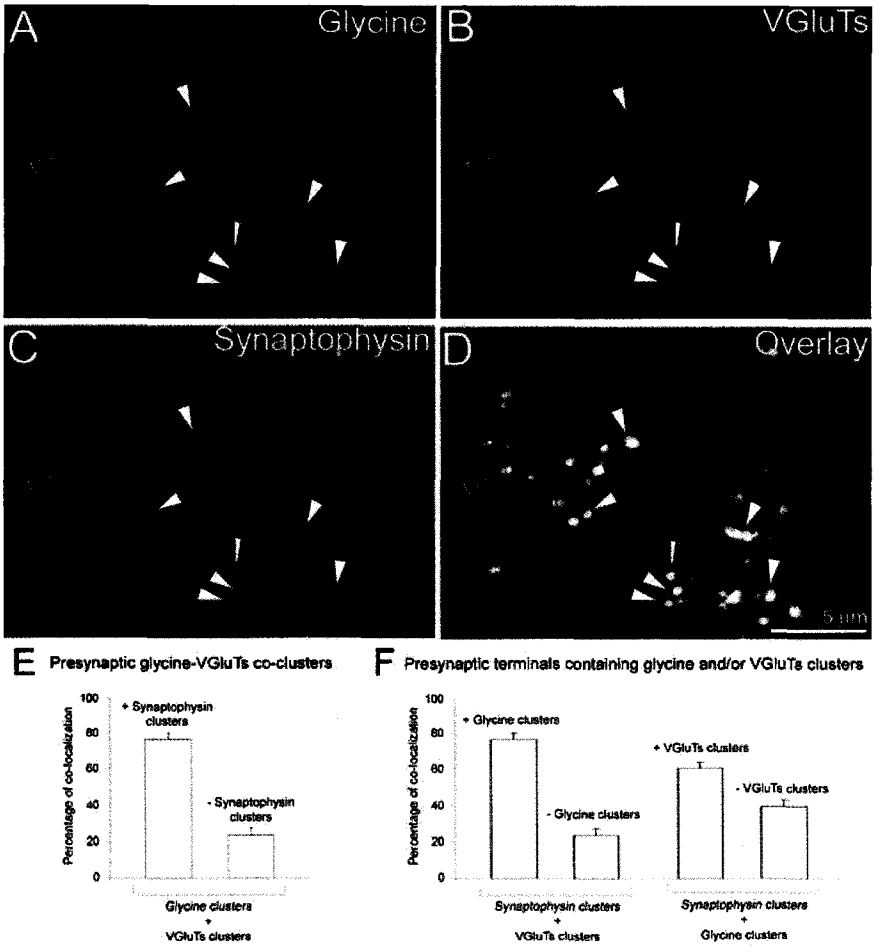
**Figure 7**




## Reconstruction of a GFAP-positive astrocyte.

Projection of 20 0.3mm-spaced confocal sections of hippocampal slices labeled for GFAP.

