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Effect of GABA$_A$ receptor clustering on phasic and tonic inhibition in the hippocampus

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List of publications

The results I collected during the four years I spent at the International School for Advanced Studies have been published in the following papers.

Part of them has been reported in this thesis.

- Barberis A., **Petrini E.M.,** Cherubini E., Mozrzymas J.W.,
  Allosteric interaction of Zn^{2+} with recombinant $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ $GABA_AR$. *Neuropharmacology* 2002; 43(4): 607-18.

- **Petrini E.M.,** Zacchi P., Barberis A., Morzyzmas J.W., Cherubini E.,
  Declusterization of $GABA_AR$ affects the kinetic properties of GABAergic currents in cultured hippocampal neurons
  Journal of Biological Chemistry 2003; 278(18): 16271-16279

- **Petrini E.M.**, Marchionni I., Zacchi P., Sieghart W., Cherubini E.
  Clusterization of extrasynaptic $GABA_A$ receptors modulates tonic inhibition in cultured hippocampal neurons
  Journal of Biological Chemistry Published On Line 17 Aug 2004

- Barberis A., **Petrini E.M.,** Cherubini E.
  Presynaptic source of quantal size variability at GABAergic synapses in rat cultured hippocampal neurons
  European Journal of Neuroscience 2004; Published On Line 14 Sep 2004

Abbreviations

[ ]:	concentration
BAPTA:	bis(2-amino-5-bromophenoxy)ethane-N,N,N’,N’-tetracetic acid
BDZ:	benzodiazepine
CF-PC:	climbing fiber-Purkinje cells
CNS:	central nervouls system
CPZ:	chlorpromazine
FZP:	flurazepam
GABA:	γ-aminobutyric acid
$GABA_AR$:	γ-aminobutyric acid type A receptor
GABA-T:	γ-aminobutyric acid transaminase
GAD:	glutamic acid decarboxylase
GAT:	γ-aminobutyric acid transporter
HEPES:	N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
IPSC:	inhibitory postsynaptic currents
MF:	mossy fiber
PBS:	phosphate buffer saline
PFA:	paraformaldehyde
P_r:	probability of release
PSD:	post synaptic density
SSA	succinic semaldehyde
TTX:	tetrodotoxin

1. Abstract

Inhibitory transmission plays a major role in information processing in the brain since it integrates excitatory signals and defines the gain between neural input and output. γ-Amino butyric acid (GABA) is the main inhibitory neurotransmitter in the adult mammalian brain. By activating $GABA_A$ and $GABA_B$ receptors this neurotransmitter inhibits neuronal firing and stabilizes the membrane potential near the resting value. In particular $GABA_A$ receptors are permeable to chloride ions and are responsible for phasic and tonic hyperpolarizing responses. GABA-mediated currents are the result of rapid, sequential events including transmitter release from the presynaptic terminal, transmitter diffusion within and outside the cleft and post-synaptic receptors gating. The kinetics of each of these processes is crucial in determining the shape of post-synaptic currents. Therefore the modulation of any of these events leads to the heterogeneity of GABAergic responses and to changes in the potency of inhibition.

In this thesis I have studied the sources of such variability at presynaptic/cleft and postsynaptic level. At presynaptic/cleft level I have focused on the influence of the agonist concentration profile in the synaptic cleft on GABA-mediated synaptic currents. Fast-off competitive antagonists and computer simulations allowed estimating the range of variability of the peak concentration and the speed of GABA clearance form the synaptic cleft. At postsynaptic level particular attention has been attributed to the impact of $GABA_A$ receptors clustering on both phasic and tonic $GABA_A$-mediated inhibition. With ultrafast applications of GABA and computer simulations it was possible to describe the modulation of $GABA_A$ receptor gating induced by clustering.

2. Introduction

2.1 Discovery of GABA as inhibitory neurotransmitter

The biochemical identification of GABA dates back to 1949 when the biochemist Eugene Roberts was performing ninhydrin-based chromatographic quantifications of the free amino acid content in the mouse brain. He found by chance an unidentified and previously unobserved ninhydrin-positive compound, which later proved to be GABA (Roberts & Frankel, 1950; Awapara *et al.*, 1950). A further and absolute characterization of GABA came from Udenfriend (1950) with the isotope-derivative method he had developed. The careful and dedicated work of Roberts and colleagues lead to a thorough analysis of GABA metabolism (Roberts *et al.*, 1960). In particular it was found that in the nervous tissue GABA is mainly synthesized from glutamate by glutamic acid decarboxylase (GAD) and it is degradated by GABA transaminase to glutamate and succinic semialdehyde (SSA), the latter being rapidly converted to succinate.

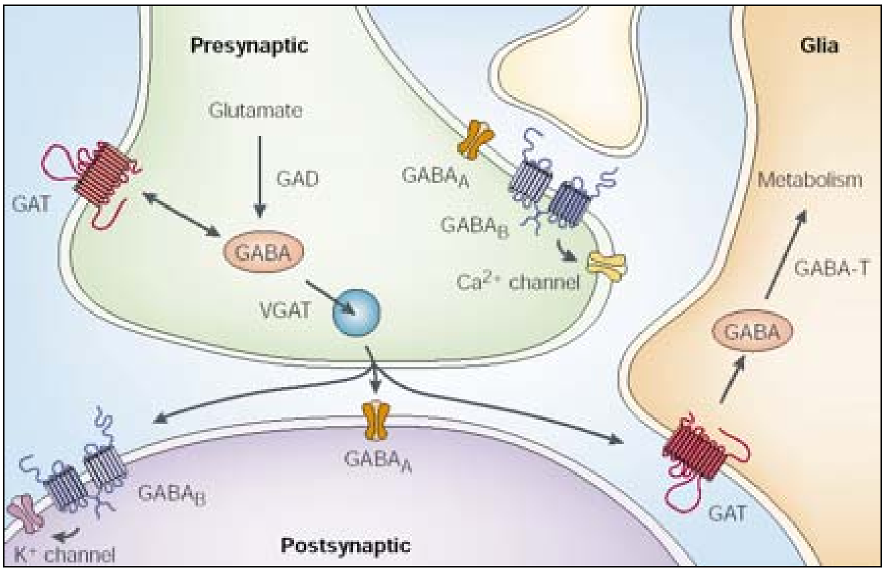
Only several years after the biochemical identification of GABA, came its functional characterization. The first suggestion that GABA might have an inhibitory function in the vertebrate nervous system came from studies in which it was found that topically applied solutions of GABA exerted inhibitory effects on electrical activity in the brain (Hayashi & Suhara, 1956). Definitive evidence for an inhibitory function of GABA came from the finding that GABA increases the membrane conductance by opening chloride channels (Krnjevic & Schwartz, 1966) and from the identification of the hyperpolarizing effect of GABA through an influx of Cl^- ions (ten Bruggencate & Engberg, 1971) in different species and brain regions.

2.2 GABAergic transmission

Synaptic signaling in the central nervous system is the final result of the counterbalance between excitatory and inhibitory stimuli. In particular the inhibitory system is devoted to tune the excitability of the cells either by shifting the resting potential towards more negative values or by lowering the cell membrane resistance.

An inhibitory current can be elicited by the inflow of negative charged ions or by the outflow of positive charges through ligand- or voltage-activated channels.

In the adult brain GABA is the main inhibitory neurotransmitter (Sivilotti & Nistri, 1991; Kaila, 1994). GABA is synthesized in the presynaptic terminals from glutamate through a catabolic process catalyzed by two glutamic acid decarboxylases (GAD), namely GAD65 and GAD67 (Erlander *et al.*, 1991). GABA is then loaded into synaptic vesicles by the vesicular inhibitory aminoacid transporter VIAAT (Gasnier, 2000) from which it can be released upon nerve stimulation. Once released through calcium-dependent exocytosis, GABA activates ionotropic Cl^- permeable channels ($GABA_A$ receptors) (Bormann *et al.*, 1987; Schofield *et al.*, 1987; Polenzani *et al.*, 1991) and metabotropic G-coupled receptors ($GABA_B$ receptors) (Wilkin *et al.*, 1981; Kaupmann *et al.*, 1997), which in turn indirectly increase K^+ currents (Gage, 1992; Mott & Lewis, 1994; Misgeld *et al.*, 1995) and/or reduce voltage-sensitive Ca^{2+} currents (Kamatchi & Ticku, 1990). GABA is cleared from the synaptic cleft by the uptake of specific transporters (GAT) located at nerve terminals and at glial cells (Cherubini & Conti, 2001). Thereafter GABA is finally metabolized by transamination of GABA-T (Roberts, 1988).



Owens D.F. and Kriegstein A.R,. Nat Rev Neurosci (2002)

$GABA_B$ autoreceptors located at presynaptic terminal are thought to contribute to a fine control of GABA-mediated inhibition through a negative feedback on the release machinery (Thompson & Gahwiler, 1989; Misgeld *et al.*, 1995; Jarolimek & Misgeld, 1997).

On the contrary $GABA_A$ receptors are mainly expressed on postsynaptic neurons, although they have been recently supposed to be present also at presynaptic sites (Ruiz *et al.*, 2003). In fact action potentials evoked with antidromic stimulation of mossy fibers are sensitive to muscimol application, thus suggesting that presynaptic $GABA_A$ receptors contribute to regulating neuronal excitability. Depending on the concentration of GABA they see and on the duration of the exposure, these ionotropic receptors can elicit either phasic or tonic currents. The main differences between these two forms of GABA-mediated inhibition basically are the duration and the amplitude of the currents. Several drugs have been identified in order to pharmacologically distinguish them (Bai *et al.*, 2001; Stell & Mody, 2002). However, although for sake of clarity phasic and tonic currents are usually described separately, they are tightly related and mutually influenced.

2.2.1 Phasic GABAergic currents

Phasic GABAergic activity (synaptic transmission) is a point-to-point inhibition consisting of a series of anionic mediated currents, namely inhibitory postsynaptic currents (IPSCs), that, after reaching a peak in less than 1 ms, decay in tens of milliseconds. These currents result from the release of GABA-containing vesicles and the subsequent activation of synaptic $GABA_A$ receptors located opposite the release site.

Peculiar characteristics of this fast form of transmission are the high concentration of agonist available to activate the receptors (in the submillimolar range) and the extremely brief time of exposure of the receptors to GABA (hundreds of microseconds). Direct consequences are that the receptors are activated in strong non-equilibrium conditions, usually at subsaturating level, for short periods of time before becoming available again for subsequent activation. The presence of clusters of $GABA_A$ receptors opposite the release site is an essential requirement to guarantee efficient synaptic inhibitory transmission.

The variability of phasic synaptic currents depends on several parameters occurring at different levels. Basically it is possible to distinguish between presynaptic/cleft and postsynaptic levels. At presynaptic level neurotransmitter release is assumed to be mainly a “quantal” process (Jonas *et al.*, 1993; Jack *et al.*, 1994; Edwards *et al.*, 1990). This means that neurotransmitter is released from presynaptic terminals in discrete units thought to be equal to the amount of neurotransmitter packed within a single presynaptic vesicle (the so-called “quanta”) (Katz, 1969). Assuming that the release of a single quantum elicits a unitary current (quantal size, q), the amplitude of synaptic currents should vary as multiples of quantal size. However, large variability has been found in synaptic currents. This is due to many other parameters included in the "quantal theory of release" proposed by Del Castillo & Katz (1954), such as the probability of release (p), the number of release sites (n). Moreover many other parameters can represent presynaptic sources of synaptic variability, such as the concentration of neurotransmitter in the vesicles, the size and the number of vesicles released and the kinetics of release mechanisms. Nevertheless, structural and molecular features of the release site could influence synaptic currents as well. Among them it is worth mentioning the number and type of Ca^{2+} channels, their localization respect to the vesicles, presynaptic proteins that allow the vesicles to be released (vesicle priming). Once the transmitter is released in the cleft, different processes (*i.e.*, diffusion of transmitter molecules and uptake) and the synapse geometry can influence its the concentration profile in the cleft and consequently synaptic currents (Atwood & Karunanithi, 2002).

At postsynaptic level morphological, electrical and chemical features can relevantly provide further sources of variability of synaptic currents. In fact the number, localization, type, affinity and gating properties of postsynaptic receptors in addition to stochastic channel gating, can provide different responses to the same quantum.

Postsynaptic determination of quantal size relies on the variability of the receptor number and properties. At a single synapse, if the number of receptors is limited, then the transmitter content of a vesicle could saturate them. Therefore any change in vesicular content would not be detected, so quantal size would only reflect postsynaptic parameters. On the contrary, at non saturated synapses, when receptor availability is not a limiting factor, quantal size is mainly dependent on presynaptic factors (*e.g.*, the amount of transmitter released from the vesicle, that rate at which it leaves the vesicle) (Atwood & Karunanithi, 2002) (see also paragraph 2.5.1).

These topics will be described in details in the next paragraphs and will be implemented with the experimental results reported in this thesis.

2.2.2 Tonic GABAergic inhibition

Tonic inhibition includes tonic currents and shunting inhibition. Tonic current is a persistent inhibitory conductance due to the activation of extrasynaptic $GABA_A$ receptors continuously exposed to submicromolar concentrations of GABA. From the Ohm's law it is rather intuitive that continuous activation of anionic channels leads to a decrease of the membrane resistance (shunting inhibition) thus reducing the gain between neural input and output (Blomfield, 1974).

The concentration of GABA that elicits tonic inhibition is at least three order of magnitude lower than that responsible for phasic transmission and it is called "ambient GABA" since it represents the amount of neurotransmitter present in the extracellular space. Ambient GABA originates from spillover of the neurotransmitter released at neighboring synapses (Brickley *et al.*, 1996; Mitchell & Silver, 2000; Hamann *et al.*, 2002), from astrocytes (Liu *et al.*, 2000; Schousboe, 2003) or from non-vesicular release (Attwell *et al.*, 1993; Wu *et al.*, 2001). For instance it has been demonstrated that rat hippocampal neurons co-cultured on a monolayer of astrocytes or exposed to astrocyte-conditioned medium exhibit larger bicuculline-sensitive currents than control neurons, demonstrating that GABA is actually released from astrocytes (Liu *et al.*, 1996; Liu *et al.*, 1997; Liu *et al.*, 2000). Further sources of GABA can be the reverse operation of GABA transporters, also known as non-vesicular release (Attwell *et al.*, 1993). In fact GABA transporters on surrounding neurons and/or glia can reverse either in physiological or pathological conditions upon increase in the cytosolic concentration of GABA or mild depolarization (Wu *et al.*, 2001; Wu *et al.*, 2003). Moreover, the efficiency of GABA reuptake system contributes to regulating the concentration of ambient GABA (Isaacson *et al.*, 1993; Semyanov *et al.*, 2003; Jensen *et al.*, 2003).

It is almost widely accepted that tonic inhibition is mainly mediated by extrasynaptic receptors, thus they act as high affinity sensors for ambient GABA. In fact the contribution of synaptic receptors to tonic currents can be almost excluded, since the number of extrasynaptic receptors largely exceeds that of synaptic ones (Nusser *et al.*, 1995; Banks & Pearce, 2000). Moreover, synaptic receptors would be less exposed to ambient GABA since their location within the cleft should hinder spillover.

2.3 Presynaptic modulation of synaptic transmission

At presynaptic level the concentration of transmitter in the cleft promptly increases after exocytosis, reaches a maximum value (peak) and quickly decreases (clearance). These factors in turn reflect the mechanisms of exocytosis, the number and the content of the vesicles released and the diffusion process across the cleft.

2.3.1 Mechanisms of exocytosis

For many years exocytosis has been supposed to consist of the collapse of the vesicle into the presynaptic plasma membrane and consequent immediate dump of neurotransmitter in the cleft. In the late ‘70s the immediate dump hypothesis was regarded as an oversimplification of exocytosis. In fact an alternative mechanisms implying the formation of a fusion pore connecting the vesicle lumen with the synaptic cleft was proposed (Chi *et al.*, 1976). After the tethering or docking of the vesicles to the appropriate presynaptic site, the exocytotic event basically occurs in two steps, *i.e.* the formation of a narrow fusion pore persisting for a variable time (priming) and the subsequent rapid expansion of the fusion pore that leads to the complete fusion of the synaptic vesicle with the presynaptic terminal (Breckenridge & Almers, 1987). These two steps are kinetically separated. The kinetic behavior of the exocytotic process can be described by a Markovian scheme, including a fusion pore closed state (C), open state (O) and a dilation state (D) (Wang *et al.*, 2001).

$$\xrightarrow{k_a} C \underset{k_c}{\overset{k_o}{\rightleftarrows}} O \xrightarrow{k_d} D \qquad (1)$$

The rates of the transitions between different states are defined by the rate constants ka, ko, kc and kd. The kinetics of the fusion pore can be described by its flickering between the closed and open state (allowing a partial release of neurotransmitter) before its dilation leads to the complete release of the vesicle content (Alvarez *et al.*, 1993). Since the transmitter can be released through the fusion pore, the rate at which the transmitter is released in the cleft is strongly dependent on the persistence of the fusion pore before its dilation. If the fusion pore persists for a relatively long time, the whole content of the vesicle will be released through a narrow fusion pore at a relatively low rate (continuous release). Otherwise, if the fusion pore quickly undergoes dilation, the rate of release will be much faster (instantaneous release) (Kleinle *et al.*, 1996). It has been found that a threshold exists for discriminating between the flickering and the dilating behavior of the fusion pore (Albillos *et al.*, 1997). In fact a stable fusion pore can exist only below a critical pore size. Below this critical value the pore is able to fully close and re-open, while at pore size larger than the critical value the pore expands quickly and irreversibly (Lollike *et al.*, 1998). This critical value has been estimated in chromaffin cells to be ~ 3 nm (Albillos *et al.*, 1997).

Different approaches have been developed to correlate the fusion process and the rate of efflux of the transmitter during exocytosis (Lindau & Alvarez, 2003; Westerink, 2004). For technical reasons, most of the experiments addressing this issue have been performed in non-neuronal secretory cells such as neutrophil, chromaffin cells, mast cells since they bear large secretory granules (Alvarez *et al.*, 1993; Lollike *et al.*, 1995; Leszczyszyn *et al.*, 1990; Lollike & Lindau, 1999). It has been assumed that the basic mechanisms of exocytosis in these cell types are similar to those occurring in neurons as several proteins involved in the exocytotic machinery in non-neuronal secretory cells are also present in neurons (Li & Chin, 2003; Rothman, 1994; Jahn & Sudhof, 1999). One approach to follow neurotransmitter release is measuring the capacitance of the presynaptic cell membrane (Gillis, 1995). In fact during exocytosis the vesicles are fused with the plasma membrane resulting in an increased membrane area, which can be resolved as an increase in the capacitance. Although this technique allows estimating the overall neurotransmitter released, it does not clarify the amount released per vesicle. An alternative approach to investigate single vesicle release is amperometry (Chow & von Ru¨den, 1995; Chow *et al.*, 1992; Wightman *et al.*, 1991). Briefly, a carbon fiber microelectrode is placed against the surface of a secretory cell in order to oxidize the neurotransmitter molecules (provided that they must be electron donor – *e.g.* catecholamine and serotonin). In this way when exocytosis occurs, the transfer of electrons during the oxidation of the secreted neurotransmitter can be recorded as an electrochemical current. During the exocytotic event, the amperometric signal shows a slight increase (foot) followed by a large spike.

According to the kinetic model (1), the increase of the amperometric signal before the spike (foot) is due to the transmitter released through the fusion pore and corresponds to the open state (O) (Alvarez *et al.*, 1993). The subsequent large amperometric spike is ascribed to the irreversible full fusion of the vesicle with the plasma membrane and corresponds to the dilation state (D). Occasionally, the fusion pore can persist for a time sufficient to allow the complete release of the vesicle content without full fusion of the vesicle (Albillos *et al.*, 1997). Furthermore imaging techniques have been developed to follow the fate of the vesicles during exocytosis. In recent years a number of specific dyes such as membrane-fluorescent styryl dyes (belonging to the FM dyes family) with different kinetics of dissociation from the membrane have been used to distinguish between the complete or incomplete depletion of synaptic vesicles (Aravanis *et al.*, 2003a; Aravanis *et al.*, 2003b). Moreover the question if transmitter release from synaptic vesicles is associated with full incorporation of the vesicle into the plasma membrane or if only a transient fusion pore is formed under physiological conditions has been controversial for many years (Heuser & Reese, 1973; Ceccarelli *et al.*, 1972; Ceccarelli *et al.*, 1973). Full fusion means that, following its formation, the fusion pore irreversibly expands to a large size and the vesicle membrane collapse into the plasma membrane. Transient fusion (usually also referred as “kiss-and-run”) means that the pore opens and possibly expands, but then closes again such that the vesicle retains its integrity when it discharges its content (Klingauf *et al.*, 1998; Stevens & Williams, 2000). The latter mechanism has recently gained more evidence but still its predominance during exocytotic events is a matter of debate (Stevens & Williams, 2000; Aravanis *et al.*, 2003a; Aravanis *et al.*, 2003b; Lindau & Alvarez, 2003). In fact some authors have demonstrated that in physiological conditions the kiss-and-run events are prevalent (~ 85 %) (Aravanis *et al.*, 2003a; Aravanis *et al.*, 2003b), while some others have observed a lower occurrence of this exocytotic mechanisms, although it can become more frequent under particular conditions. In fact an increase in the kiss-and-run mode from ~ 20% to over 80 % has been recorded during the superfusion of the synapses with a hypertonic solutions (Stevens & Williams, 2000) or in the presence of an elevated concentration of Ca^{2+} (Ales *et al.*, 1999). It has also been demonstrated that the "kiss and run" type of release is enhanced by the kinase inhibitor staurosporine, suggesting that this secretion mode is regulated by protein phosphorylation (Henkel *et al.*, 2001). Furthermore, it is known that stability of the fusion pore kinetics is oppositely regulated by two different isoforms of synaptotagmin, a protein intimately associated with the fusion pore itself (Wang *et al.*, 2001). In particular the overexpression of synaptotagmin I increased while synaptotagmin IV reduced the duration of the amperometric foot current indicating respectively an increased and a reduced lifetime of the fusion pore.

2.3.2 Amount of neurotransmitter in the cleft

Among the factors contributing to the presynaptic modulation of synaptic transmission such as the release mechanisms and the diffusion process, there is the variability of the amount of neurotransmitter present in the cleft.

This parameter is strongly influenced by the volume and the number of vesicles released and by the concentration of neurotransmitter per vesicle.

As clearly demonstrated for catecholamine-containing granules in chromaffin cells (Albillos *et al.*, 1997) the neurotransmitter content is proportional to the volume of the vesicle. With amperometric measurements the catecholamine concentration inside the vesicles was calculated from the current recorded. The linear relation between the granule volume and the amperometric charge suggested that vesicles of different size had a constant catecholamine concentration (Albillos *et al.*, 1997). Thus the larger the volume of the vesicle, the more is neurotransmitter released in the cleft. The same indication was also found in cultured neurons of the leech (Bruns & Jahn, 1995; Bruns *et al.*, 2000). It was estimated that in that preparation large vesicles were filled with 16-fold more serotonin than small ones. Therefore since the vesicle diameters vary, even if the molecular mechanisms for loading transmitter into the vesicles operated to achieve a fixed final concentration, the quantity of transmitter released would vary in proportion to the cube of the vesicle diameter (Bekkers *et al.*, 1990).

Although amperometric techniques have helped identifying of the correlation between the size and the content of catecholamine-containing vesicles, similar results have been found also for excitatory and inhibitory neurotransmitter (Bekkers *et al.*, 1990; Frerking *et al.*, 1995). Recently the linear relationship between quantal size and vesicle volume has been univocally determined in *Drosophila* glutamatergic neuromuscular synapses (Karunanithi *et al.*, 2002). In fact naturally occurring and genetically-induced variations in vesicle size was correlated with quantal size fluctuations, thus reflecting variations in the vesicle content.

