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DISTRIBUCION CEREBRAL Y CLONACIÓN MOLECULAR DEL RECEPTOR GABA RHO1 BOVINO

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Abstract

$GABA_C$ receptors were originally found in the mammalian retina and recent evidence shows that they are also expressed in several areas of the brain, including caudate nucleus, brain stem, pons and corpus callosum. In this study plasma membranes from the caudate nucleus were microinjected into *X. laevis* oocytes. This led the oocyte plasma membrane to incorporate functional bicuculline-resistant, Cl^- conducting bovine GABA receptors, similar to those of the retina. Immunolocalization of the GABA ρ1 subunit revealed its expression in bovine neurons in the head of the caudate as well as in the olive, cuneiform and reticular nuclei of the brain stem. Interestingly, the same antibodies failed to show expression in the callosum and pons, where the GABA ρ1 mRNA was previously detected.

The bovine GABA ρ1 receptor was cloned and its cDNA sequence predicts a protein with 478 amino acids and 90-95% similarity to other GABA ρ1 subunits. Oocytes injected with the cDNA express a non-desensitizing, homomeric receptor with a GABA EC_{50}=6.0 μM and a Hill coefficient of 1.8.

The results confirm the presence of $GABA_C$ receptor mRNAs in several areas of the mammalian brain and show that some of these areas express functional GABA ρ1 receptors that have the classic $GABA_C$ receptor characteristics. All this suggests future studies to determine the role played by these receptors in brain functions.

1. Introduction

The $GABA_C$ receptors were first described as a bicuculline-resistant, baclofen-insensitive and Cl^--conducting component of the currents generated by GABA acting on GABA receptors expressed by mRNA from the bovine retina (Polenzani et al, 1991). These receptors are highly expressed in the bipolar neurons, where they determine the time course and extent of inhibition which, in turn, modulates the excitatory transmission from bipolar to ganglion cells (Lukasiewicz et al, 2004). Recent evidence indicates that the receptor is expressed also in the brain, where some neurons of the cerebellum, hippocampus and vision-related areas express the mRNA of at least one of the three subunits (ρ1-ρ3) that compose the $GABA_C$ receptor family (Alakuijala et al 2006, Boller & Schmitd 2003, Liu et al 2004, López-Chavez et al 2005, Rozzo et al 2002). Although $GABA_A$ receptors play a central role in these areas, the expression of $GABA_C$ subunits suggests they are probably also involved in GABAergic neurotransmission; acting alone or in combination with $GABA_A$ subunits. Strong evidence for the heteromeric assembly of $GABA_A$ and $GABA_C$ subunits points to their potential to form receptors, with mixed pharmacological and electrophysiological properties, such as those found in the brainstem and lateral geniculate nucleus of the rat (Milligan et al 2004, Schlicker et al 2004). It is thus very important to precise the cellular distribution of the $GABA_C$ subunits in the brain in order to understand their functional role in the GABAergic system.

We recently found mRNA transcripts of the ρ1 and ρ2 subunits in the bovine brain stem, caudate, pons, cerebellum, pituitary and corpus callosum (López-Chávez et al 2005). Considering that, in several animal species,

electrophysiological recordings from these areas are consistent with a $GABA_A$-like class of receptor, we questioned if the ρ1 and ρ2 mRNAs found, and/or the receptors they encoded, were functional. We have already reported the molecular cloning of the ρ2 subunit from bovine cerebellum and showed that the encoded receptors, expressed in *Xenopus* oocytes, generate GABA-currents with properties typical of $GABA_C$ receptors (Lopez-Chavez et al 2005). In this work we found that oocytes injected with plasma membranes isolated from the bovine retina and caudate nucleus generate $GABA_C$-currents; and show also the cellular immunolocalization of the GABA ρ1 subunit in the caudate nucleus and brain stem. We also cloned the ρ1 receptor and provide evidence that its cDNA expresses fully functional receptors.