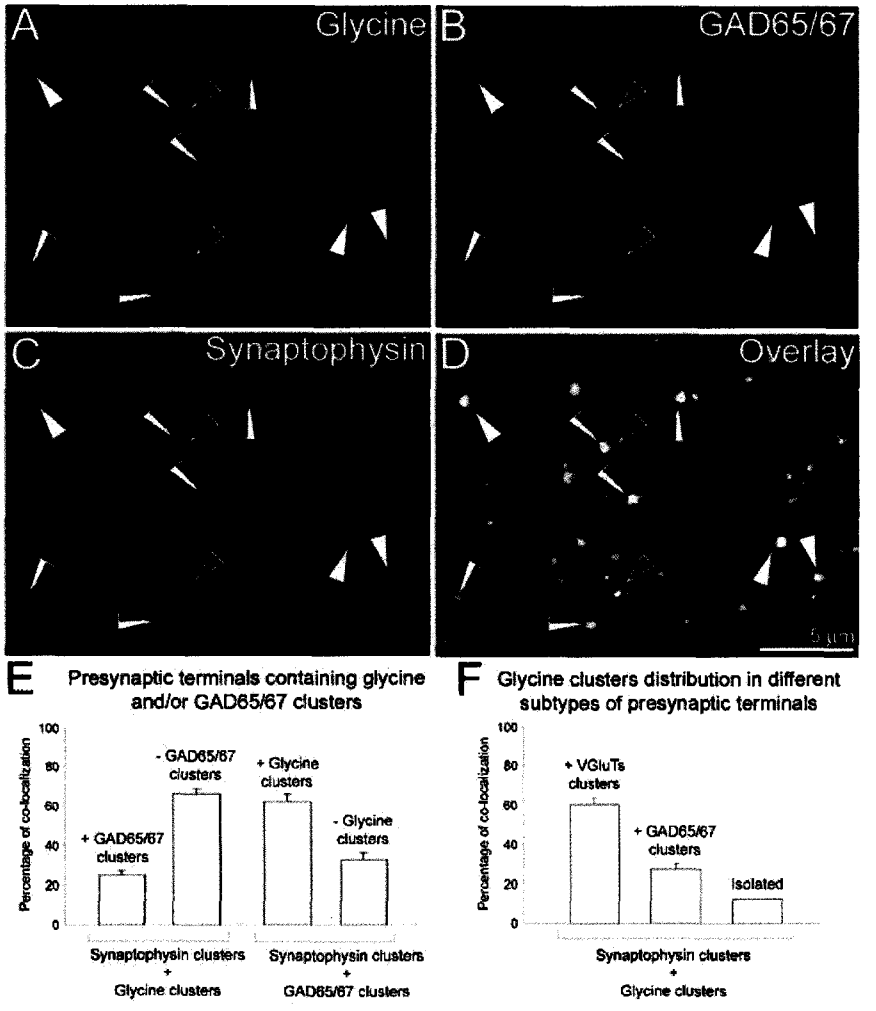
**Figure 7S**




# Immunocytochemical detection of glycine in glutamatergic presynaptic terminals in the *stratum radiatum* of the CA1 region of mouse hippocampus

(A-D) Immunofluorescent confocal sections of hippocampal slices (*stratum radiatum*) triple labeled for glycine (A), VGluTs (B), synaptophysin (C), and merged images (D). Glycine-synaptophysin co-clusters alone (half arrowhead) or co-localized with VGluTs clusters (white arrowheads). Synaptophysin-VGluTs co-clusters (black arrowhead). (E) Quantitative analysis of the percentage of glycine-VGluTs co-clusters alone or co-localized with synaptophysin clusters. Note the high proportions of triple co-localization. (F) Quantitative analysis of the percentage of synaptophysin-VGluTs co-clusters alone or co-localized with glycine clusters, and the percentage of synaptophysin-glycine clusters alone or co-localized with VGluTs clusters. Data are from 10 confocal images from 2 different animals. Values are mean ± SEM.

