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Upregulation of high affinity GABA_A receptors in cultured rat dorsal root ganglion neurons

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Abstract

Despite evidence that high-affinity GABA_A receptor subunit mRNA and protein are present in dorsal root ganglia (DRG), low affinity currents dominate those detected in acutely dissociated DRG neurons *in vitro*. This observation raises the possibility that high affinity receptors are normally trafficked out of the DRG toward central and peripheral terminals. We therefore hypothesized that with time in culture, there would be an increase in high-affinity GABA_A currents in DRG neurons. To test this hypothesis, we studied dissociated DRG neurons 2 hrs (acute) and 24 hrs (cultured) after plating with whole cell patch clamp techniques, western blot and qRT-PCR analysis. GABA_A current density increases dramatically with time in culture in association with the emergence of two persistent currents with EC₅₀'s of $0.25 \pm 0.01 \mu\text{M}$ and $3.2 \pm 0.02 \mu\text{M}$ for GABA activation. In a subpopulation of neurons, there was also an increase in the potency of GABA activation of the transient current from an EC₅₀ of $78.16 \pm 10.1 \mu\text{M}$ to $9.56 \pm 1.3 \mu\text{M}$ with time in culture. A fraction of the high affinity current was potentiated by δ -subunit agonist 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol (THIP). δ -subunit immunoreactivity was largely restricted to the cytosolic fraction in acute but the membrane fraction in cultured DRG neurons with no detectable change in δ -subunit mRNA. However, the emergence of a high affinity current blocked by THIP and insensitive to bicuculline was detected in a subpopulation of cultured neurons as well in association with an increase in ρ_2 and γ_3 -subunit mRNA in cultured DRG neurons. Our results suggest that high-affinity δ -subunit containing GABA_A receptors are normally trafficked out of the DRG where they are targeted to peripheral and central processes. They also highlight that the interpretation of data obtained from cultured DRG neurons should be made with caution.

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Introduction

γ -aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the adult mammalian nervous system, mediating its principal effects via anion selective ionotropic GABA_A receptors. In the central nervous system (CNS), GABA_A receptor activation leads to a net anion influx resulting in membrane hyperpolarization. Recent evidence indicates that GABA_A receptors underlie at least two distinct modes of inhibition. Classical synaptic inhibition is phasic, reflecting rapid increases in GABA in to the millimolar range, and is mediated by relatively rapidly desensitizing GABA_A receptors which have low affinity for GABA (Mody et al., 1994). A second mode of inhibition is tonic, mediated by high affinity extrasynaptic GABA_A receptors which are activated by GABA concentrations in the tens of nanomolar to a few micromolar range (Farrant and Nusser, 2005). GABA_A receptor subunit composition dictates the biophysical and pharmacological properties of the receptor as well as the surface distribution. For example, γ_2 subunit containing receptors are transient low affinity receptors targeted to synapses (Essrich et al., 1998) while δ subunit containing receptors are sustained high affinity extrasynaptic receptors (Nusser et al., 1998, Belelli et al., 2009).

GABA_A signaling in primary afferent neurons is distinct in several respects from that observed in the CNS. First, because of the differential regulation of Cl⁻, GABA_A receptor activation in primary afferents results in membrane depolarization (Sung et al., 2000). Second, there appears to be disagreement between mRNA and functional data, and anatomical evidence in support of presynaptic GABA_A receptors, particularly on putative nociceptive afferents. On the one hand, a number of GABA_A receptor subunits are expressed at relatively high levels in virtually all DRG neurons (Persohn et al., 1991). These expression data are consistent with electrophysiological data indicating that there is a relatively high GABA_A current density in ~100% of neurons tested (Oyelese et al., 1997, White, 1990). On the other hand, anatomical evidence of presynaptic GABA_A receptors on putative nociceptive afferents is relatively sparse (Todd, 1996). Third, there is evidence that GABA_A receptors are transported to the periphery in primary afferents (Carlton et al., 1999), despite the absence of a source for high concentrations of GABA such as that released at synapses from GABAergic neurons. These observations raise the distinct possibility that extrasynaptic GABA_A receptors are the dominant GABA_A receptor subtype in nociceptive primary afferents.