Interestingly, the amount of neurotransmitter released per vesicle at individual boutons appears to change considerably (Liu *et al.*, 1999). Variability of the vesicle transmitter concentration can arise by the modulation of vesicle loading. This process is influenced by several factors and leads to further heterogeneity in the amount of neurotransmitter released at each exocytocic event (Li & Chin, 2003). For instance the electrochemical gradient across the vesicle membrane is essential for adequate filling. Zhou *et al.*, (2000) have demonstrated that the lack of the appropriate pH environment and electrical gradient due to the block of the vacuolar proton pump (V-ATPase) leads to a reduced activity of the neurotransmitter vesicular transporters. This in turn implies that the vesicle transmitter content is decreased despite unaltered release mechanisms. The heterogeneity of the number, the type and the bioenergetics of vesicular transporters also brings further variability to the amount of neurotransmitter concentrated in the vesicles (McIntire *et al.*, 1997; Reimer *et al.*, 1998; Takamori *et al.*, 2000; Bellocchio *et al.*, 2000). In addition the efficacy of vesicle filling can also be influenced by modifications of the cytoplasmic neurotransmitter concentration. For instance the direct injection of glutamate in the presynaptic cytoplasm has been reported as an efficient tool to directly manipulate the transmitter cytoplasmic gradient (Ishikawa *et al.*, 2002). This alteration produced a potentiation of evoked and spontaneous EPSCs, providing a clear proof that a larger cytoplasmic concentration of transmitter allows a more efficient loading of the vesicles. Several efforts have been made to estimate the concentration of transmitter present in the vesicles. In the rat neocortex different approaches of vesicle purification led to the estimation that each glutamatergic vesicle contained on average 3640 glutamate molecules to an equivalent intravesicular concentration up to ~ 0.21 M (Riveros *et al.*, 1986; Burger *et al.*, 1989). Similar estimations were found for serotonin concentration in cultured neurons of the leech. Extrapolations from amperometric measurements showed that small synaptic vesicles (SSV) and large dense-core vesicles (LDCV) contained 4700 and approximately 80000 serotonin molecules, respectively at a concentration of ~ 0.27 M (Bruns & Jahn, 1995; Bruns *et al.*, 2000). An even higher vesicular transmitter concentration was estimated in chromaffin cells (0.7 M catecholamine) (Albillos *et al.*, 1997). A still highly debated parameter influencing the amount of neurotransmitter released in the cleft is the number of vesicles released per synaptic bouton (Liu *et al.*, 1999), which in turn depends on the number of active zones contained in each bouton and the number of vesicles released from each active zone .

Structural studies using freeze-fracture technique and electron microscopy (Tremblay *et al.*, 1983; Forti *et al.*, 1997; Schikorski & Stevens, 1997) combined with quantal analysis (Korn *et al.*, 1993) have found that multiple active zones can be present opposite postsynaptic receptor matrices. Interestingly, it has been reported that the number of active zones is linearly related to bouton size (Hamos *et al.*, 1987). Thus, in large boutons it is possible that neurotransmitter relesed from adjacent active zones can activate the same postsynaptic terminal. Therefore the morphology of the synapse relevantly contributes to its functional properties, since the presence of one or many active zones can significantly modify synaptic strength. For many years the so-called "one site - one vesicle" theory described the well-accepted notion that at maximum one vesicle per release site is released in a probabilistic manner with each action potential (Korn *et al.*, 1994) despite the excess of vesicles apparently docked at the presynaptic membrane. This evidence was supported by the interesting coincidence of the number of the release sites histologically identified in Mauthner cells, and those estimated by fitting with a binomial release model the amplitude distribution of spontaneous currents recorded from the same cells (Korn *et al.*, 1981). However mounting evidence supported the hypothesis (called "multivesicular release") that, upon a stimulus, more than one vesicle could be released from a single active zone (Triller & Korn, 1982; Tong & Jahr, 1994; Auger *et al.*, 1998). The existence of multivesicular release brings further variability to the concentration of the neurotransmitter in the cleft, by varying the number of the packets of transmitter released. However this hypothesis is heavily dependent on the assumption that postsynaptic receptors are far from saturation following the release of a single vesicle (Clements, 1996). Multivesicular release was first hypothesized at excitatory synapses of cultured hippocampal neurons (Tong & Jahr, 1994). Here, fast-off competitive antagonists unraveled a larger glutamate cleft concentration in conditions of increased probability of release (P_r), *e.g.* elevated calcium levels, decreased presynaptic inhibition or paired-pulse facilitation. However these experiments did not provide clear evidence for multivesicular release, since the same results could also be explained in terms of spillover and pooling of glutamate molecules released at neighboring synapses (Auger & Marty, 2000). In fact recently Scimemi *et al.* (2004) have estimated that up to approximately 30% of NMDARs contributing to EPSCs are activated by glutamate released from neighboring synapses. If glutamate can readily diffuse from one synapse to the neighbors, higher cleft concentrations will occur when adjacent synapses release simultaneously than when only one does. Although this hypothesis is reasonable, Wadiche & Jahr (2001) have demonstrated that at least at climbing fiber-Purkinje cell (CF-PC) synapses multivesicular release is the only cause responsible for increasing glutamate concentration. The authors chose climbing fiber-Purkinje cell synapses since these synapses exhibit both physical and chemical barriers against spillover and therefore tend to be isolated. The analysis of non-equilibrium inhibition of AMPA-mediated currents by fast-off antagonists in conditions of high and low P_r and reuptake blockade demonstrated that the peak concentration of glutamate correlates with P_r but it is not affected by spillover. Probably the clearest evidence for multivesicular release was found at GABAergic synapses (Auger *et al.*, 1998). An accurate kinetic analysis of closely timed events observed in spontaneous synaptic currents and the study of the amplitude distribution of evoked IPSCs during high and low P_r, demonstrated that at least 30% of synaptic events are multiple.

2.3.3 Diffusion of neurotransmitter in the cleft

The average movement of transmitter molecules within the cleft obeys diffusion dynamics. The propagation of the neurotransmitter concentration profile in the synaptic cleft over time can be described by a function *C(x,y,z,t)*. This diffusion equation (also known as Fick's second law) is defined as:

$$\frac{\partial}{\partial t}C(x,y,z,t)=D\left(\frac{\partial^2}{\partial x^2}+\frac{\partial^2}{\partial y^2}+\frac{\partial^2}{\partial z^2}\right)C(x,y,z,t) \qquad (2)$$

where C is the transmitter concentration, x, y and z are the three spatial variables, t the time and D the diffusion coefficient. In order to apply this equation to predict the dynamics of the transmitter concentration, the initial conditions (*i.e.*, the concentration profile at t=0) and boundary conditions (*i.e.*, the geometry of the cleft) have to be defined. Therefore also the kinetics of release and the efficiency of the uptake system should be adequately taken into account (see also paragraph 2.5 "Shaping synaptic currents").

The diffusion coefficient of neurotransmitters in the cleft is still unknown and only rough estimations of its value can be provided. The first quantification of a diffusion coefficient was that for glutamine in bulk aqueous solution *i.e.* 7.6×10^{-6} cm^2/s (Longsworth, 1953). The same value is assumed to be valid also for glutamate in water, provided that the cleft environment is far from being an aqueous solution (Rusakov & Kullmann, 1998). It is widely accepted that the diffusion process within the synaptic cleft is slower than in a bulk solution because of the viscosity of the extracellular fluid, the interactions with the extracellular matrix and the basal membrane and limited space (Kleinle *et al.*, 1996). Rice *et al.* (1985) estimated that the diffusion value should be reduced at least three times to be adapted for the synaptic cleft. Kleinle *et al.*, (1996) have proposed a kinetic model for release and diffusion of glutamate in the synaptic cleft in order to quantify the impact of the variability of the diffusion coefficient on the neurotransmitter transient in the cleft. The authors adopted the value previously used in modeling studies of diffusion in the cleft, *i.e.* 3×10^{-6} cm^2 sec^{-1} (Wathey *et al.*, 1979; Busch & Sakmann, 1990; Holmes, 1995). The simulated profile of glutamate in the cleft reached its maximum (0.37 mM) after 250 μs and declined to 10% after 2 ms. Lowering 10 times the value of the diffusion coefficient to 3×10^{-6} cm^2 sec^{-1} led to a higher simulated peak concentration of glutamate (1.93 mM). Moreover both the time needed to reach the maximum of concentration and the concentration decay (clearance) were slower (290 μs and 2.7 ms, respectively). Such dependence of the agonist profile on diffusion coefficient is reasonable since lower values of this parameter implicate slower and shorter lateral diffusion with consequent increase of peak concentration and slower rate of transmitter clearance. However it is interesting to note that also the instantaneous or continuous mechanism of release *i.e.* the rate of efflux of the transmitter from the vesicles is crucial for determining the transmitter peak concentration and time course seen by the post synaptic receptors (Kleinle *et al.*, 1996). Since the transmitter invades the whole cleft within the first tens of microseconds after release, the concentration at pre and postsynaptic sites is comparable, even at lower values of diffusion coefficient. Therefore release mechanisms would influence the time needed to reach the peak of transmitter concentration and together with diffusion they would mainly regulate the early phase of transmitter clearance from the cleft. On the contrary the slower kinetics of the uptake process make it suitable to regulate transmitter clearance at a longer time scale (> 1 ms), when enough time has allowed the molecules to reach specific transporters (Clements, 1996).

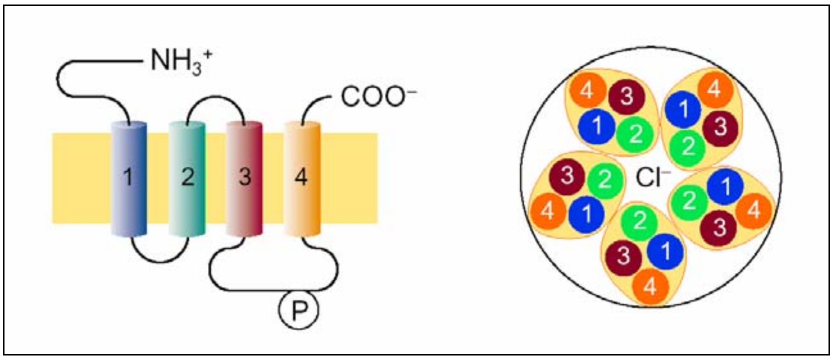
Among the boundary conditions implied in the Fick's equation there is the geometry of the synapse. So far the synaptic cleft has been usually approximated to a planar geometry. However a large heterogeneity is known to be present among different types of synapses. For instance in the hippocampus, mossy fibres (MFs) form large synaptic contacts at branched spiny structures in CA3 pyramidal cell dendrites. Three-dimensional electron microscopy indicates that each MF bouton normally encloses a system of branched dendritic spines forming up to 50 synaptic active zones (Frotscher *et al.*, 1988; Chicurel & Harris, 1992; Henze *et al.*, 2002). This implies a large and tortuous synaptic cleft with the properties that cannot be easily represented by the planar geometry, thus making it difficult to assess the time course of intracleft glutamate released at this synapse (Savtchenko & Rusakov, 2004). Therefore the geometry of the environment in which diffusion occurs is important for regulating the exit of neurotransmitter molecules from the cleft (Barbour & Hausser, 1997).

In conclusion, it is clear that the agonist time course critically shapes postsynaptic currents. Moreover it is worth noting that the speed of lateral diffusion together with the efficiency of uptake proteins, are also important for allowing transmitter spill-over, thus tonic transmission.

2.4 Postsynaptic modulation of synaptic transmission

2.4.1 GABA$_A$ receptors: structure and subunit composition

GABA$_A$ receptors are transmembrane proteins belonging to the ligand-gated ion channels superfamily. GABA$_A$ receptor channel is a heteroligomeric protein presumably composed of five subunits. Each subunit shows a large N-terminus, four hydrophobic transmembrane domains (M1–M4) connected by 2 intracellular loops (between M1-M2 and M3-M4) and one extracellular loop (between M2-M3) and a short C-terminus exposed to the extracellular space.



Bormann J. Trends Pharmacol Sci. 2000 Jan;21(1):16-9.

The large extracellular N-terminus contains the binding site, a cysteine bridge and glycosylation sites. The M2 transmembrane domain of each of the five subunits lines the pore, thus determining anion permeability (Xu *et al.*, 1995; Smith & Olsen, 1995). The intracellular loop between M3 and M4 contains phosphorylation sites for protein kinase A (PKA), protein kinase C (PKC), and tyrosine kinase. So far, nineteen subunit sub-types have been cloned. These subunits are divided, according to their degree of homology in seven sub-families (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, π, ρ_{1-3}) (Luddens & Wisden, 1991; Macdonald & Olsen, 1994; Whiting *et al.*, 2001). Further diversity among subunits can originate from alternative splicing. For instance, γ_{2S} and γ_{2L} are two different splicing forms of γ_2 subunit (Whiting *et al.*, 1990). In theory thousands of different combinations could result form the assembly of all receptor subunits and their splice variants. However GABA$_A$ receptors do form preferred assemblies, leading to only dozens of distinct subunit combinations actually present in the brain (Sieghart & Sperk, 2002; Whiting, 2003). Although the most common arrangement found in the brain is two α, two β and one $\gamma/\delta/\varepsilon$ subunits (Sieghart, 1995), a large heterogeneity of $GABA_A$ receptors with different stoichiometry and subunit combination could be present.

Several studies in recombinant systems, together with KO mice and pharmacology have contributed to identify the characteristics imparted by individual subunits to the whole receptor (Fisher & Macdonald, 1997; Bianchi *et al.*, 2002; Goldstein *et al.*, 2002). For instance it was possible to assess that the substitution of the δ subunit with the γ_2, conferred lower apparent affinity to the receptors and endowed the corresponding currents with faster onset, slower decay and larger extent of desensitization (Fisher & Macdonald, 1997; Haas & Macdonald, 1999). KO mice lacking the α_1 subunit were extremely useful to identify the contribution of that subunit to mIPSCs decay and amplitude and to clearly localize them in the hippocampal slices (Goldstein *et al.*, 2002). In fact mIPSCs recorded from α_1 KO mice were detected less frequently, were smaller in amplitude and decayed more slowly than mIPSCs recorded in neurons from WT mice. Moreover these differences could be observed only in interneurons and pyramidal cells, thus suggesting the localization of α_1 subunit.

A further source in the variability of $GABA_A$ receptor subunit composition is the differential expression of specific subunits during development (Thompson & Stephenson, 1994; Lavoie *et al.*, 1997). For instance it has been proposed that changes in decay kinetics of GABAergic mIPSCs observed in the hippocampus during development can be attributed to the replacement of α_2 subunit by α_1. In fact in neonates the presence of α_2 is associated with GABAergic mIPSCs exhibiting slow decay, while in adults faster decay is related to the presence of α_1 subunit (Hollrigel & Soltesz, 1997; Lavoie *et al.*, 1997). Recently a similar correlation between the accelerated kinetics of mIPSCs and the shifted expression of the alpha subunit from

α_2 and α_3 to α_1 has been observed during maturation of primary cultures of cerebellar granule cells (Ortinski *et al.*, 2004).

2.4.2 GABA$_A$ receptors: localization

Diverse GABA$_A$ receptors subtypes are known to be differently expressed not only over time (during development), but also in space (in different brain regions and in different subcellular areas, *e.g.* synaptic and extrasynaptic).

Immunocytochemistry, immunogold and immunoprecipitation techniques have been used to establish the differential distribution of native GABA$_A$ receptor subtypes in different regions of the brain and at subcellular level (Thompson & Stephenson, 1994; Nusser *et al.*, 1995; Khan *et al.*, 1996; Nusser *et al.*, 1998; Kannenberg *et al.*, 1999; Christie *et al.*, 2002a). While some subunits such as the α, β and γ exhibit heterogeneous distribution across different anatomical areas, others are more confined. For instance the ρ subunit is predominantly expressed in the retina and the ε subunit is in the subthalamic nucleus (Davies *et al.*, 1997). Detailed studies have demonstrated that α_6-containing receptors are exclusively expressed in the cerebellar granule cells and that they preferentially coassemble with α_1, $\beta_{2/3}$, γ_2, and δ (Luddens *et al.*, 1990; Gao & Fritschy, 1995; Rossi & Hamann, 1998). In the hippocampus α_1 and β_2 subunits show an almost uniform distribution, while α_2 and β_3 subunits preferentially accumulate in the dentate gyrus and in the CA3 area and α_5 and β_1 subunits are highly expressed in the CA1 and in the dentate gyrus (Laurie *et al.*, 1992; Christie *et al.*, 2002a). On the contrary low levels of α_4, δ, γ_1 and γ_3 subunits are found in the hippocampus (Christie *et al.*, 2002a).

The immunogold technique has been widely used for identifying receptors subtypes targeted to functionally different parts of a neuron. In hippocampal pyramidal cells α_2 subunits are present only in a subset of synapses at the axon initial segment, whereas α_1 are uniformly distributed in synapses over the axo-somato-dendritic domains (Nusser *et al.*, 1996; Essrich *et al.*, 1998). The immunolabeling of the γ_2 subunit has shown that it is present in hippocampal basket cell synapses on the somata and proximal dendrites and in axo-axonic cell synapses on the axon initial segment of pyramidal and granule cells (Nusser *et al.*, 1996).

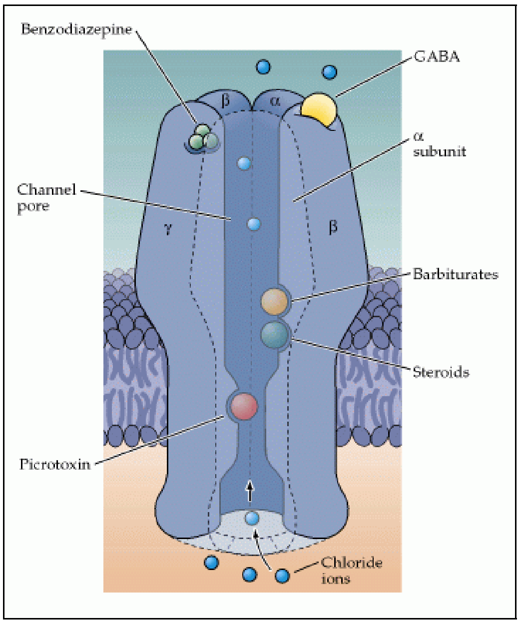
The different localization of $GABA_A$ receptor subtypes can be further classified among synaptic and extrasynaptic sites. In fact both immunolabeling and immunogold approaches have allowed elucidating the presence of some $GABA_A$ receptor subunits also at extrasynaptic locations (Nusser *et al.*, 1995; Nusser *et al.*, 1998; Christie *et al.*, 2002b; Danglot *et al.*, 2003). The most compelling evidence for extrasynaptic subtypes comes from the localization of the δ subunit in the hippocampus and in the cerebellum (Fritschy & Mohler, 1995; Saxena & Macdonald, 1996; Sperk *et al.*, 1997; Nusser *et al.*, 1998; Wei *et al.*, 2003). While the δ subunit is exclusively expressed at extrasynaptic sites, other subunits can be found both at synaptic and at extrasynaptic locations. For instance, different approaches have decorated γ_2 subunits both inside and outside synapses in cultured hippocampal neurons (Nusser *et al.*, 1998; Danglot *et al.*, 2003). It is almost widely accepted that extrasynaptic receptors are those mediating tonic inhibition (Kaneda *et al.*, 1995; Brickley *et al.*, 1996; Nusser *et al.*, 1998; Rossi & Hamann, 1998). Therefore the assembly of specific subunits at extrasynpatic sites can endow tonic currents with peculiar characteristics. It has been also demonstrated that extrasynaptic receptors exhibit lower conductance levels compared to synaptic ones, thus probably making them more suitable to mediate a persistent inhibitory conductance (De Koninck & Mody, 1994; Brickley *et al.*, 1999).

2.4.3 GABA$_A$ receptors: pharmacology

The activity of GABA$_A$ receptors can be influenced by a number of endogenous and exogenous modulators, often in a subunit dependent manner (Moss & Smart, 1996; Hevers & Luddens, 1998). Among endogenous factors, protein kinases have been reported to play an important role in regulating the action of GABA$_A$ receptors. It has been demonstrated that protein kinase A (PKA) and protein kinase C (PKC) are able to reduce and enhance respectively GABA$_A$ receptors mediated currents in a subunit and cell-dependent way (Poisbeau *et al.*, 1999). For instance, PKA but not PKC, reduces mIPCSs amplitude in pyramidal cells, whereas in granule cells PKC but not PKA enhances the peak amplitude of mIPSCs (Poisbeau *et al.*, 1999). Moreover, PKA-mediated phosphorylation strongly depends on the β subunit (McDonald *et al.*, 1998). PKA enhances mIPCSs amplitude in olfactory granule cells that express only β_3 as the β variant (Nusser *et al.*, 1999), but reduces it in hippocampal CA1 pyramidal cells that express β_1 subunit (Poisbeau *et al.*, 1999). Since it has been demonstrated that effects of phosphorylation on synaptic currents are detectable in the time scale of minutes, it is conceivable that this process (as well as dephosphorylation) can contribute to long-term changes of synaptic efficacy (Roche *et al.*, 1994; Swope *et al.*, 1999). In addition, protons and neurosteroids are also potent endogenous GABA$_A$ receptor modulators (Krishek *et al.*, 1996; Zhu & Vicini, 1997; Mozrzymas *et al.*, 2003a). The physiological relevance of the modulation of GABA$_A$ receptor by protons is due to the fact that activation of GABA$_A$ receptors leads to a net efflux of HCO_3^- that can transiently change the extracellular pH (Kaila, 1994). In an accurate kinetic study protons proved to significantly alter the gating of GABA$_A$ receptors by increasing their desensitization and affinity (Mozrzymas *et al.*, 2003a). The modulation of GABA$_A$ receptors by protons is strongly dependent on the subunit-composition. While protons increase the $\alpha_1\beta_1$ and $\alpha_1\beta_1\delta$-mediated currents, they are ineffective on $\alpha_1\beta_1\gamma_{2S}\delta$ currents. Moreover, protons decrease currents mediated by $\alpha_1\beta_1\gamma_{2S}$ receptors (Krishek *et al.*, 1996).

Neurosteroids have been demonstrated to potentiate GABA$_A$-mediated currents by prolonging their deactivation process (Zhu & Vicini, 1997). Also neurosteroids exhibit a subunit-dependent efficacy. In particular, the presence of α_6 and δ subunits has been shown to increase and decrease the sensitivity of GABA$_A$ receptor to neurosteroids, respectively (Lambert *et al.*, 1999).

GABA$_A$ receptors can be allosterically modulated by a number of exogenous molecules including benzodiazepines, barbiturates, anesthetics, ethanol and Zn^{2+}.



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Benzodiazepines (BDZ) and barbiturates are widely used for the treatment of neuropsychiatric disorders such as anxiety, epilepsy etc. It is widely accepted that benzodiazepines enhance GABAergic transmission by increasing the probability of channel opening (Macdonald *et al.*, 1989; Vicini *et al.*, 1987). Benzodiazepines have also been shown to increase $GABA_A$ receptor single channel conductance, although this effect is still under debate (Eghbali *et al.*, 1997; Rang *et al.*, 2003). Benzodiazepine sensitivity is highly dependent on $GABA_A$ receptor subunit composition. Essential requirement is the presence of the γ subunit (Pritchett *et al.*, 1989; Luddens *et al.*, 1995; Wingrove *et al.*, 1997), since the BDZ-specific binding site lies between γ and α subunits. Moreover several studies have demonstrated that the different subunits can modulate benzodiazepine sensitivity of the whole receptors. For instance α_4, α_6 and ρ subunits appear to confer insensitivity to these drugs (Mohler & et al., 2000).

Barbiturates are known to enhance the effects of $GABA_A$ receptor agonists by increasing the mean open time of the channels *i.e.*, to enhance the probability and duration of channel opening without altering receptor conductance or opening frequency (Study & Barker, 1981; Macdonald & Twyman R.E., 1992; Macdonald & Olsen, 1994; Steinbach & Akk, 2001). At high concentrations (>50 μM) which are reached in plasma during anesthesia with pentobarbital (Franks & Lieb, 1994) barbiturates are able to directly open the channels in the absence of GABA (Inomata *et al.*, 1988). Several anestetics, anxyolitic drugs, sedative hypnotic and anticonvulsant commonly used in clinics belong to the family of steroids and interact with $GABA_A$ receptors (Sieghart, 1995). At low concentrations (30 to 300 nM) they enhance GABA-mediated chloride conductance (Majewska, 1992). At higher concentrations (> 1 μM), which occur during surgical anesthesia, these compounds produce a direct opening of $GABA_A$ receptors (Callachan *et al.*, 1987). Although barbiturates and neurosteroids exert similar effects on $GABA_A$ receptors, they clearly act through different sites (McKernan & Whiting, 1996).

Another $GABA_A$ receptor enhancing compound is ethanol (Davies, 2003). A number of experiments have assessed that ethanol increases GABA-mediated current thus leading to a greater hyperpolarization of the postsynaptic membrane and a further decrease in neuronal excitability (Suzdak & Paul, 1987; Siggins *et al.*, 1987; Frye *et al.*, 1994). So far, it appears that ethanol effect on $GABA_A$ receptors is subunit independent (Mihic *et al.*, 1994). However the $\alpha_4\beta_1\delta$ subtype seems to be more sensitive to ethanol than other receptor subtypes tested (Sundstrom-Poromaa *et al.*, 2002).