2. Material and Methods

Adult cow tissues were obtained from local slaughterhouses, either in Santa Ana, California or Querétaro, México and transported on ice. The retinas, brain stem and caudate nucleus were selected for RNA extraction, membrane preparation and immunostaining.

2.1 RNA extraction and PCR cloning. Total RNA from the retina was obtained by the standard phenol-guanidinium method of Chomczynski P. and Sacchi N. (1987). A partial sequence of the ρ1 subunit, previously obtained (López-Chávez et al 2005), was used as starting point to isolate the whole open reading frame by using consensus primers corresponding to the amino-and carboxi-terminus of other mammalian ρ1 subunits. The amplified cDNA was cloned into pGEM-T-Easy (Promega), sequenced and then shuttled as a BamH1-Xho1 fragment into pcDNA3 (Invitrogen). For expression, 50 nl of *in vitro* transcribed cRNA (1μg/μl) was injected directly into the nucleus of *X. laevis* oocytes.

2.2 Preparation of plasma membranes. The tissue (0.5-1.0 g) was homogenized in a tefflon tissue grinder in cold dissociation-buffer plus protease inhibitor (Miledi et al 2002, 2004). The homogenate was centrifuged and the resulting supernatant ultracentrifugated at 100,000*g* for two hours. The pellet was resuspended in 5 mM glycine and ultrasonicated for 1 min before microinjection into frog oocytes.

2.3 Electrophysiological recordings. Handling of oocytes and injection of plasmids, RNA or plasma membranes have been previoulsy reported (Miledi et al 2002, 2004). Membrane currents generated by oocytes exposed to GABA, or other neurotransmitters, were recorded using the standard two-microelectrode voltage-clamp technique (Miledi 1982). To obtain the equilibrium membrane potential for GABA receptor action, current-voltage relations were constructed by stepping the oocyte's membrane potential from -60mV to -120mV and to +40mV (in 0.2s steps) in the absence and in the presence of 1 μM GABA.

2.4 Immunohistochemical localization of ρ1. This technique is based in different authors (Matute et al, 1993; Enz et al, 1996; Deuchars et al, 2001) Tissue blocks of about 1 cm were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer for 4 hours, cryoprotected by serial immersions in sucrose at 10, 20 and 30% for 3 days, and then frozen at -80ºC in tissue freezing-^{TM} (superfrost plus ®). Sections (30 μm) were obtained with a Leica cryostat (CM 1850), washed 3 times in 1% Triton in PBS for 10 min and blocked with 1% bovine serum albumin and 1% hydrogen peroxide at 37ºC for 30 minutes. The sections were incubated overnight in the primary antibody solution (1:200 dilution, goat anti-GABA ρ1 IgG from Santa Cruz Technologies); washed three times for 30 min in 1% Triton in phosphate buffer, and then incubated with a secondary antibody (rabbit anti-goat labeled with either FITC or HRP) at a 1:1000 dilution. The reaction of HRP was developed with diaminobenzidine in the presence of hydrogen peroxide. Images were obtained in a microscope Olympus BX60 with a DP70 camera and a FITC filter.

2.5 Rapid Golgi staining. Modifications to the method (Cintra et al, 1997) were adapted to process the head of caudate nucleus, that was cut in 1cm^2 blocks and fixed in 4.5% potassium dichromate, 1% osmium tetroxide and incubated in this solution for fourteen days on a linear shaker in the dark at room temperature. The blocks were washed for a few seconds in distillated water and placed in 0.75% silver nitrate for 24 h.

The tissue was dehydratated in alcohol gradient (50, 70, 80, 96, 100%, and a mix 1:1 ethanol–ether), embedded in nitrocellulose and sectioned (120µm) on a microtome. Tissue sections were dehydrated in alcohol and embedded in terpineol and xilene, placed on microscope slides and fixed with Entellan® (Merck) for further observation in the light microscope.

