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Patch Clamp Study of Neurotransmission at Single Mammalian CNS Synapses

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1. Introduction

Single mammalian CNS neurons can be acutely isolated with adherent and functional excitatory and inhibitory synaptic nerve terminals (boutons) using a mechanical dissociation procedure without any enzyme treatment (Vorobjev, 1991; Haage & Johansson, 1998; Rhee et al., 1999). This 'synaptic bouton' preparation is particularly suitable for physiological and pharmacological investigations of mammalian CNS synaptic transduction mechanisms, and the properties of both the receptors, transporters, and 2nd messengers present in the presynaptic terminals (boutons), and the synaptic and extrasynaptic receptors on the postsynaptic membrane, can be studied.

The truncated dissociated potsynaptic neurons are well space-clamped allowing accurate measurements of synaptic currents, and the isolated neurons are devoid of complications arising from surrounding cells such as other neurons or astrocytes (glial cells). The acute, mechanical dissociation avoids possible changes in protein distribution and/or function as result of either enzyme treatment or *in vitro* culture. Yet, the extremely small size of typical mammalian presynaptic terminals (< 1μm) have presented a challenge for functional studies on neurotransmitter release. In the synaptic bouton preparation, neurotransmitter is released from these adherent terminals giving rise to spontaneous synaptic potentials. Furthermore, a single presynaptic nerve terminal (bouton) can be focally stimulated with electrical pulses (Akaike et al., 2002; Murakami et al., 2002; Akaike & Moorhouse, 2003) to result in evoked synaptic potentials. Therefore, this 'synapse bouton' preparation has helped to unravel the mechanisms and modulation of synaptic transmission in the mammalian CNS. In this article, the general properties of this preparation are described, along with some typical examples of its applications to the study of synaptic transmission.

2. Mechanical dissociation of mammalian CNS neurons

The synaptic bouton has to date been prepared from mature (1~3 months old) and immature (12~18 days old) rats, immature and juvenile mice, and guinea-pigs (10 days~1 month old) (e.g. Akaike et al, 2002) using the following approach. Animals were decapitated under pentobarbital anesthesia (50 mg kg^{-1} *i.p.*), and the brain quickly removed and immersed in an ice-cold physiological incubation solution, saturated with 95 % O_2 and 5 % CO_2. Brain or spinal cord slices containing the region of interest were cut using a vibrating microtome (VT 1200S; Leica, Nussloch, Germany) at a thickness of ~400 μm. The brain slices were then incubated in a medium oxygenated with 95 % O_2 and 5 % CO_2 at room

temperature (21-24 °C), for at least 1h before mechanical dissociation. For mechanical dissociation, the brain slice was transferred into a 35 mm culture dish (Primaria 3801, Becton Dickinson, Rutherford, NJ, USA) containing a HEPES-buffered standard external solution, and fixed by an anchor made from a platinum frame and nylon thread (Fig. 1A). The region of interest was identified under a binocular microscope (SMZ645, Nikon Tokyo) and the tip of a fire-polished glass pipette was lightly placed on the slice surface above the target neurons and vibrated horizontally (0.2-2 mm displacement) at 50-60 Hz using the manufactured device (S1-10 cell isolator, K.T. Labs, Tokyo) (Fig. 1B). The dish is typically also moved horizontally along the target region by hand to enable isolation of neurons from more than a single spot. Thereafter, the slices were removed from the dish, and the mechanically dissociated neurons left to settle and adhere to the bottom of the dish for at least 15 min before electrophysiological measurements.

An electron microscopy study was done to confirm that boutons are attached to dissociated CNS neurons. Fifteen boutons were observed on a single dissociated rat hippocampal neuron with an averaged diameter of 0.6±0.04 μm and were clearly separated from the closest neighboring bouton, as shown in Figure 1C (Akaike et al., 2002). These adherent synaptic boutons contain functional voltage-dependent ionic channels, various chemo-receptors, transporters, and 2nd messenger pathways, as also found in the postsynaptic neuron. To date, this 'synaptic bouton' preparation has been obtained from various brain regions in our laboratory, including: the Meynert's nuclei (Rhee et al., 1999 ; Arima et al., 2001), the basolateral amygdala (Koyama et al., 1999), the hippocampal CA1 (Matsumoto et al., 2002) and CA3 (Yamamoto et al., 2011) regions, the periaqueductal gray (Kishimoto et al., 2001), the ventromedial hypothalamus (Jang et al, 2001), and the spinal sacral dorsal commissural nucleus (Akaike et al., 2010) and many other regions.

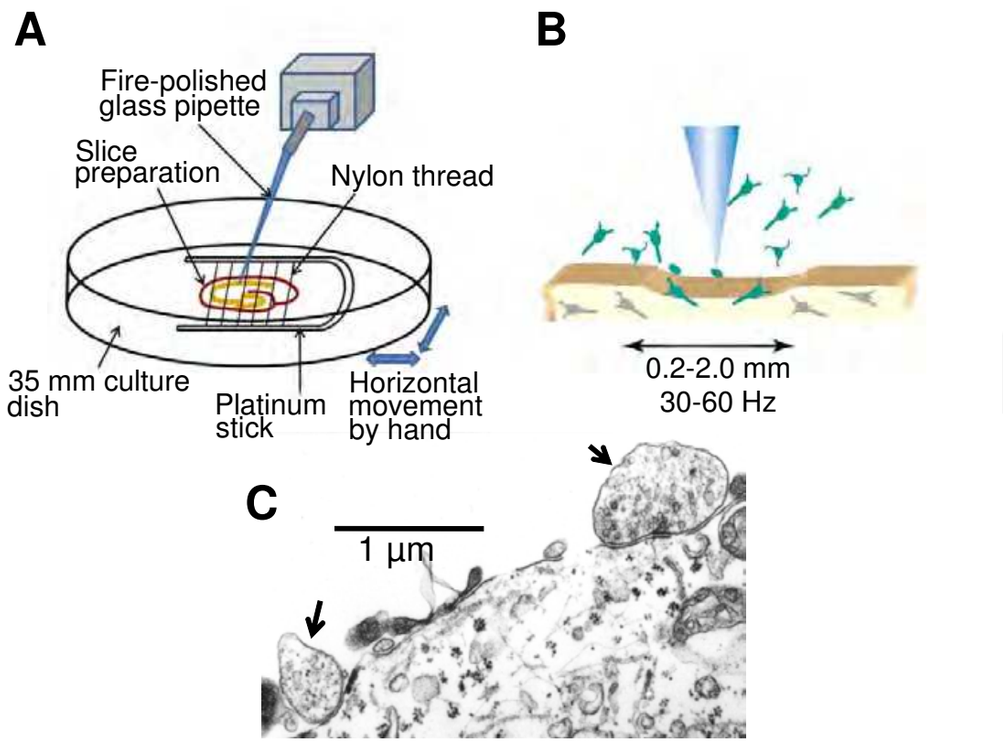

Fig. 1. Mechanical isolation of single CNS neurons

A, B: Schematic illustration of the mechanical dissociation of rat hippocampal CA1 pyramidal neurons. A fire-polished glass pipette is vibrated horizontally across the surface of the CA1 area at 50~60 Hz. Successful liberation of viable neurons results in a fine mist originating from the dissociation site. The treated slice is then removed and the liberated neurons are left to adhere to the base of the culture dish. C: Electron microscopy image showing two presynaptic boutons (arrowheads) adherent to a dissociated hippocampal CA1 neuron. Part C was used, with permission, from (Akaike et al.2002).

