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Microtransplantation of Cellular Membranes From Squid Stellate Ganglion Reveals Ionotropic GABA Receptors

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Abstract. The squid has been the most studied cephalopod, and it has served as a very useful model for investigating the events associated with nerve impulse generation and synaptic transmission. While the physiology of squid giant axons has been extensively studied, very little is known about the distribution and function of the neurotransmitters and receptors that mediate inhibitory transmission at the synapses. In this study we investigated whether γ -aminobutyric acid (GABA) activates neurotransmitter receptors in stellate ganglia membranes. To overcome the low abundance of GABA-like mRNAs in invertebrates and the low expression of GABA in cephalopods, we used a two-electrode voltage clamp technique to determine if *Xenopus laevis* oocytes injected with cell membranes from squid stellate ganglia responded to GABA. Using this method, membrane patches containing proteins and ion channels from the squid’s stellate ganglion were incorporated into the surface of oocytes. We demonstrated that GABA activates membrane receptors in cellular membranes isolated from squid stellate ganglia. Using the same approach, we were able to record native glutamate-evoked currents. The squid’s GABA receptors showed an EC_{50} of $98 \mu\text{mol l}^{-1}$ to GABA and were inhibited by zinc ($IC_{50} = 356 \mu\text{mol l}^{-1}$). Interestingly, GABA receptors from the squid were only partially blocked by bicuculline. These results indicate that

the microtransplantation of native cell membranes is useful to identify and characterize scarce membrane proteins. Moreover, our data also support the role of GABA as an ionotropic neurotransmitter in cephalopods, acting through chloride-permeable membrane receptors.

Introduction

Since its description by John Zachary Young in 1938, the squid giant synapse has been extensively studied. However, little information about the neurotransmitter receptors involved in the stellate ganglion synapses or in the brain of the squid was available. Evidence indicates that glutamate is an excitatory neurotransmitter (Miledi, 1967; DeSantis *et al.*, 1978; Eusebi *et al.*, 1985; Corrie *et al.*, 1993) that activates alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type receptors in post-synaptic terminals of the squid synapses (Messenger, 1996; Di Cosmo *et al.*, 2006). The cloning of the glutamate receptor subunit SqGluR from the stellate ganglion of *Loligo opalescens* provides further support for the excitatory role of glutamate in squid (Battaglia *et al.*, 2003). Conversely, the inhibitory neurotransmission in squid and other cephalopods is largely unexplored.

Gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter in mammals, has been found at low levels in the octopus brain (Osborne, 1971), and immunohistochemical evidence indicates that within the whole brain the presence of GABA-containing neurons is limited to certain areas (Cornwell *et al.*, 1993). Moreover, mRNA for GABA-

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like receptors in invertebrates is also limited (Harvey *et al.*, 1991; Darlison, 1992). Nevertheless, inhibitory mechanisms in the squid seem to be important for learning processes. For example, the experience-dependent behavior of prey capture and the escape response observed throughout development of the squid is highly dependent on the inhibitory control of the giant synapse output (Preuss and Gilly, 2000). Additionally, GABA reduces the resting electrical activity of the squid statocyst (Tu and Budelmann, 2000).

Although spontaneous and evoked inhibitory synaptic potentials due to chloride fluxes have been recorded in the stellate ganglion of squids (Miledi, 1972) and in slices of the cuttlefish optic lobe (Chrachri and Williamson, 2003), so far neither the neurotransmitter mediating those potentials nor their membrane receptors have been characterized. To examine the presence of membrane receptors for GABA, we injected membrane vesicles containing neurotransmitter receptors and accessory proteins from squid's stellate ganglia into *Xenopus* oocytes. As previously shown (Miledi *et al.*, 2006; Eusebi *et al.*, 2009), membrane patches are incorporated into the oocyte's surface a few hours after injection. The main advantage of this technique is the possibility of investigating neurotransmitter receptors by using already-assembled receptors, without interfering with the transcriptional and transductional host machinery. In this work we demonstrate for the first time the presence of functional ionotropic GABA receptors (GABARs) in cellular membranes microtransplanted from the squid stellate ganglion to *Xenopus* oocytes.