3. Results

3.1 Injection of plasma membranes in frog oocytes. Incorparation of the plasma membranes isolated from retina and caudate nucleus lead to the expression of several neurotransmitter receptors and ion channels, including glutamate, acetylcholine (not shown) and ionotropic GABA-receptors. The figure 1 contrasts the GABA-currents generated by oocytes injected with either retina or caudate nucleus. The fast-inward desensitizing-component is typical of a $GABA_A$ receptor; however the $GABA_C$ receptor is not quite obvious and was evidenced only after a high concentration of bicuculline (0.1 mM) inhibited the $GABA_A$ receptor. Clearly, activation with smaller GABA-concentration (5 μM) in the presence of bicuculline (0.1 mM) revealed a non-desensitizing slow-inactivating component, corresponding to the $GABA_C$ receptor. This GABA response was similar in two preparations of retina and one of caudate nucleus and the $GABA_C$ currents were considerably smaller, yielding only about 10% of the total GABA-current generated.

We previously provided evidence for the expression of ρ1 and ρ2 mRNAs in the caudate nucleus (López-Chavez et al 2005). Considering that we identified the $GABA_C$ subunit transcripts only by RT-PCR we decided to clone and express the cDNAs to probe their functional properties.

3.2 Cloning and expression of a bovine ρ1 (bρ1) cDNA. The bovine ρ1 clone that we obtained shares between 74-93% aminoacidic sequence homology with other vertebrate ρ1 genes. Analysis of the primary aminoacid sequence (Fig. 2) revealed the classic characteristic of the Cys-loop family,

including a signal peptide, four hydrophobic domains corresponding to the transmembrane passes and the two cysteines that are presumed to form the neurotransmitter binding site. Fig. 2 compares the sequence of several vertebrate ρ1 receptors and highlights some of the common traits of this familiy. During the course of this investigation a cDNA sequence corresponding to the bρ1 was uploaded to the genebank (id XP_549434). This cDNA sequence was derived by computer analysis (GNOMON) of the genomic sequence NW_978593. When compared to our cDNA, few nucleotide sequence differences were found and these did not alter the amino acid sequence: an open reading frame of 1467 bp predicting for 489 amino acid residues (Fig. 2).

Oocytes injected with bρ1 cDNA generated currents typical of those elicited by $GABA_C$ receptors: *ie* non-desensitizing slow-inactivating currents not blocked by bicuculline (Fig. 3A). The EC_{50} for this receptor was 6μM and revealed a Hill coefficient of 1.8 (*n*=6 Fig. 3B). Current-voltage relations gave a reversal membrane potential around -20 mV (Kusano et al 1977) (Fig. 3C), indicating that Cl^- is the main ion transported through the channel opened by GABA (*cf*. Kusano et al 1977). Moreover, as for other ρ1 receptor expressed (Polenzani et al 1991, Martínez-Torres et al 1998) the bρ1 did not rectify over the range of membrane-potentials explored (-120 to +40mV; *n*=6). An exhaustive characterization of the properties of the bρ1 receptor is still necessary, for now suffice to say that glycine and β-alanine can efficiently gate the receptor-channel, similar to other ρ1 receptors.

3.3 Immunolocalization of the receptor and rapid Golgi staining.

The vertebrate caudate nucleus is characterized by spiny and non-spiny GABAergic neurons. Since there is scant information on the cytoarchitecture of the bovine (*B. taurus*) caudate, we decided to determine if such neurons are also the predominant classes in this animal. Fig. 4 shows neurons stained with the silver impregnation technique. In 7 different brains examined we found some pyramidal and fusiform neurons with or without spines.

Immunoreaction to the anti-GABAρ1 antibody was heavier in the most internal area of the head of the caudate. In all the preparations studied (7 samples and 55 slices), the stained neurons belonged to the pyramidal and fusiform classes (Figure 5); and were distributed randomly without an apparent order. Such a distribution of neurons is typical of this brain area which is characterized by a non-layered cellular structure.

The medulla oblongata was immunopositive (11 samples and 151 slices) to GABAρ1 in the olivar, reticular, cuneiform and accessory cuneiform nuclei and the labeled neurons belonged to the pyramidal, oval and fusiform classes (Fig 6). Corpus callosum (5 samples and 50 slices) and pons (7 samples and 140 slices) did not show any positive signal, although they express the mRNA for bρ1.