However, previous analysis of GABA_A-receptor mediated currents in acutely isolated sensory neuron somata suggests that these currents are dominated by relatively rapidly desensitizing low affinity receptors (Oyelese et al., 1997, Sung et al., 2000). Even currents evoked with low concentrations of GABA (i.e., 2.5 μ M) desensitize (White, 1990). There is also evidence that these currents are potentiated by benzodiazepines (Witschi et al., 2011), which act at γ -subunit containing receptors that are normally targeted to synapses (McKernan et al., 1995). Furthermore, while it remains to be determined whether the amount of GABA released within the DRG is sufficient to activate low affinity receptors, it has been suggested that GABA signaling within the ganglia constitutes a normal component of sensory processing (Vit et al., 2009). This raises the possibility that the GABA_A currents present in acutely isolated sensory neurons are the currents normally present in sensory somata *in vivo*. Thus, despite evidence that high affinity GABA_A receptor subunits are expressed in DRG (Ma et al., 1993), there is little evidence that these receptors are detectable in acutely isolated DRG neurons. We therefore hypothesized that high affinity GABA_A receptors are normally trafficked out of the ganglia. A key prediction of this hypothesis is that high affinity receptors should be detectable with time in culture as receptors normally targeted for peripheral and central processes are inserted in the plasma membrane as is observed with other transducers (Kirillova et al., 2011). The present study

was designed to test this hypothesis. The biophysical and pharmacological properties of GABA receptor mediated currents were assessed in dissociated DRG neurons 2 hrs (acute) and 24 hrs (cultured) after plating with conventional whole cell patch clamp techniques. Semi-quantitative reverse-transcriptase polymerase chain reaction (sqRT-PCR) and Western blot analysis were used to further assess the basis for culture dependent changes in GABA_A currents. Our results were generally consistent with our hypothesis that high affinity GABA_A receptors are normally trafficked out of the ganglia, but also suggested that there are additional changes in subunit expression which appear to be associated with placing the neurons in culture.

Experimental procedures

Animals

Adult (180–280g) male Sprague-Dawley rats (Harlan, Indianapolis, IN) were used for all experiments. Rats were housed in the University of Pittsburgh animal facility in groups of two on a 12:12 light dark schedule. Food and water were available *ad libitum*. All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines as well as guidelines established by the International Association of the Study of Pain for the use of laboratory animals in research.

Tissue preparation

Rats were deeply anesthetized with a subcutaneous injection (1mg/kg) of a cocktail containing ketamine (55mg/kg), xylazine (5.5 mg/kg), and acepromazine (1.1mg/kg) (ketamine was from Fort Dodge Animal Health, Fort Dodge, WI, USA; xylazine and acepromazine were from Phoenix Scientific Inc., St. Joseph, MO, USA). L4–5 DRG were harvested, enzymatically treated, mechanically dissociated, and plated on laminin and ornithine-coated (Invitrogen) cover-slips as previously described (Lu et al., 2006). After plating for two hours, cells were either flooded with a L-15 (Invitrogen) based media containing 10% fetal bovine serum, 5 mM HEPES, 5 mM glucose and 1000 units of penicillin-streptomycin (Invitrogen) or flooded with Minimal Essential Media (MEM, Invitrogen) containing 10% fetal bovine serum, 1000 units of penicillin-streptomycin, and 1x MEM vitamins (Invitrogen). Those flooded with L-15 based media were stored at room temperature and studied over the next 5–6 hours while those flooded with MEM-based media were placed in a CO₂ (3%) incubator at 37°C for 24 hours prior to study: the former were considered acutely cultured and the latter were considered cultured neurons.

Electrophysiology

Whole-cell patch-clamp recordings were performed with a HEKA EPC10 amplifier (HEKA Elektronik GmbH, Lambrecht, Germany). Unless otherwise noted, data were acquired at 10 kHz and filtered at 2 kHz. Borosilicate glass (WPI, Sarasota, FL) electrodes were 2–3 MΩ when filled with the following solution (mM): CsCl 140, MgCl₂ 1, EGTA 11, HEPES 10, Mg-ATP 2, and GTP 1; pH was adjusted to 7.2 with Tris-base and osmolality was adjusted to 310 mOsm with sucrose. The bath solution contained the following (in mM): NaCl 130, KCl 3, CaCl₂ 2.5, MgCl₂ 0.6, HEPES 10, and glucose 10; pH was adjusted to 7.4 with Tris-base and osmolality was adjusted to 320 mOsm with sucrose. Neurons were held at −60 mV. Currents were evoked by 3 or 60 seconds of focal application of GABA or GABA_A receptor agonists at different concentrations. To rule out the possibility that the KCl included in the bath solution contributed to the current evoked in response to GABA_A receptor activation, GABA currents were measured in bath solution with and without K⁺. The results of this experiment indicate that there was no detectable influence of K⁺ flux to the current evoked by GABA (1, 10, and 100 μM). Current amplitudes were 12.1±1.0, 68.5±3.1

and 128.4 ± 12.0 pA compared to 12.6 ± 5.7 , 49.7 ± 6.8 and 139.3 ± 22.3 ($n = 4$) in the presence and absence of 3 mM K^+ in the bath solution, respectively.

