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Excitatory $GABA_A$ receptor in autonomic pelvic ganglion neurons innervating bladder

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ABSTRACT

Major pelvic ganglia (MPG) are relay centers for autonomic reflexes such as micturition and penile erection. MPG innervate the urogenital system, including bladder. γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system, and may also play an important role in some peripheral autonomic ganglia, including MPG. However, the electrophysiological properties and function of $GABA_A$ receptor in MPG neurons innervating bladder remain unknown. This study examined the electrophysiological properties and functional roles of $GABA_A$ receptors in bladder-innervating neurons identified by retrograde DiI tracing. Neurons innervating bladder showed previously established parasympathetic properties, including small membrane capacitance, lack of T-type Ca^{2+} channel expression, and tyrosine-hydroxylase immunoreactivity. $GABA_A$ receptors were functionally expressed in bladder innervating neurons, but $GABA_C$ receptors were not. GABA elicited strong depolarization followed by increase of intracellular Ca^{2+} in neurons innervating bladder, supporting the hypothesis GABA may play an important role in bladder function. These results provide useful information about the autonomic function of bladder in physiological and pathological conditions.

1. Introduction

The neurons of rat major pelvic ganglia (MPG) provide autonomic innervation to urogenital systems, including urinary bladder, external genitalia, and lower bowel. The MPG is comprised of a mixture of sympathetic and parasympathetic neurons, and plays an important physiological role in a variety of autonomic reflexes, including micturition and penile erection [1]. Various transmitters such as acetylcholine, norepinephrine, and serotonin are involved in autonomic regulation of the lower urinary tract [2]. Disorders of the central nervous system and peripheral organs may involve lower urinary tract dysfunctions, including overactive bladder syndrome or detrusor overactivity. Several transmitters are pharmacological targets for the development of drugs that control micturition disorders [3].

In the central nervous system, γ-aminobutyric acid (GABA) is typically an inhibitory neurotransmitter [4]. There are three types of GABA receptors: (1) ionotropic $GABA_A$ and (2) $GABA_C$, and (3) metabotropic $GABA_B$ [4]. GABA-inhibited bladder contractions are evoked by stimulation of preganglionic nerve fibers [5,6]. Thus, GABA may play an important role in lower urinary tract function. Further, during intracellular recording in rat MPG, GABA induced biphasic responses, early depolarization, late depolarization, and hyperpolarization which were mediated by $GABA_A$ and $GABA_C$ receptors, respectively [7]. MPG provides autonomic innervation to diverse urogenital organs and the lower bowel. In addition, we previously reported $GABA_A$ receptors were expressed mostly in tyrosine hydroxylase (TH)-positive sympathetic neurons, as well as in a specific subset of non-adrenergic, non-cholinergic (NANC) neurons of rat MPG [8]. However, innervation-specific expression of GABA receptors and their functional role in pelvic ganglia have yet to be fully characterized. Therefore, this study examined the functional expression and role of ionotropic GABA receptors in MPG neurons innervating urinary bladder. $GABA_A$ receptors, not $GABA_C$ receptors, were found to be functionally expressed in MPG neurons innervating bladder, which mediated depolarization followed by intracellular Ca^{2+} increase.

2. Materials and methods

2.1. DiI labeling and isolation of MPG neurons

Adult male Sprague–Dawley rats (200–300 g) were anesthetized with pentobarbital sodium (50 mg/kg *i.p.*). A small incision

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was made into the abdominal wall. DiI (Molecular Probes, Invitrogen, USA), a retrograde axonal tracer, was injected into the wall of the urinary bladder in order to label postganglionic neurons in the MPG. A 10 μL Hamilton syringe with a 28-gauge needle was used to make three to six injections (total volume 20–30 μL) into the dorsal surface of the bladder wall, while avoiding injection of the bladder lumen, as described previously [9,10]. One week after tracer injection, the rats were again anesthetized. MGP neurons were isolated using enzymatic dissociation, as previously described [11]. Isolated MPG neurons were plated onto 12-mm coverglass coated with poly-L-lysine and maintained with minimal essential medium (MEM) containing 10% fetal bovine serum and 1% penicillin–streptomycin (all from Life Technologies, Grand Island, NY, USA), in a humidified 95% air −5% CO_2 incubator at 37 °C. Current recordings were performed within 6 h after plating. Animal use and care procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Wonju College of Medicine.