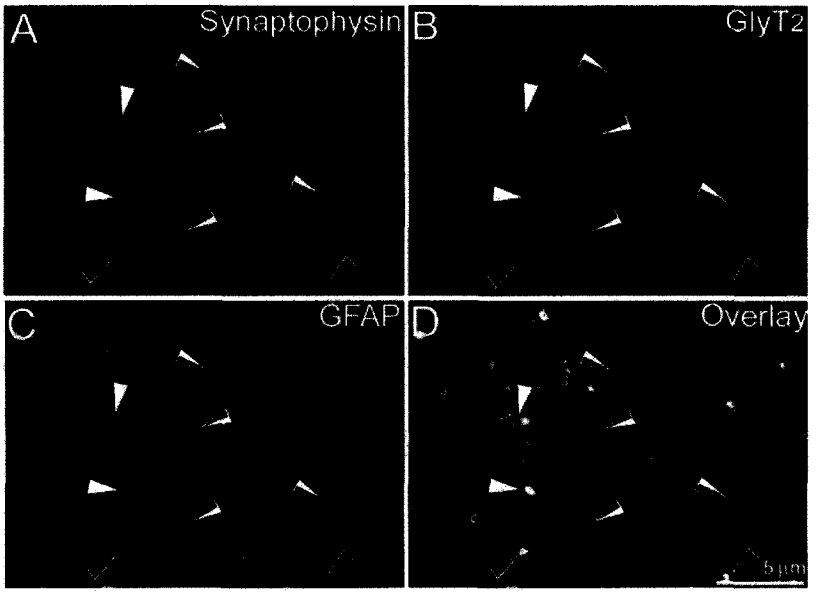
## Figure 8

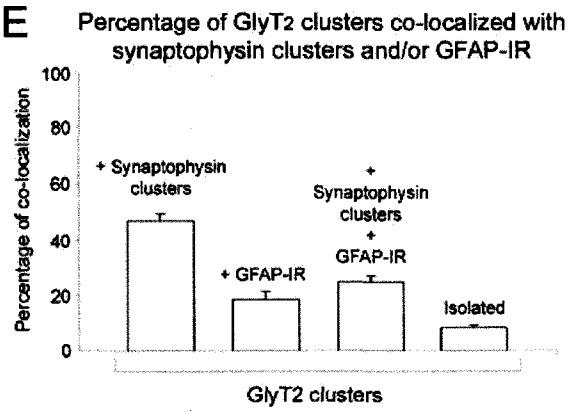



## Immunocytochemical detection of glycine in GABAergic presynaptic terminals in the *stratum radiatum* of the CA1 region of mouse hippocampus

(A-D) Immunofluorescent confocal sections of *stratum radiatum* hippocampus triple labeled for glycine (A), GAD65/67 (B), synaptophysin (C), and merged images (D). Synaptophysin-glycine co-clusters alone (half arrowheads) and co-localized with GAD65/67 clusters (white arrowheads). Synaptophysin-GAD65/67 co-clusters associated to glycine clusters (black arrowheads). (E) Quantitative analysis of synaptophysin clusters co-localized with GAD65/67 and/or glycine clusters. (F) Quantitative analysis of presynaptic glycine clusters distribution in terminals containing VGluTs, GAD65/67 or none. Note that ~13% of synaptophysin-glycine co-clusters were isolated. Data are from 10 confocal images from 2 different animals. Values are mean ± SEM.