Materials and Methods

Microtransplantation of squid membranes

To dissect the stellate ganglia, the squid (*Doryteuthis pealei*) mantle was opened and each stellate ganglion with its nerves was exposed. The stellate ganglion, which contains neuronal somatas, stellate nerves, presynaptic second-order giant fibers, and postsynaptic third-order giant fibers, was cut out under a stereomicroscope, lifted from the mantle, and then transferred to a Falcon tube containing liquid nitrogen. The frozen samples were stored at -70°C until the membranes were prepared. This preparation contains, in addition to the neuronal somatas and nerve fibers mentioned above, the connective tissue capsule surrounding the ganglion.

Membranes for oocyte injection were prepared as previously described (Miledi *et al.*, 2006). Tissue from squids was homogenized in ice-cold buffer (pH 9.0) with 200 mmol l^{-1} glycine, 150 mmol l^{-1} NaCl, 50 mmol l^{-1} EDTA, 50 mmol l^{-1} EGTA, and 300 mmol l^{-1} sucrose and protease inhibitors (Sigma P2714; Sigma, St. Louis, MO). Samples were homogenized with an electric rotor and centrifuged at $9500 \times g$ for 15 min (4°C). The supernatant was ultracentrifuged in a Beckman SW41 rotor at $100,000 \times g$

for 2 h at 4°C . The resultant pellet was resuspended in sterile distilled water and stored at -70°C . Stage V–VI *Xenopus* oocytes were injected with 50 nl of a membrane preparation. Injected oocytes were kept in Barth's solution [88 mmol l^{-1} NaCl, 0.33 mmol l^{-1} $\text{Ca}(\text{NO}_3)_2$, 0.41 mmol l^{-1} CaCl_2 , 1 mmol l^{-1} KCl, 0.82 mmol l^{-1} MgSO_4 , 2.4 mmol l^{-1} NaHCO_3 , 10 mmol l^{-1} HEPES (pH 7.4)] at $16\text{--}17^{\circ}\text{C}$ until the moment of recording.

Electrophysiology

After 12 to 48 h postinjection, membrane currents were recorded from voltage-clamped oocytes using two microelectrodes filled with 3 mol l^{-1} KCl and continuously perfused with oocyte Ringer's solution (Miledi, 1982). Solution exchange was achieved by using electromagnetic valves and a computer-controlled perfusion system (Warner Instruments).

Current-voltage (*I-V*) relationships were constructed holding the oocytes at -70 mV and stepping the membrane potential for 2–4 min to the desired value before applying 1 mmol l^{-1} GABA. To determine the equilibrium potential for GABA (E_{GABA}) a second-order polynomial curve was fitted to *I-V* relationships (pClamp 10). All results are given as mean \pm S.E.M. Two data sets were considered statistically different when $P < 0.05$ (ANOVA).

Chemicals and solutions

Oocyte Ringer's solution (OR) had the following composition (in mmol l^{-1}): NaCl 82.5; KCl 2.5; CaCl_2 2.5; MgCl_2 1; HEPES 5, adjusted to pH 7.4 with NaOH. Kainic acid, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl), propanoic acid (AMPA), and cyclothiazide (CTZ) were purchased from Tocris Biosciences (UK). GABA and bicuculline were purchased from Sigma (St. Louis, MO). ZnCl_2 was purchased from Fisher Scientific (Fair Lawn, NJ). GABA, AMPA, and bicuculline were dissolved in bidistilled water. CTZ was dissolved to 100 mmol l^{-1} in DMSO. The ZnCl_2 stock solution was prepared by serial dilutions with bidistilled water of a solution containing 100 mmol l^{-1} ZnCl_2 and 10 mmol l^{-1} HCl (Paoletti *et al.*, 1997) and the pH adjusted to 7.4 in the final solution. The concentrated stock solutions were dissolved in OR prior to use.