2.2. Patch clamp analysis

Bladder MPG neuron ion currents were recorded using whole-cell ruptured patch clamp methods, as previously described [12,13]. Patch electrodes were fabricated from a borosilicate glass capillary (BF150-117-15, Sutter Instrument, San Rafael, CA, USA) using a P-97 Flaming Brown micropipette puller (Sutter Instrument). Electrodes were coated with Sylgard 184 (Dow Corning, Midland, MI, USA) and fire-polished on a microforge (Narishige, Tokyo, Japan). Electrode tip resistance was 2–3 MΩ, when filled with the solution described above. An Ag/AgCl wire was used to ground the bath. Cell membrane capacitance and series resistance were compensated for electronically (>80%) using a patch clamp amplifier (EPC-9, Heka Electronik, Lambrecht, Germany). Voltage protocol generation and data acquisition were performed using Pulse/Pulsefit (v8.50) software (Heka Elektronik, Lambrecht, Germany). Current traces were low-pass filtered using a 4-pole 2.5 kHz Bessel filter then digitally stored for later analysis.

To measure membrane potential, current-clamp recordings were performed with the gramicidin-perforated whole cell configuration patch-clamp technique using an EPC-9 amplifier and Pulse/Pulsefit (v8.50) software. A stock solution of gramicidin D was prepared at 50 mg/ml in dimethylsulfoxide and diluted in the pipette solution to a final concentration of 50 μg/ml before use. All electrophysiological recordings were performed at room temperature (~20–24 °C).

2.3. Solution and drugs

Pipettes used to record $GABA_A$ current contained (in mM) 20 KCl, 115 K-aspartate (potassium aspartate), 10 HEPES, 10 ethylene glycol bis (2-aminoethyl ether)-N, N,N′,N′-tetraacetic acid (EGTA), 2.5 $CaPO_4$, 5 MgATP, and 0.1 Na_2-GTP (pH 7.2). The bath solution contained normal physiological salts (PSS) in mM : 135 NaCl, 5.4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 HEPES, and 10 glucose (pH 7.4). To isolate Ca^{2+} currents, the pipette and bath solution contained (in mM) 120 *N*-methyl-D-glucamine methanesulfonate (MS), 20 tetraethylammonium (TEA) MS, 20 HCl, 11 EGTA, 1 CaCl2, 10 HEPES, 4 Mg-ATP, 0.3 Na_2-GTP, and 14 creatine phosphate (pH 7.2), and 145 TEA-MS, 10 HEPES, 10 CaCl2, 15 glucose, and 0.00001 tetradodoxin (pH 7.4), respectively.

For current-clamp recordings, patch pipettes were filled with a solution containing (in mM) 140 KCl, 5 EGTA, 10 HEPES, 0.5 $CaCl_2$, and 5 NaCl (pH 7.2). A normal PSS was used for the bath solution.

Drugs used in the experiments were purchased as follows: Collagenase type D and trypsin from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA), mucimol, Bicucculin, and TPMPA from Tocris (Cookson Inc., Bristol, UK), DNAse type I and all other media from Sigma (St. Louis, MO, USA). For stock solutions (10 mM to 1 M), all drugs were dissolved in distilled water.

2.4. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurement

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using the Lambda DG-4 (Sutter Instruments, Novato, CA, USA) fluorescence measurement system. MPG neurons were placed on glass coverslips and loaded with fura-2/AM in normal PSS in darkness for 1 h at room temperature. After tracer loading, neurons were washed and transferred to the perfusion chamber of a fluorescence microscope. Fura-2 signals were obtained by alternating excitation at 340 or 380 nm, and detection of emission at 510 nm. Data were analyzed using MetaFluor (Sutter Instrument) software.