Western blot

For the separation of membrane and cytosol fraction, the cells or tissue were ground in RIPA buffer, let it sit on ice for 20 minutes, and then spun down in a table-top centrifuge at 13,000rpm for 10 minutes. The supernatant was then loaded onto a sucrose buffer and spun in an ultra centrifuge at approximately 100,000g for an hour. The top 3/4 of the supernatant was used as the cytoplasm-enriched fraction. A pellet of membrane-enriched protein formed at the bottom of the tube. One μ l of 10% SDS was added to the remaining supernatant and used to resuspend the pellet. Protein was separated on a 7.5–10% SDS-PAGE gel and blotted to nitrocellulose membrane (Amersham) with a Trans-Blot Transfer Cell system (Bio-Rad). Blots were blocked with 5% milk in TBS buffer (20 mM Tris, 150 mM NaCl, pH 7.4) at room temperature for 30 min. After decanting the blocking buffer, the blot was incubated with an antibody against the δ -subunit of the GABA_A receptor (AB9752, Millipore: 1:500). The specificity of this antibody has been determined previously (Korpi et al., 2002). Membranes were incubated with the δ -subunit antibody overnight at 4°C at a 1:200 dilution. Immunoreactivity was detected using Enhanced Chemiluminescence (ECL, Amersham). Chemiluminescence was captured with a CCD camera (Las-3000, Fujifilm) and analyzed with Fuji software Multi Gauge.

RT-PCR

Total RNA was extracted from two groups of cells (2 hr (acute) and 24 hr (cultured) after plating) using Trizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out using 1 μ g total RNA with SuperScript II reverse transcriptase and an anchored oligo(dT) primer. SYBR Green was used to monitor amplification of template with primers on a real-time thermal cycler (Applied Biosciences) controlled by a PC running Prism 7000 SCS software. A melting curve was generated at the end of each experiment to assess for the presence of contamination. Gel electrophoresis was used to confirm the generation of a single PCR product of the expected size. The Standard curve and $\Delta\Delta CT$ methods were used to assess differences in relative expression levels following confirmation that 24hr culturing has no effect on comparator gene (e.g., GAPDH) expression levels. Primers for amplification of $\rho 1,2,3$ genes were as follows: $\rho 1$ F-GCAACATGGACTTCAGCCGGTTT, R-GGTCTGCTTCTGTGTAGGCATAGCTT, $\rho 2$ F-TACCTGAGGCATTACTGGAGGGATGA, R-TTCTTCACCAGCCTGCCATCGAA, $\rho 3$ F-TGGATCACACTGATGCTGGATGCT, R-GAATGCGGGTTTGTTCGGTGAAGAG, those for amplification of δ were F-TCC CTG GGC TTT ACC TCA ATT, R-ACT AAT GGC CCT CTG TGC AAA while those for GAPDH were: F-GGCCTACATGGCCTCCAA, R-TGGAATTGTGAGGGAGATGCT.

Statistical analysis

Data are expressed as mean $\pm$ SEM unless otherwise stated. Differences between groups were assessed with a one-way ANOVA for multiple group comparisons (i.e., concentration response data) or a Students t-test to compare results from acute and cultured neurons, where $p < 0.05$ was considered statistically significant.

Results

GABA_A currents in DRG neurons increase with time in culture

Because we are ultimately interested in the role of GABA signaling in primary afferent neurons in nociceptive processing, the focus of this study was on small diameter neurons, a

population enriched in putative nociceptors (Lawson SN, 2002). Therefore the membrane capacitance of the neurons included in this study were less than 45 pF. Increasing concentrations of GABA (1, 10, and 100 μ M) was used to evoke GABA currents in acutely dissociated DRG neurons (2hr after plating) and those cultured for 24 hours (Fig 1). While acutely dissociated neurons had no response to a brief (3 sec) application of 1 μ M of GABA (0 of 6), after 24hrs in culture, a bicuculline-sensitive current was present in 10 of 15 DRG neurons tested, where the average current density was 2.1 ± 0.5 pA/pF (Fig. 1B, n=10). A response to 10 μ M GABA was detectable in only 10 of 29 acutely dissociated DRG neurons, but was clearly manifest in all of the neurons studied after 24 hrs in culture: the current density in cultured neurons was significantly (n=10, $p < 0.05$) greater than that in acutely dissociated neurons, even when only data from responsive neurons were included in this analysis (Figure 1B). Likewise, while all of the acutely dissociated neurons tested (n = 10) were responsive to 100 μ M GABA, current density in cultured neurons (128.5 ± 12.0 pA/pF) was significantly ($p < 0.01$) greater than in acutely dissociated neurons (20.5 ± 1.9 pA/pF, n = 10). While GABA_B receptors are clearly present in DRG neurons (Menon-Johansson et al., 1993), and a GABA_B receptor antagonist was not used in the majority of experiments, control experiments (n = 9 acute and 5 cultured) indicated that presence of the GABA_B receptor antagonist, CGP35348 (10 μ M) had no influence on the magnitude or kinetics of inward currents evoked with GABA (data not shown).