4. Discussion

The molecular and functional properties of $GABA_C$ receptors are very interesting and considerably different from those of $GABA_A$ receptors. Because

of their high GABA sensitivity and low rate of desensitization the $GABA_C$ receptors are bound to play important roles in the functions of the brain. Therefore, knowledge of the cellular distribution of $GABA_C$ receptors is necessary to understand their function. In this work we describe the distribution of the ρ1 subunit in the bovine caudate nucleus and brain stem and provide evidence that the cloned ρ1 subunit expresses functional, presumably homomeric, $GABA_C$ receptors when injected alone into *Xenopus* oocytes. We show also that microtransplantation of caudate nucleus cell membranes to frog oocytes leads to the incorporation of functional $GABA_A$ and $GABA_C$ receptors which had been previously assembled in their native bovine cell membranes. Although small, the $GABA_C$ component was clearly identified because of its resistance to block by bicuculline, similar to the GABA-currents generated here by oocyte injection of retina membranes or by injection of retina mRNA (Polenzani et al, 1991).

Since the $GABA_C$ currents, elicited by oocytes injected with caudate nucleus cell membranes were quite small, it was possible that the bovine subunit was somehow incapable of generating large currents. To answer this question we cloned the bρ1 subunit. Expression of this cDNA in *Xenopus* oocytes yielded bρ1-receptors that generated large currents; similar to the typical slow-desensitizing, bicuculline-resistant, non-rectifying Cl^- currents elicited by other GABAρ1 receptors (Calvo et al, 1994, Cutting et al 1991, Martínez-Torres et al, 1998, Polenzani et al 1991). In contrast, the current-voltage relation of bρ2-receptors shows some rectification around -100mV (López-Chávez et al 2005). The molecular bases of these properties are still unknown, although structural

motifs playing a role in these characteristics may be deduced from experimental approaches involving chimeric $GABA_C$ receptors. In this context, the residue K322 which occupies the same position in ρ1 and ρ2 has been held responsible for rectification of the $GABA_A$ receptors that include the β3 subunit (Fisher, 2002).

GABA plays a central role in the function of the mammalian brain, including the basal ganglia (Tisch et al, 2004). The caudate nucleus is divided in two main regions: the striosome and the matrix compartment, with both areas possesing GABAergic neurons as well as $GABA_A$ receptors; and many studies have established that the $GABA_A$ receptors mediate the signaling from the cortex and to the globus pallidus (Chan et al, 2005, Flores-Hernández, 1994, Hitoshi et al, 2006, Tepper et al, 2004). Our work here shows that $GABA_C$ receptors have to be taken also into account when dealing with neuronal circuits because the presence of $GABA_C$ receptors in the brain, functioning either alone or in combination with $GABA_A$ subunits, may confer synaptic properties not previously considered. For example, the high GABA-sensitivity and low desensitization of $GABA\rho1$ receptors may permit some pathways to be closed for long periods, and new pathways to open rapidly, without having to lay down new neuronal wiring. The possibility of having heteromeric $GABA_A/GABA_C$ receptors, with new physiological and pharmacological properties, adds further complexity to the brain's circuitry.

Figure 1. Transplanting plasma membranes in *X. laevis* oocytes. GABA-currents generated by oocytes injected with plasma membranes from A) retina or B) caudate nucleus. The $GABA_A$ component generated larger currents that desensitized rapidly and were antagonized by bicuculline (Bic). The $GABA_C$ receptor was identified by its resistance to Bic and slow rate of desensitization.

Figure 2. Primary sequence of GABA ρ1. Alignment of four mammalian (rat, human, mouse and bovine) and two fish (*Morone Americana* and *Fugu*) GABA ρ1 subunits. Conserved regions such as the signal peptide (SP), the cys-loop and the four transmembrane domains (M1-4) are shaded. Three serine residues in the large intracellular loop are absent in the fish receptors.