2.5. Immunohistochemistry

Immunohistochemistry on MPG was described previously [8]. Briefly, rats were anesthetized with ketamine, then MPGs were removed under a surgical microscope. MPGs were immersed in 4% paraformaldehye (0.1 M phosphate buffer, pH 7.4) for 2 h, washed using phosphate buffered saline, followed by cryoprotected overnight storage in 30% sucrose solution. Cryosections (10 μM thick) were made from whole MPG, and every sixth section was mounted on the same slide coated with Vectabond™ (Vector Labs, USA). Sections were treated with 5% normal sera (Vector labs) for 1 h and then incubated overnight in a humidified chamber with rabbit anti-tyrosine hydroxylase (Chemicon; 1:500) antibody at 4 °C. Double labeled images of DiI and thyrosine hydroxylase were captured with a DXM 1200CCD camera (Nikon, Japan) attached to a light microscope (Optiphot, Nikon).

2.6. Data analysis

Data were presented as mean ± SEM. Groups were compared using a two-tailed unpaired Student's *t*-test. A one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were used for multiple comparisons. Statistical significance was defined as $p < 0.05$ for a single comparison and $p < 0.01$ for multiple comparisons.

3. Results

3.1. Characteristics of MPG neurons innervating bladder

Injection of DiI into the bladder wall of the adult rat resulted in retrograde labeling of MPG neurons (Fig. 1A). Male rat MPG is a large, unique autonomic ganglion containing both sympathetic and parasympathetic neurons. DiI-labeled neurons were mostly TH-negative (Fig. 1A). However, some DiI-labeled neurons were co-localized with TH (Fig. 1A, arrow). Electrophysiological properties of DiI-positive single MPG neurons were examined. DiI-positive cells were identified under a fluorescent microscope (Fig. 1B). MPG neuron cell types were recognized by their electrophysiological properties, including cell membrane capacitance and T-type Ca^{2+} channels [14,15]. A voltage ramp from −100 mV to +60 mV evoked Ca^{2+} channels. T-type Ca^{2+} currents were detected as a prominent hump at low-voltage range (−50 mV to −20 mV) (Fig. 1C). T-type Ca^{2+} currents were detected in DiI-negative neurons but not in DiI-positive neurons (Fig. 1C). Mean membrane capacitance of DiI-positive neurons was 26.1 ± 9.7 pF. Neuron firing patterns were classified as tonic or phasic according to their rate of accommodation during depolarizing current injection. In addition, rebound action potential spikes after anodal break in