Among the substances that inhibit $GABA_A$ receptors, zinc is probably the most studied (Westbrook & Mayer, 1987; Smart *et al.*, 1994). Several mechanisms have been put forward to account for the zinc-induced reduction of GABA-mediated currents. It has been proposed that zinc affects GABA-induced currents by reducing the frequency of channel openings (Legendre & Westbrook, 1991), an effect that could be achieved either by decreasing or increasing GABA binding or unbinding rate constants, respectively. However, these possibilities have been considered unlikely due to the lack of zinc effects on $GABA_AR$ single-channel kinetics (Legendre & Westbrook, 1991; Smart, 1992; Smart *et al.*, 1994). The possibility that binding of Zn^{2+} might allosterically trigger a transition to a long-lived non conducting state has been suggested (Celentano *et al.*, 1991; Smart, 1992; Smart *et al.*, 1994; Gingrich & Burkat, 1998) but the mechanism of such modulatory effect has not been fully clarified. Alternatively, Zn^{2+} could increase the onset of desensitization, a phenomenon that may be responsible for the acceleration of GABA-induced current deactivation (Berger *et al.*, 1998). Modulation of $GABA_A$ receptors by endogenous Zn^{2+} has profound implications for developmental and pathological processes, in particular, epilepsy. Experimental status epilepticus has been associated with a decreased Zn^{2+} sensitivity of $GABA_A$ receptors (Banerjee *et al.*, 1999; Kapur & Macdonald, 1997). Recently Zn^{2+} modulation on $GABA_A$ receptors has been described also in physiological conditions. Ruiz *et al.*, (2004) have reported that endogenously released Zn^{2+} reduces the amplitude of GABA-mediated IPSCs in the CA3 area, although this effect is significantly smaller than in pathological conditions. It has been assessed in our laboratory that the block of mIPSCs by zinc is not due to a direct block of the channel pore but rather to an allosteric modulation of $GABA_A$ receptors, *i.e.* alteration of their affinity, efficacy and desensitization (Barberis *et al.*, 2000). The action of Zn^{++} on $GABA_A$ receptor is dependent on subunit composition. In fact the presence of the γ subunit drastically reduces the sensitivity of $GABA_A$ receptors for zinc ions (Saxena & Macdonald, 1994). Furthermore the different α subunits also seem to play an important role in the sensitivity of $GABA_A$ receptors to zinc ions (Saxena & Macdonald, 1994). Recently we demonstrated in a recombinant system that the absence of the γ_2 subunit from $GABA_A$ receptors, allows also a direct block of the channel pore by zinc (Barberis *et al.*, 2002).

2.4.4 $GABA_A$ receptor gating

Del Castillo & Katz (1957) have defined the basic gating scheme for ligand-gated ion channel as:

$$\mathrm{R} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{AR} \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} \mathrm{AR^*} \qquad (3)$$

where R is the receptor in the unbound state, A is the ligand molecule, AR is the receptor bound closed, AR* is the receptor bound open. The rate constants governing these processes are k_{on}, k_{off}, β and α. This gating scheme shows that the receptor in the unbound state reaches the open state in two steps: the binding of the agonist molecule and the transition from bound-closed to bound-open states. This can be considered a basic characteristic shared by all ligand-gated receptor channels. Thus, binding of the agonist molecule(s) facilitates the conformational transition to the open state. However, the gating of $\mathrm{GABA_A}$ receptor (as any other fast-ligand gated receptor) appears to be more complex than that proposed in scheme (3). In fact, in order to describe at least the basic properties of the $\mathrm{GABA_A}$-mediated current, desensitization has to be taken into account. The most general definition of desensitized state is a non-conductive state of the receptor that is kinetically distinct from the unbound state and bound-closed state. Usually, the rate of exit from desensitized states (resensitization rate, *r*) is rather slow implying that the receptors remain trapped in this state for relatively long periods. Thus, the observed fading of the macroscopic current in the presence of agonist is due to the progressive trapping of the receptors in the desensitized state. It is not clear if the receptor can enter the desensitized state from the bound, unbound, open or closed states. Despite intense study, the conformational modifications (induced by the agonist binding) underlying the transition of the receptor in the different states are not precisely defined yet. Recently, it has been proposed that the open state can be achieved by a rotation of the M2 segments induced by agonist binding (Horenstein *et al.*, 2001). This conformational change probably promotes the removal of the closed channel gates from the pore leaving it free for ions permeation.

Gating of $GABA_A$ receptors shows peculiar properties. Unlike other ligand-gated receptors such as ACh receptor, binding of the agonist molecule to the receptor is not a diffusion-limited process (Colquhoun & Sakmann, 1985). In that case the affinity of the agonist for its receptor (k_{off}/k_{on}) would be mainly due to the unbinding rate constant (k_{off}) since the binding rate is thought to be faster than diffusion and therefore it should not be the rate-limiting step of the binding process. On the contrary Jones *et al.*, (1998) have demonstrated that the binding rate of GABA for $GABA_A$ receptor is orders of magnitude slower than that expected for free diffusion. This fact implies that the binding of the GABA molecule to $GABA_A$ receptor requires a significant amount of energy. The same authors, using agonists with different length, have also shown that the binding rate constant (k_{on}) is inversely correlated with the unbinding rate constant (k_{off}). This behavior can be explained if short agonists (such as β-alanine) require large activation energy (slow binding rate) and only slightly stabilize the agonist-receptor complex (fast unbinding rate). On the contrary longer agonists (such as GABA) exhibit a smaller activation barrier energy (relatively fast binding rate) and significant stabilization (slow unbinding rate). These data allowed Jones *et al.*, (1998) to propose a “flexible binding site” as a representative model for the binding site of $GABA_A$ receptors. In fact the binding event would consist of the stretching of two flexible “arms” that bind the agonist molecule. Therefore the longer the agonist, the smaller would be the barrier of activation energy. After the binding, the stretched conformation of the site determines the stability of the complex. Jones *et al.* (1998) have demonstrated that longer agonists exhibit higher stabilization after the binding.

This scenario shows that the affinity of GABA for $GABA_A$ receptors is strongly affected by the binding process and that the ligand-specific energy barrier for binding determines the agonist selectivity of $GABA_A$ receptors. It is interesting to point out that, as demonstrated by Jones *et al.* (2001) $GABA_A$ receptor competitive antagonists differ from agonists since they do not show the correlation between binding, unbinding and affinity. In particular, antagonists exhibit faster binding rates and lower activation energies than the agonists. Thus the energy required for the binding process is somehow related to the ability of the ligand to drive the channel gating. This is consistent with the hypothesis that the agonist binding promotes movements within the binding site that lead to channel gating (Williams & Akabas, 1999; Jones *et al.*, 2001), while antagonists binding do not.

In addition, the gating of $GABA_A$ receptor shows other peculiarities. It is known that, after a pulse of agonist, current deactivation is much slower than that predicted by the unbinding rate constant. This is basically due to the fact that deactivation, desensitization and opening processes are functionally coupled since they have comparable rate constants (Mozrzymas *et al.*, 2003b). Hence, immediately after the agonist pulse is over the unbinding is not the only process that takes place, but also multiple entrances in the desensitized state and reopening can be observed before the unbinding. This implies that the deactivation process is strongly prolonged. Taken together all these data confirm that gating of $GABA_A$ receptor is a very complex process.

However, in spite of this high level of complexity, the transitions between different conformational states of $GABA_A$ receptor fulfill the criterion of Markovian processes following the mass action law. Taking advantage of this property it would be possible to provide a representative simplification of $GABA_A$ receptor gating. In fact, once the number of conformational states and the value of the rate constants governing the transitions between different states have been defined, it would be possible to simulate the gating of the receptor. Applying the mass action law, every transition can be described by a differential equation and the gating scheme can be converted into a set of differential equations. By solving this differential equation system it is possible to know the time course of the occupancies of all the states. The occupancy of the different states is given in terms of probability and it concerns the average behavior of the system. Given the large number of equations involved, an integrated solution would be difficult to obtain; therefore this systems are usually solved numerically.

Several models have been proposed to describe the gating of ligand-gated ion channels. As already mentioned, Del Castillo & Katz (1957) first suggested that the activation of ligand gated channels occur in at least two steps: the binding of the agonist to the receptor and consequent conformational change of the 'agonist-receptor' complex that finally determines the opening of the channel (see eq. 3).

Based on the work of Colquhoun & Hawkes (1981), Bormann & Kettenmann (1988) have proposed a gating scheme for GABA$_A$ receptor that included the sequential binding of two molecules of GABA to the receptor (as suggested from the Hill coefficient ~ 2). This scheme also includes the possibility of opening from a singly bound state:

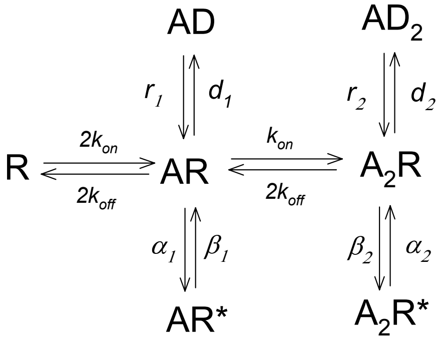
$$\begin{tikzcd}
R \arrow[r, shift left, "2k_{on}"] & AR \arrow[l, shift left, "k_{off}"] \arrow[r, shift left, "k_{on}"] \arrow[d, shift right, "\alpha_1"'] & A_2R \arrow[l, shift left, "2k_{off}"] \arrow[d, shift right, "\alpha_2"'] \\
 & AR^* \arrow[u, shift right, "\beta_1"'] & A_2R^* \arrow[u, shift right, "\beta_2"']
\end{tikzcd} \tag{4}$$

where R is the unbound receptor, AR the singly bound-closed receptor, AR* the singly bound-open while A_2R and A_2R^* respectively the double bound closed and open state.

Analyzing the single channel properties of $GABA_A$ receptors, Macdonald *et al.* (1989) have suggested a different kinetic scheme in which three kinetically distinct open states are present and cyclically connected. In 1995 Gingrich *et al.* have proposed a kinetic model that includes desensitization. By the time passing many more kinetic schemes have been hypothesized, all exhibiting increasing complexity.

The kinetic model that probably represents the best compromise between low level of complexity and satisfactory capability of describing $GABA_A$-mediated macroscopic currents is the one proposed by Jones & Westbrook (1995).

It contains a small number of conformational states thus reducing its degeneration, but still allowing a good representation of $GABA_A$ receptor gating. This simplified scheme is defined as:



(5)

where R is the unbound receptor. One or two molecules of agonist(s) (A) can bind to the receptor. From singly- and doubly- bound closed states (AR and A_2R, respectively) originate both open and desensitized states (AR*, AD, A_2R^*, A_2D). Since at saturating GABA concentrations the singly-bound states are poorly occupied, according to this scheme the onset of desensitization induced by a prolonged application of saturating GABA concentration will be mono-exponential (only one desensitized state originates from the doubly-bound state). This behavior is different from that experimentally observed. In fact the onset of desensitization induced by a prolonged application of saturating GABA can be fitted by several exponential components, indicating that more than one desensitized states is present. In particular, a very fast component (τ = 3-10 msec) and slower components have been described (Celentano & Wong, 1994; Overstreet & Westbrook, 2001). However, it has to be emphasized that, very likely, currents evoked by a brief pulse of agonist ($\leq$ 2ms) are poorly affected by the slow desensitization components. Thus this model seems to be adequate to describe the $GABA_A$ currents evoked by brief pulses of agonist at high concentration, as occurs for instance in the synaptic cleft after synaptic release. Nevertheless this model appears appropriate also for studying the behavior of $GABA_A$ receptor at low dose of agonist. In particular, the presence of a desensitized state originating from the singly bound closed state is very important to explain the gating of $GABA_A$ receptor at tonic and low agonist concentrations.

However it must be pointed out that this model was first developed to provide a general gating scheme able to account for GABAergic synaptic currents. In particular, the authors have referred to GABA-evoked and synaptic currents recorded from cultured hippocampal neurons, which were inherently elicited by a heterogeneous population of $GABA_A$ receptor subtypes. Therefore, when referring to this model it must be taken into account that it does not aim at describing the gating properties of a specific $GABA_A$ receptor subtype, rather the “average” behavior of $GABA_A$ receptors in the hippocampus.

2.5 Shaping synaptic currents

As already mentioned synaptic current results from a series of events occurring in rapid succession including transmitter release, transmitter diffusion, transmitter binding to the receptor and post-synaptic receptors gating. In the previous paragraphs most of the factors involved in generating and regulating phasic transmission have been described, both at presynaptic/cleft and postsynaptic level. While discussing each of these events, it has already pointed out that their variability can generate different synaptic responses. However the influence of the kinetics and the variability of the processes described above in influencing the concentration profile of the transmitter in the cleft (transient) and the gating properties of the post-synaptic receptors cannot be analyzed separately. Therefore in this section an implemented and integrated view of the shaping of postsynaptic currents will be presented.

The shape of the post-synaptic currents is crucial for both the spatial and the temporal summation of excitatory and inhibitory inputs determining thus the capacity of the membrane to fire action potentials. This implies that, by interfering with the time course of post-synaptic currents it is possible to substantially change synaptic output, with potentially very important consequences for the network coding.

2.5.1 Postsynaptic receptor saturation

The synaptic agonist transient is thought to be extremely fast (1-5 mM with clearance time constant of ~ 100 μs) (Clements, 1996). Such a brief transmitter transient is even faster than $GABA_A$ receptor opening, implying that synaptic receptor activation occurs in conditions of non-equilibrium. However, it is not clear whether this transmitter pulse (assumed to be due to the release of a single transmitter vesicle) is able to saturate post-synaptic receptors (*i.e.*, to maximally activate the receptors so that larger concentrations of agonist would produce the same response) (Frerking & Wilson, 1996). Receptor saturation is a particularly interesting issue, since it suggests if the variability of synaptic currents originates at pre- and/or post-synaptic level. This in turn becomes physiologically relevant for identifying pre- and/or post-synaptic substrates for the short- and long-term changes in synaptic strength.

If the repetitive release of synaptic vesicles (during synaptic transmission) does not saturate receptors, the heterogeneity of postsynaptic currents would depend on both pre- and post-synaptic sources of variability. On the other hand if receptors were saturated, synaptic currents would not reflect any change of the transmitter concentration and time course in the cleft. They would rather be influenced by postsynaptic parameters such as the stochastic nature of receptor gating (Faber *et al.*, 1992). In fact, since receptor gating is known to be a Markovian process, stochastic variation of single channel currents upon the same stimulus would turn out in macroscopic currents as well. Therefore receptor openings can be only expressed in term of probability. The more P_o approaches $P_o$max, the more would saturation be approached and current variability reduced. It is worth mentioning that during repetitive release of synaptic vesicles also receptor desensitization could significantly contribute to the variability of synaptic currents, in particular if the instantaneous frequency of release is comparable to (or higher than) the rate constant describing the entry and the exit from the desensitized state (Rigo *et al.*, 2003). Therefore, for a given kinetics of desensitization, the higher the frequency of release, the lower would be the probability that receptors exit from desensitized states in the time gap between two consecutive release events and the smaller would be the response to the subsequent release. In order to address the issue of receptor saturation, the degree of occupancy of post-synaptic receptors following release of a single transmitter vesicle has been analyzed with several approaches. With the "occlusion method" (Tang *et al.*, 1994; Auger & Marty, 1997) studied consecutive mIPSC separated by a time interval δt during high frequency burst evoked by α-latrotoxin in order to estimate receptor occupancy. After the peak, the occupancy (ω) of a first synaptic event due to unbinding of transmitter from the receptors, relaxes back to zero and will be $\omega(\delta t)$ at the time δt. Thus, if a second event occurs at the time δt, the fraction of available receptors at that time will be 1 - $\omega(\delta t)$. Being I_1 the amplitude of the first synaptic event and I_2 the amplitude increment of the second event, the fraction of available receptors can also be expressed as I_2/I_1, so that:

$$\frac{I_2}{I_1} = 1 - \omega(\delta t) \qquad (6)$$

Plotting I_2/I_1 as a function of δt, it is possible to extrapolate the value I_2/I_1 at the time δt = 0 and calculate the value of the peak occupancy ω_0. The value (Auger & Marty, 1997) found was 0.76. They suggested that $GABA_A$ receptors were not saturated. However, desensitization and contamination by GABA coming from neighboring release sites may lead to possible errors while using this method. At inhibitory synapses the degree of occupancy of the post-synaptic receptors following the release of a transmitter quantum has been also studied taking advantage of the pharmacological effect of benzodiazepines (BDZ). These drugs are believed to increase the affinity of GABA for $GABA_A$ receptor. Thus, if the post-synaptic receptor cluster is not saturated, application of BZD is expected to increase the mIPSC amplitude. On the other hand if saturation occurs, BZD are expected to fail to increase the mIPSC amplitude. Using this method, conflicting results have been reported. De Koninck & Mody (1994) and Poncer *et al.* (1996) studying mIPSCs in hippocampus (CA3 pyramidal cells) found that BDZ do not increase the peak amplitude of mIPSCs indicating that in these synapses $GABA_A$ receptors are saturated. According to the increase in the GABA affinity for its receptor, the decay time of miniature currents was slowed down. In contrast, in other preparations including cerebellar stellate cells (Nusser *et al.*, 1997), cultured cerebellar granule cells (Mellor & Randall, 1997), cortical slices (Perrais & Ropert, 1999) and cultured retinal amacrine cells (Frerking *et al.*, 1995) BDZ increased the mIPSCs peak amplitude. Moreover the effect of BDZ has been reported to be temperature dependent. In fact Perrais & Ropert (1999) found that zolpidem enhanced mIPSCs only at room temperature and not at physiological temperatures. It has to be pointed out that the effect of BDZ has not been fully elucidated yet and their action depends on the subunit composition of $GABA_A$ receptors. Thus the observed variability could be due to the different $GABA_A$ receptors subunit composition at different preparations.

Another method to assess receptor saturation is to estimate the degree of occupancy of post-synaptic receptors by studying the coefficient of variation (CV) of the amplitude of miniature post-synaptic currents. This method is conceptually based on the fact that a full occupancy of the post-synaptic receptors (saturation) determines a very low coefficient of variation while a partial occupancy presupposes higher coefficient of variation values. Thus, measuring the coefficient of variation at a single release site it is possible to estimate the value of occupancy of the post-synaptic receptor cluster. More precisely, the value of the coefficient value is defined as:

$$CV = \sqrt{\frac{1-P_0}{N_0}} \qquad (7)$$

where P_0 is the peak opening probability (defined as the product of the peak occupancy ω_0 and the probability that bound channels are open p_0), N_0 is the product between P_0 and the total number of channels in the receptor cluster. At central synapses N_0 is in the range of 10-100. For small and medium values of P_0 (≤ 0.5), application of eq. (7) predicts CV values of 7 – 32 %. CV values below 7 % indicate P_0 values above 0.5 and even higher occupancy values (Auger & Marty, 2000). Assuming the values of open probability and number of channels (per receptor cluster) estimated in previous works, the full occupancy of the post-synaptic receptors after quantal transmitter release would provide a much smaller coefficient of variation value than that experimentally observed at a single release site. However this method has been predominantly applied to excitatory synapses to demonstrate the incomplete occupancy of postsynaptic receptor clusters (CV = 28 % and 50%, from Liu & Tsien, 1995 and Forti *et al.*, 1997, respectively).

Nowadays the hypothesis that post-synaptic receptors are not saturated by the release of a single transmitter vesicle is progressively more supported (Liu, 2003). Nevertheless, it is difficult to assess whether this assumption can be generalized to all synapses. It is in fact possible that in given conditions (receptor affinity, synapse geometry etc.), post-synaptic receptors work in condition of saturation (Frerking & Wilson, 1996). This is probably the reason why several fully contradictory papers exist on this topic (Poncer *et al.*, 1996; Perrais & Ropert, 1999).

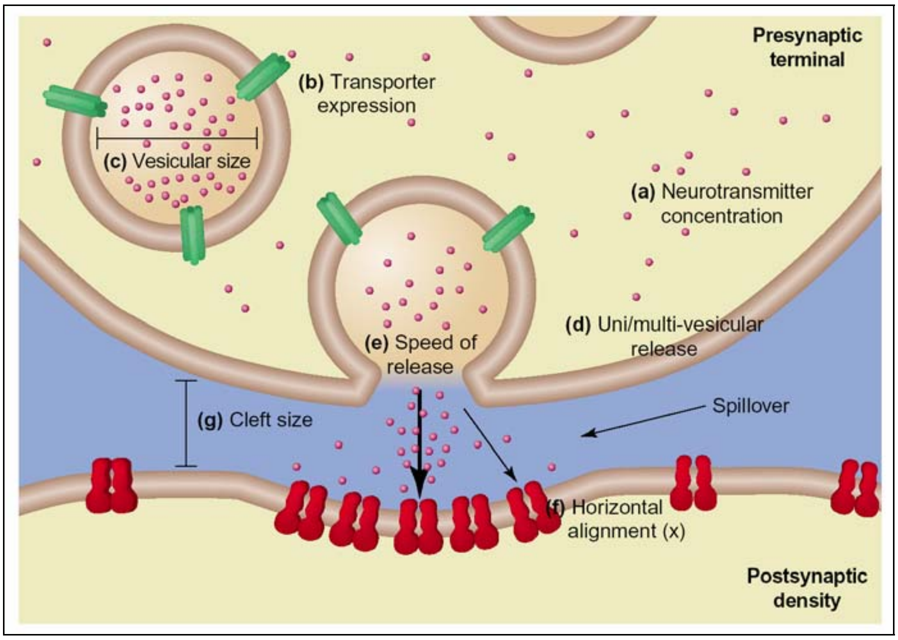
2.5.2 Factors contributing to the variability of the agonist transient in the cleft

Assuming the non-saturation of postsynaptic receptors, it is reasonable to expect that slight changes in the transmitter peak and time course in the cleft may result in large variability of postsynaptic current properties. The peak and time course of transmitter concentration depend on several parameters (most of which have been already described in previous paragraphs) including 1) the concentration of transmitter in the vesicle, 2) the rate of transmitter efflux through the synaptic vesicle fusion pore, 3) the number of vesicles simultaneously released in the cleft, 4) synchronous or asynchronous modality of transmitter release, 5) the transmitter diffusion coefficient, 6) the geometry of the cleft and the adjacent extrasynaptic space, 7) the distribution and the affinity of transmitter binding sites (channels and transporters) and 8) GABA transporters.

Although most of these factors have been discussed before, it is worth mentioning other possible parameters that can further contribute to the variability of synaptic currents. For instance it has been reported that quantal release can switch form a highly synchronized, action-potential dependent mode, to an asynchronous one upon persistent high frequency stimulation (Lu & Trussell, 2000) or application of Sr^{2+} (Oliet *et al.*, 1996). This phenomenon has been ascribed to a raise in the intraterminal Ca^{2+} concentration, facilitation of vesicle release and short-term depletion of available vesicles (Lu & Trussell, 2000; Kirischuk & Grantyn, 2003)

A further source of variability is the possibility that mIPSCs originate from single or different release sites from the same synaptic bouton. In the latter case different characteristics among the release sites including their variable electronic distance from the recording electrode may contribute to further variability. In fact currents are usually recorded from the soma, therefore raising the possibility that cable attenuation may occur. The comparison between the variance of synaptic currents coming from a single release site and that of events coming from all the release sites is a good tool to detect the presence of additional variability due to multiple-site release. As reviewed by Auger & Marty (2000) different approaches have been used to record miniature synaptic currents coming from a single release site. These methods have lead to contradictory results and interpretations. For instance Liu & Tsien (1995) triggered release at putative single release sites (localized with the fluorescent indicator FM1-43) by focally applying a high potassium containing solution in the presence of TTX. The single site variance of the post-synaptic currents was very large (40-50%) and comparable with the variance obtained considering post-synaptic currents coming from all the release sites (40-50%). These experiments suggest that variability depends on the properties of the single release site. In contrast, Forti *et al.* (1997) proposed that the main source of variability could be due to the difference among release sites and/or electrical distance from the recording electrode. In fact they found that the coefficient of variation at the single release site was smaller than that observed considering the population of all the release sites (28% and 72%, respectively). However, it must be pointed out that different approaches to record from single sites might lead to bias. In fact, similarly to Auger & Marty (1997), Forti *et al.* (1997) localized single sites with FM1-43, but then they recorded from single synaptic boutons contained in the recording pipette ("loose patch clamp") in the presence of TTX. Taken together these results indicate that also at single release sites some variability in the amplitude of miniature synaptic current is present.