3. 'Synaptic bouton' preparations preserve functional presynaptic terminals (boutons)

Neurons mechanically isolated as described above show spontaneous synaptic potentials, as shown and described below. Fluorescence was also used to visualize functional presynaptic boutons. FM1-43 (Molecular probes, OR, USA) was applied to dissociated rat sacral dorsal commissural nucleus (SDCN) neurons, at a concentration of 10μM and in a depolarizing external solution containing high (45mM) K^+ for 30 sec, before the neurons were well washed with a standard external solution. FM1-43 flourescent spots, representing putative presynaptic boutons, were seen under the inverted microscope attached to an SDCN neuron at the soma and proximal dendrites (Fig. 2Aa). These fluorescent spots quickly distained when a second high $[K^+]_o$ (15~20mM) external solution was applied for 30 sec (Fig. 2Ab), indicating these are functional boutons undergoing endocytosis and exocytosis of the

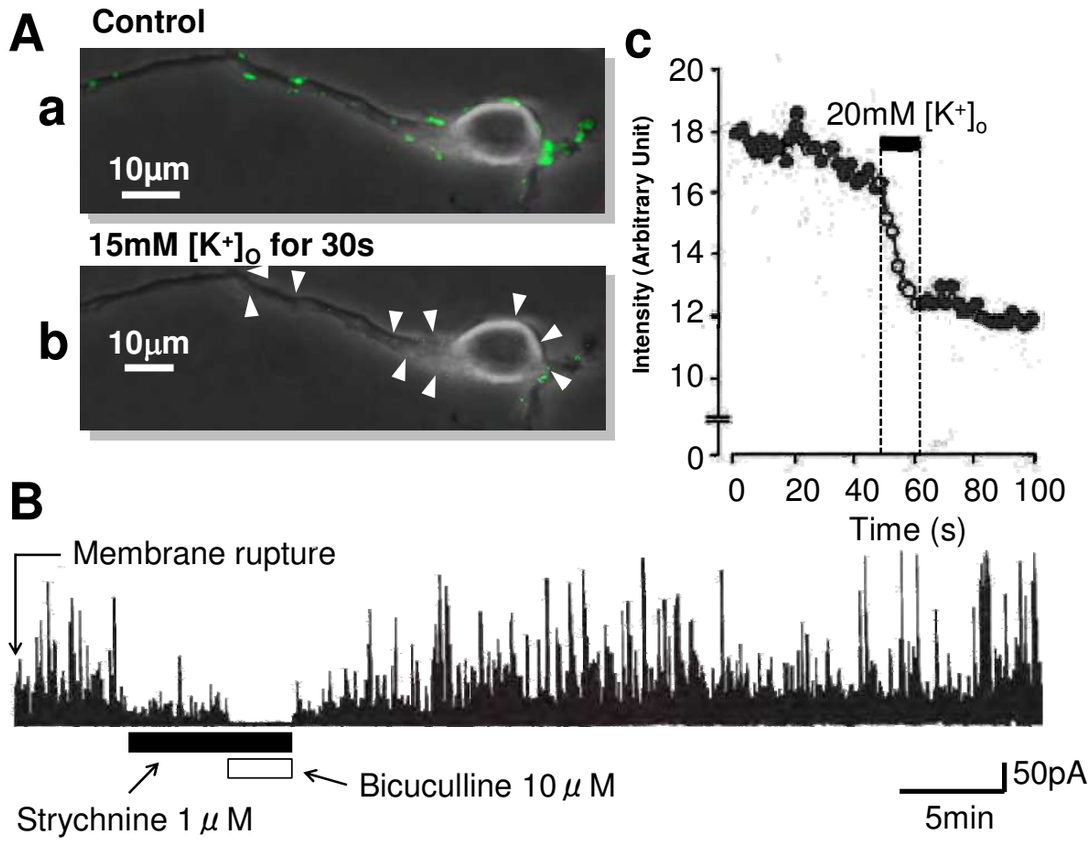

Fig. 2. The 'synaptic bouton' preparation.

fluorescent dye. Figure 2Ac shows the time course of one of the fluorescent spots before, during, and after application of a 20mM K^+ external solution.

Spinal SDCN neurons receive the projections of two kinds of inhibitory nerves, glycinergic and GABAergic ones, and hence the sIPSC is completely ceased by cumulative application of strychinine (a selective glycine receptor antagonist) and bicuculline (a selective $GABA_A$ receptor antagonist) (Fig. 2B). These representative results suggest that mechanically dissociated spinal neurons maintain functional presynaptic nerve terminals, which are sensitive to $[K^+]_o$ dipolarization, and useful for pharmacological studies of release and/or synaptic receptors.

Aa~c: A dissociated hippocampal CA1 neuron with presynaptic boutons stained by FM1-43 fluorescence (arrowheads). The intensity of the fluorescent signal in the boutons disappeared after adding of 15 mM K^+ to external solution (a, b). (c) A typical time course of the fluorescence intensity of a presynaptic bouton during the addition of 20mM K^+. B: Effects of strychnine and bicuculline on spontaneous inhibitory (glycinergic and GABAergic) currents recorded from a single rat spinal interneuron from the sacral dorsal commissural nucleus (SDCN) region. The result indicates that these neurons receive both glycinergic and GABAergic projections. Parts A and B were adapted with permission, from (Akaike et al., 2002 ; Jang et al., 2002)

4. Focal electrical stimuli of a single bouton using *a "θ"* glass pipette

The stimulating pipette for focal electrical stimulation of a single bouton adherent to mechanically dissociated CNS neurons was made fromθglass tube (φ= OD 2mm, ID 1.4mm, WPI) filled with normal external test solution. A θ glass pipette is separated down the centre by a wall to give rise to two adjacent and separated compartments. Both compartments are filled with external solution and electrical wires, thereby acting like a bipolar electrode. The pipette was placed closer as possible to the postsynaptic soma membrane of a single CNS neuron during a whole-cell patch recording (Fig. 3A). The stimulating pipette was then carefully moved along the surface membrane of the soma or dendrites while applying stimulation pulses and monitoring for responses. Paired pulses are typically used if investigating presynaptic mechanisms, and stimuli applied typically once every 5-10 sec and applied via a stimulus isolator (SS–202 J, Nihon Koden, Tokyo). In individual neurons, the stimulus paradigms used are 100 μs duration, 0.1–0.3 mA intensity and 30–60 ms inter-stimulus intervals for evoked IPSCs (eIPSCs), and 100 μs duration, 0.05–0.08 mA intensity and 20–30 ms inter-stimulus intervals for evoked EPSCs (eEPSCs).

To determine whether GABAergic IPSCs were really evoked from a single bouton or, alternatively, from multiple separate boutons, the stimulus-amplitude and stimulus-distance relationships were examined. When a GABAergic eIPSC was identified, it appeared in an all-or-none fashion as stimulus strength increased or decreased (Fig.3B), indicating that the stimulating pipette was positioned just above a single GABAergic bouton. Furthermore, when the stimulus pipette was moved horizontally along the surface of a dissociated neuron, the eIPSC again appeared or disappeared in an all-or-none fashion. With shifts in distance of less than 0.4μm, the eIPSCs were maintained in the majority of boutons tested. The shift in the electrode did not affect the mean amplitude of eIPSCs but increased the failure rate of eIPSCs (Fig. 3Bb). In the case of #4 in Figure 3Bb, however, the

eIPSCs were still elicited even when the stimulus electrode was shifted ±0.4μm (totally about 0.8μm), suggesting that the eIPSCs were elicited from several boutons. Hence, studies on 'single boutons' seem to require stimuli locations that fail to elicit the eIPSC response if shifted by more than 0.4μm.