Figure 3. Some properties of cloned bρ1 receptors. A) GABA-dose / current response of (n=6) oocytes injected with bρ1 cDNA. The inset shows sample currents recorded from one oocyte. B) current-voltage relation (n=6). The inset shows currents recorded previous and during application of 1μM GABA. Calibration bars= vertical: 100 nA, horizontal: 10s.

Figure 4. Pyramidal neurons of the bovine caudate nucleus. A and B) Spiny neuron (arrow). The arrowheads indicate the spines along the dendritic tree of this neuron. C and D) an aspiny neuron, the arrow indicating a dendritic arborization. The discontinuos rectangles indicate the amplified areas. Bar=50μm for A and C; 3μm for B and D.

Figure 5. Immunolocalization of GABA ρ1 in the caudate nucleus. Positive signal of anti-ρ1 antibody was identified in neurons (arrowheads) towards the internal region of the head of the caudate. A) Several pyramidal and fusiform neurons with immunoreaction. B) and C) Fusiform and D) Pyramidal neurons showed immunoreaction anti-ρ1. E) Control. Bar=20μm A. 10 μm B-E.

Figure 6. Immunolocalization of GABA ρ1 in the brain stem. A) Horizontal cut showing the olive (ON), reticular (RN) and cuneiform (CuN) nuclei. B-F arrowheads indicating pyramidal, fusiforms and oval neurons. B, CuN; C, accesory to the CuN, D-F, RN. Bar= 30μm B -D; E and F=20μm.

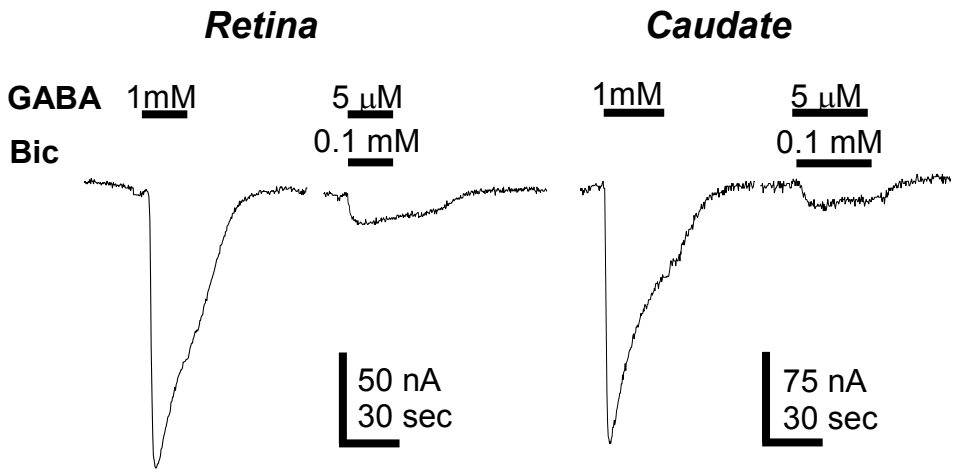

Retina
Caudate
GABA
Bic
1mM
5 μM
0.1 mM
1mM
5 μM
0.1 mM
50 nA
30 sec
75 nA
30 sec