It is also worth noting that the stochastic nature of synaptic receptor activity can represent a source of synaptic variability. This possibility becomes more evident when the number of channels available at the synapse is small, thus the inherent fluctuations in channel behavior can significantly increase the variability of the macroscopic postsynaptic currents. With Monte Carlo simulations and the analysis of inhibitory quanta in embryonic and adult Mauthner cells (involving a small and large number of channels, respectively) Faber *et al.* (1992) demonstrated that the stochastic behavior of the receptors produces significant intrinsic variance in the current amplitude and kinetics, particularly when few receptors are present at a synapse. Monte Carlo simulations have been used to reproduce the whole process of fast excitatory transmission at glutamatergic synapses by simulating three-dimensional random walk diffusion of transmitter molecules and bimolecular reaction kinetics in complex spatial environments reflecting realistic cellular ultrastructures (Franks *et al.*, 2002). This approach allowed investigating and comparing the effect of the variability of single steps of synaptic transmission on the overall result (*e.g.*, synaptic currents). For instance, Franks *et al.* (2002) demonstrated that peak amplitude responses are more sensitive to increases in the number of receptors than in the amount of transmitter released, since in the latter case receptors progressively approach saturation. A more accurate investigation of the influence of morphological parameters on synaptic variability has been recently provided (Franks *et al.*, 2003). The authors have studied the contribution of the cleft size and the PSD density and shape on the variability of simulated activation of a postsynaptic receptor matrix after quantal release. They found that receptor activation progressively decreased with the increase of the cleft size, until the furthest limit of 300 nm. Moreover the density and spatial arrangement of receptors at the synapse are important determinants of the variability of receptor activation. According to Monte Carlo simulations, smaller, denser PSD are more efficacious than larger ones containing the same number of receptors, since the same amount of transmitter would be released over a reduced area. On the contrary, increase in the number of receptors (with constant density) requires increased PSD size and therefore reduces the average receptor efficacy for a given release event (Franks *et al.*, 2003). It is also worth mentioning that the influence of PSD shape on the variability of synaptic currents has been addressed by including circular, rectangular and annular PSD (same area and the same receptor density) in Monte Carlo simulations of synaptic transmission. Interestingly, decreasing levels of receptor open probability was achieved with the circular, rectangular and annular configurations, respectively, because of the increasing distance of the edge receptors from the release site (Franks *et al.*, 2003).



Liu, Current opinion in neurobiology (2003), 13:324-331

2.5.3 Theoretical and experimental estimations of the agonist transient in the cleft

Since most of the parameters responsible for the variability of GABAergic currents can significantly change in different synapses and only few of them can be accurately measured, the transmitter time course can be provided in terms of average behavior, bearing in mind the expected sources and effects of variability.

The transmitter concentration dynamics in the cleft (agonist transient) has been estimated with theoretical and experimental approaches. Theoretical modeling consists of solving the Fick's equation (Eq. 2) for the boundary conditions reflecting the geometry of the synapse or applying the Monte Carlo method that simulates the random movement of single neurotransmitter particles within the synapse. The application of the diffusion equation requires accurate estimations of the diffusion coefficients, of the radius of the cleft, corrections for the geometry of the synapse.

Therefore the lack of precise quantification of these parameters may represent a source of error in studying transmitter diffusion in the cleft. Kleinle *et al.* (1996) have quantified the impact of different release mechanisms (instantaneous or continuous release) and different diffusion constants on the dynamics of the transmitter concentration in the cleft. In the case of instantaneous release, it was found that, assuming a concentration of the transmitter in the vesicle of ~ 100 mM, the transmitter concentration at the post-synaptic side peaked at a very high concentration (18 mM) within 1 µs and declined to 10% of its value in 25 µs. In contrast when the continuous release was simulated (by introducing a release function calculated by Bruns & Jahn (1995), the release function peaked after ~ 100 µs and after ~ 0.5 ms declined to 50% of its maximum value. Including the release function in the diffusion process, the concentration of transmitter at the post-synaptic site was found to reach a peak value of 0.37 mM only after 250 µs and to decline to 10% of its maximum value after 2 ms. These simulations confirm that the rate of efflux of the transmitter from the vesicle is crucial for determining the transmitter concentration peak and time course seen by the post-synaptic receptors. In fact the rate of rise, the peak and the decay of the concentration of neurotransmitter in the cleft appear to be considerably different in the instantaneous or continuous release model.

The dynamics of the transmitter in the cleft can be theoretically predicted also by using Monte Carlo simulation. This technique, although less accurate, is more versatile than the modeling based on Fick's equation. For a diffusion process occurring in one dimension, Monte Carlo simulation allows every transmitter molecule to perform 2 steps of length $\Delta x/2$ in a time Δt. After the time Δt, the transmitter molecule has 50% probability to remain in the same place and 25% probability to be displaced both on the left and on the right side for the length Δx.

Considering the *x, y* and *z* dimensions the diffusion process can be simulated in the space. The synaptic cleft space is thought to be divided in a grid of squared solids with dimension Δx. Every step Δt, a transmitter molecule can leave or remain in the squared solid according to a fixed probability. As in the Fick's equation, also in Monte Carlo simulation a diffusion coefficient has to be defined. An interesting property of this approach is that, unlike equation modeling, the variability due to the stochastic nature of diffusion can be considered and quantified. In fact, since the number of molecules and the time steps are limited, starting from the same initial conditions, a trial-to-trial variability may occur. Assuming instantaneous release, Wahl *et al.* (1996) obtained results very similar to those observed with the approach based on the diffusion equation. In particular, they reported that the transmitter concentration peaked in few µs at a concentration of 12 mM and quickly decayed at the 10% of its maximum value in ~ 25 µs. Monte Carlo simulation appears to be adequate to study diffusion in complex geometry.

In order to assess whether a given estimated transmitter concentration profile in the cleft can actually account for the experimental data on post-synaptic currents, these diffusion models were added with a kinetic model simulating the gating of the post-synaptic receptors. Using either modeling (based on diffusion equation or Monte Carlo simulation), a peak concentration value > 0.37 mM and < 12 mM reached in < 100 µs with a half-time fast clearance > 25 µs and < 2 ms seems to be a reasonable estimation of the transmitter concentration time course in the cleft. The theoretic modeling also shows that in addition to the reported fast clearance, a slower component is also present. The impact of this slower clearance component should strongly depend on the presence and the activity of the transmitter transporters.

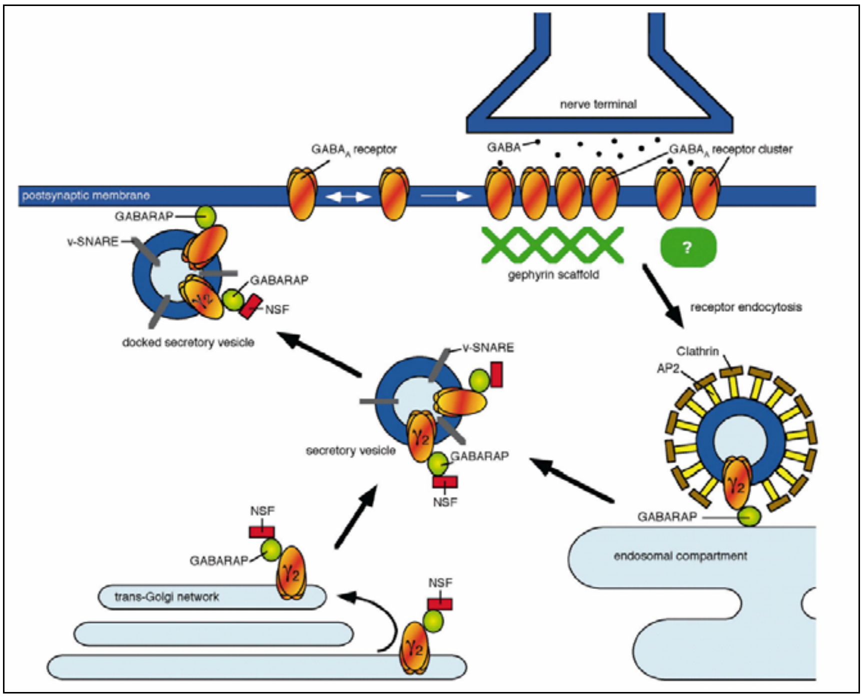
The experimental approach for estimating the agonist transient in the cleft is based on artificially inducing an attenuation of synaptic currents. With a quantitative processing of the attenuation it is possible to infer the peak concentration and the time course of the synaptic agonist transient in the cleft. Recently allosteric modifiers of gating have been used to induce the attenuation of postsynaptic currents (Mozrzymas *et al.*, 1999; Mozrzymas *et al.*, 2003a). In particular, agents down regulating the binding rate (such as chlorpromazine or pH) turned out to be particularly useful. The main idea of such approach is that the reduction of the binding rate (by CPZ or acidic pH) requires a longer time of agonist exposure to achieve a complete binding. If the agonist transient is very fast, in the presence of modifiers of gating (CPZ or acidic pH) its duration becomes insufficient to complete the binding step. Thus, incomplete binding gives rise a to reduced current amplitude. It is worth noting that the shorter the agonist transient, the larger is the sensitivity of synaptic currents to reduction of the binding rate. On the contrary, for relatively slow clearance the considered modification of the binding rate gives rise to a very small change in current amplitude. A satisfactory reproduction of the attenuation induced by CPZ and pH was achieved by simulating the agonist concentration ~ 3 mM and a clearance time constant (τ_t) ~ 100 μs, thus providing an estimation of the synaptic transient (Mozrzymas *et al.*, 1999; Mozrzymas *et al.*, 2003a). Altogether these findings lead to the important conclusion that saturation of the receptors depends not only on the concentration of the transmitter in the cleft but also on the time duration of its clearance.

An alternative experimental approach to estimate the concentration profile of neurotransmitter in the cleft is the use of low-affinity competitive antagonists. The rationale that sustains this approach relies on the comparable kinetics of the agonist transient and the antagonists unbinding. In fact when post-synaptic receptors are pre-equilibrated with a low-affinity (fast-off) competitive antagonist, the transmitter released would progressively displace the antagonist molecules from the binding site. If the duration of the synaptic agonist transient is comparable to that of the low-affinity antagonist (non-equilibrium displacement) the displacement of the antagonist would generate a current flowing through the unblocked receptors, thus attenuating the effect of the antagonist. Since the displacement of the antagonist (and the degree of attenuation) depends on both the transmitter concentration and the time it lasts in the cleft, with model simulations it is possible to infer the properties of the transmitter transient. Therefore a longer permanence and/or a higher concentration of transmitter in the cleft will reduce the antagonist-induced attenuation. On the contrary, at low transmitter concentrations and/or brief transients, a large attenuation is expected. This method was first applied to study the dynamics of glutamate concentration in the cleft by using low-affinity competitive antagonist for NMDA, receptors D-aminoadipate (D-AA) (Clements *et al.*, 1992; Clements, 1996). More recently, the method based on the use low-affinity antagonist has been applied to give a quantification of the variability of glutamate transient in the cleft and to correlate it with the variability of AMPA-mediated mEPSCs (Liu *et al.*, 1999).

With a similar approach it was also possible to estimate the time course of agonist at GABAergic synapses (Overstreet *et al.*, 2002). The use of SR95531 as a fast-off competitive antagonist of $GABA_A$ receptors allowed concluding that synaptic GABA peaks at 3-5 mM and is cleared out within 300-600 μs. To our knowledge the fluctuations of GABA transient in the cleft and the influence of this variability on GABA-mediated mIPSCs have never been specifically addressed, thus the study reported in this thesis it the first attempt in that direction.

2.6 GABA$_A$ receptor clustering

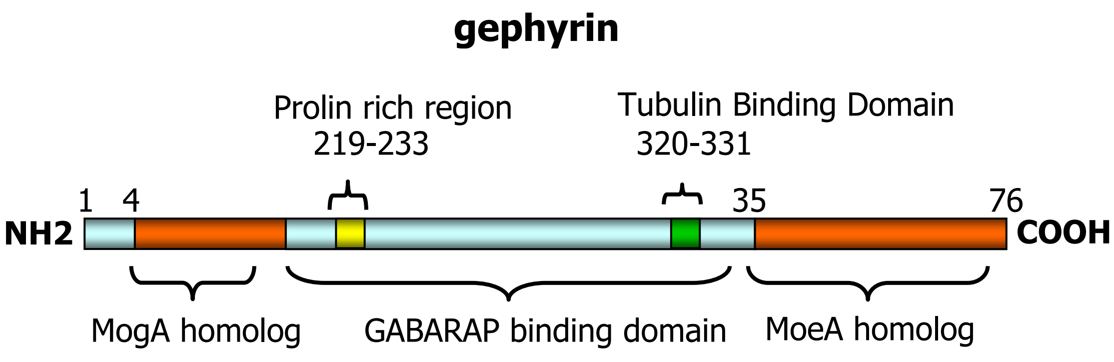
Clustering of neurotransmitter receptors at the postsynaptic membrane is a critical requirement for efficient synaptic transmission. For instance defects in GABA$_A$ receptor clustering are associated with cellular dysfunction and anxiety disorders in mice (Crestani *et al.*, 1999). Neurotransmitter receptor clustering appears to be mediated by a number of intracellular proteins that can, either individually or as part of multiprotein complexes, physically link the membrane-localized receptors to the cytoskeleton (Colledge & Froehner, 1998; Moss & Smart, 2001). Currently the best understood clustering machinery is the one relative to glutamate receptors (reviewed in Bolton *et al.* (2000). On the contrary although several proteins have been identified to be directly or indirectly involved in the sorting, trafficking, synaptic targeting and clustering of GABA$_A$ receptors, until now little is known about the molecular mechanisms that concentrate GABA$_A$ receptors in the postsynaptic membrane (Coyle & Nikolov, 2003).



Kneussel M. *Brain Res Brain Res Rev.* (2002) Jun;39(1):74-83

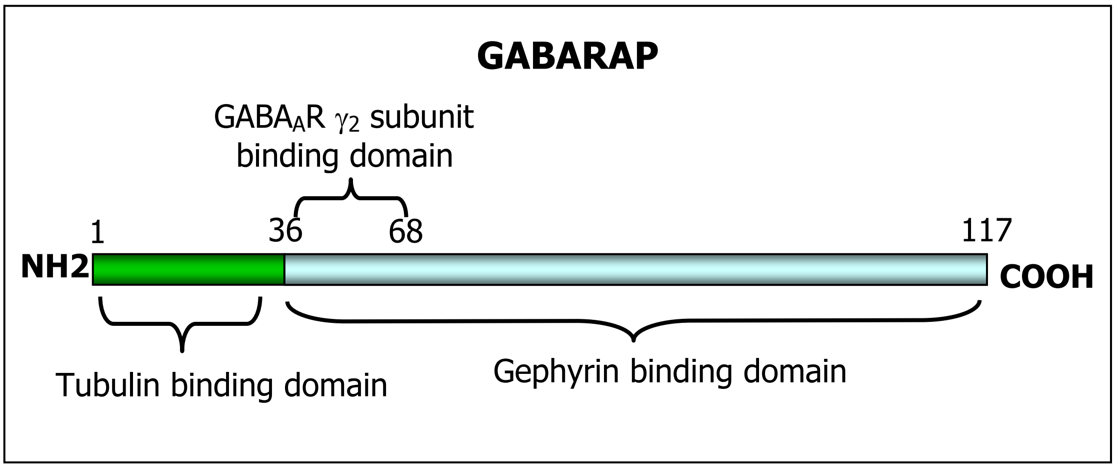
A well-assessed requirement for the clustering of virtually all GABA$_A$ receptor subtypes is the presence of the γ_2 subunit. In fact Essrich *et al.* (1998) found that, in primary cultures from cerebral cortex of $\gamma_2^{-/-}$ mice there was a strong reduction of the puntuate staining of GABA$_A$ receptors. Similarly, the deletion of the γ_2 subunit with the Cre-loxP strategy resulted in the loss of benzodiazepine-binding sites and parallel loss of punctate immunoreactivity of postsynaptic GABA$_A$ receptors in the hippocampus (Schweizer *et al.*, 2003).

It has also been reported that the clustering of GABA$_A$ receptors is associated with gephyrin, a tubulin-binding protein known to directly anchor glycine receptors to the cytoskeleton (Kirsch & Betz, 1995; Feng *et al.*, 1998; Crestani *et al.*, 1999; Meier & Grantyn, 2004; Sola *et al.*, 2004). In particular gephyrin is thought to be involved in the stabilization of GABA$_A$ receptor clusters at the membrane (Sassoe-Pognetto 2000), although not in the direct anchoring, since efforts to demonstrate a direct interaction of gephyrin with GABA$_A$R have failed (Meyer *et al.*, 1995; Kannenberg *et al.*, 1997).



In favor of a gephyrin-mediated stabilization of GABA$_A$ receptor clusters it has been reported that, in wild type mice, GABA$_A$ receptor clusters colocalized with gephyrin clusters (Christie *et al.*, 2002a; Schweizer *et al.*, 2003; Danglot *et al.*, 2003), while in mice lacking γ_2 subunit there was a significant reduction of gephyrin puncta (Essrich *et al.*, 1998). Further evidence for the involvement of gephyrin in mediating the stabilization of $GABA_A$ receptor clusters came from the decrease in the punctate staining of the γ_2 and the α_2 subunits after the inhibition of gephyrin synthesis in cortical neurons with anti-sense oligonucleotides (Essrich *et al.*, 1998). The final demonstration of a gephyrin-dependent stabilization of $GABA_A$ receptor clusters came from the total loss of postsynaptic γ_2- and α_2-subunit immunoreactivity observed in spinal cord sections and hippocampal neurons of gephyrin knock-out mice (Kneussel *et al.*, 1999). Notably in the absence of gephyrin the number of functional $GABA_A$ receptors was only marginally reduced, while that of intracellular microclusters was increased (Kneussel *et al.*, 1999). These observations indicate that gephyrin is important both for receptor localization and stabilization (or “non-direct anchoring”) at postsynaptic sites (Kneussel & Betz, 2000). Speculations on the anchoring of $GABA_A$ receptors can be put forward from a model for GlyR anchoring/clustering recently proposed (Sola *et al.*, 2004). This model relies on the ability of trimeric gephyrin to dimerize upon appropriate stimulus and potentially form a hexagonal network that could mediate GlyR clustering. Combining this hypothesis with the recent evidence of a PKC-enhanced preferential accumulation of $GABA_A$ receptor γ_2 subunit intracellular loop at inhibitory synapses in association with gephyrin (Meier 2004) opens the possibility of undiscovered interactions, which could potentially account for the direct anchoring of $GABA_A$ receptors. Nevertheless it is worth noting that a gephyrin independent clustering has been identified (Kneussel *et al.*, 2001; Levi *et al.*, 2004; Fritschy *et al.*, 2003). In fact clusters of different types of $GABA_A$ receptors could still be detected in gephyrin knock out mice, suggesting that an alternative mechanism for $GABA_A$ receptor clustering must exist.

An intriguing protein found after using the yeast two-hybrid approach to search for interactors of the $GABA_A$ receptor γ_2 subunit is GABARAP ($GABA_A$ receptor-associated protein). This protein not only shows tubulin binding activity (Wang & Olsen, 2000) but also interacts with the γ_2 subunit of $GABA_A$ receptors (Wang *et al.*, 1999) and binds a truncated form of gephyrin (Kneussel *et al.*, 2000).



Its colocalization with surface $GABA_A$ receptors and gephyrin first suggested that GABARAP could be a putative anchoring protein located beneath the plasma membrane (Wang *et al.*, 1999; Wang & Olsen, 2000; Kneussel *et al.*, 2000). Recently several studies have demonstrated that GABARAP is rarely found beneath the membrane, rather the majority of GABARAP puncta are located in intracellular compartments such as putative ER and Golgi structures (Kneussel *et al.*, 2000; Okazaki *et al.*, 2000; Kittler *et al.*, 2001) thus rising the possibility that this protein is mainly involved in receptor trafficking. However it is worth noting that receptor trafficking includes several mechanisms such as the assembly of subunits into functional receptors, targeting to synaptic or extrasynaptic membranes, endocytosis, recycling and degradation (Coyle & Nikolov, 2003). The possible role of GABARAP in $GABA_A$ receptors trafficking is strengthened by its sequence and structural similarities with mammals and yeast proteins involved in membrane dynamics and vesicular transport such as Golgi-associated ATPase enhancer (GATE-16, (Sagiv *et al.*, 2000), Apg8p/Aut7 (Lang *et al.*, 1998; Kirisako *et al.*, 1999), MAP1B (Passafaro & Sheng, 1999; Wang *et al.*, 1999). Moreover GABARAP also binds N-ethylmalemide sensitive factor (NSF), a protein that plays an essential role in intracellular membrane trafficking events (Rothman, 1994) and ULK-1 (Okazaki *et al.*, 2000), an Unc-51-like serine-threonine kinase involved in neurite extension. All together these evidences suggest that GABARAP could be specialized to recruit $GABA_A$ receptors into vesicles targeted to and from the postsynaptic membrane (Kneussel, 2002).

Whatever is the precise function of GABARAP in $GABA_A$ receptor dynamics, it seems that its final effect is to promote $GABA_A$ receptor clustering, even though the native protein poorly colocalyzed with synaptic clusters of $GABA_AR$ (Kneussel *et al.*, 2000; Kittler *et al.*, 2001). In fact there is compelling evidence that coexpression of GABARAP with subunits of $GABA_A$ receptors does result in increased clustering of recombinant receptors in many cells (Chen *et al.*, 2000; Everitt *et al.*, 2004). An effort to reconcile coexpression studies with in vivo localization of GABARAP has been made recently. From the structural studies on GABARAP (Knight *et al.*, 2002; Coyle *et al.*, 2002), Phillips & Froehner (2002) speculated that polymerized forms of GABARAP bound to γ_2 subunit, to tubulin and possibly to gephyrin can have such a packed structure that becomes inaccessible to antibody probes. This can be the reason why GABARAP was almost undetectable beneath the membrane with traditional immunofluorescence methods (Kneussel *et al.*, 2000; Kittler *et al.*, 2001).

Another essential requirement for proper receptor clustering is the intact cytoskeleton (Chen *et al.*, 2000). In fact the depolymerization of microtubules with nocodazole impaired the clustering of recombinant $GABA_A$ receptors in QT6 cells and seemed to affect receptor function (Wang & Olsen, 2000; Chen *et al.*, 2000).

The evidence of the essential role of the cytoskeleton in allowing neurotransmitter receptor clustering was also demonstrated by the loss of postsynaptic gephyrin and glycine receptor clusters in cultured neurons after treatment with alkaloids that destroy microtubules (Kirsch & Betz, 1995; Sheng & Pak, 2000).

3. Methods

3.1 Cell culture

Hippocampal cell culture was prepared according to the method previously described by Andjus *et al.* (1997). Briefly, Hippocampi were taken from postnatal day 2 (P2)–P4 Wistar rats after being anesthetized with an intraperitoneal injection of urethane (2 gm/kg). This procedure is in accordance with the regulation of the Italian Animal Welfare Act and was approved by the local authority veterinary service. Hippocampi were dissected free, sliced, and digested with trypsin, mechanically triturated, centrifuged twice at 40 X g, plated in the Petri dishes, and cultured for up to 14 d. Experiments were performed on cells cultured for at least 7 days.

3.1 Nocodazole treatment

Nocodazole (purchased from Sigma, Milano, Italy) was used to disrupt microtubules. It was applied at the concentration of 10 µg/ml (Bueno & Leidenheimer, 1998) from a 100% Dimethyl Sulfoxide (DMSO) stock solution. The final concentration of DMSO in the working solutions was 0.1% (v/v). Nocodazole was applied in two different ways: in the culture medium (bath treatment) and *via* the patch pipette (intrapipette application). Bath treatment consisted of adding the drug to the neuronal culture medium and incubating the cells at 37° C for 2 hours. In order to verify whether DMSO alone could affect GABAergic transmission, some electrophysiological experiments were performed also on cells incubated for 2 hours with DMSO alone. DMSO 0.1% (v/v) did not produce any change in the kinetic properties of miniature inhibitory synaptic Intrapipette application consisted of adding nocodazole to the intracellular solution in order to apply the drug only to the recorded cell *via* the patch pipette (Meyer *et al.*, 2000; van Zundert *et al.*, 2002).

3.2 Immunofluorescence

Immunofluorescence labeling of surface antigens was performed before fixation, by incubating living neurons for 15 minutes at 4^oC with primary affinity-purified rabbit polyclonal antibody raised against the δ subunit (1-44 region) and γ_2 subunits (1-33 region) of $GABA_A$ receptors. The antibodies, kindly provided by Prof. W. Sieghart (University of Wien, Austria), were diluted in the external solution (also used in electrophysiology experiments; in mM: NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 20, and HEPES 10, pH 7.4, with NaOH) supplemented with 0.1% BSA (Sigma). After washing in the same medium, hippocampal neurons were fixed in 4% paraformaldehyde-4% sucrose (w/v) in PBS, blocked with 10% normal serum and sequentially incubated with FITC-labeled goat anti-rabbit IgG or biotinylated goat anti-rabbit IgG followed by FITC-labeled streptavidin. In order to decorate intracellular antigens such as microtubules and the presynaptic marker, immunocytochemistry for tubulin and synaptophysin was performed on the same hippocampal neurons. Cells were therefore permeabilized with 0.1% NP40, washed with PBS, blocked with 10% normal serum, and incubated with a in house polyclonal antibody against tubulin (rat) and a monoclonal mouse anti-synaptophysin antibody (Chemicon). The resulting immune complexes were incubated for 1 hour at room temperature with appropriate secondary antibodies conjugated with different fluorophores (*i.e.*, TRICT-labeled rabbit anti-rat IgG or TRITC-labeled rabbit anti-mouse IgG). All secondary antibodies were from Sigma (Milano, Italy). These experiments were performed both on untreated and on nocodazole-treated neurons.