Time dependent changes in high and low affinity GABA_A currents

Our initial screen of GABA currents in DRG neurons suggested that there was an increase in GABA potency with time in culture. One of the unique features of extrasynaptic high affinity GABA receptors is that the currents are persistent. Therefore, to better characterize time-dependent changes in GABA potency as well as the presence of persistent currents in cultured DRG neurons, another group of neurons was studied with prolonged (1 min) application of additional concentrations of GABA. Ten μ M GABA was used as starting dose on acutely dissociated neurons as none were responsive to 1 μ M, while doses from 0.1 μ M were tested on cultured DRG neurons. There was a concentration dependent increase in the rate of current desensitization in acutely cultured DRG neurons (Figure 2A). Nevertheless even with 10 μ M GABA, peak current ($I_{\text{peak}} = 198 \pm 22.9$ pA) was reduced by >98% by the end of a 1 minute GABA application ($I_{\text{sustain}} = 3.4 \pm 0.2$ pA, n = 5). In contrast, there was a significant ($p < 0.05$) increase in the sustained current ($I_{\text{sustain}} = 55.5 \pm 6.8$ pA) evoked with 10 μ M GABA in cultured DRG neurons. This increase in sustained current also constituted a significantly larger fraction (15.5 ± 3.2 %) of peak current amplitude ($I_{\text{peak}} = 359 \pm 126$ pA, n=7, $p < 0.05$), despite the increase in peak current. Furthermore, there was little desensitization of the current evoked in cultured DRG neurons with 1 μ M GABA (n = 7, Figure 2B and C). Dose response analysis of peak and sustained currents revealed up to three populations of DRG neurons defined by the presence transient and sustained currents with different potencies for GABA. One had sustained currents EC_{50} of 0.25 ± 0.01 μ M (Figure 2B & D, n = 3) while the other had sustained currents with an EC_{50} of 3.2 ± 0.02 μ M (Figure 2C & D, n = 4).

There were also two transient currents detected in DRG neurons. One was a relatively slowly desensitizing current ($I_{\text{S-Transient}}$) present in both acutely dissociated and cultured DRG neurons (Figure 2A and B). The second was a rapidly desensitizing current ($I_{\text{F-Transient}}$) detectable in all cultured DRG neurons (Figure 2B and C). The EC_{50} for $I_{\text{F-Transient}}$ appeared to be consistent among cultured DRG neurons but was not determined because of contamination by $I_{\text{S-Transient}}$ in a significant number of neurons. The EC_{50} for activation of $I_{\text{S-Transient}}$ in acutely dissociated neurons was also consistent (78.16 ± 10.1 μ M, Figure 2E). However, there were at least two populations of cultured neurons defined by the threshold for the activation of the $I_{\text{S-Transient}}$. One was activated by GABA at concentrations

as low as 1 μM (Figure 2B) while the other required considerably higher concentrations of GABA (Figure 2C, inset). The EC_{50} for the high affinity $\text{I}_{\text{S-Transient}}$ in cultured neurons ($9.56 \pm 1.3 \mu\text{M}$, $n = 7$) was significantly ($p < 0.01$) lower than that of the lower affinity $\text{I}_{\text{S-Transient}}$ ($91.5 \pm 12.1 \mu\text{M}$, $n = 6$) in cultured neurons or the transient current in acutely dissociated neurons ($78.16 \pm 10.1 \mu\text{M}$, $n=4$, Figure 2E). There was no significant difference between acutely dissociated or cultured neurons with respect to the EC_{50} of the low affinity transient current. There did not appear to be a differential association of high and low affinity transient currents with high and low affinity sustained currents in cultured DRG neurons.

We did not pursue a pharmacological approach to tease apart the three components of the transient GABA current in cultured neurons. That said, because $\text{I}_{\text{F-Transient}}$ is activated at lower concentrations of GABA than either of the two $\text{I}_{\text{S-Transient}}$ currents and $\text{I}_{\text{F-Transient}}$ was not present in acutely dissociated neurons, we do not believe that $\text{I}_{\text{F-Transient}}$ is an artifact. As the decay of $\text{I}_{\text{F-Transient}}$ was obscured by $\text{I}_{\text{S-Transient}}$, it was not possible to accurately determine the rate of decay of this current and consequently, no additional statistical analyses were performed. However, the decay rate of $\text{I}_{\text{S-Transient}}$ was comparable in acute and cultured neurons: $130 \pm 10 \text{ ms}$ vs $103 \pm 8 \text{ ms}$, respectively.