Figure 1

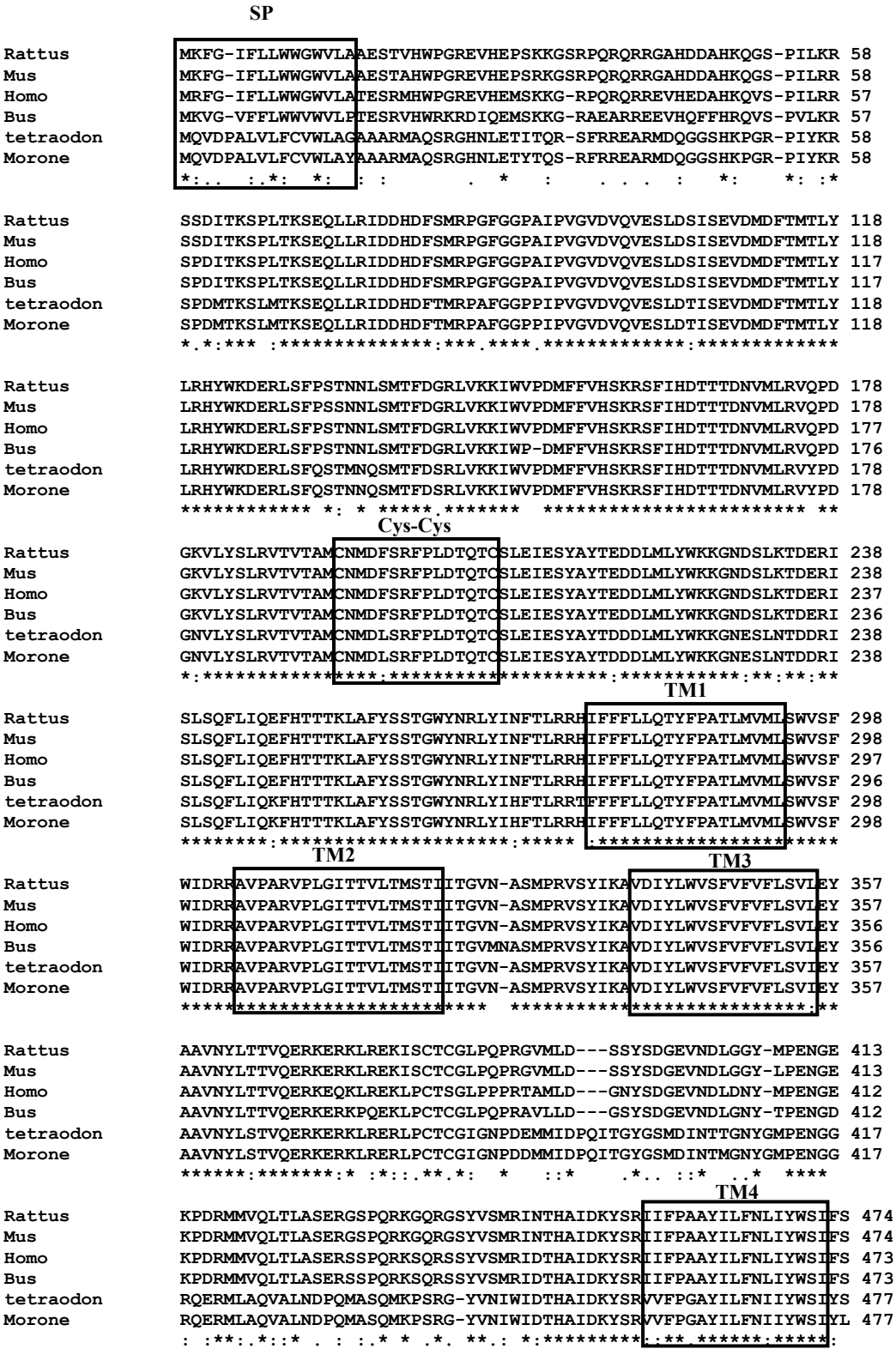

SP
Cys-Cys
TM1
TM2
TM3
TM4
Rattus
Mus
Homo
Bus
tetraodon
Morone

Fig 2

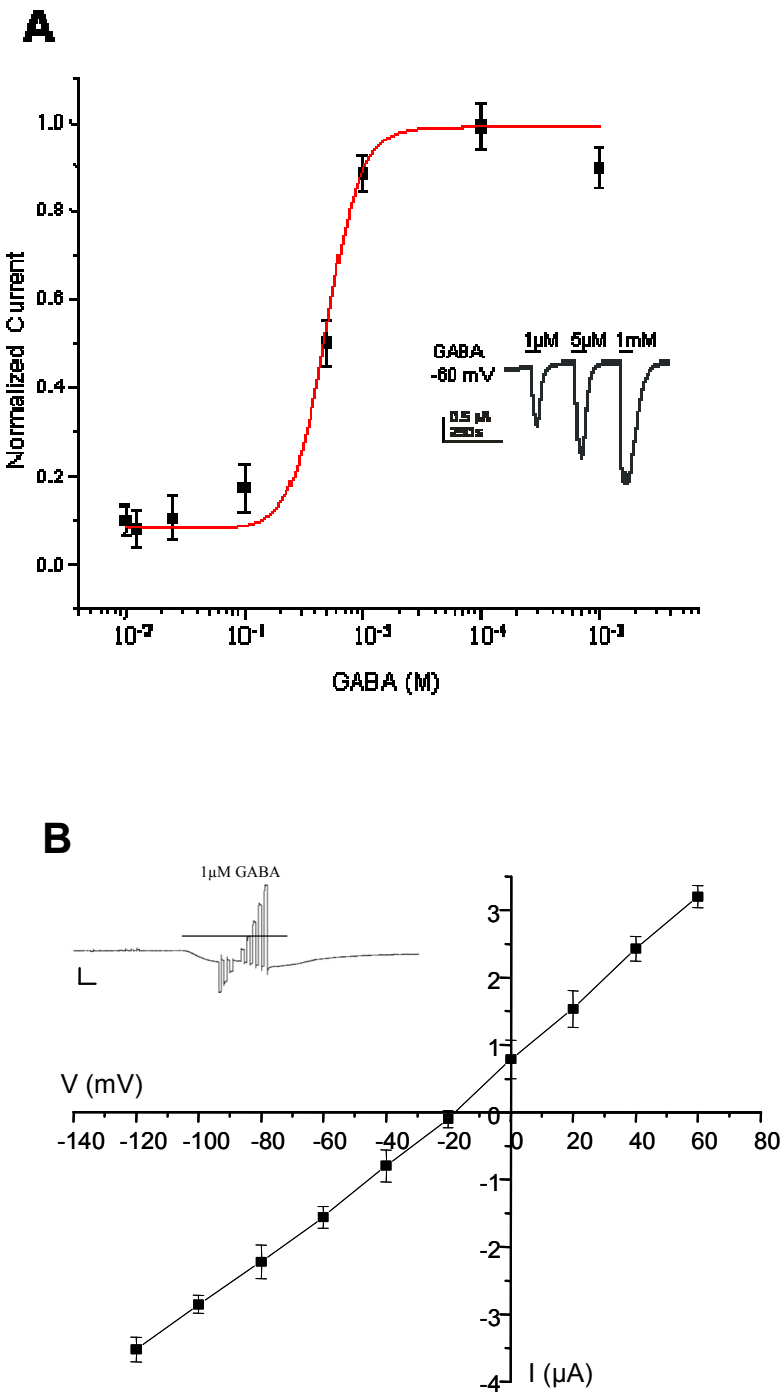

A
Normalized Current
1.0
0.8
0.6
0.4
0.2
0.0
GABA (M)
GABA
-60 mV
1μM 5μM 1mM
B
1μM GABA
V (mV)
-140 -120 -100 -80 -60 -40 -20 0 20 40 60 80
3
2
1
0
-1
-2
-3
-4
I (μA)