**Figure 9**

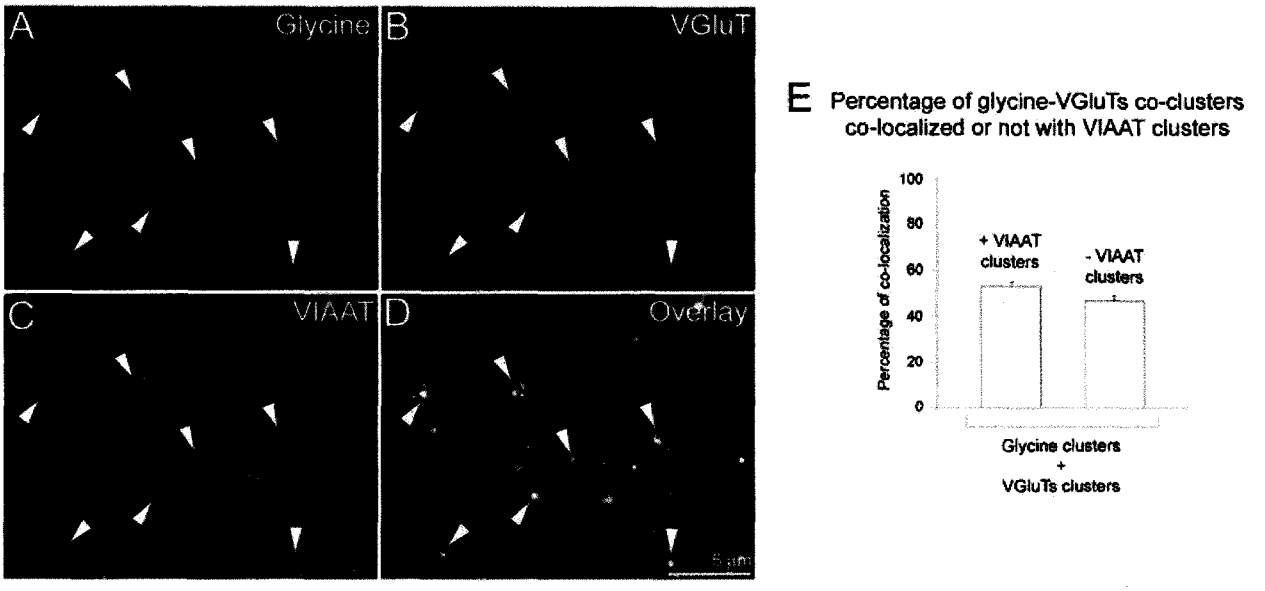



# Immunocytochemical detection of GlyT2 transporters: synaptic or astrocytic distribution in the *stratum radiatum* of the CA1 region of the mouse hippocampus

(A-D) Immunofluorescent confocal sections of *stratum radiatum* hippocampus triple labeled for synaptophysin (A), GlyT2 (B), GFAP (C) and merged images (D). GlyT2 clusters co-localized with synaptophysin clusters (black arrowheads), GFAP-IR (half arrowheads) or both (white arrowheads). (E) Quantitative analysis of GlyT2 clusters co-localized with synaptophysin clusters and/or GFAP-IR. Note that the proportion of GlyT2 clusters co-localized with synaptophysin clusters alone was similar to the proportion of GlyT2 clusters co-localized with GFAP alone plus those co-localized with both GFAP-IR and synaptophysin clusters. Data are from 10 confocal images from 2 different animals. Values are mean ± SEM.

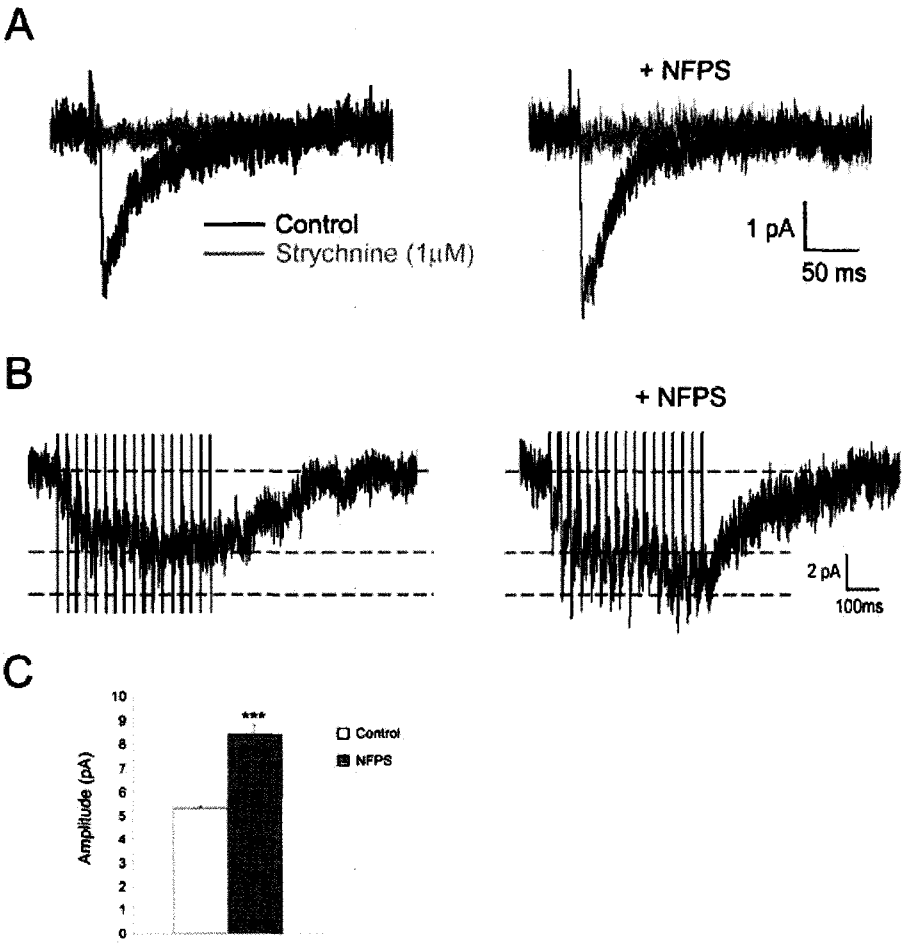
**Figure 10**




# Immunocytochemical detection of VIAAT in glutamatergic presynaptic terminals containing glycine in the *stratum radiatum* of the CA1 region of the mouse hippocampus