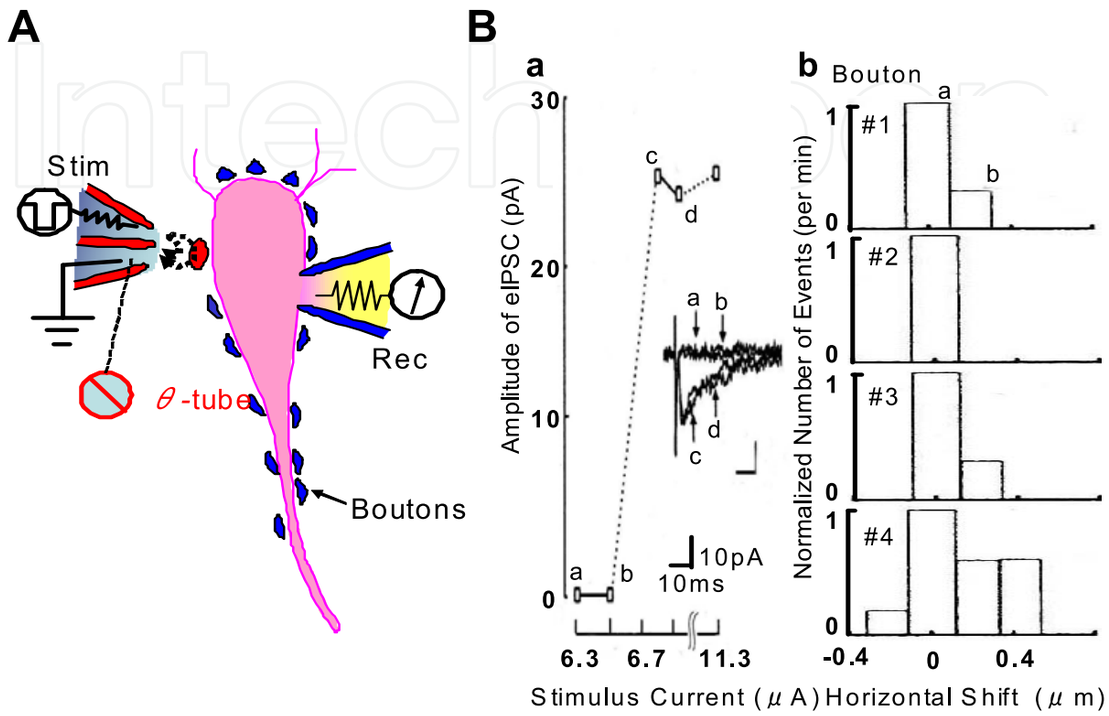

Fig. 3. Focal electrical stimulation of a single bouton

A: Schematic representation of a 'synaptic bouton' preparation and focal electrical stimulation of a single nerve ending (bouton). Ba: The relationship between stimulus strength and the amplitude of GABAergic eIPSCs, which appeared in an 'all or none' nature as stimulus strength varied. Inset shows two failure responses and two successfully evoked responses. Bb: The relationship between the extent of lateral displacement of the stimulating pipette (from a starting position, 0μm, that induced a minimum failure rate) and the proportion of successfully evoked eIPSCs. Shifts greater than 0.4~0.6μm abolished the response. Each of the four panels (#1~4) comes from four different boutons. The Y-axis shows the number of failures relative to the maximum incidence of successfully evoked responses, obtained at position 0μm. Part B was adapted with permission, from (Akaike et al., 2002).

5. General properties of spontaneous and evoked transmitter release in the 'synaptic bouton' preparation

The frequency of spontaneous IPSCs (sIPSC) and spontaneous EPSCs (sEPSCs) recorded from different CNS regions is between 1 and 10Hz, and the variability presumably reflects the differences in the number and excitability and adherent boutons. The spontaneous synaptic currents are both action potential-dependent and –independent (TTX; tetrodotoxin resistant). The addition of TTX, a selective Na channel blocker, decreases the frequency of

GABAergic or glycinergic sIPSC (Rhee et al., 1999) and glutamatergic sEPSC (Jang et al., 2001) by about 50 % at least. In the presence of Ca channel blockers (Rhee et al., 1999; Koyama et al., 1999; Shoudai et al., 2007) or in nominal Ca-free solution (Maeda et al., 2009), glycinergic sIPSC frequency decreases 30 ~40% of control. A large proportion of these TTX-resistant miniature IPSCs (mIPSCs) are independent from Ca^{2+} influx (Emptage et al., 2001; Miller et al., 1998; Scholtz et al., 1992), as reported by others. Both Ca^{2+} release from internal Ca stores and store – depleted Ca^{2+} influx contribute to these mIPSCs (Emptage et al., 2001). Consequently, many 'minis' remain in the absence of external Ca^{2+} influx. The ability to dissect transmitter release into such spontaneous and miniature postsynaptic currents, Ca^{2+} influx resistant or sensitive, is useful for examining the locus of action of presynaptic neuromodulators.

As indicated above, the selective activation of a single excitatory glutamatergic (Yamamoto et al., 2011; Akaike et al., 2010) or inhibitory GABAergic (Akaike et al., 2002; Murakami et al., 2002; Akaike et al., 2010; Ogawa et al., 2011) and glycinergic (Akaike et al., 2010; Nonaka et al., 2010) nerve terminals (boutons) are possible in the 'synaptic bouton' preparation by using focal electrical stimulation technique. The current amplitudes of glutamatergic eEPSCs, glycinergic eIPSCs, and GABAergic eIPSCs recorded from SDCN neurons are on average about 140, 250, and 80 pA, respectively. On the other hand, the average failure rates of glutamatergic, glycinergic and GABAergic single bouton synaptic responses are 18, 37, and 32%, respectively.

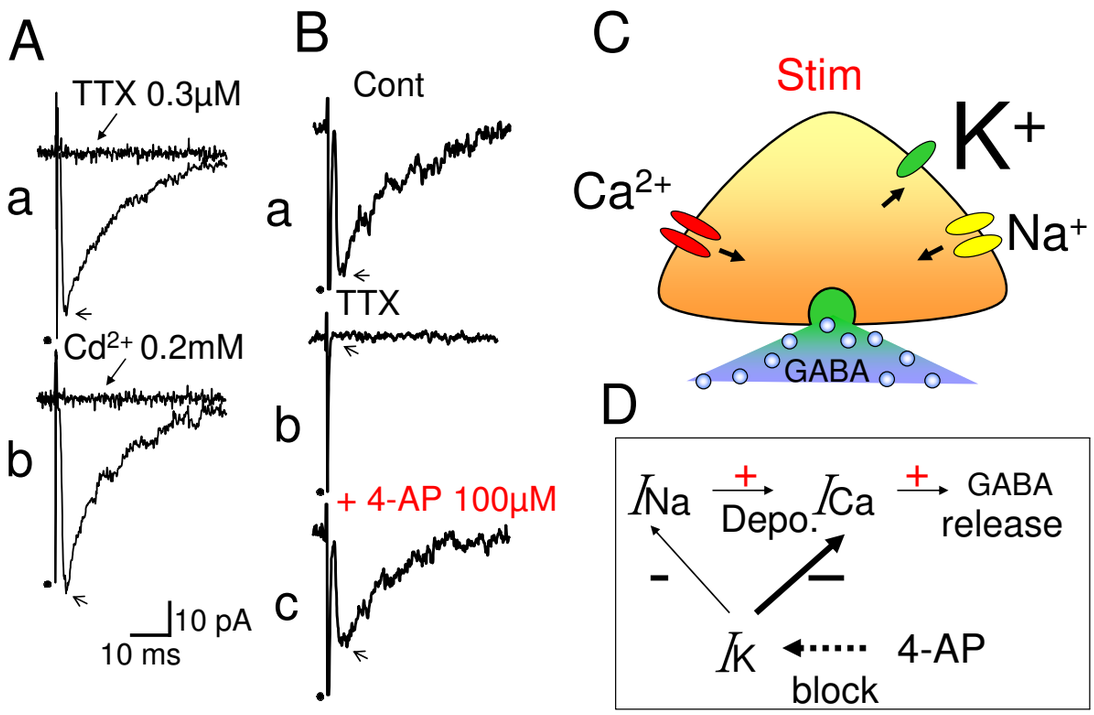

Fig. 4. Focal stimulation of a single GABAergic bouton

A,B: GABAergic eIPSCs in the presence and absence of 0.3μM tetrodotoxin (TTX), 0.2mM Cd^{2+}, and 0.3μM TTX + 100μM 4-AP as indicated. All traces were recorded from the same hippocampal CA1 pyramidal neuron. C: A schematic illustration of voltage-dependent Na^{+}, K^{+} and Ca^{2+} channels in single GABAergic boutons. D: Diagram of the functional interaction

among Na^+, K^+ and Ca^{2+} currents in single boutons. Parts A and B were adapted with permission, from (Akaike et al., 2002).