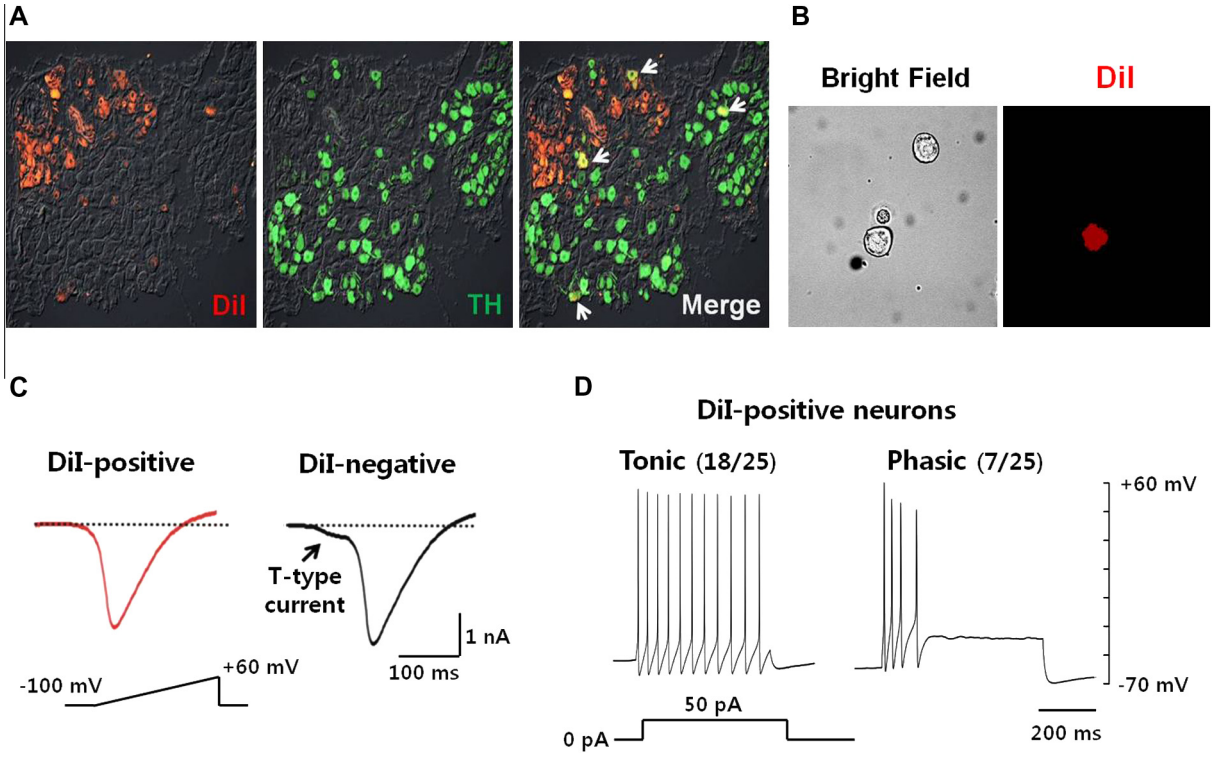

Fig. 1. Characteristics of MPG neurons innervating the bladder. (A) Double immunofluorescence labeling of DiI and tyrosine hyroxylase (TH). DiI-labeled neurons are not colocalised with TH-immunoreactivity. Few DiI-positive neurons are labeled with TH (arrows). (B) Identification of DiI-labeled MPG neurons using an immunofluorescence microscope. (C) Representative traces of inward Ca^{2+} currents in DiI-positive and -negative MPG neurons measured with the whole-cell ruptured patch clamp technique. Ca^{2+} currents were evoked by ramp pulses from −100 to +60 mV as indicated. Ramp *I–V* curves of current showed typical inward Ca^{2+} currents. T-type Ca^{2+} currents were detected by hump. (D) Action potential firing during a 500 ms current injection were measured using current-clamp recording with the gramicidin-perforated whole-cell configuration patch-clamp technique in normal PSS.

DiI-labeled neurons were examined. The majority of neurons showed tonic firing (18 out of 25) in response to a long depolarizing current injection, which evoked action potentials as often as 14.1 ± 2.6 beats/0.5 s (n = 18), while 28% of neurons exhibited a phasic firing pattern (n = 7, evoked discharge: 1.3 ± 0.5 beat/0.5 s).

3.2. Characterization of ionotropic GABA receptors

It has been reported that $GABA_A$ and $GABA_C$ receptors mediate biphasic GABA responses in male rat MPG neurons [7]. Thus, we examined which GABA receptors were functionally expressed in MPG neurons innervating bladder. GABA-evoked currents were measured in symmetrical Cl^- concentration using ruptured whole-cell patch clamp recordings. Focal application of GABA elicited inward current (Fig. 2A and D). GABA-induced inward current was completely blocked by bicuculline, a selective $GABA_A$ receptor antagonist, but not by TPMPA, a selective inhibitor of $GABA_C$ receptors (Fig. 2B–D). Application of mucimol, a selective $GABA_A$ agonist, also evoked inward current (Fig. 2E and H). The effect of mucimol was also completely prevented by bicuculline but not by TPMPA (Fig. 2F–H), supporting the hypothesis that the GABA-elicited inward current was mediated by $GABA_A$ receptors in MPG neurons innervating bladder.

3.3. GABA-induced depolarization increases Ca^{2+} in DiI-labeled MPG neurons

GABA effect on membrane potential was examined continuously in real-time using gramicidin-perforated whole-cell patch clamp recording. The $GABA_A$ receptor is a Cl^--permeable channel. Intracellular milieu and Cl^- concentration is preserved in gramicidin-perforated whole-cell recording, unlike in ruptured whole-cell mode. The resting membrane potential of DiI-labeled neurons was −64.0 ± 20.6 mV. Application of GABA elicited strong depolarization (Fig. 3A). GABA-mediated depolarization can cause Ca^{2+} influx via voltage-gated Ca^{2+} channels (VGCC). Next, if GABA-mediated depolarization evoked intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) increase was examined with Fura-2 fluorescence in MPG neurons labeled with or without DiI (Fig. 3B). Bath application of 100 μM GABA increased intracellular $[Ca^{2+}]_i$ in a DiI-positive but not DiI-negative neurons (Fig. 3C and D), supporting the hypothesis that $GABA_A$ receptor activation increases intracellular $[Ca^{2+}]_i$ via VGCC. Interestingly, a majority of DiI-positive MPG neurons showed GABA-mediated intracellular $[Ca^{2+}]_i$ increase, but some neurons did not respond to GABA (Fig. 3E).