(A-D) Immunofluorescent confocal sections of *stratum radiatum* hippocampus triple labeled for glycine (A), VGluTs (B), VIAAT (C), and merged images (D). Glycine-VGluTs co-clusters co-localized with VIAAT clusters (white arrowheads). (E) Quantitative analysis of glycine-VGluTs co-clusters alone or co-localized with VIAAT clusters. Data are from 10 confocal images from 2 different animals. Values are mean ± SEM.

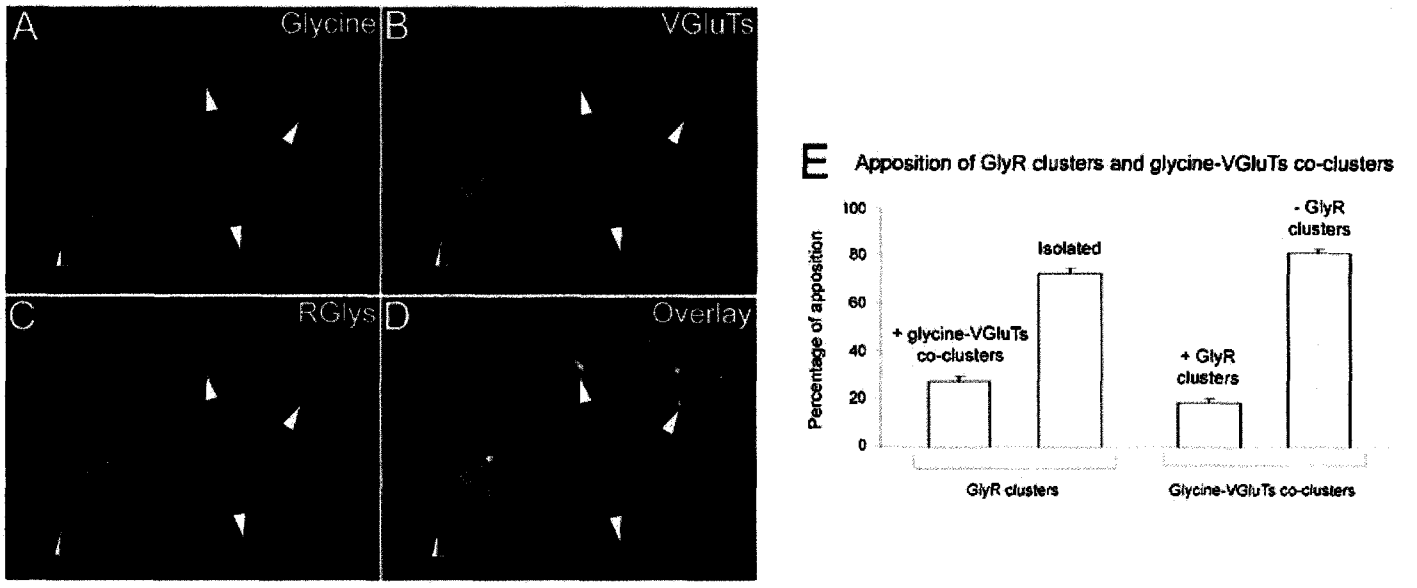
**Figure 11**




## Frequency-dependent release of glycine in the *stratum radiatum* of the CA1 region of the mouse hippocampus

(A) GlyRs-mediated currents (black trace) recorded in absence (left) and presence (right) of NFPS. Note that 1μM strychnine abolished this current in both conditions (gray trace). (B) GlyRs-mediated currents evoked by a 33Hz train (500 ms) in absence (left) and presence (right) of NFPS. Note the progressive increase in the current amplitude. (C) Amplitude of GlyRs-mediated currents measured at the end of the 500ms stimulation train, in presence or in absence of NFPS. Each trace is an average of 5 traces.