Results

GABA and glutamate receptors in the stellate ganglion of squid

We applied 1 mmol l^{-1} GABA to oocytes not injected with membranes from squid stellate ganglia, and the lack of responses to GABA in these oocytes confirmed that native *Xenopus* oocytes do not express endogenous GABARs (Fig. 1A). In contrast, 80% of all the oocytes injected with the membranes elicited GABA-activated currents (I_{GABA}) of

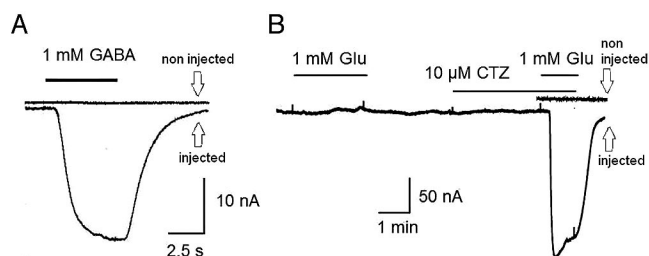


Figure 1. GABA and glutamate receptors from squid stellate ganglia. Current traces of responses to 1 mmol l⁻¹ GABA and to 1 mmol l⁻¹ glutamate plus 10 μmol l⁻¹ cyclothiazide (CTZ) of an oocyte injected with cellular membranes isolated from squid stellate ganglia. Superimposed: GABA or glutamate responses in non-injected oocytes. Bars indicate the perfusion time of the agonists.

-48.3 ± 18.0 nA (range -2.7 to -106.8 nA), clearly indicating that squids express ionotropic GABARs that were successfully transplanted to *Xenopus* oocytes (Fig. 1A). The activation of I_{GABA} was adjusted with a time constant of 1.9 ± 0.4 s (*n* = 19). Interestingly, I_{GABA} showed little desensitization at maximal concentrations of GABA. The oocytes that exhibited I_{GABA} also responded to 100 μmol l⁻¹ kainate, an agonist of ionotropic glutamate receptors (GluRs). However, 1 mmol l⁻¹ glutamate did not elicit responses in the same oocytes. To determine if the lack of responses to glutamate was a consequence of a fast and strong desensitization of GluRs, we co-applied 1 mmol l⁻¹ glutamate with 10 μmol l⁻¹ CTZ, the latter a compound that stabilizes the non-desensitized state of AMPA-type receptors. In these conditions glutamate elicited non-desensitizing responses of -10.4 ± 5.1 nA (range -3.0 to -20.8 nA, *n* = 8; Fig. 1B). Similar non-desensitizing currents were observed with co-application of 50 μmol l⁻¹ AMPA and 20 μmol l⁻¹ CTZ (-22.9 ± 7.3 nA, *n* = 7). The potentiation of glutamate currents by CTZ indicates that the kinetic properties of glutamate receptors from the stellate ganglion are quite different from those of ionotropic receptors activated by GABA, which showed a limited level of desensitization. No responses to 1 mmol l⁻¹ glycine (*n* = 6) or 1 mmol l⁻¹ carbachol (*n* = 30), the latter an agonist of ionotropic and metabotropic cholinergic receptors, were detected in oocytes injected with membranes from the squid stellate ganglia.

Physiological properties of GABARs from the squid stellate ganglion

We further characterized GABARs from squid stellate ganglia. The perfusion of 1 mmol l⁻¹ GABA to microtransplanted oocytes held at different voltages was used to determine the current-voltage (*I-V*) relationship of I_{GABA}. The current inverted its polarity at -18.3 ± 0.9 mV (*n* = 6, Fig. 2), a value near the predicted equilibrium potential of chloride of -26 mV in *Xenopus* oocytes, with an external chlo-