4. Discussion

MPG neurons provide autonomic innervation to various urogenital systems and the lower bowel [9,16]. Previous electrophysiological studies have been performed on intact MPG or single isolated neurons. However, the specific electrophysiological properties of MPG neurons remained unclear. The MPG contains at least two subpopulations of postganglionic neurons, including sympathetic and parasympathetic neurons with distinct electrophysiological properties [13,14,17]. Electrophysiological properties of sympathetic neurons include expression of low-voltage activated T-type Ca^{2+} channels, large electrical capacitance, TH-immunoreactivity (TH-IR) [14,15], and a tonic firing pattern [18,19]. On the other hand, the properties of parasympathetic neurons include lack of T-type Ca^{2+} channels, small electrical capacitance, and a phasic firing pattern [14,18,19].

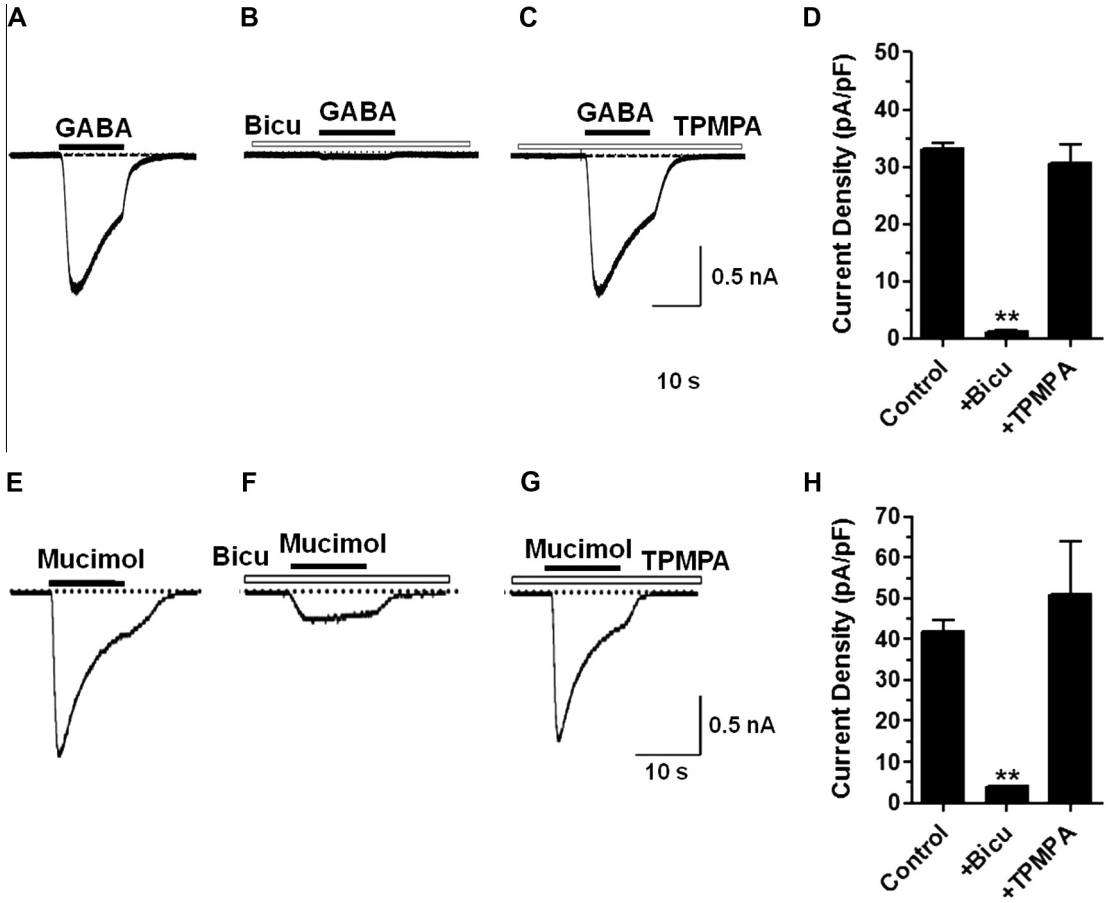

Fig. 2. Characterization of ionotropic GABA receptors in DiI-labeled MPG neurons. (A–C) Representative traces of inward Cl^- currents evoked by 100 μM GABA without or with selective $GABA_A$ or $GABA_C$ receptor blockers, bicuculline (Bicu, 100 μM) and TPMPA (100 μM), respectively. (D) Summary of results in panel 2A–C. (E–G) Representative traces of inward Cl^- currents evoked by 100 μM mucimol, a selective $GABA_A$ receptor agonist without or with bicuculline (Bicu, 100 μM) and TPMPA (100 μM), respectively. (H) summary of results in panel 2A–C. Data points (D and H) are mean ± *SEM* (n = 5–10). Double asterisk denotes $p < 0.01$ versus control.

Immunohistochemical data showed that DiI-labeled neurons and TH-IR-positive neurons were not co-localized (Fig. 1A). If TH-IR is considered a marker for sympathetic neurons, most neurons innervating bladder are not sympathetic neurons. DiI-positive neurons in MPG showed properties of parasympathetic neurons, such as lack of T-type Ca^{2+} channels and a small membrane capacitance (26.1 ± 9.7 pF). Interestingly, most DiI-positive MPG neurons exhibited a tonic firing pattern, which was previously described as a property of sympathetic neurons [18,19]. In addition, a small subset of DiI-labeled neurons was colocalized with TH-IR (Fig. 1A, arrow) and showed a phasic firing pattern, supporting the hypothesis that MPG neurons innervating bladder are heterogeneous.