The depolarization of the nerve terminals triggered by Na channel activation results in subsequent Ca^{2+} influx passing through voltage-dependent Ca channels (Nonaka et al., 2010; Jackson & Zhang, 1995). The application of 0.3μM TTX reversibly abolished GABAergic eIPSCs. In the presence of TTX, eIPSCs could not be evoked even after increases in the stimulus strength. However, in the presence of TTX, eIPSC reappears when 100μM 4-AP (a nonselective K channel blocker) is applied (Fig.4A,B). The application of the voltage-dependent Ca^{2+} channel blocker, Cd^{2+}, reversibly blocks eIPSCs *in either in control conditions or in the presence of TTX and 4-AP*. This suggests that eIPSCs are evoked by Ca^{2+} influx through voltage-dependent Ca^{2+} channels that typically activate due to the opening of voltage-dependent Na^+ channels. In the presence of TTX, electrical stimulation are unable to directly depolarize the terminals sufficiently to activate Ca^{2+} influx and release, seemingly due to a high density and/or lower activation threshold of presynaptic K channels. Once these K^+ channels are blocked (by adding 4-AP) the nerve terminals can be directly activated by focal stimulation (Akaike et al., 2002) (Fig. 4C,D).

6. Voltage-dependent Ca channel subtypes on single GABAergic and glycinergic nerve terminals

Varying the external Ca^{2+} concentration ($[Ca^{2+}]_o$) changes both the current amplitude and the failure rate (Rf) of eIPSCs elicited by focal stimulation of a single glycinergic bouton (Fig.5). Raising $[Ca^{2+}]_o$ from 2 to 5mM slightly increases the eIPSC current amplitude while decreasing Rf. Decreasing $[Ca^{2+}]_o$ from 2 mM to 1mM substantially decreases the current amplitude and increases Rf. The eIPSCs are abolished at 0.3mM $[Ca^{2+}]_o$ (Fig.5). These results indicate glycine release is highly dependent on Ca^{2+} influx into single glycinergic nerve terminals, via both changes in release probability (changing Rf), and changes in the amount of glycine released (changing eIPSC amplitude).

Voltage-dependent Ca^{2+} channels are distributed throughout the CNS and play a key role in many neuronal functions including synaptic transmission. Ca^{2+} entering into the presynaptic terminal through different Ca^{2+} channel subtypes result in local intra-terminal "hot-spots" in which Ca^{2+} binds to various Ca^{2+} -binding proteins located at the release sites to trigger exocytosis of neurotransmitter vesicles (Borst & Sakmann, 1996; Seager et al., 1999). In these brain slice and cultured neuronal preparations, different Ca^{2+} channel subtypes coexist and co-regulate transmitter release.

The precise functional arrangement of Ca channel subtypes on small CNS nerve terminals is technically challenging, and focal electrical stimulation of single GABAergic and glycinergic nerve terminals in nerve-bouton preparations of hippocampal CA1 and spinal SDCN neurons, respectively, has addressed this question. The L-, N- and P/Q subtypes of Ca^{2+} channels were identified on the CA1 GABAergic nerve terminals (Murakami et al., 2002) and P- and R Ca^{2+} channel subtypes on the SDCN glycinergic terminals (Nonaka et al., 2010). There is some Ca^{2+} channel cooperativity in the individual terminals, and the different subtypes present all contribute to determining the total Ca^{2+} influx associated with synaptic vesicle release.

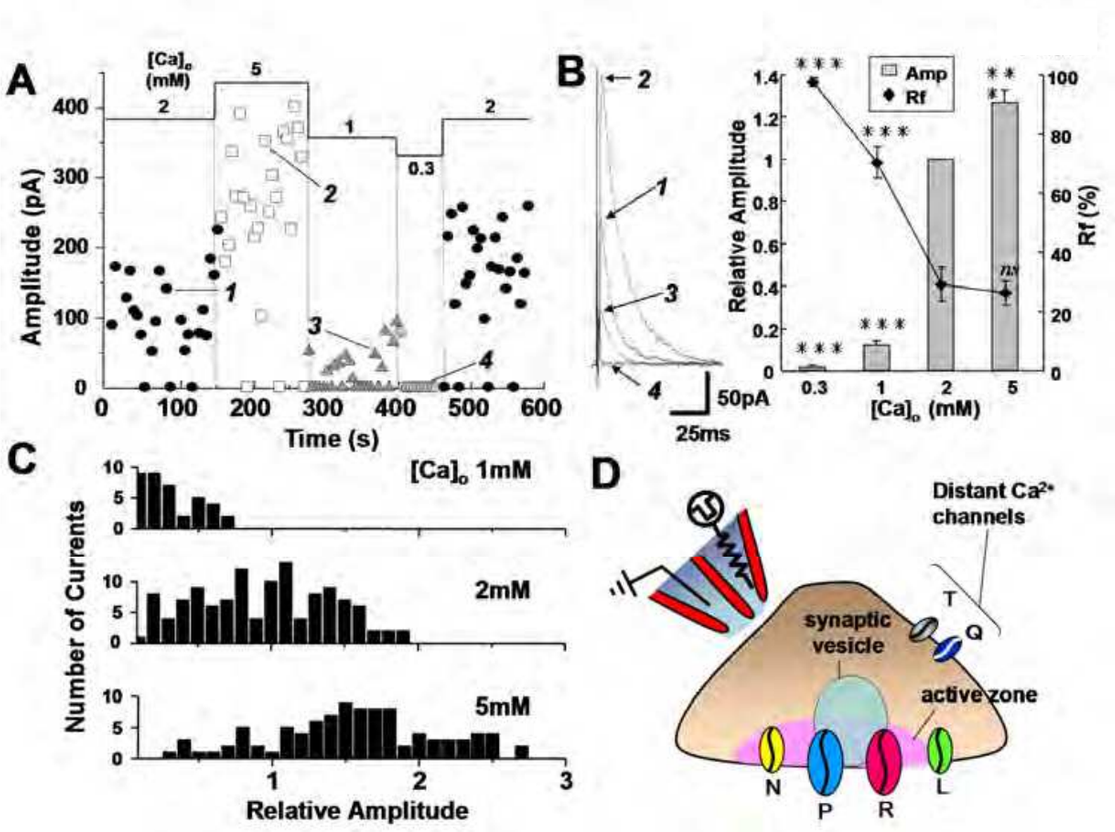

Fig. 5. Effects of changes in the external Ca^{2+} concentration ($[Ca^{2+}]_o$) on glycinergic eIPSCs from the spinal SDCN neuron

A: A plot of eIPSC amplitude (evoked at a frequency of 0.2 Hz) in different $[Ca^{2+}]_o$ concentrations as indicated (0.3~5mM). B: Left panel, Representative current traces (1~4) appeared at times and in A. Right panel, Relationship between the averaged current amplitude and failure rate (Rf), and $[Ca^{2+}]_o$. ** P<0.01, *** P<0.001, ns, no significant. Each data point represents the mean ± SEM of 6 neurons. C: Histogram of the relative current amplitudes of eIPSCs at 1, 2 and 5mM $[Ca^{2+}]_o$. All current amplitudes in different $[Ca^{2+}]_o$ were normalized to the average current amplitude obtained from each bouton in control external solution with 2mM Ca^{2+}. D: Schematic diagram of the proposed distribution of high- and low-voltage threshold Ca^{2+} channels on a single glycinergic bouton projecting to rat spinal SDCN neurons. Dominant control of glycine release depends on P- and R-type Ca^{2+} channels. Parts A~C were obtained, with permission, from (Nonaka et al., 2010).