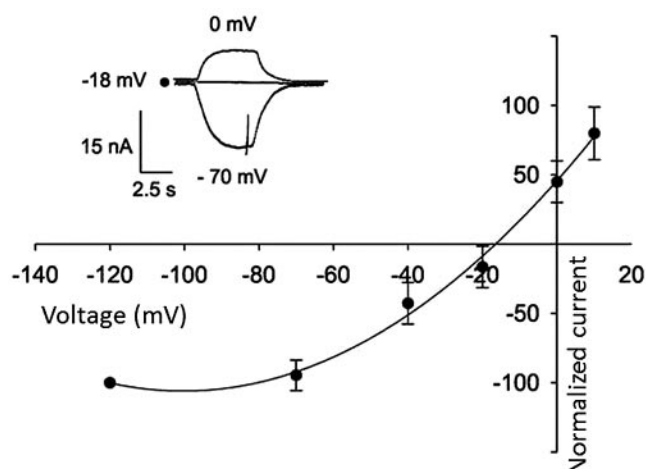


Figure 2. Reversal potential of GABA-evoked current. Current-voltage (*I-V*) relationship from oocytes injected with membranes of stellate ganglia of squids. Points represent means (± SEM) of peak GABA currents that inverted at -18.3 ± 0.9 mV (6 oocytes, 2 frogs, 30 squids). The GABA concentration applied was 1 mmol l⁻¹. Currents were normalized to those induced at -120 mV (average of GABA currents = -21.7 ± 9.8 nA, 6 oocytes, 2 frogs, 30 squids). Note the strong GABA current rectification at very negative potentials.

ride concentration of 92 mmol l⁻¹ (Ringer's in this study) and an internal concentration of 33.4 mmol l⁻¹ (Barish, 1983). This result indicates that chloride is the main carrier of I_{GABA}. The apparent affinity of GABARs for GABA was calculated from concentration response curves. Application of each concentration of GABA was done every 5 min to allow for full recovery of the response. I_{GABA} had EC₅₀ and *n*_H values of 98.0 ± 0.7 μmol l⁻¹ and 1.5 ± 0.1 (8 oocytes, 2 frogs, 30 squids; Fig. 3). GABARs were reversibly inhibited by Zn²⁺ in a concentration-dependent manner. The IC₅₀ of Zn²⁺ on I_{GABA}, elicited by 250 μmol l⁻¹ (EC₈₀ value for GABA), was 356 ± 6 μmol l⁻¹ (10 oocytes, 2 frogs, 30 squids; Fig. 4). We also examined the effects of 100 μmol l⁻¹ bicuculline, a competitive antagonist of GABA_A receptors, in batches of oocytes from two different frogs. Interestingly, bicuculline reduced the maximum amplitude of I_{GABA} by 23%, from -64.4 ± 14.7 nA to 51.5 ± 11.7 nA. The reduction in the amplitude of I_{GABA} by bicuculline did not affect the temporal course of the activation or the level of desensitization of I_{GABA} (6 oocytes, 2 frogs; data not shown).

Discussion

Since no information about the GABA receptors in squid is available and mRNAs of GABA-like subunits are at very low abundance in invertebrates (Harvey *et al.*, 1991; Darlison, 1992), we used the method of microtransplantation of cell membranes to determine the presence of GABARs in the squid stellate ganglia. This method has been used suc-