Since it is known that the microtubular network is temperature sensitive (Machu, 1998) immunocytochemical experiments were performed in parallel at room temperature and at 4°C in order to verify cytoskeleton integrity after in vivo labeling. Speciments were observed with a 63X oil immersion objective, followed by a 2X and 4.5X digital zoom magnification. Images were acquired on an Olympus (BX51WI) confocal system by using sequential dual channel recording of double-labeled cells.

3.3 Electrophysiological recordings

Currents were recorded in the whole-cell, excised-patch and nucleated-patch configurations of the patch-clamp technique using an EPC-7 amplifier (List Medical, Darmstadt, Germany). The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiments. Cells exhibiting more than 15% changes during the experiment were excluded from the analysis. The series resistance (R_s) was 4 - 8 MΩ and it was compensated by 70 – 80 %. All the experiments were performed at room temperature (22–24°C). The intrapipette solution contained (in mM): CsCl 137, $CaCl_2$ 1, $MgCl_2$ 2, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA) 11, ATP 2, and HEPES 10, pH 7.2 with CsOH. The composition of the external solution was (in mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 20, and HEPES 10, pH 7.4, with NaOH.

During whole-cell recordings the holding potential was -70 mV. The external solution was supplemented with tetrodotoxin (TTX; 1 μM), kynurenic acid (1 mM) and CGP 55845 (1 μM), to block voltage activated Na^+ channels, ionotropic glutamate receptors and $GABA_B$ receptors, respectively. In some cases tetrodotoxin (TTX) omitted from the solution in order to allow action potentials to occur and thus promote a larger release of GABA.

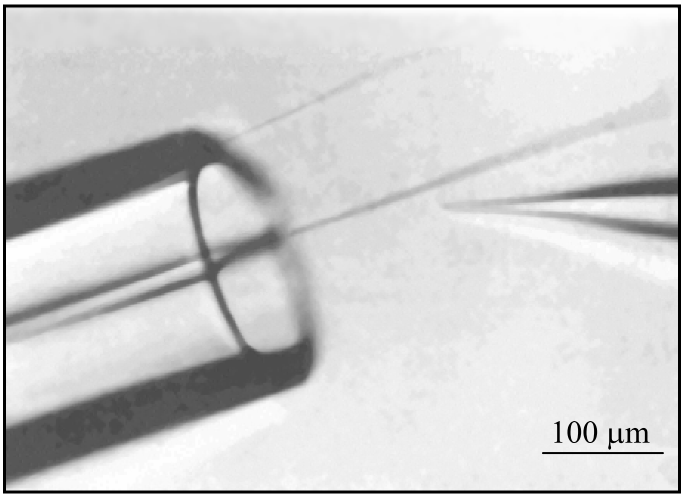
In the excised-patch and nucleated-patch configurations the holding voltage was –70 mV and – 30 mV, respectively.

Currents were sampled at 50–100 kHz, digitized, low-pass filtered at 3.15 kHz with a Butterworth filter and stored on the computer hard disk. The acquisition softwares used were Clampex 9 (Axon Laboratory) and WCP, kindly given by Dr. J. Dempster (Strathclyde University, Glasgow, UK). Since it has been previously observed that the amplitude of tonic GABAergic currents in the hippocampus can be reduced when the cells are perfused with a stream of saline (Valeyev *et al.*, 1993; Bai *et al.*, 2001), except otherwise stated our experiments were performed with a constant low perfusion rate.

Two different perfusion systems for drug applications were used: the multibarrel RSC-200 perfusion system (Bio-Logic, Grenoble, France) and the ultrafast system based on the use of a piezoelectric-driven theta glass application pipette (Colquhoun *et al.*, 1992). The head of the multibarrel system was positioned close to the soma of neurons either to evoke whole-cell GABA-induced currents or to exchange drug-containing solutions around the cell from which synaptic activity was recorded in the whole-cell configuration. The time course of the solution exchange was estimated by liquid junction potential measurements. The application of a 10%-diluted external solution to the open tip patch pipette evoked a junctional current. The establishment of this current represents the complete solution exchange around the patch pipette. The 10-90% of this process occurred within 10-20 msec (10-90% solution exchange time). A better indication of the exchange time around the cell was given by the rise time of whole-cell responses evoked by high concentrations of GABA (>1 mM).

Since it is known that with such high concentrations of GABA the rise time of the current responses is less than or close to 1 msec the observed rise time of the whole-cell current (15-30 msec) was mainly determined by the speed of the solution exchange.

The piezoelectric translator used for ultrafast perfusion system was from Physik Instrumente (Waldbronn, Germany), and theta glass tubing was from Hilgenberg (Malsfeld, Germany). Judging from the onset of the liquid junction potentials, the 10-90% solution exchange time was 40-80 µsec. The speed of the solution exchange was also estimated around the excised patch by the 10-90 % onset of the membrane depolarization induced by application of high (25 mM) potassium saline. In this case the 10-90 % rise time value (60-90 µs) was very close to that found for the open tip recordings.



From Jonas P., Single Channel Recording. Plenum Ed.

3.4 Drugs

Picrotoxin and CGP 55845 were purchased from Tocris (Bristol, UK). BAPTA was purchased from Molecular Probes (Leidens, The Netherlands). Flurazepam was a kind gift of Dr.Vicini, SR-95103 a gift of Dr. Héaulme (Sanofi), TPMPA a gift of Dr.

Nistri (SISSA), Dextran a gift of Dr. Liut (University of Trieste). All the other drugs were purchased from Sigma (Milano, Italy).

3.5 Data Analysis

Synaptic currents were analyzed with the AxoGraph 4.9 program (Axon Instruments, Foster City, CA). This program uses a detection algorithm based on a sliding template. The template did not induce any bias in the sampling of events since it was moved along the data trace one point at a time and was optimally scaled to fit the data at each position. The detection criterion was calculated from the template scaling factor and from how closely the scaled template fitted the data. The threshold for detection was set at 3.5 times the standard deviation of the baseline noise. Using the same program, the rise time of averaged mIPSCs was estimated as the time needed for 10 to 90 % increase of the peak current response and the decay time constants were taken from multiexponential fit of the decay.

Deactivation time courses of mIPSCs and GABA-evoked currents were fitted with a sum of two and three exponentials, respectively.

$$y(t) = \sum_{i=1}^{n} A_i * \exp(-t/\tau_i), \qquad (1)$$

where, A_i are the fractions of respective components, τ_i are the time constants and A_s is the steady-state current.

In the case of analysis of normalized currents, the fractions of kinetic components fulfilled the normalization condition:

$$\sum_{i=1}^{n} A_{\mathrm{i}} = 1. \tag{2}$$

The decaying phase of the currents was also estimated using the τ_{mean} defined as:

$$\tau_{\mathrm{mean}} = \sum_{i=1}^{n} A_{\mathrm{i}} * \tau_{\mathrm{i}}, \tag{3}$$

where A_i and τ_i are respectively the relative fraction and time constant of the different components.

The desensitization onset was described by:

$$y(t) = A_{\mathrm{fast}} * \exp(- t / \tau_{\mathrm{fast})} + A_{\mathrm{slow}} * \exp(- t / \tau_{\mathrm{slow}}) + A_{\mathrm{s}} \tag{4}$$

where A_{fast} and A_{slow} are the fractions of the fast and the slow component, respectively, τ_{fast} and τ_{slow} are the fast and the slow time constants and A_{s} is the steady state current.

Brief (1-2 ms) paired pulses separated by a variable time interval (paired-pulse protocols) were used to test whether or not the entrance of bound receptors into the desensitised state proceeded after the agonist removal. The parameter R was calculated according to the formula:

$$R = \frac{I_2 - I_{end}}{I_1 - I_{end}} \tag{5}$$

where I_l is the first peak amplitude, I_{end} is the current value immediately before the application of the second pulse, I_2 is the second peak amplitude. During 1-2 ms pulse the onset of the use-dependent desensitization is minimal. Thus, in the case of continued entrance into the desensitized state after the first short agonist pulse, the peak of the second response (I_2) was smaller than the first one resulting in $R < 1$.

The goodness of the fit was assessed by minimizing the sum of the squared differences.

The amplitude of the tonic current was estimated by the outward shift of the baseline current after the application of the $\mathrm{GABA_A}$ receptor antagonists bicuculline (100 μM) or picrotoxin (100 μM) (Wall & Usowicz, 1997). Four epochs of 500 ms each, were pooled together to calculate the baseline current amplitude and its standard deviation. The resulting all-point histogram was fitted with a Gaussian function. Only current recordings that exhibited a stable baseline were included in the analysis. During the experiments in the whole cell configuration spontaneous inhibitory postsynaptic currents (IPSCs) were recorded but were avoided during the analysis of tonic currents. The analysis of the variance of the baseline current (σ^2) allowed estimating the single channel current (i) and the single channel conductance (γ) of the receptors mediating tonic currents (I) (Valeyev *et al.*, 1993; Bai *et al.*, 2001; Wisden *et al.*, 2002; Yeung *et al.*, 2003).

Plotting σ^2 *vs* I gives rise to a parabolic function

$$\sigma^2 = i\,(1 - P_o)\,I \qquad (6)$$

where P_o is the channel open probability which varies from 0 to 1. Assuming that at extremely low concentrations of GABA (0.1 – 1 μM) the open probability of the channels mediating tonic currents is extremely small, a simplified eq. (6) suggests that

$$i = \sigma^2 / I \qquad (7)$$

However in the presence of GABA_A receptor antagonist there is still a variance in the baseline current (σ^2_{bic}). Therefore eq. (7) must be adjusted for the intrinsic variability of tonic currents:

$$i = (\sigma^2 - \sigma^2_{bic}) / I \qquad (8)$$

Single channel conductance of the receptors mediating tonic currents was estimated with the following equation:

$$\gamma = I / (|V_m| - |E_{Cl^-}|) \qquad (9)$$

where V_m is the holding potential and E_{Cl^-} is the reversal potential of Cl^- (in our experimental conditions these values were approximately -70 mV and 0 mV).

Data are expressed as mean ± SEM, and all the values included in the statistics represent recordings from separate cells. Statistical comparisons were made with the use of paired and unpaired *t* test, Wilcoxon signed rank test and Kolmogorov-Smirnov test ($p < 0.05$ was taken as significant).

3.6 Kinetic model simulations

The kinetic modelling was performed with the ChanneLab2 software (Synaptosoft Inc, Synaptosoft Inc., USA). The ChanneLab2 software converted the kinetic model into a set of differential equations and solved them numerically. Since in the absence of agonist, receptors can spontaneously open at very low probability (Birnir *et al.*, 2000; Birnir *et al.*, 2001; Bianchi & Macdonald, 2001), for simulation convenience it was assumed as initial condition, *i.e.* at $t = 0$, no bound or open receptors were present. Various experimental protocols were investigated by "clamping" the agonist concentration time course in the form of square-like pulses (ultrafast perfusion experiments). The solution of such equations yielded the time courses of probabilities of all the states assumed in the model. The fit to the experimental data was performed by optimizing the values of rate constants to reproduce the current time course for a given experimental protocol. The procedure for the rate constants optimization was based on the comparison of the time course of recorded currents and that of simulated responses.

The model chosen for the simulations was that proposed by Jones & Westbrook (1995) (see Introduction, ch 2.4.4). The quality of the fit was assessed by measuring the relative distance (*RD*) of the investigated parameter (e.g. current rise time or amplitude) from that in the model prediction:

$$RD = \frac{1}{n}\sum_{i=1}^{n} Abs(y_i - m_i) \qquad (10)$$

where *n* is the number of data points, y_I is the experimentally measured value, m_i is the model prediction and *Abs(x)* is the absolute value function.

-Paper 1-

Presynaptic source of quantal size variability at GABAergic synapses in rat hippocampal neurons in culture

European Journal of Neuroscience, ***published on line*** **14 Sept 2004**

Andrea Barberis, Enrica Maria Petrini and Enrico Cherubini

-Paper 2-

Declusterization of $GABA_A$ receptors affects the kinetic properties of GABAergic currents in cultured hippocampal neurons

Journal of Biological Chemistry (2003), 278 (18):16271-16279

Enrica Maria Petrini, Paola Zacchi, Andrea Barberis, Jerzy W. Mozrzymas
and Enrico Cherubini

-Paper 3-

Clustering of extrasynaptic $GABA_A$ receptors modulates tonic inhibition in cultured hippocampal neurons

Journal of Biological Chemistry, ***published on line*** **17 Aug 2004**

Enrica Maria Petrini, Ivan Marchionni, Paola Zacchi, Werner Sieghart
and Enrico Cherubini

Summary

Tonic inhibition plays a crucial role in regulating neuronal excitability since it sets the threshold for action potential generation and integrates excitatory signals. Tonic currents are known to be largely mediated by extrasynaptic $GABA_A$ receptors that are persistently activated by submicromolar concentrations of ambient GABA. Recently we have reported that, in cultured hippocampal neurons, the clustering of synaptic $GABA_A$ receptors significantly affects synaptic transmission. In the present work we have demonstrated that the clustering of extrasynaptic $GABA_A$ receptor modulates tonic inhibition. The depolymerization of the cytoskeleton with nocodazole promoted the disassembly of extrasynaptic clusters of δ and γ_2 subunit-containing $GABA_A$ receptors. This effect was associated with a reduction in the amplitude of tonic currents and a diminished shunting inhibition. Moreover diffuse $GABA_A$ receptors were less sensitive to the GAT-1 inhibitor NO-711 and to flurazepam. A quantitative analysis of GABA-evoked currents after a prolonged exposure to submicromolar concentrations of GABA and model simulations suggest that clustering affects the gating properties of extrasynaptic $GABA_A$ receptors. In particular a larger occupancy of the singly- and doubly- bound desensitized states can account for the modulation of tonic inhibition recorded after nocodazole treatment. Moreover the comparison between tonic currents recorded during spontaneous activity and those elicited by exogenously applied low concentrations of agonist allow estimating the concentration of ambient GABA. In conclusion receptor clustering appears to be an additional regulating factor for tonic inhibition.

Introduction

Similarly to many other neurotransmitter receptors, $GABA_A$ receptors are localized at synaptic and extrasynaptic level. While synaptic $GABA_A$ receptors are involved in phasic inhibition (1), extrasynaptic ones are responsible for tonic inhibition (2-9). Tonic inhibition is due to a persistent inhibitory conductance that contributes to “signal integration” in the brain, since it sets the threshold for action potential generation (10, 11) and shunts excitatory synaptic inputs (2, 12-15). This conductance is maintained by “ambient” GABA, which represents the amount of neurotransmitter present in the extracellular space. “Ambient” GABA originates from spillover of the neurotransmitter released at neighboring synapses (3, 5, 11), from astrocytes (16, 17) or from non-vesicular release (18, 19). Tonic inhibition has been well characterized in the cerebellum where α_6 subunit-containing receptors act as high affinity sensors for GABA (4, 11, 20, 21). A persistent GABA conductance has been identified also in other brain regions, including the hippocampus (6, 8, 9, 22, 23). However, in this structure the subunits composition of the receptors involved has not been fully elucidated. In the past years immunocytochemical and *in situ* hybridization approaches (20, 24-26) have demonstrated that $GABA_A$ receptors are clustered not only at synaptic but also extrasynaptic level. However, at present the influence of the clustering of extrasynaptic $GABA_A$ receptors on tonic current is still unclear while it is well established that clustering of synaptic receptors ensures proper synaptic signaling. In line with a previous study (27), here we have pharmacologically induced the declusterization of $GABA_A$ receptors by means of nocodazole, a microtubule disrupting agent and we have analyzed tonic inhibition in cultured hippocampal neurons. With immunocytochemical experiments we found clusters of δ subunit-containing $GABA_A$ receptors exclusively at extrasynaptic locations, while clusters of

γ_2 subunit-containing receptors were detectable either at synaptic and extrasynaptic sites. Nocodazole treatment induced the disassembly of all the clusters, thus promoting a uniform distribution of δ– and γ_2- containing $GABA_A$ receptors on the cell surface. This effect was associated with a reduced tonic current. Moreover, diffuse $GABA_A$ receptors were less sensitive to the GAT-1 inhibitor NO-711 and to flurazepam (FZP). Model simulations suggest that a larger occupancy of singly bound desensitized state of declustered receptors may account for the reduction of the tonic current after nocodazole treatment.

Experimental Procedures

Cell culture

Hippocampal cell cultures were prepared as previously described (28). Briefly, 2-4 days old (P2-P4) Wistar rats were decapitated after being anesthetized with an intraperitoneal injection of urethane (2 mg/kg). This procedure is in accordance with the regulation of the Italian Animal Welfare Act and was approved by the local authority veterinary service. Hippocampus were dissected free, sliced, and digested with trypsin, mechanically triturated, centrifuged twice at 40 x *g*, plated in Petri dishes, and cultured for up to 14 days. Experiments were performed on cells cultured for at least 7 days.

Nocodazole treatment

Nocodazole (purchased from Sigma, Milano, Italy) was used to disrupt microtubules. It was applied at the concentration of 10 μg/ml (29) from a 100% Dimethyl Sulfoxide (DMSO) stock solution. The final concentration of DMSO in the working solutions was 0.1% (v/v). Nocodazole treatment consisted of incubating the neurons with the drug for at least 2 hours in the culture medium. In order to verify whether DMSO alone could affect GABAergic transmission, some electrophysiological experiments (n = 12) were performed also on cells incubated for 2 hours with DMSO alone. DMSO 0.1% (v/v) did not produce any change in the kinetic properties of miniature inhibitory synaptic currents (data not shown).

Immunofluorescence staining and confocal microscopy

Immunofluorescence labeling (30) of surface $GABA_A$ receptor δ and γ_2 was performed before fixation, first by incubating living neurons for 15 minutes at 4^oC

with affinity-purified rabbit polyclonal antibodies raised against the extracellular amino terminal region of the corresponding subunits (*i.e.* 1-44 region of the δ subunit and 1-33 region of the γ_2 subunit), diluted in the external solution (also used in electrophysiological experiments; in mM: NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 20, and HEPES 10, pH 7.4, with NaOH) supplemented with 0.1% BSA (Sigma). After washing in the same medium, hippocampal neurons were fixed in 4% paraformaldehyde-4% sucrose (w/v) in PBS, blocked with 10% normal serum and sequentially incubated with biotinylated goat anti-rabbit IgG followed by FITC-labeled streptavidin. To evaluate the synaptic and extrasynaptic distribution of $GABA_A$ receptors, immunocytochemistry for synaptophysin was performed on the same hippocampal neurons. Cells were therefore permeabilized with 0.1% NP40, washed with PBS, blocked with 10% normal serum, and incubated with a monoclonal anti-synaptophysin antibody (Chemicon). The resulting immune complexes were visualized with TRITC-labeled rabbit anti-mouse IgG (Sigma, Milano, Italy). All secondary antibodies were from Sigma (Milano, Italy). These experiments were performed both on untreated and on nocodazole-treated neurons.

Since it is known that the microtubular network is temperature sensitive (31) some control experiments were performed in parallel to our main immocytochemical analysis in order to verify the cytoskeleton integrity after in vivo labeling. Briefly, the same protocol described above was followed with the exception that, after permeabilization, a rat anti-tubulin monoclonal antibody was used instead of synaptophysin. Confocal analysis performed on these neurons demonstrated that the typical branched microtubular bundles were intact after in vivo labeling and comparable with similar experiments performed at room temperature (data not shown). Moreover the same type of control experiments were also performed on nocodazole treated neurons in order to verify the expected (27) drug induced depolymerization of the microtubules (data not shown).

Speciments were observed with a 63X oil immersion objective, followed by a 2X and 4.5X digital zoom magnification. Images were acquired on a Olympus (BX51WI) confocal system by using sequential dual channel recording of double-labeled cells.

Electrophysiological recordings

Currents were recorded in the whole-cell and nucleated-patch configurations of the patch-clamp technique using an EPC-7 amplifier (List Medical, Darmstadt, Germany). The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiments. Cells exhibiting more than 15% changes during the experiment were excluded from the analysis. The series resistance (R_s) was 5 - 7 MΩ and it was compensated by 70 – 80 %. All the experiments were performed at room temperature (22–24°C). The intrapipette solution contained (in mM): CsCl 137, $CaCl_2$ 1, $MgCl_2$ 2, 1,2-bis(2-aminophenoxy)ethane-*N,N,N'*-tetra-acetic acid (BAPTA) 11, ATP 2, and HEPES 10, pH 7.2 with CsOH. The composition of the external solution was (in mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 20, and HEPES 10, pH 7.4, with NaOH. During whole-cell recordings the holding potential was -70 mV. The external solution was supplemented with tetrodotoxin (TTX; 1 μM), kynurenic acid (1 mM) and CGP 55845 (1 μM), to block voltage activated Na^+ channels, ionotropic glutamate receptors and $GABA_B$ receptors, respectively. In some cases tetrodotoxin (TTX) omitted from the solution in order to allow action potentials to occur and thus promote a larger release of GABA. Currents were sampled at 50–100 kHz, digitized, low-pass filtered at 3.15 kHz with a Butterworth filter and stored on the computer hard disk. The acquisition softwares used were Clampex 9 (Axon

Laboratory) and WCP, kindly given by Dr. J. Dempster (Strathclyde University, Glasgow, UK). Control and drug-containing solutions were delivered to the recorded neurons with a perfusion systems consisting of glass barrels positioned close to the soma of the recorded cell (multibarrel RSC-200 perfusion system, Bio-Logic, Grenoble, France). Judging from the onset of the liquid junction potentials, a complete exchange of the solution around the open-tip electrode occurred within 10–20 msec.

Since it has been previously observed that the amplitude of tonic GABAergic currents in the hippocampus can be reduced when the cells are perfused with a stream of saline (6, 32), our experiments were performed with a constant low perfusion rate. However in the experiments with NO-711, the perfusion was turned off in order to allow a larger accumulation of GABA. The drug was first injected into the recording chamber through the glass barrels previously described. After the recording solution was completely replaced by the NO-711-containing solution (around 4 min), the perfusion system was turned off in order to avoid that the stream of fluids partially diffused away ambient GABA.

Picrotoxin and CGP 55845 were purchased from Tocris (Bristol, UK). Flurazepam was a kind gift of Dr.Vicini. All the other drugs were purchased from Sigma (Milano, Italy).

In nucleated patch configuration the holding voltage was –30 mV. GABA-containing solutions were applied to nucleated patches using an ultrafast perfusion system based on piezoelectric driven theta-glass application pipette (33). The piezoelectric translator was from Physik Instrumente (Waldbronn, Germany), and theta glass tubing was from Hilgenberg (Malsfeld, Germany). The open tip recordings of the liquid junction potentials revealed that the 10–90% exchange of the solution occurred within

40–80 μsec. The speed of the solution exchange was also estimated around the excised patch by the 10–90% onset of the membrane depolarization induced by application of high (25 mM) potassium saline. In this case the 10–90% rise time value (60–90 μsec) was very close to that found for the open tip recordings.

Data analysis

The amplitude of the tonic current was estimated by the outward shift of the baseline current after the application of the $GABA_A$ receptor antagonists bicuculline (100 μM) or picrotoxin (100 μM) (1). Four epochs of 500 ms each, were pooled together to calculate the baseline current amplitude and its standard deviation. The resulting all-point histogram was fitted with a Gaussian function. Only current recordings that exhibited a stable baseline were included in the analysis. During the experiments in the whole cell configuration spontaneous inhibitory postsynaptic currents (IPSCs) were recorded. In particular depending on the presence or the absence of TTX, spontaneous miniature (mIPSCs) or action potential-dependent synaptic (sIPSCs) GABAergic currents were identified. Synaptic currents were analyzed with the AxoGraph 4.9 program (Axon Instruments, Foster City, CA). This program uses a detection algorithm based on a sliding template. The template did not induce any bias in the sampling of events because it was moved along the data trace one point at a time and was optimally scaled to fit the data at each position. The detection criterion was calculated from the template-scaling factor and from how closely the scaled template fitted the data. The threshold for detection was set at 3.5 times the SD of the baseline noise. Using the same program, the decay time constant of averaged mIPSCs was taken from the biexponential fit of the decay time.