7. $GABA_A$ receptor-mediated 'autoinhibition' and 'presynaptic inhibition' in single CNS nerve terminals

GABA is accepted as a major inhibitory neurotransmitter, and can act at $GABA_A$ receptors to cause both postsynaptic and/or presynaptic inhibition. Presynaptic $GABA_A$ receptors can inhibit GABA release from GABAergic nerve terminals, as an example of classical auto-inhibition, or can presynaptic inhibition in glutamatergic nerve terminals including the classically studied primary-afferent depolarization in the spinal cord. To investigate how

presynaptic $GABA_A$ receptors modulate spontaneous and action potential mediated GABA release, 'synaptic bouton' preparations isolated from hipppocampal CA3 region were used. Muscimol, a selective $GABA_A$ receptor agonist, increased spontaneous GABAergic IPSCs (sIPSCs) in a concentration-dependent manner, without affecting the current amplitude, indicating that muscimol acts on $GABA_A$ receptors in the presynaptic GABAergic nerve terminals. The increase in sIPSC frequency is reversibly prevented by the addition of Cd^{2+}, or in Ca^{2+}-free external solution, suggesting that muscimol depolarizes nerve terminals to induce Ca^{2+} influx through voltage-dependent Ca channels (Jang et al., 2002; Jang et al., 2006; Yamamoto et al., 2011). The depolarization indicates a GABA-induced Cl^- efflux and hence a higher Cl^- concentration in presynaptic nerve terminals than prediction by a passive distribution. In neuronal soma and in nerve terminals, this results from the activity of the Na^+, K^+, $2Cl^-$ cotransporter type1 (NKCC-1) (Jang et al., 2001; Kakazu et al., 1999) and in fact blocking this transporter with bumetanide prevents the GABA-induced increase in sIPSC frequency (Jang et al., 2001). The functional role of presynaptic $GABA_A$ receptors on eIPCS at GABAergic hippocampal CA1 synapses was also studied. Muscimol (3μM) decreased the eIPSC amplitude, and increased the Rf (Fig.6), with this inhibitory effect being completely abolished by bicuculline, confirming the role of $GABA_A$ receptors.

Similar inhibitory effects were also seen for the presynaptic inhibition of excitatory responses at Glutamatergic hippocampal CA3 synapses by muscimol. At a concentration range of between about 0.3~10 μM, muscimol decreased eEPSC amplitude, and increased the Rf (Fig 6A), and this effect was also sensitve to bicuculline (Fig. 6C). At a lower concentration (0.03μM), muscimol had an excitatory effects, increase of eEPSC amplitude and decrease of the Rf (Fig. 6Ab). This presynaptic action also depends on NKCC-1 mediated Cl- uptake (Kakazu et al., 1999; Payne et al., 2003) into the glutamatergic nerve terminals, as bumetanide (10μM), a blocker of NKCC-1, completely blocks the muscimol effects on eEPSCs (Figure 6D). Consequently, activation of presynaptic $GABA_A$ receptors induces a small or large depolarization of the terminals, which induces a sustained increase in eEPSCs or a decrease in eEPSC, respectively. The decrease in evoked glutamate release may result from either or both of the blockade of action potentials as a consequence of inactivation of voltage-dependent Na channels (Sasaki et al., 2008) or a depolarization-induced shunt of the membrane conductance (Yamamoto et al., 2011; Jang et al., 2002; Cattaert et al., 1994; Graham et al., 1994).

We have also examined the effects of muscimol on short-term synaptic plasticity, including paired-pulse facilitation responses (PPF). The response to the second stimulus in a paired stimulus paradigm depends on residual intracellular Ca^{2+} and remaining vesicles available for release after the initial stimulus evoked response. Hence paired pulse responses are a measure of PPF is considered to be a presynaptic phenomenon which is regulated by presynaptic vesicle and intracellular Ca^{2+} homeostasis (Zucker, 2002). Muscimol caused a significant enhancement of the paired-pluse ratio (PPR = response P_2/ response P_1) suggesting either an alteration of presynaptic Ca^{2+} homeostasis or an increase in the number of vesicles available for release induced by the cation (Fig. 6B). The result supports the presynaptic locus of effect, with the probable likely mechanism being reason is that muscimol of high concentration causes some inactivation of voltage-dependent Na^+ channels, hence a less effective action potential, a reduced activation could inhibit Ca^{2+} influx into presynaptic terminals through voltage-dependent Ca channels (VDCC) to a reduced Ca^{2+} influx into presynaptic terminals by causing presynaptic inhibition resulting from the inactivation of voltage-dependent Na channels, then under an intraterminal lower Ca^{2+} condition and a reduced presynaptic release probability (P_r). Therefore, relatively more

vesicles are available for the second response, which would potentiate PPR (Fig. 6B). This is consistent with previous studies showing an inverse relationship between P_r and PPR (Asztely et al., 1996; Murthy et al., 1997).

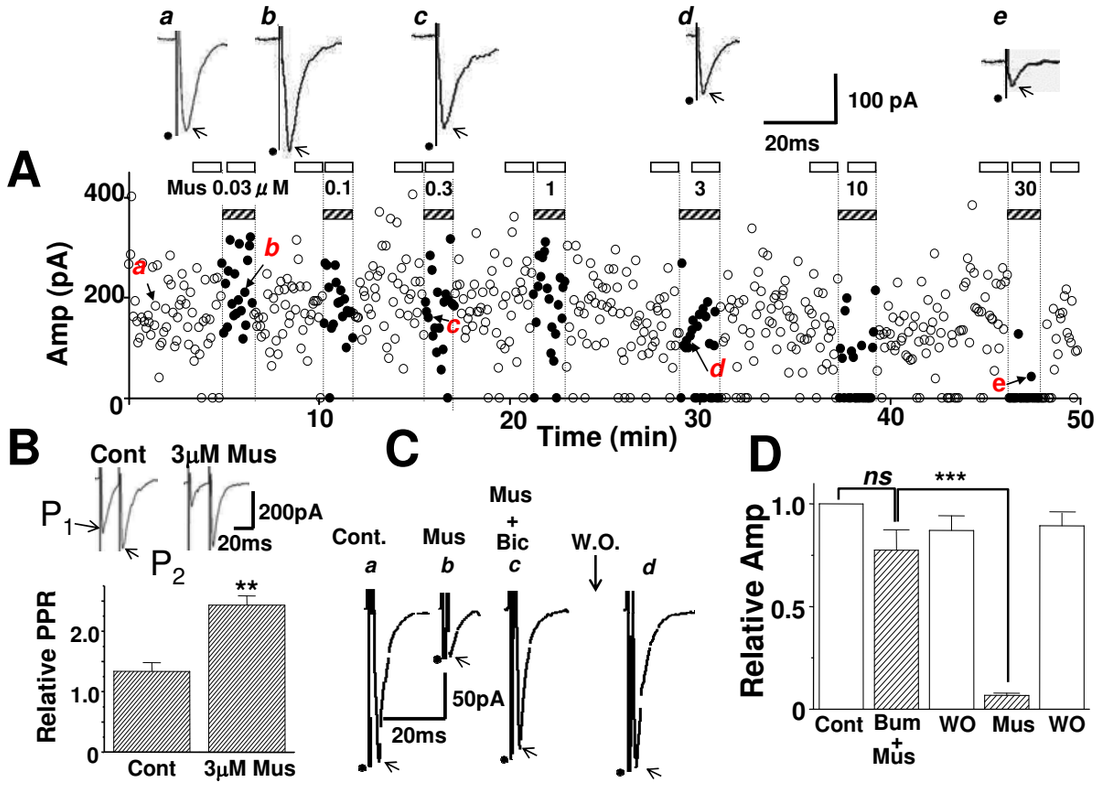

Fig. 6. Glutamatergic evoked EPSCs

Effects of muscimol on glutamatergic eEPSCs recorded from CA3 pyramidal neurons. A: All eEPSCs were evoked from a single glutamatergic bouton stimulated every 5s while the postsynaptic hippocampal CA3 neuron was voltage-clamped at a holding potential (V_H) of –65mV. The amplitude of the eEPSCs gradually decreased with increasing muscimol concentration. The insets a~e show sample single eEPSCs at the time points a~e indicated in the graph.