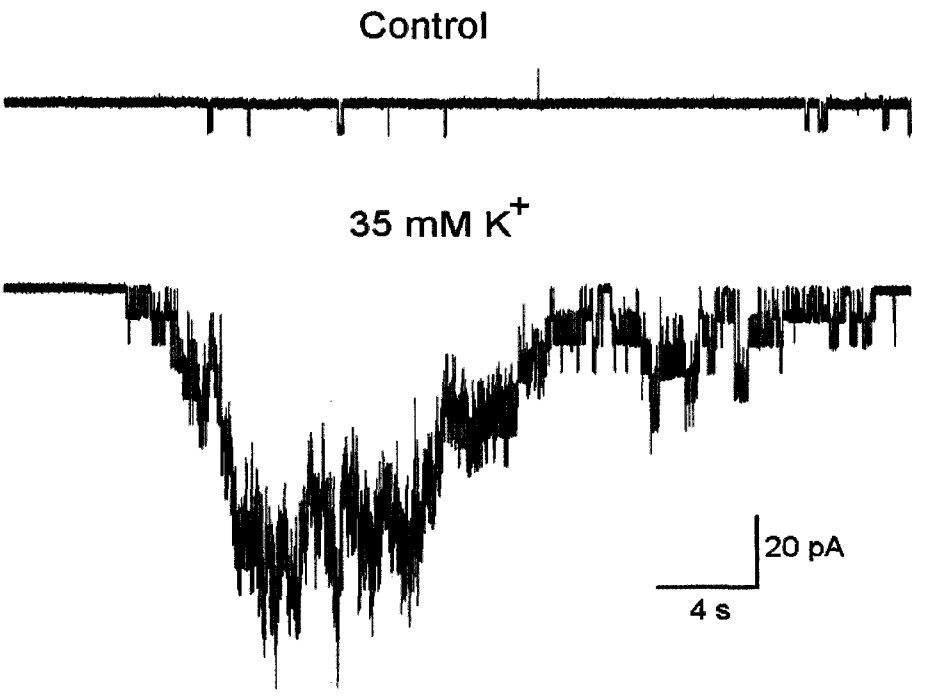
**Figure 12**




## Immunocytochemical detection of GlyRs facing glutamatergic presynaptic terminals containing glycine in the *stratum radiatum* of the CA1 region of the mouse hippocampus

(A-D) Immunofluorescent confocal sections of *stratum radiatum* hippocampus triple labeled for glycine (A), VGluTs (B), GlyRs (C) and merged images (D). GlyRs-IR mainly provided weak diffuse staining. GlyR clusters alone (half arrowhead) or co-localized with glycine-VGluTs co-clusters (black arrowhead). Glycine-VGluTs co-clusters apposed to GlyR clusters (white arrowheads). (E) Quantitative analysis of GlyR clusters *vs.* glycine-VGluTs co-clusters apposition. Data are from 10 confocal images from 2 different animals. Values are mean ± SEM.