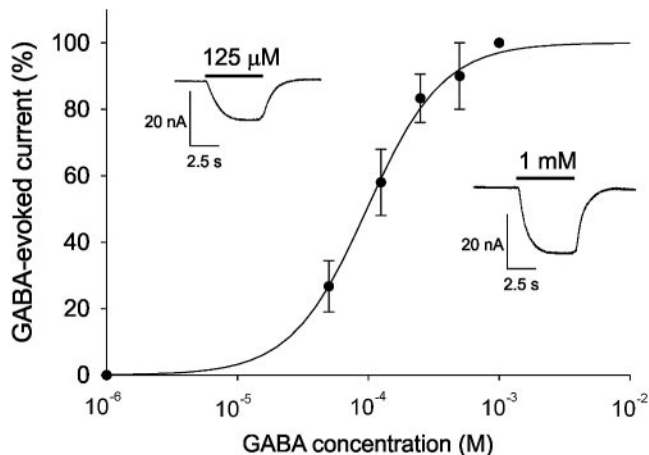


Figure 3. Concentration-dependent responses of squid GABA receptors. Log dose–response curve showing peak GABA currents versus GABA concentrations, fitted using the Hill equation. The parameters fitted by least squares analysis were $EC_{50} = 98.0 \pm 0.7 \mu\text{mol l}^{-1}$ and $n_H = 1.5 \pm 0.1$. The average of peak GABA (1 mmol l^{-1})-currents was $-65.3 \pm 9.6 \text{ nA}$ (8 oocytes, 2 frogs, 30 squids). Points represent means (\pm SEM).

cessfully in different species, from Torpedo fish to human, but it had not been tried on the nervous system of an invertebrate organism (Marsal *et al.*, 1995; Miledi *et al.*, 2002; Palma *et al.*, 2005; Limon *et al.*, 2008; Eusebi *et al.*, 2009). In this study we show that squid membranes were able to fuse into the oocyte's membrane, and as evidenced by ion currents elicited by glutamate and GABA, squids express native ionotropic GluRs and GABARs.

Squid GluRs displayed a strong desensitization to glutamate that was removed by cyclothiazide (CTZ), which is a

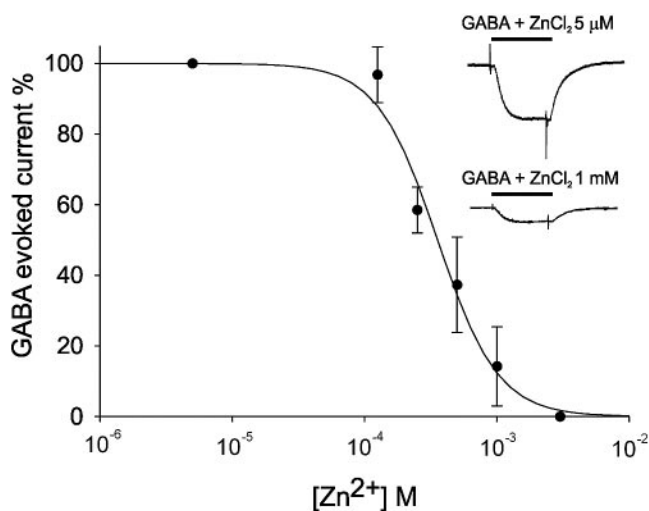


Figure 4. Squid GABA receptors are sensitive to zinc. Currents evoked by $250 \mu\text{mol l}^{-1}$ GABA co-applied with different Zn^{2+} concentrations in oocytes injected with squid stellate ganglion (6 oocytes, 2 frogs, 50 squids). Data were fitted by least squares analysis, giving $IC_{50} = 356 \pm 6 \mu\text{mol l}^{-1}$. Points represent means (\pm SEM).

compound that stabilizes the non-desensitized state of AMPA receptors in vertebrates (Partin *et al.*, 1994). The potentiation of glutamate currents by CTZ suggests that SqGluR, with a 44%–46% amino acid identity to the mammalian AMPA-subunits GluR1–GluR4, is the subunit making up the native glutamate receptors (Battaglia *et al.*, 2003).