The emergence of a THIP sensitive current in cultured DRG neurons

The preceding data indicate the emergence of high affinity non-desensitizing GABA_A receptors with time in culture. The biophysical and pharmacological properties of these currents are consistent with those of δ -subunit containing GABA receptors that are located extrasynaptically in the CNS. To further explore the possibility that δ -subunit containing receptors contribute to the high affinity current observed in cultured DRG neurons, we assessed the impact of δ -subunit agonist, THIP, on acutely dissociated and cultured DRG neurons. Application of THIP alone for 3 sec resulted on a concentration-dependent increase in evoked current in cultured neurons, where the threshold for activation was $\sim 10 \mu\text{M}$ (Figure 3A, $n=4$). In contrast, acutely dissociated DRG neurons were largely unresponsive to THIP at concentrations as high as 1mM (Figure 3A). The results of co-application of THIP with GABA were more complicated suggesting that in a subpopulation of neurons (6/11) a low concentration of THIP (1 μM) inhibited the GABA evoked current. However, at concentrations of THIP above 1 μM , THIP potentiated the GABA evoked current in every neuron tested. The THIP-induced potentiation of 3 μM of GABA was concentration dependent (Figure 3A and B).

Redistribution of GABA δ -subunit with time in culture

To further explore the possibility that the increase in high affinity GABA current with time in culture is due to an increase in δ -subunit containing receptors in the plasma membrane and whether this change reflects *de novo* expression of this subunit and/or translocation of receptors already present in acutely dissociated neurons, we performed two additional experiments. In the first, we employed sqRT-PCR to assess changes in δ subunit mRNA in acutely dissociated and cultured DRG neurons. Results from this analysis indicated that there was no detectable increase in δ -subunit expression (Figure 4A). Using the $\Delta\Delta\text{CT}$ method to compare the change in δ -subunit expression relative to the housekeeping gene GAPDH, the fold change at 24 hrs relative to acutely dissociated DRG was ~ 1 ($n = 4$). In the second experiment, we assessed δ -subunit like immunoreactivity levels in membrane and cytosolic fractions of the total protein harvested from acute and cultured DRG neurons. Results of this analysis revealed a shift in the distribution of the protein from cytosol at 2 hrs in culture to membrane at 24 hrs in culture (Figure 4B). This shift was significant ($p < 0.01$, $n = 4$).

A ρ containing subunit may also contribute to the increase in GABA current with time in culture

To further explore the properties of the high affinity GABA current blocked by THIP (Fig 5), we first quantified the fraction of current blocked in acute and cultured DRG neurons. THIP had no detectable influence on the response of acutely dissociated neurons to 10 μ M GABA ($n = 4$). In contrast, in the 6 of 11 cultured neurons in which a THIP induced block of GABA current was detected, 1 μ M THIP blocked 68.2 ± 9.4 % of current density evoked with 3 μ M GABA ($n=6$). Because of the competing impact of inhibition and potentiation, only 13.4 ± 5.2 % of 3 μ M GABA evoked current ($n=3$) was blocked by 3 μ M THIP. Because of evidence that THIP is able to block ρ -subunit containing GABA receptors (Qian and Dowling, 1994) in addition to activating δ -subunit containing receptors, we explored the possibility that the THIP-blocked current was mediated by a ρ -subunit containing receptor. This involved four additional experiments. In the first, we determined the extent to which the high affinity current was blocked by bicuculline, given evidence that ρ -subunit containing GABA_A receptors are resistant to this competitive antagonist (Chebib and Johnston, 1999). Cultured neurons with a bicuculline resistant current constituted a minority of DRG neurons, as bicuculline (50 μ M) completely blocked GABA (3 μ M) evoked current in 34 out of 50 neurons tested. The bicuculline resistant current observed in the remaining 16 neurons was 20.7 ± 5.2 % of the current evoked by GABA alone. Second, to confirm that the current blocked by THIP was resistant to bicuculline, we assessed the fraction of bicuculline resistant current evoked with 3 μ M GABA that was blocked by 1 μ M THIP. The results of this analysis indicated that the bicuculline resistant current was blocked by THIP (Figure 5C and D). Third, we tested the effects of a second, non-selective GABA_A receptor agonist *trans*-4-aminocrotonic acid (TACA) on cultured DRG neurons, given evidence that this compound activates receptors with and without the ρ subunit (Johnston et al., 1975). The TACA evoked current in acutely dissociated DRG neurons was almost completely blocked by bicuculline (97.5 ± 0.03 %, $n=7$, Figure 6A & C). In contrast, a significantly ($p < 0.05$) greater fraction (12.3 ± 3.1 %, $n = 7$) of the TACA-evoked current in cultured DRG neurons was resistant to bicuculline (Figure 6B & C). Fourth, we performed sqRT-PCR analysis on ρ -subunit expression levels in acutely dissociated and cultured DRG neurons. With time in culture, we detected significant increases in ρ_2 and ρ_3 with no detectable change in ρ_1 (Figure 7).