GABA is a typical central nervous system inhibitory neurotransmitter which may also play a role in the pelvic plexus [5,6]. In a functional intracellular recording study, GABA exerted biphasic responses, and depolarization followed by hyperpolarization in pelvic ganglia of the cat and rat [7,20]. In GABA-induced biphasic responses, early depolarization and late hyperpolarization were mediated by activation of $GABA_A$ and $GABA_C$ receptors, respectively [7]. We have previously reported that GABA evoked an inward Cl^- current in single isolated sympathetic MPG neurons expressing T-type Ca^{2+} channels, but not in parasympathetic MPG neurons [13]. As mentioned above, MPG neurons are a heterogenous population and innervate pelvic organs and the lower bowel. $GABA_A$, but not $GABA_C$, receptors were expressed in MPG neurons innervating bladder (Fig. 2). GABA caused membrane depolarization in neurons innervating bladder, but did not show biphasic responses. In previous studies, GABA-induced biphasic responses were observed in intracellular recordings performed in intact MPG tissue [7,20]. This study showed GABA-mediated depolarization in single isolated MPG neurons innervating bladder. It is also conceivable that $GABA_A$ receptor expression and functional role is innervation-specific. In our previous immunohistochemical study, $GABA_A$ receptors were expressed in TH-IR-positive adrenergic (sympathetic) neurons and a few subpopulations of cholinergic or non-adrenergic, non-cholinergic (NANC) neurons expressing $GABA_A$ receptors [8], supporting the hypothesis that $GABA_A$ receptor expression may be innervation- and/or phenotype-specific. The present study also showed GABA-induced membrane depolarization followed by an increase of intracellular Ca^{2+} in MPG neurons innervating bladder. In DiI-labeled neurons, GABA-stimulated Ca^{2+} influx occurred in most but not all neurons, indicating that functional expression of $GABA_A$ receptors was diverse in MPG neurons innervating bladder.

In summary, MPG neurons innervating bladder showed distinct electrophysiological properties including low membrane capacitance, tonic firing pattern, lack of T-type Ca^{2+} current, and $GABA_A$ receptor current. GABA caused membrane depolarization followed by stimulation of Ca^{2+} influx, supporting the hypothesis GABA may play an important role in bladder function. Thus, identification of the electrophysiological properties and functional roles of $GABA_A$ receptors in MPG neurons innervating bladder may provide useful information regarding autonomic functions of endogenous and exogenous modulators of the $GABA_A$ receptor in diseases of the bladder.