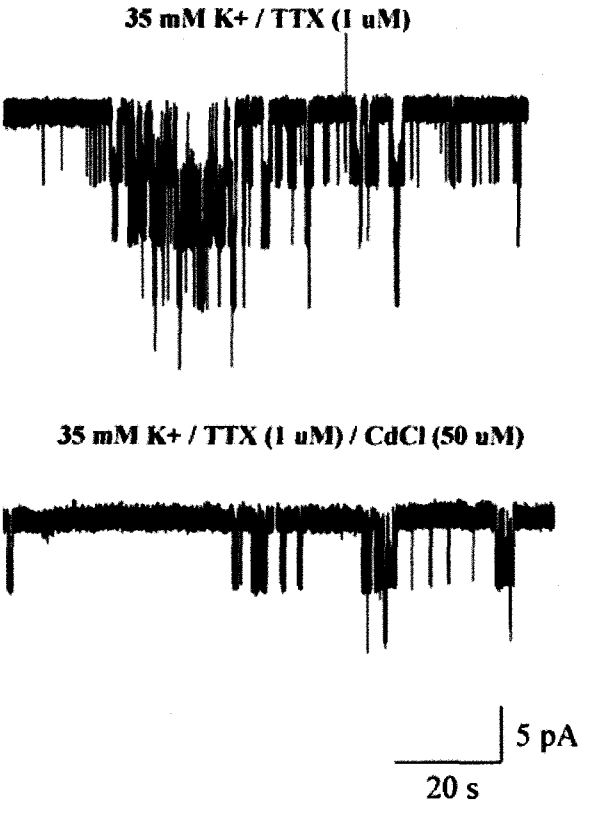
**Figure 13**




## Potassium-dependent glycine release in the *stratum radiatum* of the CA1 region of mouse hippocampus

Single channel recordings from sniffer obtained from CHO cells expressing glycine receptors (GlyRs) in absence (Control; upper trace) and presence (35mM $K^+$; lower trace) of 35mM of potassium. Note that the addition of 35mM of potassium to the ACSF caused a 7-fold increase in activity of the GlyRs.

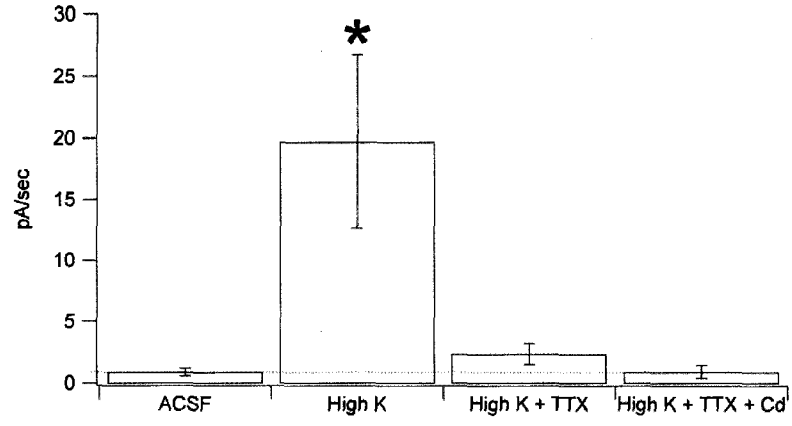
**Figure 14**




## Potassium-induced glycine release depends partially on synaptic activity and on extracellular calcium

Single channel recordings from sniffer obtained from CHO cells expressing glycine receptors (GlyRs) in presence of 35mM $K^+$ and 1μM TTX (upper trace) and 35mM $K^+$, 1μM TTX and 50 μM CdCl (lower trace). Note that the addition of TTX and TTX + CdCl, significantly reduced the potassium-induced glycine release.

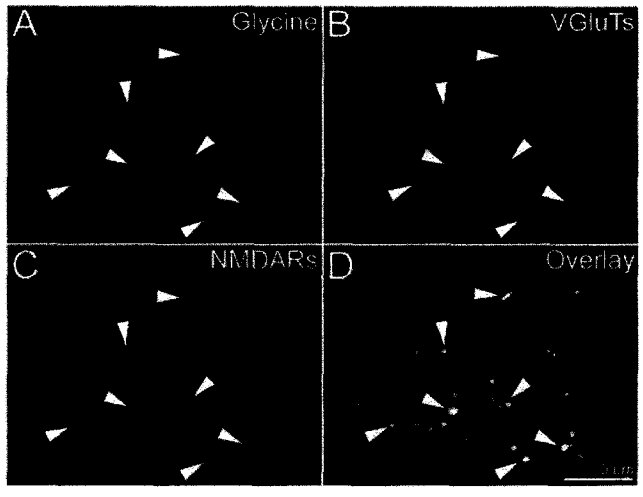
**Figure 15**

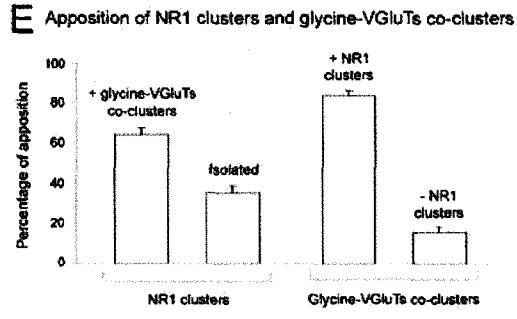



## Effects of exogenous application of high potassium, TTX and CdCl on glycine release in the CA1 region of the mouse hippocampus

Quantitative analysis of glycine release in the stratum radiatum of the CA1 region of the mouse hippocampus upon treatment with high K alone, high K + TTX and high $K^+$ TTX + Cd. Values are mean ± SEM.