Discussion

The present experiment was designed to begin to test the hypothesis that a high affinity extrasynaptic GABA_A receptor is normally targeted to central and peripheral processes of primary afferents. Toward that end, we studied acutely dissociated and cultured DRG neurons. The major observations of this study were as follows: 1) there is a dramatic increase in GABA current density with time in culture; 2) there is a dramatic leftward shift in the concentration-response curve to GABA with time in culture; 3) the high affinity GABA evoked currents observed after 24 hrs in culture are non-desensitizing and are activated by the δ -subunit containing receptor agonist THIP; 4) despite no detectable change in δ -subunit expression, there was a shift in the distribution of δ -subunit-like immunoreactivity from the cytosolic fraction at 2 hr in culture to the membrane fraction at 24 hr in culture; 5) there is an increase in bicuculline-insensitive high affinity GABA current with time in culture that is also activated by the mixed agonist TACA; 6) there is an increase in expression of ρ_2 - and ρ_3 -subunits in cultured DRG neurons. These results are consistent with our central hypothesis, but suggest that there are also cell culture-induced changes in GABA currents detectable after 24 hrs in culture.

The increase in current density, changes in biophysical and pharmacological properties of the GABA evoked currents, and δ -subunit movement from cytosol to cell membrane with

time in culture, are all consistent with our initial hypothesis that high-affinity extrasynaptic GABA_A receptors are normally expressed by DRG neurons but trafficked out of the cell body. Nevertheless, the dissociation process clearly involves tissue damage, which might also be the cause of the changes observed with time in culture. Previous data indicate that ATF3 mRNA, a marker for peripheral nerve injury, is detectable within hours of culturing sensory neurons (Dussor et al., 2003). Others have used longer term (days) cultures of neurons as a model for peripheral nerve injury, documenting changes in proteins, such as voltage-gated Na⁺ channels, in cultured neurons that are also observed following peripheral nerve injury (Fjell et al., 1999). We also obtained evidence in the present study that at least some of the increase in high affinity GABA_A current observed in culture was due to an increase in the expression of ρ -subunits (see below). Preliminary analysis of ρ subunit mRNA levels in L5 ganglia harvested 7 days after L5 spinal nerve ligation revealed no significant changes in mRNA levels for any of the 3 ρ subunits (data not shown), suggesting that either the time course for the changes in ρ subunit expression are rapid and transient or that changes in ρ -subunit expression detected in culture are due to unique aspects of cell culture (such as exposure to bovine serum, etc). Nevertheless, the apparent impact of ρ -subunit containing receptors was relatively small (between 12 and 20% of the total GABA_A current). Furthermore, there are a number of lines of evidence to suggest that the dissociated sensory neuron cell body is a reasonable model of the afferent terminal, given evidence that receptors, second messenger cascades and effector proteins that appear to contribute to the activation and/or sensitization of afferent terminals *in situ*, are present in the dissociated cell body (Spigelman et al. 2001). The results of the present study suggest that while potential changes in protein expression due to cell culture per se cannot be ignored, and that acutely cultured neurons is a viable way to mitigate the impact of injury-induced changes in afferent properties, the use of even longer term culture can be used to assess the properties of proteins not normally targeted and/or rapidly inserted in the plasma membrane of the sensory neuron cell body.

Available evidence suggests that extrasynaptic GABA_A receptors are composed of two α subunits ($\alpha 4$ or $\alpha 6$) combined with two β and the δ subunit (Farrant and Nusser, 2005). The EC₅₀ for sustained GABA currents we observed in cultured DRG neurons are remarkably similar to those reported for receptors composed of $\alpha 6\beta 3\delta$ (0.49 μ M) and of $\alpha 4\beta 3\delta$ (2.28 μ M) heterologously expressed in *Xenopus* oocytes (Storustovu and Ebert, 2006). Extrapolating from heterologous expression studies should always be done with caution. However, additional evidence that THIP possesses both high potency and efficacy at receptors composed of $\alpha 4\beta 3\delta$ combination of subunits (Adkins et al., 2001, Brown et al., 2002) and that $\alpha 4$ - subunit mRNA is detected in both rat (unpublished observation) and human DRG neurons (Maddox et al., 2004), makes it tempting to speculate that at least some of the high affinity currents described in the present study reflect activity at receptors composed of this combination of subunits. On the other hand, the observation of a leftward shift of EC₅₀ for transient currents in a subpopulation of cultured neurons suggests that even low affinity GABA_A receptors are modified with time in culture reflecting either changes in subunit assembly (Peng et al., 2002) or post-translational modifications. Additional experiments will be needed to determine why this shift in transient current only occurs in a subpopulation of DRG neurons. Nevertheless, the observation highlights yet another feature of the heterogeneity among DRG neurons.