B: The effects of muscimol on the paired pulse responses. The paired pulse ratio, PPR (P2/P1), increases in the presence of 3μM muscimol. ** P<0.01. C: Muscimol (10μM) –induced inhibition of eEPSC is reversibly abolished by bicuculline (10μM). D: Bumetanide (10μM) prevents the muscimol (3μM) –induced inhibition of eEPSC. *** P<0.001, ns, no significant. Parts A~D were quoted with permission, from (Yamamoto et al., 2011)

8. Investigating the effects of neuromodulators on presynaptic nerve terminals using the 'synaptic bouton' preparation

8.1 5-HT action on GABAergic transmission

Serotonin (5-HT) is an important neurotransmitter in CNS and can modulate neuronal activities via 5-HT receptors (Bruns et al., 2000; Levkovitz & Segal, 1997), which consist of

seven families of membrane proteins comprising a total of fourteen subtypes (Barns & Sharp et al., 1999). In the rat hippocampal CA1 region, both the pyramidal neurons and the GABAergic interneurons are innervated by serotoninergic neurons originating from the midbrain raphe nuclei (Azmitia & Segal, 1978). Activation of 5-HT_{1A} and 5-HT_3 receptors in the hippocampal slice *in vitro* reduces and enhances GABAergic transmission, respectively (McMahon & Kauer , 1997; Schmitz et al., 1995). Figure 7Ab shows such serotoninergic modulation of GABAergic eIPSCs recorded from CA1 pyramidal neurons in a rat hippocampal slice preparation. Interestingly, the eIPSC amplitude is initially reduced, then followed by a gradual increase with increasing 5-HT concentration. This biphasic action is consistent with 5-HT activating at least two different receptor subtypes. In fact, a selective 5-HT_{1A} receptor agonist, 8-OH DPAT, increases eIPSC amplitude while a selective 5-HT_3 receptor agonist, mCPBG, inhibits eIPSC amplitude, both acting in a concentration-dependent fashion (Fig.7Ac). The results indicate two subtypes at least present at GABAergic terminals, but it is difficult in the slice preparation, with numerous synaptic connections on to a single neuron, to determine if both subtypes exist on the same terminal, and if they interact.

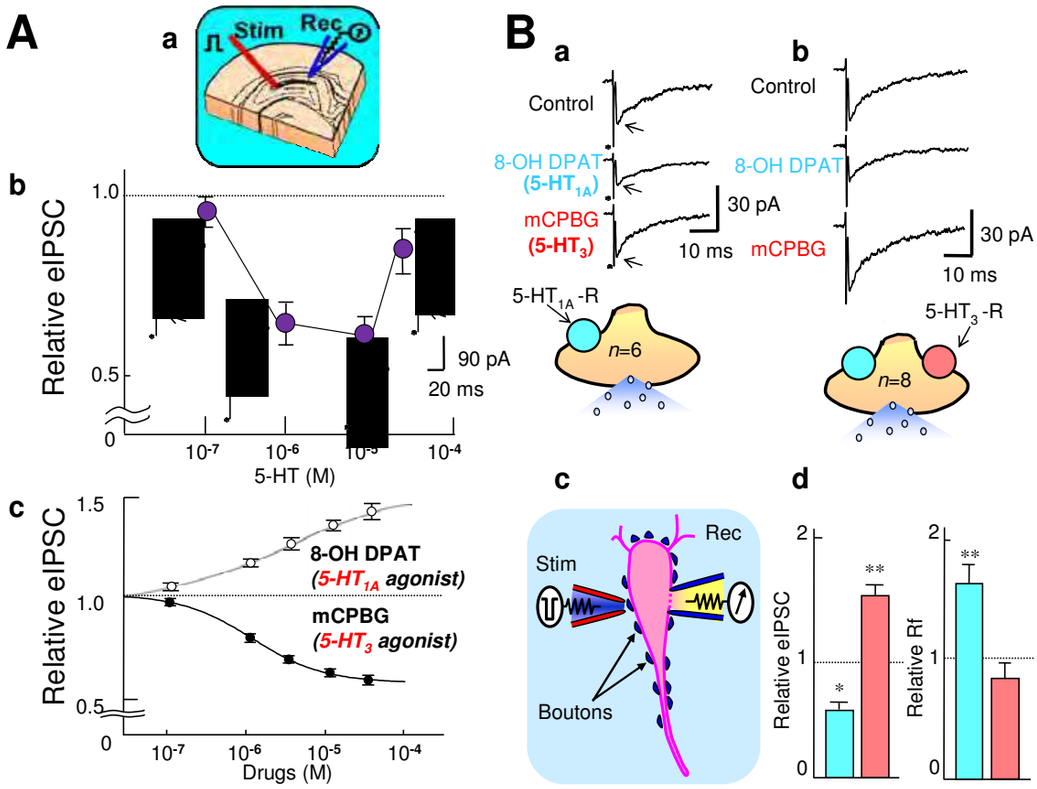

Fig. 7. Modulation of GABAergic evoked IPSCs by 5-HT

A: 5-HTergic modulation of GABAergic eIPSCs recorded in rat hippocampal CA1 pyramidal neurons in the brain slice preparation, illustrated schematically in Aa. (Ab): Concentration-response curves and sample GABAergic eIPSCs, all recorded in the presence of glutamatergic antagonists, 10μM CNQX and 10μM AP-V. 5-HT initially decreases the responses, and then increases the response as the 5-HT concentration is increased. (Ac): The effects of selective agonist of 5-HT_{1A} receptor, 8-OH DPAT, and a selective agonist of 5-HT_3 receptor, mCPBG on the mean amplitude of GABAergic eIPSCs. Each point shows the mean

±SEM of data from eight neurons. B: 5-HTergic modulation of eIPSCs elicited focally in single GABAergic boutons. Single boutons could be classified as either Type 1 boutons (Ba) that have only $5HT_{1A}$ receptors and respond only to 8-OH DPAT (1μM), or type 2 boutons (Bb) have both 5-HT_{1A} and 5-HT_3 receptors and also respond to mCPBG (1μM). (Bc): Schematic illustration of 'synaptic bouton' preparation and focal electrical stimulation. (Bd): Effects of 5-HT_{1A} (blue) and 5-HT_3 agonists (red) on the mean amplitude and Rf of GABAergic eIPSCs. The data in each column are expressed relative to the initial control value, and represent the mean ±SEM of 8~14 neurons. * P<005、** P<0.01 Parts A, Ba, b, d, were adapted, with permission, from (Katsurabayashi et al.,2003).

Using the 'synaptic bouton' preparations of single hipocampal CA1 neurons, a single GABAergic nerve terminal (bouton) was activated by focal electrical stimuli (Fig. 7Bc). At six boutons tested, 8-OH DPAT (1μM) decreased the GABAergic eIPSC amplitude and increased the Rf 1.8 fold, while mCPBG (1μM) to these cells had no effect, indicating that these six boutons have no 5-HT_3 receptors but only functional 5-HT_{1A} receptors (Fig.7 Ba,d,). However, at other eight boutons, both 8-OH DPAT and mCPBG had effects, decreasing and increasing eIPSC amplitude, respectively. Hence, the results indicate that these boutons had both 5-HT_{1A} and 5-HT_3 receptors (Fig. 7Bb,d). Interestingly, mCPBG only decreased Rf modestly at these boutons. Furthermore, there were no boutons which had only 5-HT_3 receptors, or which had neither 5-HT_{1A} nor 5-HT_3 receptors. The physiological consequences of this co-localisation of 5-HT_{1A} and 5-HT_3 receptors on single boutons is that 5-HT may cause an initial transient enhancement of GABA release, progressing into a reduction of GABA release as the 5-HT_3 receptors become desensitized and the slower actions of metabotropic 5-HT_{1A} receptors takes over. Such combination of transient excitatory receptors and persistent inhibitory receptors may have some benefits for more rapid and sustained signaling, respectively (Koyama et al., 1999; Koyama et al., 2000, Koyama et al., 2002; Katsurabayashi et al, 2003).