**Figure 16**

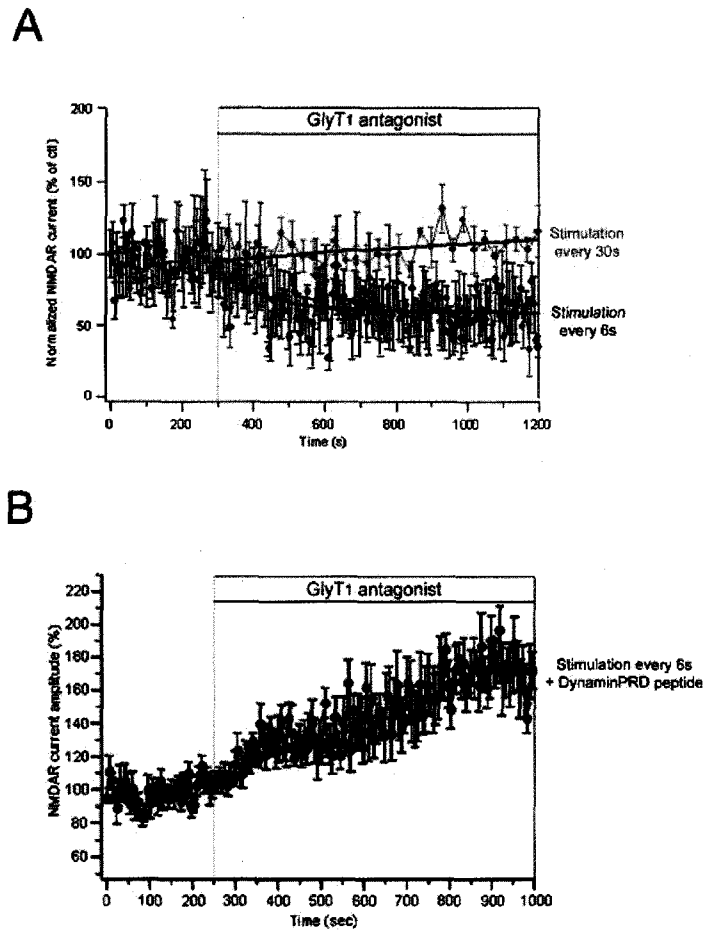



## Immunocytochemical detection of NMDARs facing glutamatergic presynaptic terminals containing glycine in the *stratum radiatum* of the CA1 region of the mouse hippocampus

(A-D) Immunofluorescent confocal sections of *stratum radiatum* hippocampus triple labeled for glycine (A), VGluTs (B) NR1-containing NMDARs (C) and merged images (D). NR1-containing NMDAR clusters apposed to glycine-VGluTs co-clusters (white arrowheads). (E) Quantitative analysis of NR1-containing NMDAR clusters *vs.* glycine-VGluTs co-clusters apposition. Data are from 10 confocal images from 2 different animals. Values are mean ± SEM.

**Figure 17**




## Effect of two different frequency of stimulation on the amplitude of NMDARs currents in presence of NFPS (300nM), a GlyT1 antagonist

(A) Graph showing the normalized amplitude of NMDAR currents (%) recorded from CA1 pyramidal cells in hippocampal slice using whole-cell patch clamp recording in voltage clamp mode, as a function of time, upon two different frequency of stimulation. Stimuli were delivered every 6s (black) or 30s (red). (B) Graph showing the normalized amplitude of NMDAR currents evoked every 6s, as a function of time, with dynaminPRD.

**Figure 18**

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