We have suggested that at least some of the high affinity current present in cultured DRG neurons reflect activity at GABA_A receptors containing a ρ -subunit based on the pharmacological properties of at least a fraction of the currents observed and the presence of ρ -subunit mRNA. Inhibition by THIP and resistance to bicuculline are characteristic of ρ -subunit containing GABA_A receptor currents described in previous studies (Qian and Dowling, 1994, Bormann and Feigenspan, 1995). ρ -subunit containing receptors have

historically been viewed as a unique subclass of ionotropic GABA receptor because this subunit is able to form functional homomeric receptors (Zhang et al., 1995, Enz and Cutting, 1999). While there is evidence that ρ -subunit containing receptors are present in the spinal cord (Johnston et al., 1975) and cerebellum (Drew et al., 1984), they have been most extensively characterized in retina bipolar cells, where they modulate the transmission of visual signals between bipolar and ganglion cells (Ichinose and Lukasiewicz, 2002, Jones and Palmer, 2009). Our data suggesting that ρ -subunits are expressed in sensory neurons in the absence of tissue injury are consistent with previous expression data (Zheng et al., 2003). That these receptors are functional in peripheral terminals of nociceptive afferents is suggested by the observation that peripheral administration of the selective agonist CACA is antinociceptive (Reis and Duarte, 2007). However, considerably more work is needed to characterize the function of these receptors both in the presence and absence of tissue injury.

There is now extensive evidence indicating that δ -subunit containing GABA_A receptors are widely expressed throughout the central nervous system (Brickley et al., 1996, Bai et al., 2001, Drasbek and Jensen, 2006, Takahashi et al., 2006) where they contribute to the persistent or tonic inhibition crucial in regulating neuronal excitability (Hausser and Clark, 1997, Hamann et al., 2002). As previously mentioned, one of the most striking ways that GABA_A signaling in primary afferent neurons is distinct from that in the CNS is due to the differential regulation of Cl⁻ which is maintained at elevated levels in sensory neurons into adulthood (Alvarez-Leefmans et al., 1988, Sung et al., 2000). The result is that GABA_A receptor activation in primary afferents drives membrane depolarization, a process referred to as primary afferent depolarization (PAD), rather than membrane hyperpolarization. In the absence of tissue injury, PAD is still thought to be inhibitory as a result of depolarization-induced inactivation of voltage-gated Na⁺ channels and membrane shunting (Price et al., 2009). However, following tissue injury there is a shift in spinal GABA signaling, such that the GABA_A receptor agonist muscimol, which is analgesic in the absence of tissue injury, exacerbates inflammatory hyperalgesia, at least at low doses, and the GABA_A receptor antagonist gabazine becomes antinociceptive (Anseloni and Gold, 2008). Interestingly, benzodiazepines, thought to act at γ -subunit containing synaptic receptors, were analgesic both in the presence and absence of tissue injury (Anseloni and Gold, 2008, Knabl et al., 2008). We hypothesized that at least two changes were needed to account for these paradoxical results: a) a depolarizing shift in the Cl⁻ equilibrium potential that was b) localized to the site of action of high affinity extrasynaptic GABA_A receptors. The presence of high affinity extrasynaptic receptors in primary afferents, as suggested by the results of the present study, is consistent with this hypothesis. Additional experiments will be needed to determine whether there are localized changes in intracellular Cl⁻ concentration in response to tissue injury.

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Highlights

GABA_A currents in DRG neurons change with time in culture

High affinity “extrasynaptic” GABA_A currents are increased

δ-subunit containing receptors already present are trafficked to the membrane

An increase in ρ-subunit expression and functional receptors is also detected

Figure 1.

GABA-evoked currents from acutely dissociated (2hr in culture) and cultured (24hr in culture) DRG neurons. **A–C:** Representative examples of GABA-evoked currents recorded from acutely dissociated (gray) and cultured (black) DRG neurons. The horizontal bars indicate application of GABA in different concentration (1, 10, 100 μM). **D:** Averaged data from 10 responsive neurons in each group. Since none of the acutely dissociated neurons were responsive to 1 μM GABA, the current “amplitude” in this group is zero. Numbers beneath each bar are the proportion of neurons responsive to GABA at each concentration.

Figure 2.