8.2 Modulation of excitatory and inhibitory presynaptic terminals by A type botulinum toxin

Botulinum toxins (BoNTs) are currently widely used to study the molecular events that are involved in exocytosis (Schiavo et al., 2000; Sudhof, 2004). Studies on brain slices, cultured neurons and synaptosomes have indicated that BoNTs can impede the release of various transmitters such as acetylcholine, glutamate, glycine, noradrenalin, dopamine and ATP (Ashton & Dolly, 1988; Capogna et al., 1997), in addition to the well documented actions on neuro-muscular transmission (Schiavo et al., 2000). Therefore, it is of interest to study the effects of BoNTs on fast neurotransmission at mammalian CNS terminals. Below, we describe the effects of A2 type botulinum toxin (A2NTX) on spontaneous and evoked neurotransmitter release at inhibitory (glycinergic or GABAergic) and excitatory (glutamatergic) synapses in rat spinal neurons using 'synaptic bouton' preparations (Akaike et al., 2010; Sakaguchi et al., 1981).

The rank order of the sensitivity of these different synapses to the inhibitory effects of A2NTX (0.1~10pM) on spontaneous transmitter release was glycinergic > GABAergic≫glutamatergic synapses (Fig. 8A). Using focal electrical stimulation to evoke eIPSCs or eEPSCs of large amplitude and with low Rf, we showed that A2NTX (0.01~1pM) completely abolishes the eIPSC and eEPSC in a time-dependent fashion and with partial reversibility. The rank order of this inhibitory effect was glycinergic eIPSC≧GABAergic eIPSC ≧glutamatergic eEPSC (Fig. 8 Ba,b). The neurotoxin sensitivity for the evoked transmitter release of three transmitters was greater than the spontaneous one. The other

striking feature of this study was that the spontaneous or evoked release of the inhibitory transmitters was 10-100 times more sensitive to A2NTX, as compared to those of the excitatory glutamate release. This observation suggests that the precise molecular events underlying excitatory and inhibitory, and spontaneous and evoked neurotransmitter release, may be different. We have also seen differences between spontaneous and evoked release in their sensitivity of divalent cations, and had previously suggested that spontaneous and evoked glycine release in SDCN neurons involved Ca^{2+} binding to different synaptotagmins (Maeda et al., 2009). Recent studies have now in fact indicated that > 95% spontaneous release is induced by Ca^{2+}-binding to synaptotagmin 1 in murine cortical neurons (Xu et al., 2009), while synaptotagmins 1, 2, and/or 9 are involved in evoked neurotransmitter release (Sollner, 2003; Rizo & Sudhof, 1998). This involvement of different synaptotagmins in spontaneous and evoked neurotransmitter release could also explain the different sensitivities of spontaneous and evoked release of glycine, GABA and glutamate to A2NTX. In addition, transmitter vesicles at CNS terminals are divided into two general pools, a ready-to-release pool, and a reserve pool (Sudhof, 2004; Schikorski & Stevens, 2001). The different vesicle pools may contribute differently to spontaneous and evoked release, and A2NTX may also acts differentially on these processes.

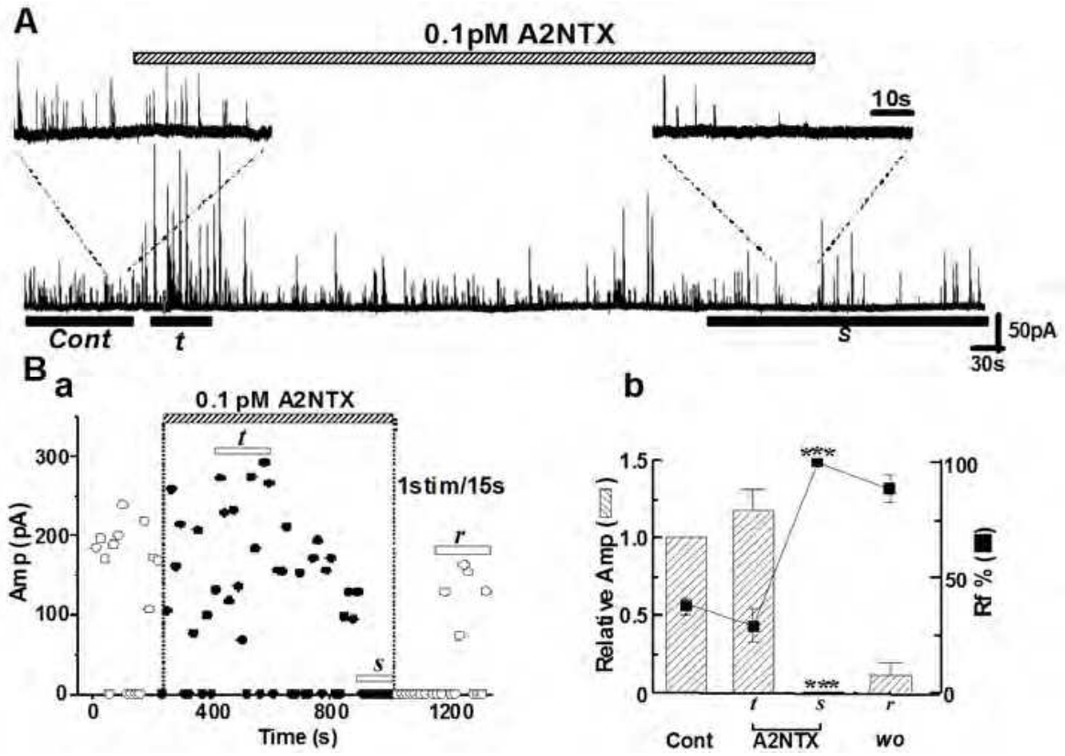

Fig. 8. Modulation of spontaneous and evoked glycinergic IPSCs by A2 type botulinum toxin

Effects of A2NTX on glycinergic spontaneous inhibitory postsynaptic currents (sIPSCs). A: Typical sIPSCs recorded from a mechanically isolated spinal SDCN neuron. The glycinergic sIPSCs are isolated by allowing the GABAergic sIPSCs to run down because the internal patch pipette solution is without ATP. Application of 0.1pM A2NTX transiently enhanced both the frequency and amplitude of glycinergic sIPSCs, before gradually decreasing them. The periods in the current trace indicated by "cont", "t", and "s" represent the control

currents, transient facilitation and steady-state inhibition of currents in the presence of A2NTX. Expanded current recordings are shown in the upper panel. B: Effects of 0.1pM A2NTX on evoked glycinergic eIPSCs. (a): A single glycinergic bouton was activated by focal electrical stimuli every 15s. Stimuli that failed to evoke a response (i.e. current amplitude=0) were referred to as failure and used to calculate the failure rate (Rf). (b): Data were analyzed during the control, transient (t), steady-state (s), and recovery (r) periods, as indicated in panel A. Each column and filled square shows the mean value of 7 experiments. *** P< 0.001 Parts A and B were quoted with permission from (Akaike et al., 2010).