The decaying phase of the IPSCs was fitted with exponential functions in the form:

$$y(t) = \sum_{i=1}^{n} A_i * \exp(-t / \tau_i), \qquad (1)$$

where τ_i and A_i are the time constants and relative fractions of the respective components.

In the case of analysis of normalized currents, the fractions of kinetic components fulfilled the normalization condition:

$$\sum_{i=1}^{n} A_i = 1. \qquad (2)$$

The deactivation time courses of IPSCs was fitted with the sum of two exponentials (n=2).

The mean time constant, calculated as:

$$\tau_{mean} = \sum_{i=1}^{n} A_i * \tau_i, \qquad (3)$$

was used to estimate the speed of the decaying process.

The goodness of the fit was assessed by minimizing the sum of the squared differences.

The analysis of the variance of the baseline current (σ^2) allowed estimating the single channel current (i) and the single channel conductance (γ) of the receptors mediating tonic currents (I) (6, 32, 34, 35).

Plotting σ^2 *vs* I gives rise to a parabolic function

$$\sigma^2 = i\,(1 - P_o)\,I \qquad (4)$$

where P_o is the channel open probability which varies from 0 to 1. Assuming that at extremely low concentrations of GABA (0.1 – 1 µM) the open probability of the channels mediating tonic currents is extremely small, a simplified eq. (4) suggests that

$$i = \sigma^2 / I \qquad (5)$$

However in the presence of $GABA_A$ receptor antagonist there is still a variance in the baseline current (σ^2_{bic}). Therefore eq. (5) must be adjusted for the intrinsic variability of tonic currents:

$$i = (\sigma^2 - \sigma^2_{bic}) / I \qquad (6)$$

Single channel conductance of the receptors mediating tonic currents was estimated with the following equation:

$$\gamma = I / (| V_m | - | E_{Cl^-} |) \qquad (7)$$

where V_m is the holding potential and E_{Cl^-} is the reversal potential of Cl^- (in our experimental conditions these values were approximately -70 mV and 0 mV).

Data are expressed as mean ± SEM, and all the values included in the statistics represent recordings from separate cells. Statistical comparisons were made with the use of paired and unpaired *t* test, Wilcoxon signed rank test and Kolmogorov-Smirnov test ($p < 0.05$ was taken as significant).

Model simulations

Simulation experiments were performed using the ChanneLab2 software (Synaptosoft). The ChanneLab2 software converted the kinetic model (see Fig. 8*A*) into a set of differential equations and solved them numerically. Since in the absence of agonist, receptors can spontaneously open at very low probability (36-38), for simulation convenience it was assumed as initial condition, *i.e.* at $t = 0$, no bound or open receptors were present. The solution of such equations yielded the time courses of probabilities of all the states assumed in the model. The fit of the experimental data was performed by optimizing the values of rate constants. The procedure for the rate constants optimization was based on the comparison of the time course of recorded currents and that of simulated responses.

Results

Delta and gamma2 subunit-containing GABA$_A$ receptors are clustered at extrasynaptic sites and become uniformly distributed after nocodazole treatment

In the present work we analyzed the influence of the clustering of extrasynaptic GABA$_A$ receptors on tonic inhibition. Taking advantage of a previous demonstration that GABA$_A$ receptor clustering can be impaired by inducing the disassembly of the cytoskeleton with the microtubule disrupting drug nocodazole (27), we first analyzed the distribution pattern and nocodazole sensitivity of δ subunit-containing receptors. In fact there is a number of experimental evidence demonstrating that the δ subunit is exclusively expressed at extrasynaptic locations, even though, to our knowledge, its clustering has not been addressed yet.

Immunocytochemical experiments were therefore performed in order to detect the surface distribution of δ subunit-containing receptors in untreated primary cultured hippocampal neurons. We interestingly found a clearly dotted staining of δ subunits (Fig. 1*A*). Delta subinits never colocalized with the presynaptic marker synaptophysin (Fig. 1*B* and *C*), thus suggesting a clustered arrangement of δ subunit-containing GABA$_A$ receptors at extrasynaptic sites. This punctate staining was significantly affected by nocodazole treatment. In fact, in nocodazole treated neurons, δ subunits were almost uniformly distributed along the cell surface although some residual puncta were still detectable (Fig. 1*D*, see inset). The double staining delta/synaptophysin in nocodazole treated neurons never showed a colocalization of the two proteins, suggesting that also declustered δ-containing receptors maintained their extrasynaptic localization (Fig. 1*F*, see inset).

Clusters of γ_2 subunit-containing GABA$_A$ receptor have been recently identified at extrasynaptic sites (26, 39) although their functional role has been poorly investigated. An antibody specific for the N-terminal extracellular domain of the γ_2 subunit was used to decorate γ_2-containing GABA$_A$ receptors on the cell surface. In untreated neurons γ_2 subunit displayed a punctate staining pattern over the neuronal somata and dendrites (Fig. 2*A*). Only some of the puncta were colocalized with synaptophysin, thus indicating that γ_2 subunits are present both at synaptic (Fig. 2*C*, see open arrowheads) and extrasynaptic sites (Fig. 2*C*, see arrows). As expected from our previous work, nocodazole treatment was associated with the declusterization of γ_2-subunit containing GABA$_A$ receptor (27). Most interestingly, we found that under these conditions colocalization between declustered γ_2 subunits and synaptophysin was still detectable, thus indicating that nocodazole treatment affected both synaptic and extrasynaptic receptors (Fig. 2*F*, see crossed arrows).

Tonic currents amplitude in untreated and nocodazole-treated neurons

Currents were recorded from cultured hippocampal neurons in the whole-cell configuration of the patch-clamp technique at a holding potential of -70 mV in the presence of kynurenic acid (1 mM) and CGP 55845 (1 mM). In these conditions it was possible to record both sIPSCs and a persistent tonic current that was identified with the application of GABA$_A$ receptor antagonists (see Experimental Procedures). The application of bicuculline (100 μM) produced the complete disappearance of sIPSCs and an outward shift of the baseline current (Fig. 3*A*).

In the attempt to test whether nocodazole treatment could affect the amplitude of the tonic current, the shift of the baseline current after the application of bicuculline (100 μM) was measured in untreated and nocodazole-treated neurons. In these two conditions tonic currents were not significantly different. In untreated neurons the baseline shift was 14.7 ± 5.3 pA, while in nocodazole-treated neurons it was 12.7 ±

2.3 pA (n=6, p>0.05, Fig. 3 *A* and *E*). All-point histograms of the baseline current in untreated and nocodazole treated neurons exhibit overlapping distributions (Fig. 3*B*). Similar results were obtained with the application of picrotoxin (100 μM). The baseline shift was 15.4 ± 5.7 pA and 11.9 ± 3.5 pA in untreated and nocodazole-treated neurons, respectively (data not shown, n=4, p>0.05). The similarity of the tonic current amplitude in untreated and nocodazole-treated neurons suggests that the depolymerization of microtubules and the associated declusterization of $GABA_A$ receptors did not reduce the number of the receptors expressed on the cell surface. This hypothesis is further supported by our previous demonstration that also the amplitude of synaptic currents and GABA-evoked currents was not significantly reduced after nocodazole treatment (27).

Nocodazole treatment attenuates NO-711-induced increase of tonic current amplitude

The lack of significant differences between tonic current amplitudes recorded in untreated and nocodazole-treated neurons could be attributed to the small concentration of ambient GABA mainly derived from spillover of the neurotransmitter from neighboring synapses. To test this hypothesis we sought to increase the concentration of ambient GABA by blocking GABA uptake with the GAT-1 antagonist, NO-711 (8, 34, 40, 41). In agreement with previous reports (34, 41), NO-711 (100 μM) slowed down the deactivation kinetics of sIPSCs both in untreated and in nocodazole treated neurons. In untreated neurons the application of NO-711 increased the values of τ_{mean} from 24.1 ± 2.5 ms to 39.4 ± 4.8 ms (n=6, p<0.05, data not shown). Consistent with a larger concentration of ambient GABA, in the presence of NO-711 both untreated and nocodazole-treated neurons exhibited larger tonic currents (Fig. 3 *C, D* and *E*). In untreated neurons, after the application of

NO-711, the tonic current was ~3.6 fold larger than in control. In fact in NO-711 (10 μM), the baseline shift induced by bicuculline was 51.5 ± 8.5 pA, while in control it was 14.7 ± 5.3 pA ($p<0.001$; $n=6$; Fig. 3 *A, C* and *E*). In contrast, in nocodazole-treated neurons, NO-711 induced a non-significant increase in the amplitude of the tonic current, from 12.7 ± 2.3 pA to 22.9 ± 7.5 pA ($p>0.05$; $n=6$; Fig. 3 *A, C* and *E*). All point histogram of tonic currents recorded in the presence of NO-711, from untreated and nocodazole treated neurons clearly showed a significant difference in their distributions ($p<0.001$, Fig. 3*D*). In conclusion, in the presence of NO-711, the difference between the amplitude of the tonic current in untreated and in nocodazole-treated neurons was statistically significant ($p<0.05$, Fig. 3*E*).

Tonic currents from untreated and nocodazole-treated neurons are sensitive to flurazepam

In order to compare the pharmacology of tonic currents in untreated and nocodazole-treated neurons and to infer the subunit composition of extrasynaptic $GABA_A$ receptors, we studied the effect of benzodiazepines, known to be effective on γ_2 subunit-containing receptors (42, 43).

The efficiency of FZP was assessed by analyzing its effect on the peak amplitude and the kinetics of sIPSCs. In agreement with previous studies (6, 44-46), in untreated neurons FZP (1 μM) significantly ($p<0.05$) prolonged the decay kinetics of sIPSCs (the value of τ_{mean} was 27.7 ± 2.1 ms in control and 33.9 ± 0.9 ms in FZP 1 μM; $n=4$) and increased their peak amplitude from 126.9 ± 26.2pA to 166.5 ± 28.9 pA ($p<0.05$; data not shown). The amplitude of the tonic current was measured in control conditions and during the application of FZP (1 μM and 3 μM). In untreated neurons FZP induced a significant dose-dependent increase of the amplitude of the tonic current (from 14.4 ± 1.5 pA in control to 27.5 ± 2.5 pA in FZP 1 μM and to 36.3 ± 3.1 pA in FZP 3 μM; $p<0.001$; n=6; Fig. 4). After nocodazole treatment the effect of FZP was attenuated. The mean amplitudes of tonic current increased from 11.6 ± 2.1 pA in control to 17.6 ± 3.4 pA and 22.9 ± 3.5 pA, in FZP 1 μM and 3 μM, respectively (n=6; $p<0.02$; Fig. 4). It is clear from Fig. 4 that all point histograms of tonic current amplitudes recorded in control conditions from untreated and nocodazole-treated neurons show two overlapping distributions ($p>0.05$; Fig. 4*B*), while those recorded in the presence of FZP (3 μM) are significantly separated ($p<0.01$; Fig. 4*C*). The susceptibility of tonic currents to benzodiazepines suggests the involvement of γ_2 subunit-containing receptors.

Nocodazole treatment reduces the amplitude of tonic currents induced by low concentrations of exogenous GABA

A widely accepted hypothesis is that the spillover of GABA from neighboring synapses contributes to the accumulation of the neurotransmitter in the extracellular space (1, 3, 32), thus influencing tonic inhibition. A direct proof of an activity-dependent modulation of tonic currents came from experiments performed in the presence of TTX where reduced synaptic activity was accompanied with a tonic current of smaller amplitude (45, 47). In a set of experiments TTX (1 μM) was added to the external solution in addition to kynurenic acid (1 mM) and CGP 55845 (1 mM). In these conditions the amplitudes of the tonic current were 7.5 ± 1.2 pA and 7.8 ± 3.3 pA (n=12-14; Fig. 5*A*), in untreated and nocodazole-treated neurons, respectively, *i.e.* ~ two fold smaller than in the absence of TTX (see Fig. 3*A*). Similar results were obtained when picrotoxin (8) was used instead of bicuculline. In the presence of TTX the amplitudes of the tonic current were 7.7 ± 3.2 pA and 7.2 ± 3.7 pA in untreated and nocodazole-treated neurons, respectively (n=4, data not shown). These results clearly indicate a direct correlation between synaptic activity and tonic current amplitude.

In order to quantitatively estimate the correlation between the concentration of GABA in the extracellular space and the amplitude of the tonic current in untreated and nocodazole-treated neurons, low concentrations of GABA (0.1 – 1 μM) were applied. In this set of experiments TTX (1 μM) was added to the external solution in order to minimize the amount of endogenously released GABA. In both untreated and nocodazole-treated neurons, GABA produced a dose-dependent downward shift of the baseline current. (Fig. 5 *A* and *C*).

However, in nocodazole-treated neurons this effect was less pronounced and the amplitude of the tonic current induced by a given concentration of GABA was always smaller than the corresponding value in untreated neurons. In particular, the difference became significant at GABA (1 μM). In untreated and nocodazole-treated neurons the amplitudes of the current were 134.7 ± 17.1 pA and 87.3 ± 9.8 pA, respectively ($p<0.05$, n = 12-14, Fig. 5*C*). The all-point histograms of the tonic current in the presence of GABA 1 μM showed distinct distributions with significantly different mean and standard deviation values (Fig. 5*B*).

In order to see whether nocodazole-induced reduction in amplitude of tonic currents could be attributed to changes in the single channel conductance of declustered receptors, a simplified form of stationary variance analysis of the baseline currents was performed (6, 32, 34, 35). The single channel conductance of clustered and declustered receptors was calculated assuming that the concentration of GABA responsible for tonic current is so low that the receptor open probability becomes negligible. The relationship between the amplitude (I) and the variance (σ^2) of the tonic current is illustrated in Fig. 5*D*. As shown in the Figure 5*D* the estimated single channel conductance of $\mathrm{GABA_A}$ receptors exposed to 0.1 - 1 µM GABA in untreated and nocodazole-treated neurons were comparable (17.1 ± 1.0 pS and 16.6 ± 2.5 pS, respectively, $p>0.05$, n=12-14). These data allow excluding nocodazole-induced changes in single channel conductance or re-assortment of $\mathrm{GABA_A}$ receptor subunits exhibiting different conductances.

Interestingly, the dose-dependent increase in the amplitude of the tonic current induced by exogenous GABA, was accompanied with a similar increase of the SD of the baseline current (Fig 6A). The S.D. has been considered a good parameter for evaluating the variability of the tonic current since it reflects the level of activation of $\mathrm{GABA_A}$ receptors (6, 8, 35).

The S.D. of the tonic currents in control and in the presence of low [GABA] was normalized to that obtained in the presence of bicuculline. In nocodazole-treated neurons these values were always smaller than in untreated neurons (Fig. 6*A*). In particular, when GABA 1 µM was applied, the normalized S.D. of the tonic current in nocodazole-treated neurons was significantly smaller than that observed in untreated ones (1.9 ± 0.1 and 2.5 ± 0.2, respectively, n=12-16, $p<0.05$). This result suggests that, in response to the same GABA concentration, $\mathrm{GABA_A}$ receptors are less activated after nocodazole treatment.

A further confirmation of this hypothesis came from the measurement of the membrane input resistance in untreated and nocodazole-treated neurons. The input resistance values obtained in the presence of bicuculline or low [GABA] were normalized to those found in control conditions (Fig. 6*B*). In bicuculline the normalized value was similar in untreated and in nocodazole-treated neurons (1.05 ± 0.04 and 1.12 ± 0.03, respectively, n= 6, $p>0.05$), and, as expected, greater than 1. At increasing concentrations of GABA, the normalized input resistance progressively decreased, reflecting a larger flow of ions through the receptors *i.e.* a larger shunting inhibition. However in nocodazole-treated neurons this effect was less pronounced and in the presence of GABA 1 μM the normalized input resistance was significantly larger than in untreated ones (0.45 ± 0.04 and 0.59 ± 0.08 in untreated and nocodazole-treated cells, respectively, n=6, $p<0.05$).

The block of GABA-evoked currents induced by a prolonged exposure to low [GABA] is smaller after nocodazole treatment

The reduction of tonic inhibition observed in nocodazole-treated neurons can be attributed either to a change in the gating properties of $GABA_A$ receptors or to a reduced number of receptor channels. To test for these possibilities, currents evoked by ultrafast applications of saturating [GABA], after a pre-equilibrating protocol with low [GABA] or with a control solution, both in untreated and nocodazole-treated neurons were examined.

If nocodazole treatment *per se* reduced the number of active receptors, all the responses, with and without pre-equilibration with low [GABA], should be smaller than those obtained in untreated neurons. The peak amplitude of the currents evoked by saturating [GABA] after nocodazole treatment was used as an index of the total number of functional declustered $GABA_A$ receptors.

Currents were recorded at –30 mV from nucleated patches. GABA pulses (10 mM for 2 ms) were applied every 2 minutes either in control or after 20'' pre-equilibration with low [GABA] (0.3 – 1 μM). Only stable recordings with no signs of run down were used for the analysis. The responses obtained in control and after pre-equilibration with low [GABA] were averaged separately. The responses evoked by saturating [GABA] in untreated and nocodazole-treated neurons in control conditions (without pre-equilibration with low [GABA] were very similar (1056.6 ± 144.3 pA and 1010.5 ± 212.2 pA, respectively, n=10-11, $p>0.05$, Fig. 7*A*). This suggests that nocodazole treatment does not affect the number of active receptors (see ref. 27). After the pre-equilibrating protocol with GABA (1 μM), the amplitude of the responses elicited by saturating pulses of GABA (10 mM) was significantly reduced. However, in comparison with untreated neurons, the responses obtained after nocodazole treatment were significantly smaller (240.2 ± 30.2 pA and 136.8 ± 47.9 pA in untreated and nocodazole-treated neurons, respectively; n=10-11; $p<0.05$; Fig. 7*A*). This indicates that pre-equilibration with GABA produces a block of GABA responses of 70.7 ± 4.3 % and 86.1 ± 2.3 % of GABA in untreated and nocodazole-treated neurons, respectively ($p<0.05$, Fig. 7*B*).

In the presence of GABA (0.3 μM) the reduction in the peak amplitude of GABA-evoked currents in untreated and nocodazole-treated neurons was significantly different (37.2 ± 3.3 % and 51.3 ± 3.4 %, respectively; n=10-11; $p< 0.05$; Fig. 7*B*). In conclusion, the pre-equilibration of nucleated patches with low [GABA] blocked more extensively GABA-evoked currents in nocodazole-treated neurons than in untreated ones.

Model simulations

The present findings demonstrate that in cultured hippocampal neurons, nocodazole-induced microtubule disruption is associated with the declusterization of extrasynaptic $GABA_A$ receptors and with a reduction of tonic inhibition. In particular, after nocodazole treatment, declustered extrasynaptic $GABA_A$ receptors are less activated by ambient (endogenous or exogenous) GABA, thus the amplitude of the tonic current is smaller. In the attempt to reconstruct the gating properties of declustered extrasynaptic $GABA_A$ receptors, model simulations were used. We referred to the kinetic model proposed by Jones and Westbrook (48) (Fig. 8*A*), which fulfils the minimum requirement to adequately reproduce the gating of $GABA_A$ receptors in different experimental protocols. We adopted and optimized the parameters we proposed previously (27) to reproduce the gating properties of declustered synaptic $GABA_A$ receptors. Since the concentrations of GABA used in that study were >30 μM, the transitions between singly-bound open and desensitized states could not be resolved (49, 50). Therefore the corresponding rate constants were merely adopted from Jones and Westbrook (48). In the present work the application of extremely low concentrations of GABA (0.1-1 μM) allowed investigating the singly bound states (50-52). The rate constants were adjusted in order to reproduce tonic and GABA-evoked currents recorded in the presence of exogenous GABA. The experimental data and our previous study (27) suggested that declustered receptors can be more susceptible to desensitization. For this reason in this study we tried to simulate an increased occupancy of the singly-bound desensitized state, besides the already assessed larger occupancy of the doubly-bound desensitized state (27). It must be pointed out that a larger occupancy of a given conformational state may be either due to a faster entry into or a slower exit from that state. Unfortunately for the singly bound states there are no specific protocols to distinguish between these two possibilities. For this reason we achieved a simulated larger occupancy of the singly-bound desensitized state of declustered receptors, simply by increasing the value of d1, the rate constant governing the transition from the singly-bound closed states to the singly-bound desensitized state (Fig. 8*B*).

With the increased value of d1 it was possible to reproduce the smaller amplitude of tonic current in nocodazole-treated neurons (Fig. 8*C*). During a simulated prolonged (10 s) exposure to GABA (1 μM and 0.3 μM) the steady state open probability (tonic current) of declustered $GABA_A$ receptors was 0.0034 and 0.0031, respectively, while that of clustered receptors was 0.0094 and 0.0047, respectively. Such small values indicate that when GABA is present in the extracellular space (ambient GABA), the open states of $GABA_A$ receptors are poorly occupied. Nevertheless after nocodazole treatment their occupancy is even smaller.

The same sets of rate constants relative to clustered and declustered receptors were used to simulate GABA-evoked currents in control and after pre-equilibration with GABA containing solutions. The reduction of the simulated open probability induced by pre-equilibration with low [GABA] was very similar to that recorded experimentally. Simulated pre-equilibration with GABA (1 μM) induced a block of 71% and 84% (Fig. 8*E*) while with GABA (0.3 μM) induced a block of 42% and 61 % for clustered and declustered $GABA_A$ receptors, respectively (Fig. 8*D*).

Model simulations suggest that after nocodazole treatment there is a larger occupancy of the singly bound desensitized state of $GABA_A$ receptors and this is responsible for reduced tonic inhibition.

Discussion

The present results clearly demonstrate that, in cultured hippocampal neurons, nocodazole treatment induces the declusterization of extrasynaptic $GABA_A$ receptors, and reduces GABA-mediated tonic inhibition. Analysis of GABA-evoked currents after pre-incubation with low concentrations of GABA and model simulations suggest that the effects of nocodazole on tonic inhibition are mainly due to changes in the gating properties of $GABA_A$ receptors. In particular we propose that nocodazole treatment promotes the accumulation of declustered receptors into singly bound desensitized state during tonic exposure to ambient GABA.

Consistent with previous reports (20, 25, 26, 39, 53) our immunocytochemical data clearly demonstrate that, in addition to synaptic receptors, also extrasynaptic ones are arranged in clusters, and that they become uniformly distributed throughout the cell surface after nocodazole treatment. In agreement with other groups we have found that extrasynaptic receptors in the hippocampus include the δ and the γ_2 subunit (39, 54-56). However it seems quite clear that the δ and the γ_2 subunits cannot be assembled within the same receptor (57, 58). Yet, the exact subunit composition of extrasynaptic $GABA_A$ receptors is still unknown although a substantial effort has been recently made in this direction (58-60). While the contribution of δ subunits has been well documented particularly in the dentate gyrus (55, 56), that of γ_2 subunits has been often neglected (20, 61). The lack of complete disappearance of the tonic current in the hippocampus of $\delta^{-/-}$ mice favored the involvement of other receptor subunits (15, 62). Possible candidates are the γ_2 subunits since they are required for the observed facilitatory effect of FZP on tonic inhibition (42, 43, 63-66). However it must be pointed out that BDZ sensitivity can also be due to α_5 subunit containing receptors (67-69) that are largely expressed in the hippocampus (68-71). On the basis of recent studies that have demonstrated the co-assembly of α_5 and γ_2 subunits and have predicted that native receptors in the hippocampus were $\alpha_5\beta_3\gamma_2$ (68, 71), we can speculate that in our preparation γ_2 subunits may belong to $\alpha_5\beta_3\gamma_2$ receptors.

In the present experiments nocodazole was used as a pharmacological tool to promote the loss of clustered arrangement of GABA_A receptors. The observation that, after nocodazole treatment, the declusterization of receptors that did not co-localise with synaptophysin (*i.e.* extrasynaptic receptors) was associated with changes in tonic inhibition suggests that the two events are correlated. Although nocodazole-treatment affected both synaptic and extrasynaptic receptors, it is conceivable that the contribution of synaptic receptors to tonic currents is very small or even negligible, since the number of extrasynaptic receptors largely exceeds that of synaptic ones (24, 72).

By comparing the amplitude of tonic currents evoked by endogenous or exogenous applications of GABA it was possible to estimate the ambient agonist concentration in our preparation. Consistent with previous reports on cerebellar granule cells (45) and cultured hippocampal neurons (73) we found a value ~ 0.1 μM. However, these estimations, may not exactly reflect the physiological value *in vivo* since they all refer to neurons in culture. In fact the loss of the anatomical arrangement and the modification of the architecture of the extracellular space in primary cultures may influence the concentration of ambient GABA. In our results differences between tonic inhibition mediated by clustered and declustered receptors could be revealed only when ambient GABA was increased (*i.e.* in the presence of NO-711 or at the highest concentration of exogenous of GABA). This evidence suggests that, in normal conditions, the modulatory effect of receptor clustering on tonic inhibition is not detectable, but it becomes effective when ambient [GABA] exceeds a threshold as during sustained GABAergic activity.