Figure 3

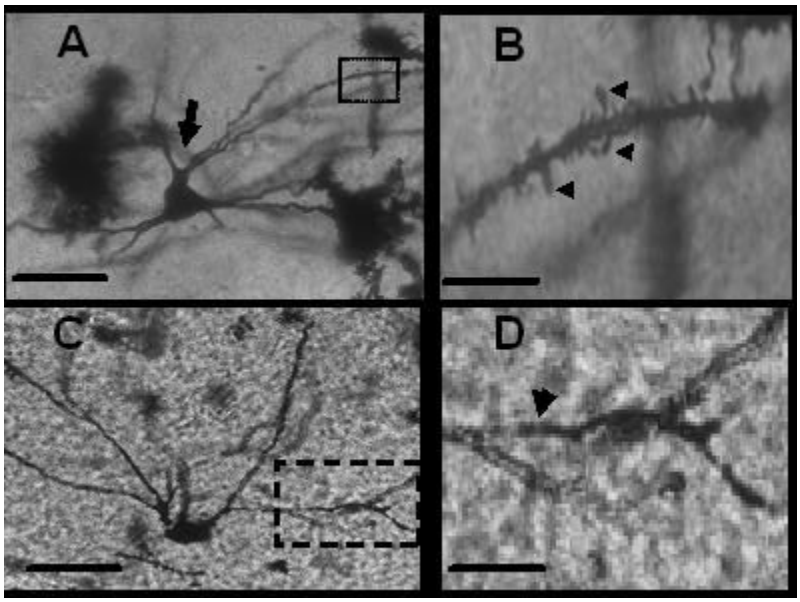
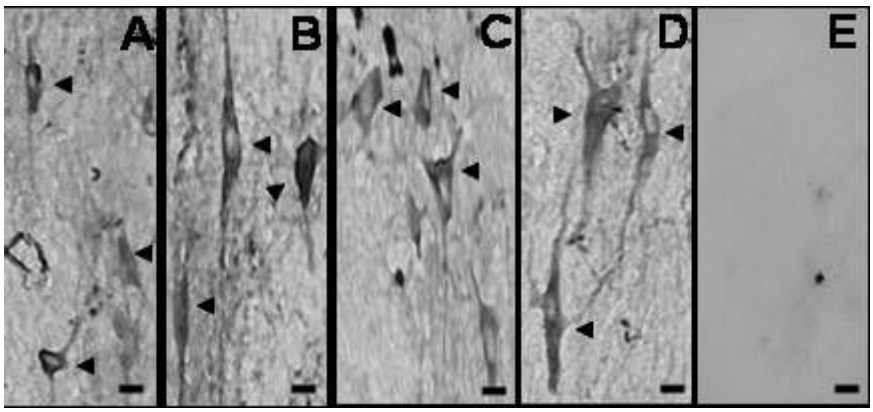


Figure 4

Figure 5



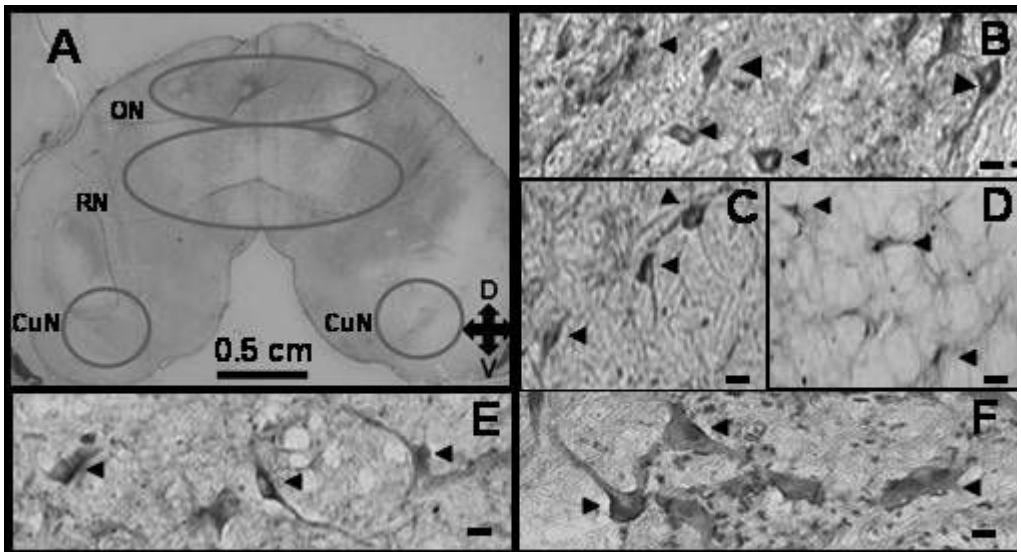


Figure 6

Acknowledgments

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