8.3 Action mechanisms of volatile anesthetics

Volatile anesthetics inhibit neuronal activity throughout the CNS, causing complex behavioral effects including sedation, analgesia, hypnosis, unconsciousness, and immobility. Many previous studies using brain slice preparations and primary cultured neurons indicated that the volatile anesthetics enhance GABAergic inhibitory transmission at both synaptic and extrasynaptic sites (Jones et al., 1992; Zimmerman et al., 1994; Nishikawa &

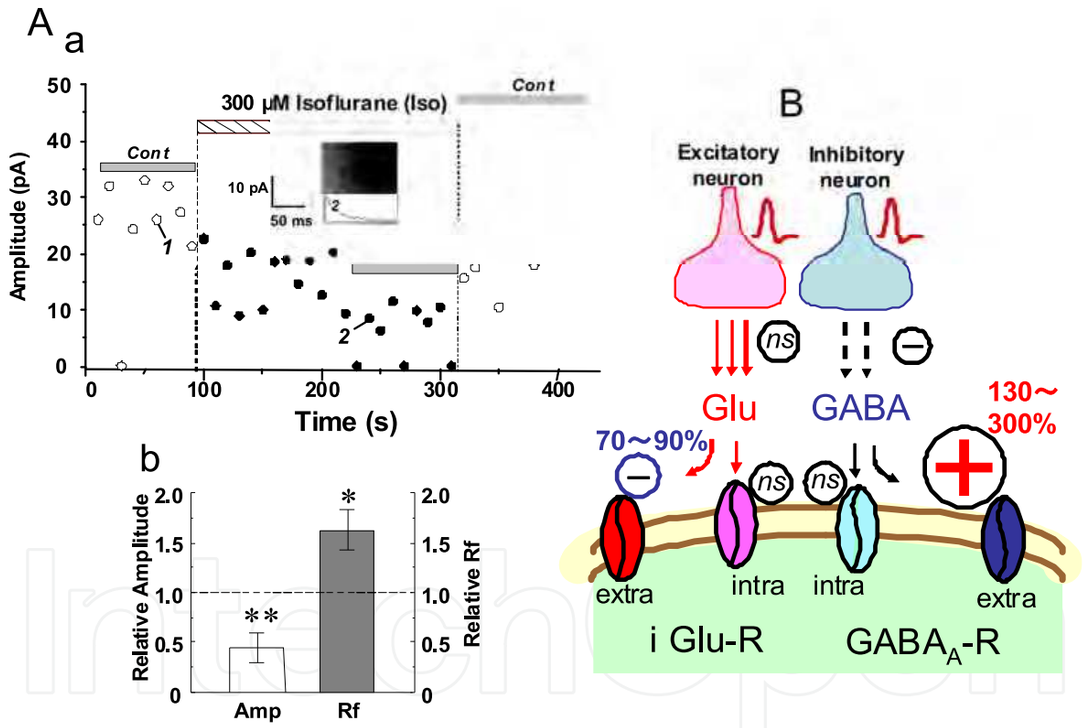

Fig. 9. Modulation of GABAergic evoked IPSCs by volatile anesthetics

A: Effects of isoflurane on GABAergic eIPSCs. (a): Plots of the eIPSC amplitude against time in the absence and presence of 300μM isoflurane. Focal electrical stimulation was applied to a single GABAergic bouton projecting to rat hippocampal CA1 neuron every 10s. (b): Relative current amplitude and Rf of eIPSCs in the presence of isoflurane. Each column shows the mean value of 4~8 neurons. Error bar represents ±SEM. * P < 0.05, ** P < 0.01. B: Schematic illustration of how volatile anesthetics modulate excitatory and inhibitory presynaptic terminals, synaptic receptors, and extrasynaptic receptors. Parts A was obtained with permission, from (Ogawa et al., 2011).

Maclver, 2005; Nishikawa et al., 2005; Bai et al., 2001; Bonin & Orser, 2008; Bieda et al., 2009). In an attempt to more clearly delineate the sites of actions of volatile anaestheics at different pre- and postsynaptic levels at single synapses, devoid of complications from surrounding cells, we used the 'synaptic bouton' preparation of rat hippocampal CA1 pyramidal neurons. As shown in Figure 9Aa, b, isoflurane (300μM) inhibited the amplitude of eIPSCs induced by focal stimuli of a single GABAergic bouton, and increased the Rf. This result, obtained at the single GABAergic synapse level, clearly indicates that volatile anesthetics such as enflurane, isoflurane and sovoflurane also act presynaptically to inhibit GABA release, and this dominates any potentiation of the postsynaptic $GABA_A$ receptors, which is often been thought to be the main site of action of these drugs. As shown in Figure9B, the volatile anaesthetics also had no effect at glutamatergic synapses. As has been reported frequently by others, the extrasynaptic $GABA_A$ receptor-mediated response (by exogenous GABA application) was greatly enhanced by the volatile anaesthetics, while the extrasynaptic glutamatergic receptor-mediated response was significantly inhibited. Thus, the behavioral effects of volatile anesthetics may result from both the enhancement of extrasynaptic $GABA_A$ responses and the suppression of extrasynaptic glutamate responses (Ogawa et al., 2011), although the synaptic responses are quite differently affected.

9. Concluding remarks

The 'synaptic bouton' preparation is a simple and convenient methodology to investigate the pharmacology, physiology and transduction mechanisms of neurotransmission at mammalian nerve terminals (boutons), the vast majority of which are small (diameter less than a few μm) and difficult to access for functional studies by other means. Spontaneous IPSCs and EPSCs mediated by the classical fast neurotransmitters can be recorded in acute preparations from many brain regions, with accurate space-clamp of the postsynaptic membrane voltage and with good control of both the cytoplasmic constitutions and the test solutions bathing single neurons. The preparation is devoid of complications arising from surrounding other neurons and glia cells, and from possible changes in protein distribution and function resulting from enzyme treatment and *in vitro* culture. A single bouton in this preparation can also be selectively activated by focal electrical stimulation and visualized by fluorescent signals. The 'synaptic bouton' preparation could be helpful to reveal further the repertoire of receptors, ion channels, transporters, and second messengers that mediate and regulate synaptic transmission in mammalian presynaptic terminals.

10. Acknowledgements

The author wish to thank Dr. A. Moorhouse for his helpful discussion and T. Yamaga for his assistance with the figures.

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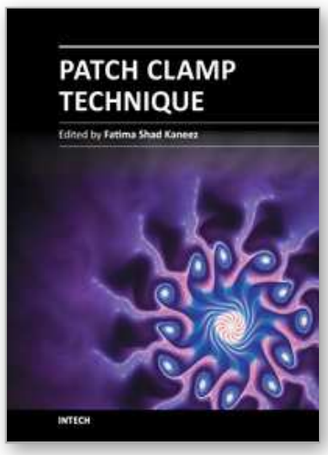

Patch Clamp Technique
Edited by Prof. Fatima Shad Kaneez

ISBN 978-953-51-0406-3
Hard cover, 356 pages
Publisher InTech
Published online 23, March, 2012
Published in print edition March, 2012

This book is a stimulating and interesting addition to the collected works on Patch clamp technique. Patch Clamping is an electrophysiological technique, which measures the electric current generated by a living cell, due to the movement of ions through the protein channels present in the cell membrane. The technique was developed by two German scientists, Erwin Neher and Bert Sakmann, who received the Nobel Prize in 1991 in Physiology for this innovative work. Patch clamp technique is used for measuring drug effect against a series of diseases and to find out the mechanism of diseases in animals and plants. It is also most useful in finding out the structure function activities of compounds and drugs, and most leading pharmaceutical companies used this technique for their drugs before bringing them for clinical trial. This book deals with the understanding of endogenous mechanisms of cells and their receptors as well as advantages of using this technique. It covers the basic principles and preparation types and also deals with the latest developments in the traditional patch clamp technique. Some chapters in this book take the technique to a next level of modulation and novel approach. This book will be of good value for students of physiology, neuroscience, cell biology and biophysics.

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