While glutamate in squid has an excitatory role, it is not known which neurotransmitter is responsible for the inhibitory modulation in the squid or in cephalopods in general. Given that cephalopods are considered to be the more advanced invertebrates in problem-solving tests, it is important to determine which components maintain the excitatory-inhibitory balance in the cephalopod nervous system. Hyperpolarizing potentials have been described in the squid stellate ganglion (Miledi, 1972), in secondary hair cells and afferent neurons of the squid statocyst (Williamson, 1989), and in central neurons of the cuttlefish (Chrachri and Williamson, 2003). In our experiments, native receptors elicited GABA currents that were carried by chloride. In squid, the extracellular chloride concentration $[\text{Cl}]_o$ is near 560 mmol l^{-1} (Hodgkin, 1951), and the internal concentration $[\text{Cl}]_i$ varies between 41 mmol l^{-1} (Steinbach, 1941) and 150 mmol l^{-1} (Keynes, 1963); therefore the equilibrium potential for chloride, and consequently the polarity of the potentials elicited by chloride fluxes, may vary over a wide range. However, it is important to note that the inversion potential for chloride is near the resting membrane potential of squid axons, suggesting that chloride is the main carrier of inhibitory currents (Miledi, 1972). Moreover, in those experiments, hyperpolarizing potentials frequently reversed sign minutes after cells were impaled with KCl-filled electrodes; when cells were impaled with electrodes filled with K citrate, the reversal of sign was slower (Miledi, 1972). Although it cannot be discounted that GABA may be excitatory under certain circumstances, it is highly probable that chloride-permeable GABA receptors are the molecular basis of hyperpolarizing potentials in squid synapses.

Native GABA receptors from the squid were less sensitive (EC_{50} of $98 \mu\text{mol l}^{-1}$) than homomeric GABA receptors composed of the *Rdl* subunit from the fruit fly (EC_{50} between 10 and $50 \mu\text{mol l}^{-1}$; Ffrench-Constant *et al.*, 1993); but similar to the sensitivity of receptors composed by one of the *Rdl* splice variants (EC_{50} of $152 \mu\text{mol l}^{-1}$; Belelli *et al.*, 1996). Interestingly, native receptors from the squid were more sensitive than the heterologously expressed GABARs of the pond snail *Lymnaea stagnalis* (EC_{50} 200–300 $\mu\text{mol l}^{-1}$; Harvey *et al.*, 1991), a mollusc that is phylogenetically closer to cephalopods than the fruit fly and that expresses a transmembrane protein with about 50% identity to vertebrate GABA_A receptor β subunits (Harvey *et al.*, 1991). Among the cephalopods there is evidence that the cuttlefish expresses an mRNA (Q9GYU4_Uniprot; Kirby *et al.*, 1997) that would produce

a protein with an identity of 66% with the GABA_AR β -like subunit in the pond snail, of 61% with the GABA_AR β -like subunit *Lcch3* in the fruit fly, and of 48% with *GABRB1* in human. However, it is not known if this protein from the cuttlefish will produce functional homomeric GABA receptors, or if it will require a heteromeric assembly with another as yet unidentified subunit to make functional channels. In this regard, the microtransplantation method provides a strong experimental platform for study of the properties of native GABA receptors still embedded in their own membranes. An initial exploration of the pharmacological profile of native squid GABA receptors showed that squid GABA receptors were sensitive to Zn²⁺, similarly to GABA receptors from some invertebrates (Smart and Constanti, 1982) and to all GABA receptors in vertebrates (Hosie *et al.*, 2003). Interestingly, the IC₅₀ for Zn²⁺ in squid GABARs (356 $\mu\text{mol l}^{-1}$) is closer to that of GABA_ARs containing a γ subunit that show a lower affinity for Zn²⁺ than the receptors lacking the γ subunit but with high affinity for Zn²⁺ (Hosie *et al.*, 2003; Palma *et al.*, 2007). Additionally, squid GABA receptors were only partially blocked by bicuculline, similar to GABA receptors in invertebrates (Lunt, 1991) and the vertebrate GABARs composed by ρ subunits (Polenzani *et al.*, 1991) in vertebrates. Future experiments using molecular biology approaches on squid stellate ganglion will be useful to identify the GABAR subunit involved in the inhibitory transmission in invertebrates. The pharmacological and biophysical properties of native GABARs and their comparison with other invertebrate and vertebrate GABAARs merit further studies.

Acknowledgments

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