Noise analysis has demonstrated that in both untreated and nocodazole treated neurons "low conductance channels" mediate tonic inhibition. The value of the single channel conductance estimated here for both clustered and declustered receptors (~16 pS) is consistent with that reported previously (32, 45, 49-51, 74) for receptors mediating tonic inhibition (74, 75). It has been proposed that receptors can be partially or fully activated depending on the concentration of agonist they "see" (76-78). Therefore, it is possible that, at ambient concentrations of GABA, tonic currents are mainly mediated by monoliganded receptors in a lower conductance state (6, 35, 50). However, it cannot be excluded that the conductance level of a given receptor can be also influenced by its subunit composition (45, 69, 74). Therefore the comparable estimation of the single channel conductance of clustered and declustered receptors suggests that, at low concentrations of agonist, the receptors are similarly activated since nocodazole treatment affects neither the molecular structure/subunit composition nor the proportion of monoliganded $GABA_A$ receptors.

The widespread action of nocodazole raises the possibility that modulation of tonic inhibition can be due to multiple indirect effects. For instance it is possible that this drug alters the release machinery and therefore influences the concentration of ambient GABA, or reduces the number of $GABA_A$ receptors, changes their subunit composition or affects their gating properties. On the basis of our previous work (27) we can exclude a presynaptic site of action of nocodazole since this drug did not affect the frequency of mIPSCs and comparable results were obtained when nocodazole was added either to the culture medium or to the intracellular solution. The similar effect of nocodazole on tonic currents induced by endogenous and exogenous applications of low concentrations of GABA argues against a reduction of ambient GABA. As previously discussed, we believe that nocodazole did not modify the number of $GABA_A$ receptor on the cell surface since the peak amplitudes of tonic and GABA-evoked currents were comparable in untreated and nocodazole-treated neurons (see also ref. 27). The similar single channel conductance estimated for clustered and declustered receptors allows excluding a re-assorted subunit composition of $GABA_A$ receptors after nocodazole treatment. In conclusion, we believe that the effect of nocodazole on tonic inhibition is due to changes in the gating properties of declustered $GABA_A$ receptors. This idea is supported by the results from the pre-equilibrating protocols combined with kinetic modelling and computer simulations.

Previous studies have demonstrated that pre-equilibration of excised patches with micromolar or submicromolar concentrations of agonist, trapped the receptors in slow and strongly absorbing desensitized states (41, 52, 79, 80). In the present work it was found that the reduction of current responses to saturating [GABA] after pre-equilibration with low [GABA] was larger in nocodazole-treated neurons than in untreated ones. This suggests a larger occupancy of slow desensitized states of declustered $GABA_A$ receptors, presumably in the singly bound conformation. For this reason, the kinetic model previously proposed to reproduce the gating of declustered receptors (27) was optimised taking into account the experimental data obtained with extremely low [GABA]. In fact it is widely accepted that the probability of occupancy of the receptors in the singly bound conformation is relevant only at very low concentrations of GABA (≤ 10 μM), while it can be neglected at saturating or subsaturating concentrations of agonist (50-52). It is for this reason that the increased occupancy of the singly bound desensitized state of declustered receptors allows mimicking the effect of nocodazole on tonic currents and the results of the pre-equilibration experiments. It is worth noting that the parameters used in the present work did not alter the simulations of previous results on mIPSCs and GABA-evoked currents (27). However the lack of specific protocols to selectively unmask the kinetics of the singly bound states allows only a qualitative estimation. In fact we cannot precisely attribute the larger occupancy of the singly bound desensitized conformation to an increased rate of entry or to a decreased rate of exit from that state. Nevertheless we cannot exclude the possibility that also slowly absorbing doubly bound desensitized states (81, 82) neglected in the Jones and Westbrook's model can also be involved.

We conclude that desensitization of $GABA_A$ receptor, in particular of extrasynaptic γ_2 containing ones, contributes to the modulation of tonic inhibition. This conclusion is supported by the data reported previously (82) demonstrating that the significant but still incomplete desensitization of γ_2-containing receptors allows a current amplitude comparable to that mediated by low desensitizing δ–containing receptors. Moreover less apparent desensitization is observed in γ_2 currents evoked by low agonist concentrations (79, 81).

The present work suggests that receptor clustering is an additional regulating factor for tonic inhibition. Since clustered extrasynaptic $GABA_A$ receptors mediate larger tonic currents, it is possible that either in physiological and pathological conditions receptor clustering can modulate tonic inhibition and in turn influence synaptic efficacy and integration (6, 10, 13, 14, 20).

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Figure legends

FIG. 1 **Extrasynaptic clusters of δ subunit-containing GABA$_A$ receptors are lost after nocodazole treatment**

Neurons were labeled with a polyclonal antibody recognizing GABA$_A$ receptor δ subunit (green), in combination with a mouse anti-synaptophysin antibody (red). In untreated neurons, δ subunit-containing GABA$_A$ receptors were arranged in clusters (*A*) localized exclusively at extrasynaptic sites, since they were never associated with synaptophysin immunoreactivity (*B* and *C*). After nocodazole treatment δ subunit-containing GABA$_A$ receptors were uniformly distributed (*D*) and they still did not overlap with synaptophysin staining (*E* and *F*) Lower panels are magnifications of the boxed windows. Panels scale bar, 10 μm; insets scale bar, 20 μm.

FIG. 2 **Nocodazole treatment promotes the disassembly of γ_2 subunit-containing GABA$_A$ receptors clusters located both at synaptic and extrasynaptic sites**

Neurons were labeled with a rabbit polyclonal antibody specifically recognizing GABA$_A$ receptor γ_2 subunit (green) in combination with mouse anti-synaptophysin (red). Lower panels are magnification of the boxed windows. In untreated neurons (*A*-*C*) γ_2 subunit-containing GABA$_A$ receptors formed clusters, that either colocalized with synaptophysin immunoreactivity (synaptic clusters, open arrowheads) or not (extrasynaptic receptors, arrows). Nocodazole treated neurons (*D-F*) displayed a diffuse γ_2-subunit immunoreactivity throughout the cell surface. However some residual puncta could be still detected. Note that some diffuse γ_2 subunit-containing $GABA_A$ receptors still colocalized with synaptophysin indicating synaptic receptors (crossed-arrows). Panels scare bar, 20 µm; insets scale bar, 10 µm.

FIG. 3 **Nocodazole attenuates the effect of NO-711 on tonic current amplitudes**

A, Currents (*lower traces*) recorded in control conditions and in the presence of bicuculline (*upper lines*) from untreated (*left panel*) and nocodazole-treated neurons (*right panel*). Some sIPSCs were present, but they were excluded from the analysis of tonic current amplitudes. The dotted line represents the holding current in the presence of bicuculline. *B*, All point histogram of a 500 ms trace recorded in control conditions from untreated and nocodazole-treated neurons (note that the two distributions overlap) and in the presence of bicuculline. The thin black lines represent the gaussian fit of the distributions. *C*, Tonic currents (*lower traces*) recorded in the presence of NO-711 and in the presence of bicuculline (*upper lines*) from untreated (*left panel*) and nocodazole-treated neurons (*right panel*). *D*, All point histogram of a 500 ms trace recorded in the presence of NO-711 from untreated and nocodazole-treated neurons (note that the two distributions are significantly shifted) and in the presence of bicuculline. *E*, Summary of the mean tonic current amplitude (baseline shift) in control and in the NO-711, for untreated and nocodazole-treated neurons (n=6). *, $p<0.05$.

FIG. 4 **Nocodazole treatment reduces the enhancing effect of FZP on tonic currents**

A, Example traces recorded from untreated (*left panel*) and nocodazole-treated (*right panel*) neurons in control conditions, in the presence of FZP (1 and 3 μM) and bicuculline (*upper lines*). The dotted lines represent the holding current in the presence of bicuculline. sIPSCs were removed during the analysis of tonic currents. *B*, All point histogram of 500 ms trace recorded in control conditions from untreated and nocodazole-treated neurons (note that the two distributions overlap) and in the presence of bicuculline. The thin black lines represent the gaussian fit of the distributions. *C*, Similar all point histogram, in the presence of FZP 3 μM. Note the different distributions between untreated and nocodazole-treated neurons. *D*, Mean tonic current amplitude recorded from untreated and nocodazole-treated neurons in control and in the presence of FZP (1 and 3 μM), n=6. *, $p<0.05$.

FIG. 5 **In nocodazole-treated neurons tonic currents evoked by exogenous applications of low [GABA] are smaller**

A, Currents recorded in control conditions and in the presence of different concentrations of exogenous GABA from untreated (*left panel*) and nocodazole-treated neurons (*right panel*). The holding current in the presence of bicuculline sets the zero level and it is represented by the dotted line. *B*, All point histogram of 500 ms trace recorded in the presence of GABA 1 μM and bicuculline from untreated and nocodazole-treated neurons. mIPSCs were excluded from the analysis. C, Mean amplitude of tonic currents recorded in control and in the presence of different concentrations of GABA from untreated and nocodazole-treated neurons. (n=12-14). *, $p<0.05$. *D*, Plots of the variance *vs* the amplitude of the tonic currents recorded from untreated (*left panel*) and nocodazole-treated (*right panel*) neurons (n=12-14). The solid lines is the linear regression fits of the all data points. The estimated mean single channel conductance was ~16 pS for untreated and nocodazole-treated neurons.

FIG. 6 **After nocodazole treatment GABA$_A$ receptors are less activated in response to low agonist concentrations**

A, Mean standard deviation of baseline currents recorded in control and in the presence of low [GABA], normalized to that recorded in the presence of bicuculline (n=12-14). *,p<0.05. *B*, Mean membrane input resistance recorded from untreated and nocodazole-treated neurons in the presence of bicuculline and low [GABA], normalized to the value recorded in control (n=6). *, p<0.05.

FIG. 7 **The block of GABA-evoked currents after pre-equilibration with low [GABA] is larger in nocodazole-treated neurons**

A, Current responses to short pulses (2 ms) of saturating GABA (10 mM, *open bars*) after 20'' pre-equilibration with a control solution (*thick line*) or with GABA 1 μM (*thin line*) in untreated (*left panel*) and nocodazole-treated (*right panel*) neurons. *B*, Mean reduction of control responses after the pre-equilibration with low [GABA], in untreated and nocodazole-treated neurons (n=10-11). *, p<0.05

FIG. 8 **Model simulations**

A, Kinetic model proposed by Jones and Westbrook (48). According to the model the receptor (R) can bind one or two molecules of agonist (A), reaching either the singly (AR) or doubly-bound-closed state (A_2R). From these states it can open (AR* and A_2R*) or desensitize (AD and A_2D). *B*, Values of the rate constants chosen to simulate tonic and GABA-evoked currents in control and nocodazole. *C*, Simulated tonic current (*lower traces*) evoked by a prolonged exposure to GABA 1 μM (*upper lines*) in untreated and nocodazole-treated neurons. *D*, Mean reduction of the amplitude of simulated GABA-evoked currents after pre-equilibration with low [GABA] in untreated and nocodazole-treated neurons. *E*, Simulated current responses (*lower traces*) to short pulses (2 ms) of saturating GABA (10 mM, *open bar*) after pre-equilibration with a control solution (*thick line*) or with GABA 1 μM (*thin line*) in untreated (*left panel*) and nocodazole-treated (*right panel*) neurons.

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Fig.1

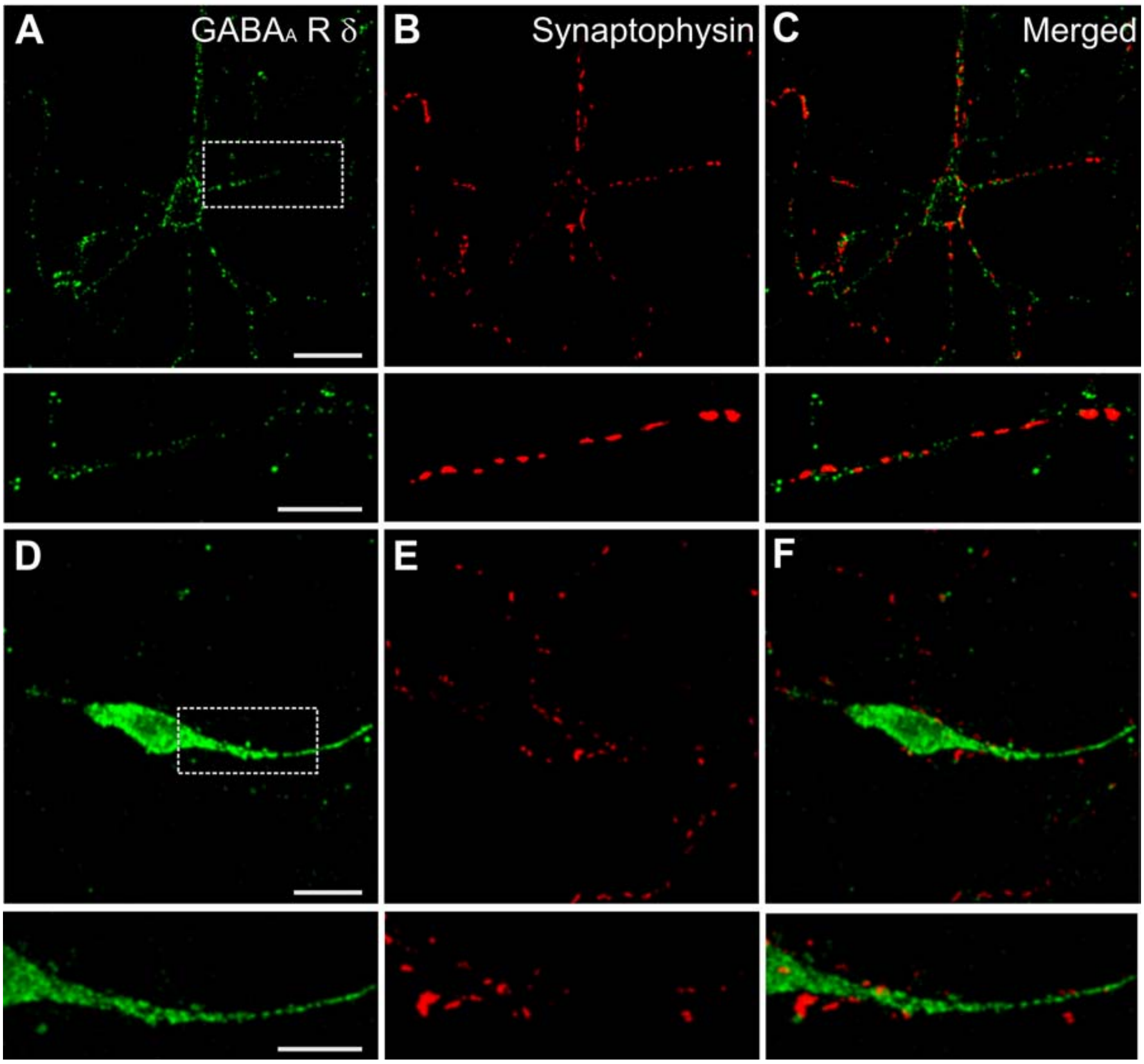


Fig.2

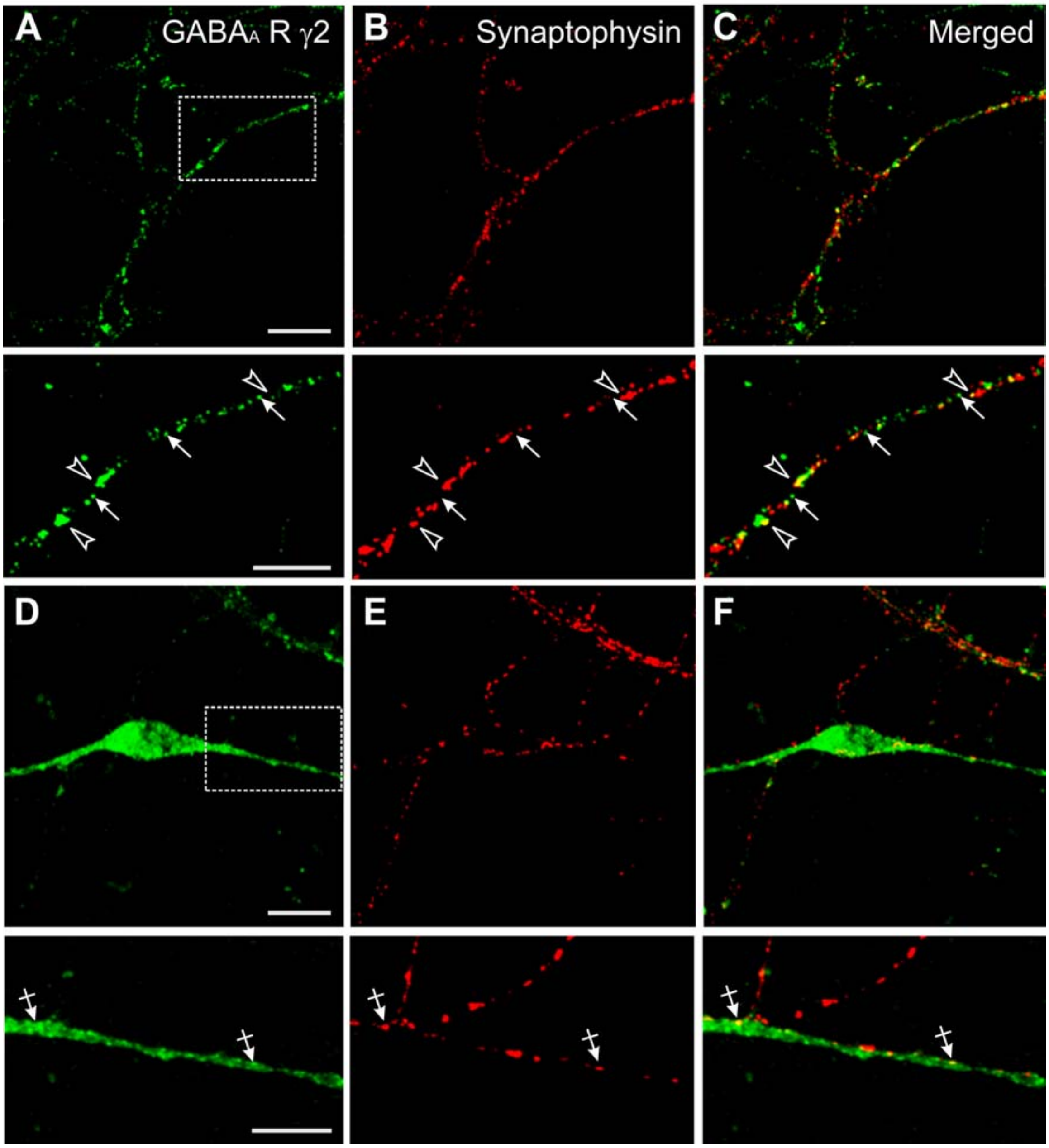


Fig.3

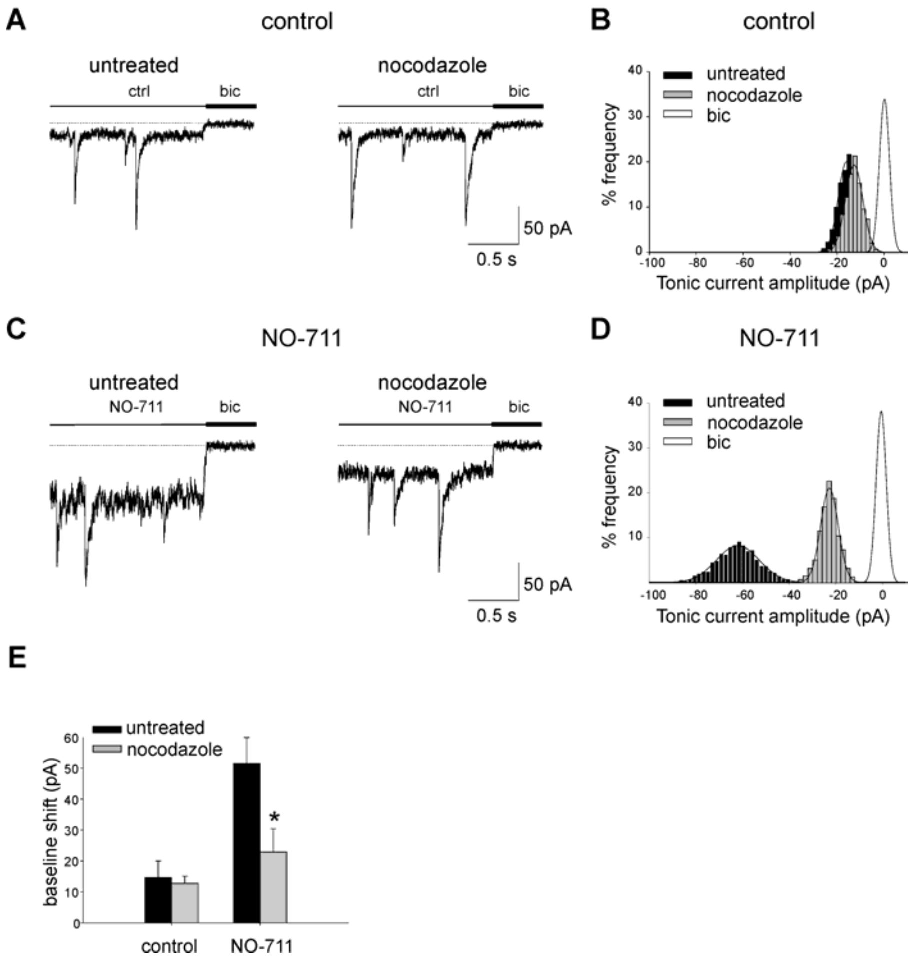

A
control
untreated
nocodazole
ctrl
bic
ctrl
bic
50 pA
0.5 s
B
control
untreated
nocodazole
bic
40
30
20
10
0
% frequency
-100
-80
-60
-40
-20
0
Tonic current amplitude (pA)
C
NO-711
untreated
nocodazole
NO-711
bic
NO-711
bic
50 pA
0.5 s
D
NO-711
untreated
nocodazole
bic
40
30
20
10
% frequency
-100
-80
-60
-40
-20
0
Tonic current amplitude (pA)
E
untreated
nocodazole
60
50
40
30
20
10
0
baseline shift (pA)
*
control
NO-711

Fig.4

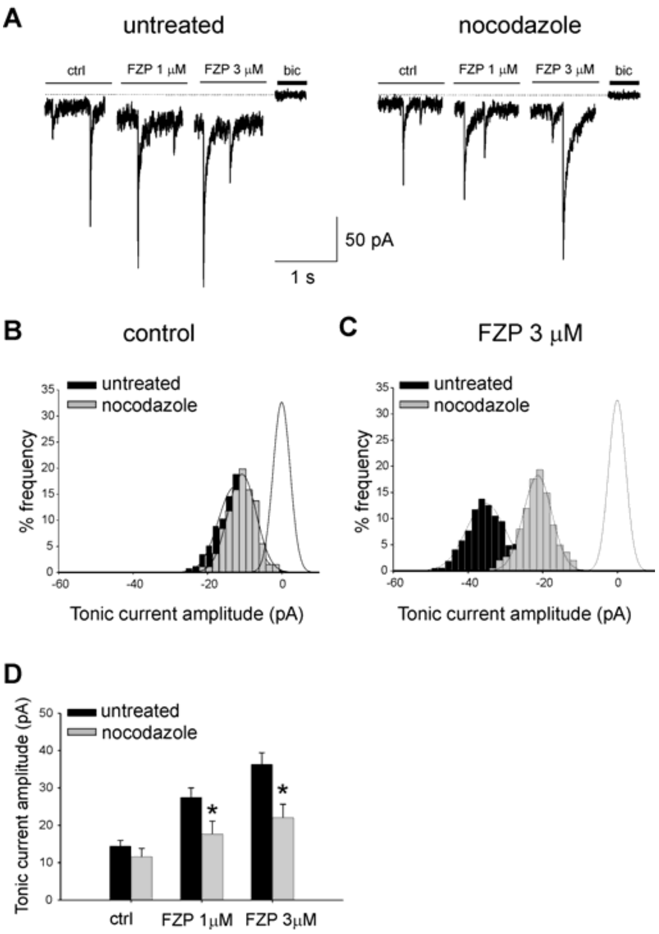

A
untreated
nocodazole
ctrl
FZP 1 μM
FZP 3 μM
bic
50 pA
1 s
B
control
C
FZP 3 μM
untreated
nocodazole
% frequency
35
30
25
20
15
10
5
0
-60
-40
-20
0
Tonic current amplitude (pA)
D
Tonic current amplitude (pA)
50
40
30
20
10
0
*
ctrl
FZP 1μM
FZP 3μM