Currents elicited by prolonged application of GABA (1 minute) on acutely dissociated (**A**), and cultured (**B** and **C**) DRG neurons. Data were acquired at 100Hz and filtered at 50 Hz. Horizontal bars indicate application of GABA (10–1000 μM in **A**, and 0.1–3 μM in **B** and **C**). Arrows indicate the transient, both fast ($I_{\text{F-Transient}}$) and slow ($I_{\text{S-Transient}}$) and sustained ($I_{\text{Sustained}}$) current. Current shown in **A** is typical of that observed in 4 other acutely dissociated neurons while those shown in **B** and **C** are typical of that observed in cultured DRG neurons with very high affinity (**B**, $\text{EC}_{50} = 0.27 \mu\text{M}$) and high affinity (**C**, $\text{EC}_{50} = 3.2 \mu\text{M}$) sustained GABA currents. Higher concentrations of GABA applied to the neuron in **C** (inset) resulted in a large transient current. Concentration-response relationships for sustained (**D**) and slow transient (**E**) currents evoked by GABA in acutely dissociated (2 hr in culture; open square, $n=4$) and cultured (24hr in culture; filled square, $n=7$) DRG neurons. Data from each neuron were normalized with respect to maximal inward current and subsequently fitted to single Hill equation to generate the EC_{50} values indicated in the legend for Panels **D** and **E**.

Figure 3.

Effects of THIP alone or in combination with GABA (3 μ M) on acutely dissociated and cultured DRG neurons. **A.** concentration-response relationships for THIP on acutely dissociated (Open triangle, n=4) and cultured (circle, n=4) DRG neurons. THIP evoked little if any detectable current in acutely dissociated DRG neurons at the highest concentration tested (1 mM). The combination of THIP with GABA (3 μ M, filled Circles, n = 4), resulted in a leftward shift in the THIP concentration response curve, relative to that obtained with THIP alone (open Circle, n = 4); a and b are representative currents evoked by THIP (30 μ M) and combination of THIP (30 μ M) with GABA (3 μ M), respectively. **B.** Representative current evoked in response to a 3 second application of 3 μ M GABA to a cultured DRG neuron in combination with increasing concentrations of THIP. The time of GABA application is indicated by the black bar. The THIP concentration, in μ M is indicated below each current trace.

Figure 4.

A change in distribution rather than a change in expression of the δ -subunit of the GABA_A receptors in cultured DRG neurons. **A.** Semiquantitative (sq) real time PCR was used to assess δ -subunit mRNA levels in acutely dissociated and cultured DRG neurons. While expression was low, there was no detectable change in δ -subunit mRNA relative to that of the comparator GAPDH. **B.** Western blot analysis of cytosolic and membrane fractions of the total protein obtained from acutely dissociated and cultured DRG neurons revealed a significant ($p < 0.01$) increase in amount of membrane associated δ -subunit like immunoreactivity relative to that detected in the cytosol in cultured neurons. Inset, example of blot from one experiment in which half the neurons were harvested at 2hr and the other half at 24hr. Total protein from each harvest was processed in parallel to separate cytosolic and membrane fractions, and equal amounts of total protein was added to each lane as indicated. The cortex specimen in which δ -subunit is highly expressed was used to facilitate comparisons between blots and as a positive control for molecular weight of δ -subunit (50kDa) like immunoreactivity.

Figure 5.

The emergence of a THIP inhibited current in cultured DRG neurons. **A.** GABA evoked-current in an acutely dissociated (10 μ M, Top Traces) and cultured (3 μ M, Bottom Traces) DRG neurons without (black traces) and with (gray traces) application of THIP (1 μ M). **B.** Pooled data, plotted as a percent of baseline (GABA alone) from acute (2hr in culture stimulated with 10 μ M GABA, n = 6) and cultured (24hr in culture, stimulated with 3 μ M GABA, n = 6) DRG neurons. **C.** Current inhibited by THIP (1 μ M) is resistant to bicuculline (50 μ M). Current evoked by GABA (3 μ M) before (black trace) or after the application of bicuculline (50 μ M, dark gray trace), or the combination of bicuculline + THIP (50 μ M + 1 μ M, light gray trace). **D.** Pooled current density data for each test condition from three cultured neurons studied with this protocol that had bicuculline resistant current. Bic is bicuculline and ** is $p < 0.01$.

Figure 6.

Bicuculline resistant current evoked by TACA in cultured DRG neurons. TACA- (100 μ M) evoked current in an acutely dissociated (**A**) and cultured (**B**) DRG neuron before (TACA, gray horizontal bar) and after (TACA+Bic, black horizontal bar) application of bicuculline (Bic, 100 μ M). **C**. The fraction of peak current evoked with the application of 100 μ M TACA resistant to bicuculline (100 μ M) was significantly ($p < 0.05$) greater in cultured DRG neurons ($n = 7$) than that in acutely dissociated ($n = 7$) DRG neurons.

Figure 7.

Increase in p-subunit expression in cultured DRG neurons. Semiquantitative real-time PCR was used to assess p-subunit expression levels in acutely dissociated and cultured DRG neurons. Pooled data revealed significant increases both in p2 and p3 with no detectable change in p1 (n = 4 per group). * is $p < 0.05$ and ** is $p < 0.01$.