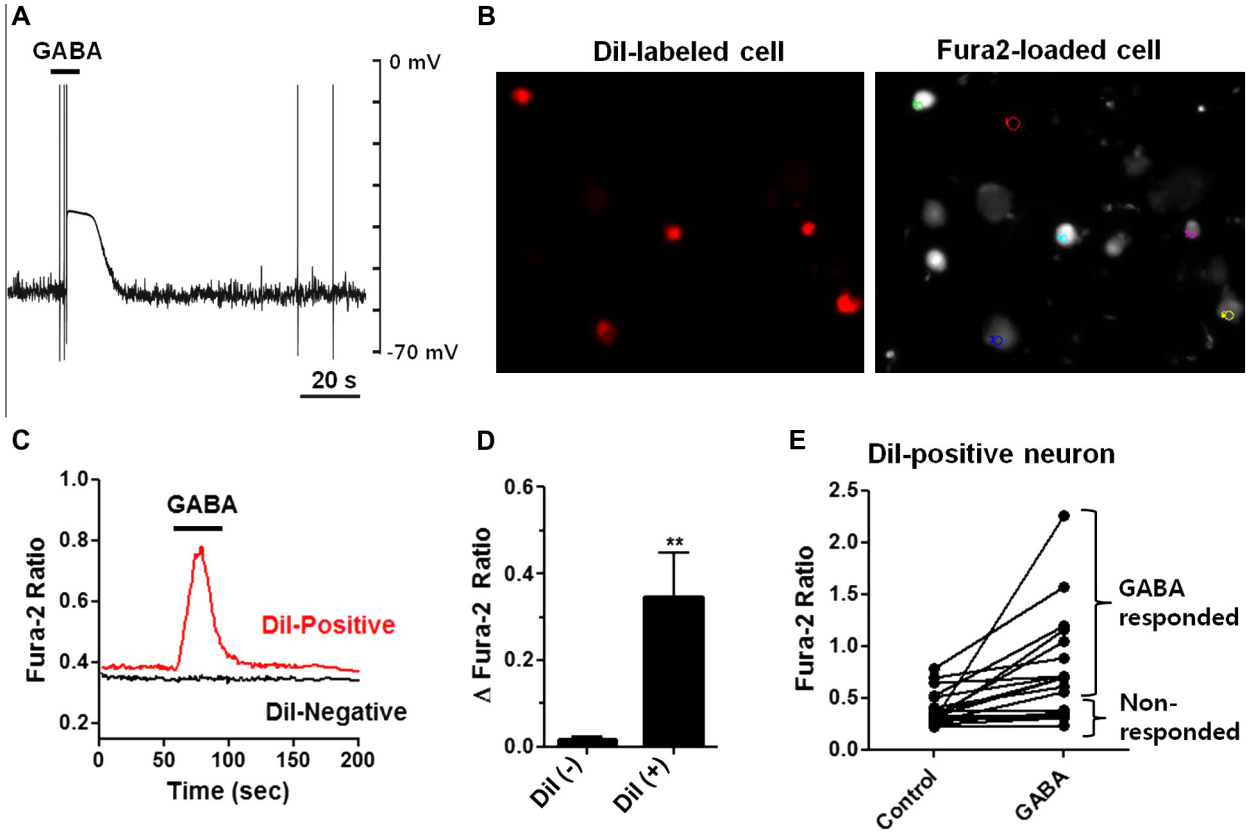

Fig. 3. GABA-induced depolarization increases Ca^{2+} in DiI-labeled MPG neurons. (A) Membrane potential was measured using current-clamp recording with the gramicidin-perforated whole-cell configuration in normal PSS. Representative traces showing effects of GABA (100 μM) on membrane potential changes in DiI-labeled MPG neurons. (B) Representative images of DiI and Fura2. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using Fura2 in DiI-postive and negative neurons. (C) Representative traces showing GABA-induced $[Ca^{2+}]_i$ responses measured in DiI-postive and -negative neurons. (D) Summary of GABA-induced change in $[Ca^{2+}]_i$, $\Delta[Ca^{2+}]_i$. Data points are mean ± SEM. Double asterisk denotes $p < 0.01$ versus DiI-negative neurons (DiI (−)). (E) Individual response to GABA in DiI-positve neurons. Fura2 ratio before (control) and after application of GABA (100 μM).

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