Fig.5

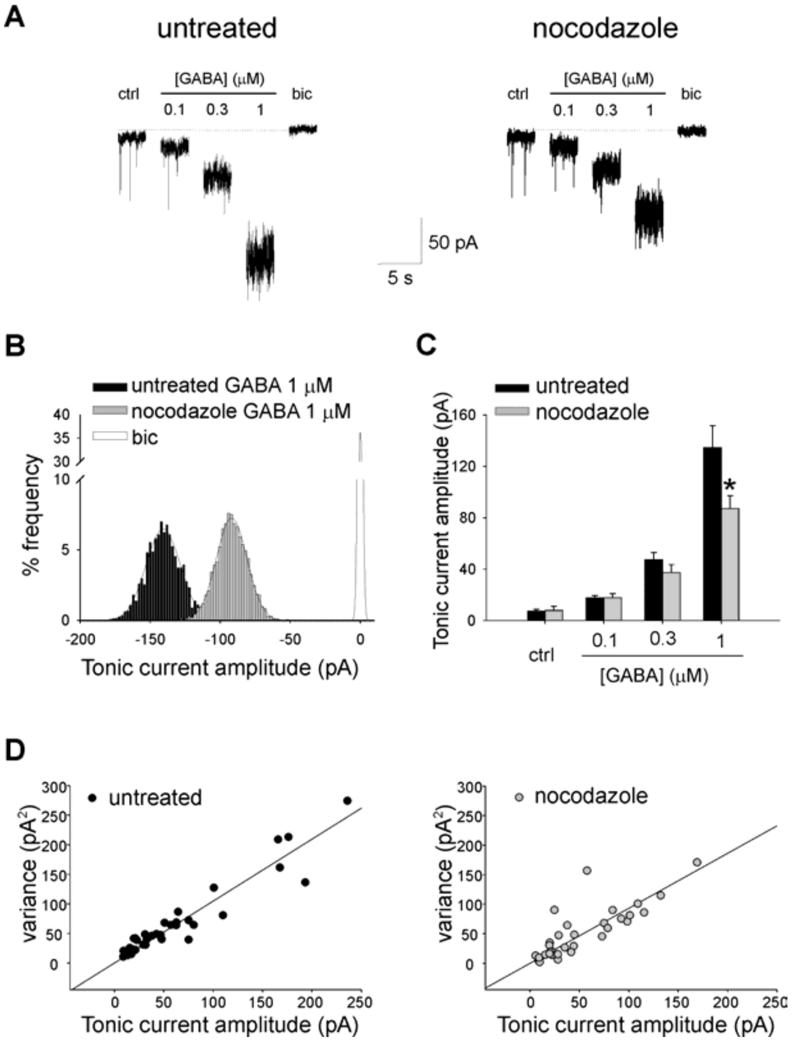

A
untreated
nocodazole
ctrl
[GABA] (μM)
0.1
0.3
1
bic
50 pA
5 s
B
untreated GABA 1 μM
nocodazole GABA 1 μM
bic
% frequency
40
35
30
10
5
0
-200
-150
-100
-50
0
Tonic current amplitude (pA)
C
untreated
nocodazole
Tonic current amplitude (pA)
160
120
80
40
0
*
ctrl
0.1
0.3
1
[GABA] (μM)
D
untreated
variance (pA^2)
300
250
200
150
100
50
0
0
50
100
150
200
250
Tonic current amplitude (pA)
nocodazole
variance (pA^2)
Tonic current amplitude (pA)

Fig.6

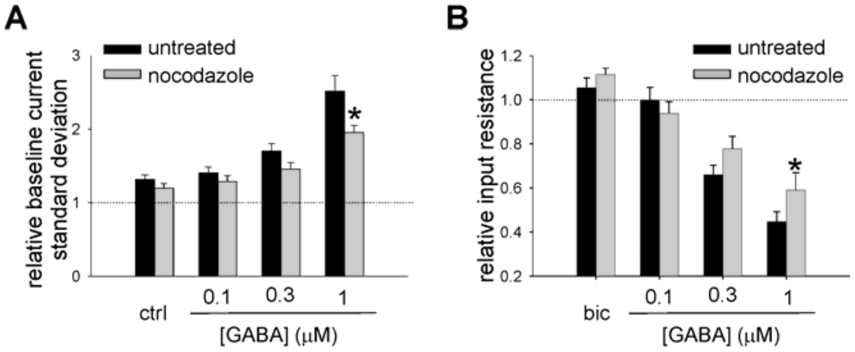

A
untreated
nocodazole
relative baseline current standard deviation
0
1
2
3
*
ctrl
0.1
0.3
1
[GABA] (μM)
B
untreated
nocodazole
relative input resistance
0.2
0.4
0.6
0.8
1.0
1.2
*
bic
0.1
0.3
1
[GABA] (μM)

Fig.7

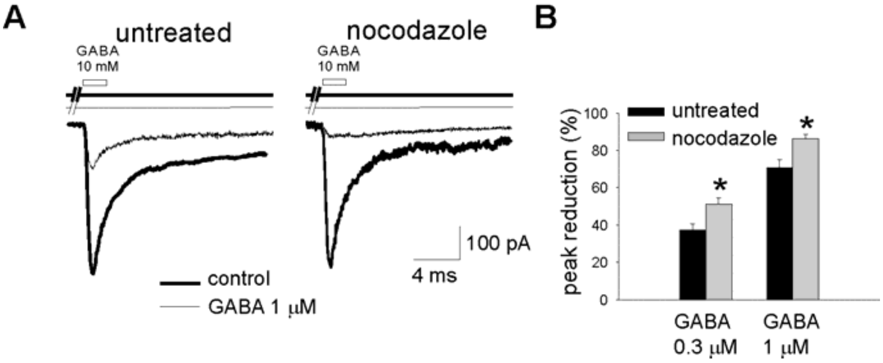
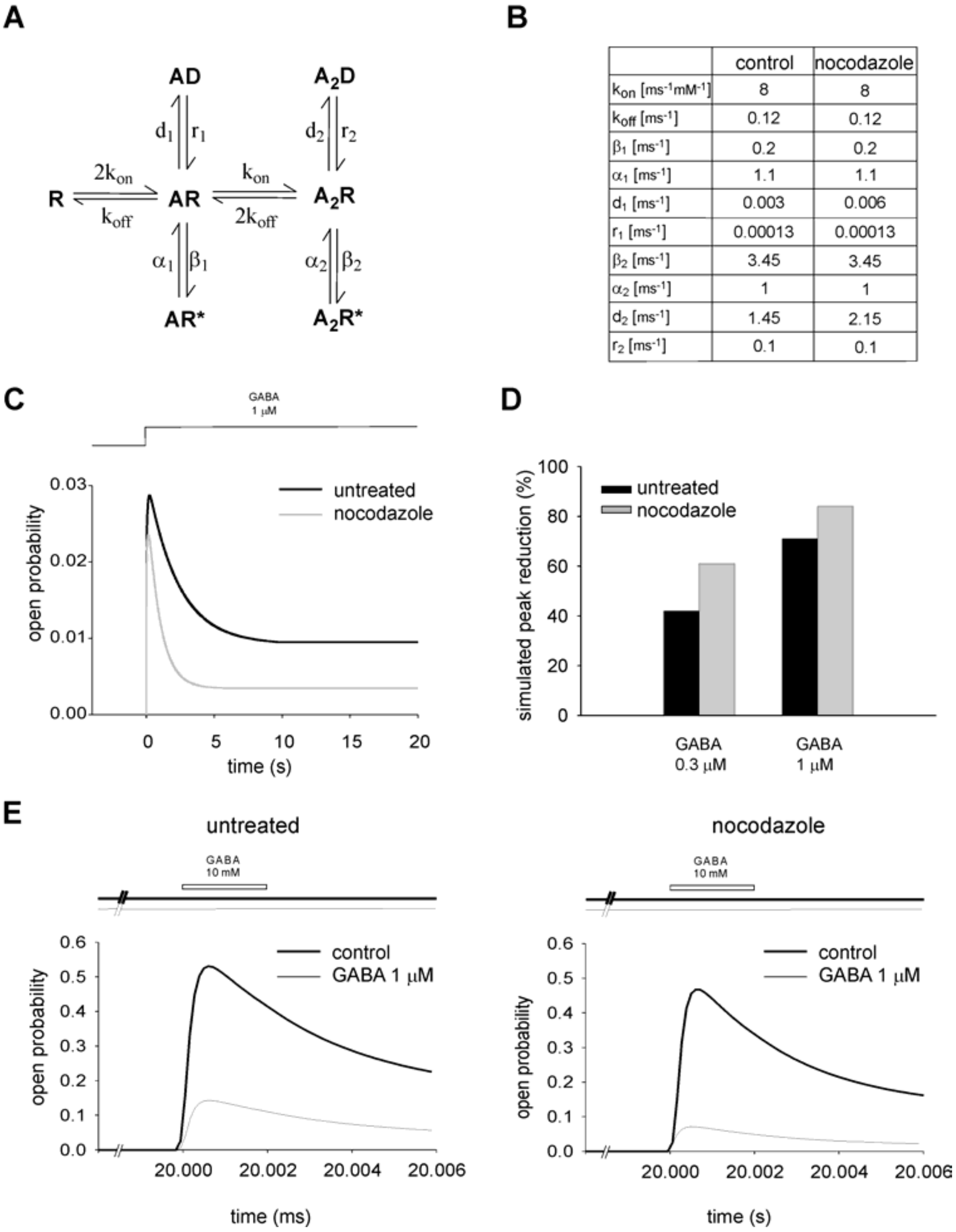

A
untreated
GABA
10 mM
nocodazole
GABA
10 mM
100 pA
4 ms
control
GABA 1 μM
B
untreated
nocodazole
peak reduction (%)
100
80
60
40
20
0
*
*
GABA
0.3 μM
GABA
1 μM

Fig.8



	control	nocodazole
k_{on} [$ms^{-1}mM^{-1}$]	8	8
k_{off} [ms^{-1}]	0.12	0.12
β_1 [ms^{-1}]	0.2	0.2
α_1 [ms^{-1}]	1.1	1.1
d_1 [ms^{-1}]	0.003	0.006
r_1 [ms^{-1}]	0.00013	0.00013
β_2 [ms^{-1}]	3.45	3.45
α_2 [ms^{-1}]	1	1
d_2 [ms^{-1}]	1.45	2.15
r_2 [ms^{-1}]	0.1	0.1

5. Conclusions and future perspectives

In this thesis I have analyzed the heterogeneity of phasic and tonic GABAergic inhibition in cultured hippocampal neurons, as a result of the presynaptic variability of GABA transient in the cleft and of the postsynaptic modulation of the gating properties of $GABA_A$ receptors induced by receptor clustering.

The heterogeneity of spontaneous miniature GABA-mediated synaptic currents is exemplified by the broad and skewed distribution of amplitudes of mIPSCs. At non-saturated synapses I found that the variability of synaptic currents was correlated with the variability of GABA concentration profile in the cleft. In particular both the concentration GABA (A_t) that activates the receptors and the speed of clearance of the agonist from the cleft (τ_t) are crucial for determining the amplitude and kinetics of synaptic currents. By modeling mIPSCs it was possible to find out a range of variability for A_t and τ_t values responsible for the observed mIPSCs variability. According to the estimation reported in this thesis, rapidly cleared low concentrations of GABA in the cleft are responsible for small and rapid mIPSCs, while large and persistent concentrations of agonist in the cleft elicit large and slow synaptic events.

Probably the most innovative result reported in this thesis is the identification of $GABA_A$ receptor clustering as a novel possibility for the modulation of GABAergic transmission. In fact an accurate analysis of the influence of $GABA_A$ receptor clustering on phasic and tonic GABA-mediated inhibition has never been reported previously. The only previous attempt in this direction was made by Chen *et al.* (2000) who described altered kinetic properties of GABA-evoked currents mediated by declusterered recombinant receptors. As pointed out by the authors, the overexpression of the receptors in a heterologous system was far from approaching physiological conditions. Moreover the limited time resolution of the agonist application system precluded the assessment of the kinetic changes occurring at submillisecond time scale.

In our preparation, *i.e.* cultured hippocampal neurons, the microtubule depolymerizing drug nocodazole proved to be a useful tool to induce the disruption of $GABA_A$ receptor clusters. Therefore it was possible to record changes in both phasic and tonic currents after nocodazole treatment. The combination of an ultra-fast agonist perfusion system and model simulations allowed concluding that receptor clustering affects the gating properties of $GABA_A$ receptors. In particular the desensitization of the receptors in the singly and doubly-bound conformations appeared to be promoted in declustered receptors. Nevertheless we cannot exclude the possibility that receptor clustering may also affect slowly absorbing doubly bound desensitized states (Haas & Macdonald, 1999; Bianchi *et al.*, 2002) neglected in our model. It is worth noting that these conclusions came from separate investigations on GABA-evoked responses mimicking phasic and tonic currents. In fact the study of currents evoked by GABA concentrations larger than 30 μM shed light only on the transitions within the doubly-bound open and desensitized states, while the effects of the persistent exposure to submicromolar concentrations of agonists allowed resolving the singly bound conformations. Regardless the number of desensitized states involved, the conclusions reported here are in line with the recently reported crucial role of receptor desensitization in shaping $GABA_A$ mediated currents (Mozrzymas *et al.*, 2003b). In fact the different conformational states of the receptors follow a Markovian scheme and this inherently implies that the temporal profile of the occupancy of any conformation depends on all the rate constants and occupancies of all other states.

Immunocytochemical approaches have shown that γ_2 subunit-containing receptors are present not only at synaptic but also at extrasynaptic sites, therefore supporting electrophysiological evidence for the possible involvement of this receptor subtype in mediating tonic inhibition. Both γ_2 and δ subunit-containing receptors (the latter found exclusively at extrasynaptic locations) are arranged in clusters and are sensitive to nocodazole.

Although at first choice nocodazole provided a decent tool to induce the declusterization of $GABA_A$ receptors, it should be used with caution. In fact microtubules are widespread throughout the neurons and are involved in many physiological processes. Therefore, in addition to the declusterization of $GABA_A$ receptors, microtubule disruption might have provided possible bias by promoting any other kind of effect. Although all the electrophysiological results collected from declustered receptors with different techniques seem to be coherent, we have been always aware of this weakness.

It is for this reason that a promising ongoing collaboration with Prof. Cattaneo's group is aiming at selecting specific tools for inducing $GABA_A$ receptors declusterization by taking advantage of a robust procedure they have developed, called Intracellular Antibody Capture Technology (IACT) (Visintin *et al.*, 2002). The rationale is to ablate proteins' function by taking advantage of antibody specificity in recognizing antigens, *i.e.* intracellular immunization (Biocca & Cattaneo, 1995).

However it is worth noting that when antibodies are expressed in the cell cytoplasm, folding and stability problems often occur. The reducing condition of the cytoplasm (Hwang *et al.*, 1992) hinders the formation of the intradomain disulphide bond in the V_H and V_L domains (Biocca & Cattaneo, 1995), resulting in low expression levels and limited half-life of antibody domains. However, some single-chain Fv (scFv), which consist of a V_H chain linked to a VL chain, have been shown to tolerate the absence of this bond (Proba *et al.*, 1997) and to maintain their function when overexpressed in cells.

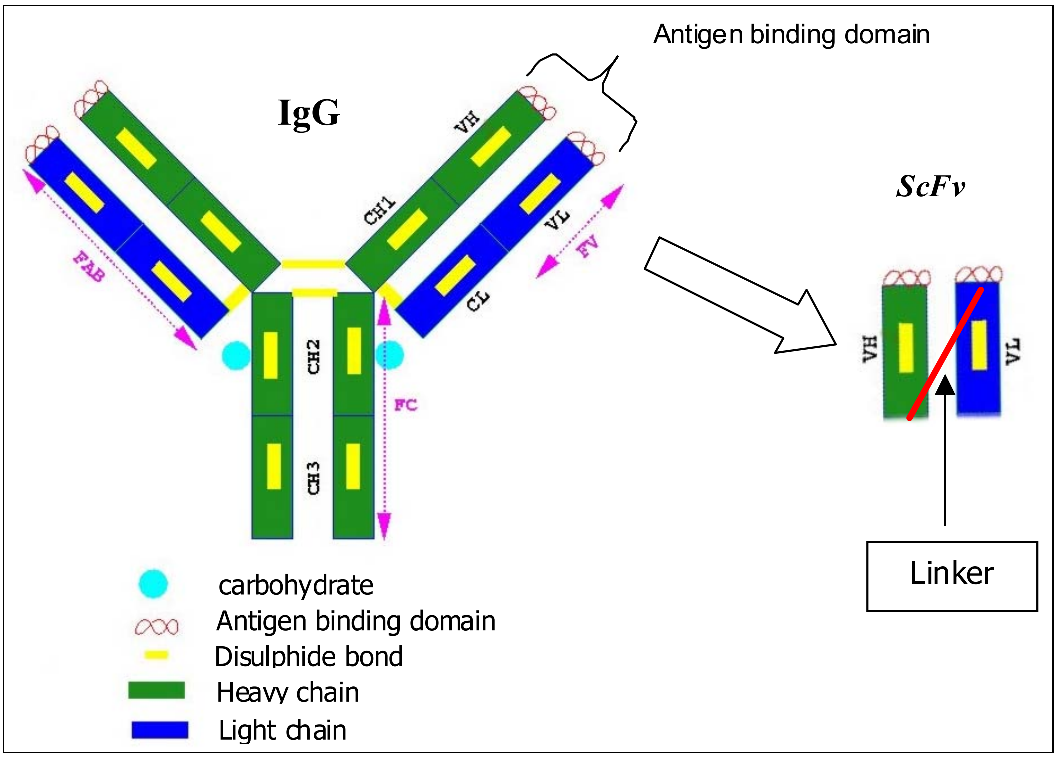


Fig.1 Schematic representation of the structure of IgG and scFv

The impairing effect of these scFv on the function of a target protein can be achieved in two different ways. One possibility is the steric hindrance of scFv that prevents the interaction of the target protein with other molecules. Another possible mechanism is that engineered scFv change the localization of the target protein. For instance an scFv modified with a nuclear localization sequence would first bind the target protein and then redirect and retain it into the nucleus.

The selection strategy for functional scFv (IACT) occurs *in vivo* and is based on a two-hybrid approach (Visintin *et al.*, 1999).

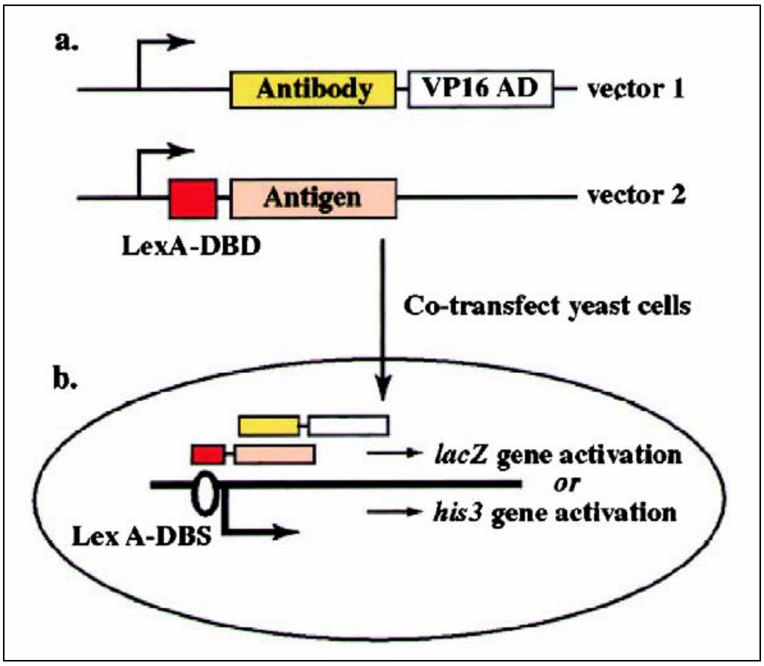


Fig.2 Diagram of the two hybrid-based IACT approach (from Visintin et al., PNAS, 1999)

Briefly the two-hybrid system is adapted to detect antibody-antigen interaction *in vivo*. Yeast expression constructs are prepared encoding either an antibody fragment, in the form of scFv, linked to the VP16 transcriptional activation domain (AD) or the LexA DNA binding domain (DBD) linked to an target antigen sequence (bait). These constructs are cotransfected into yeast cells unable to synthesize histidine and carrying either the histidine (*his*) gene or the *lacZ* gene controlled by a minimal transcription promoter with a LexA DNA binding site (DBS). If antibody-bait interaction occurs *in vivo*, the resulting complex can bind to the LexA DBS upstream of *his* or *lacZ* genes and transcription of these genes occurs (the VP16 activation domain is thus brought close to the DNA transcription start site and can recruit accessory factors needed for transcription). The transcriptional activation of the *his* gene facilitates growth of yeast in growth media lacking histidine and activation of the *lacZ* gene produces β-gal, which can be assayed with 5-bromo-4-chloro-3-indolyl β-D-galactoside to yield blue yeast colonies. Neither feature of the transfected yeast will occur if the antibody fragment does not functional inside cells. Once the positive scFvs have been isolated, their specific interaction with the bait protein is confirmed again both in yeast and in vitro. Then the ability of maintaining appropriate folding is verified in mammalian cells and finally scFvs must be functionally tested for their ability to impair the function of the bait protein.

In the attempt to select specific tools for studying $\mathrm{GABA_A}$ receptor clustering two different proteins involved in receptor trafficking and/or clustering (GABARAP and gephyrin) have been used as baits for the IACT approach. Untill now scFv specifically recognizing GABARAP and gephyrin have been already selected and their interaction with these two bait proteins confirmed. Moreover successful expression of anti gephyrin scFv has been detected in both neuronal and non-neuronal cells.

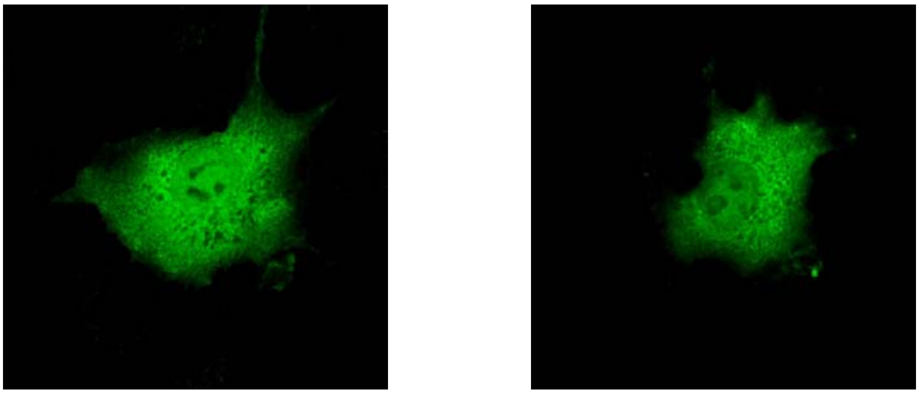


Fig.3 Immunofluorescence microscopy of anti-gephyrin-FLAG scFv transiently trasnfected in COS cells (non neuronal type), incubated with an anti-FLAG primary antibody and a FITC-conjugated secondary antibody.

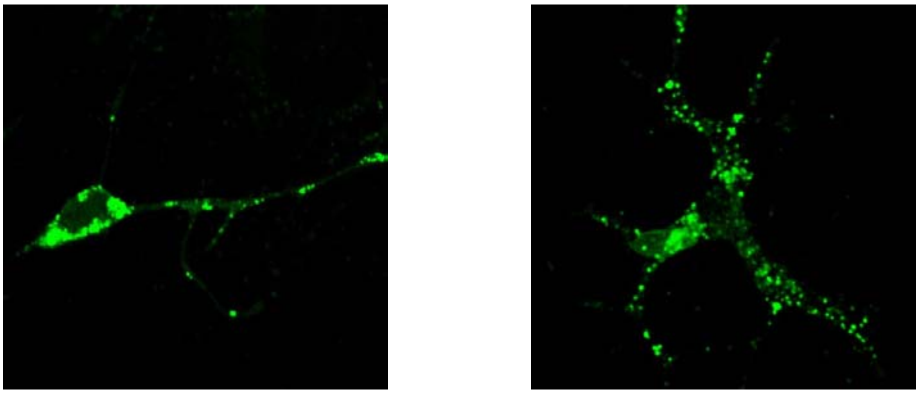


Fig.4 Immunofluorescence microscopy of anti-gephyrin-FLAG scFv transiently trasnfected inprimary cultured hippcampal neturons, incubated with an anti-FLAG primary antibody and a FITC-conjugated secondary antibody.

Anti-GABARAP and anti-gephyrin scFv will be tested on neurons soon for their ability to induce the declusterization of native $GABA_A$ receptors.

6. References

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