GABA<sub>B</sub> receptor-mediated tonic inhibition of noradrenergic A7 neurons in the rat

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The A7 catecholamine cell group consists of noradrenergic A7 neurons (referred to as NAergic A7 neurons) that project their axonal terminals to the dorsal horn of the spinal cord to modulate nociceptive signaling. NAergic A7 neurons that project axonal terminals to the dorsal horn of the spinal cord to modulate nociceptive signaling are suggested to receive tonic inhibition from local GABAergic interneurons, which are under the regulation of descending analgesic pathways. In support of this argument, we presently report GABA<sub>B</sub> receptors, which are under the regulation of descending analgesic pathways, that are present on axonal terminals of NAergic A7 neurons. Bath application of baclofen induced an outward current (I<sub>bac</sub>) in NAergic A7 neurons that was blocked by CGP 54626, a GABA<sub>B</sub>R blocker. The I<sub>bac</sub> was reversed at about −99 mV, displayed inward rectification, and was blocked by Ba<sup>2+</sup> or Tertiapin-Q, showing it was mediated by G protein-activated inward-rectifying K<sup>+</sup> (GIRK) channels. Single-cell RT-PCR results suggested that GIRK1/3 heterotramers might dominate functional GIRK channels in NAergic A7 neurons. Under conditions in which GABA<sub>A</sub> and glycine receptors were blocked, bath application of GABA inhibited the spontaneous firing of NAergic A7 neurons in a dose-dependent manner. Interestingly, CGP 54626 application not only blocked the effect of GABA but also increased the firing rate to 126.9% of the control level, showing that GABA<sub>B</sub>Rs were constitutively active at an ambient GABA concentration of 2.8 μM and inhibited NAergic A7 neurons. GABA<sub>B</sub>Rs were also found at presynaptic excitatory and inhibitory axonal terminals in the A7 area. Pharmacological activation of these GABA<sub>B</sub>Rs inhibited the release of neurotransmitters. No physiological role was found for GABA<sub>B</sub>Rs on excitatory terminals, whereas those on the inhibitory terminals were found to exert autoregulatory control of GABA release.

baclofen; descending analgesic pathway; G protein-activated inward-rectifying K<sup>+</sup> channels; pain

The A7 catecholamine cell group consists of noradrenergic neurons that project their axonal terminals to the dorsal horn of the spinal cord (Clark and Proudfit 1991; Jones 1991), and norepinephrine (NE) release in the dorsal horn results in marked analgesia (Hodge et al. 1986; Pertovaara 2006). NE has been shown to inhibit nociceptive neurons located in the substantia gelatinosa area of the dorsal horn by activation of α<sub>2</sub>-adrenoceptors. It has also been shown to promote GABA and glycine release by activation of α<sub>1</sub>-adrenoceptors located at the axonal terminals of interneurons and/or to depress glutamate release by activation of α<sub>2</sub>A-adrenoceptors at the terminals of Aδ- and C-fibers in the dorsal horn (Baba et al. 2000a, 2000b; North and Yoshimura 1984; Yoshimura and Jessell 1989; Yoshimura and Nishi 1993). Another role for NE in inhibiting nociceptive signaling is seen in the synergistic interactions between α<sub>2</sub>A-adrenoceptors and δ-opioid receptors in the spinal cord. α<sub>2</sub>A Adrenoceptors and δ-opioid receptors are colocalized at substance P (SubP)-releasing terminals in the dorsal horn (Arvidsson et al. 1995; Cheng et al. 1997; Dado et al. 1993; Zhang et al. 1998), and intrathecal coadministration of α<sub>2</sub>A-adrenoceptor and δ-opioid receptor agonists can produce profound analgesia (Ossipov et al. 1990; Stone et al. 1997). A recent report (Overland et al. 2009) showed that the dosage of δ-opioid receptor agonist required to produce effective analgesia is dramatically reduced by more than 10-fold when an equal amount of α<sub>2</sub>A-adrenoceptor agonist is coadministered. These observations suggest a strategy for achieving efficient analgesia using a low dose of morphine, thereby avoiding the development of associated side effects, such as addiction, tolerance, and dependence (Angst and Clark 2006), which have always been a major problem when using morphine for long-term pain treatment.

Physiologically, synergistic interactions between α<sub>2</sub>A-adrenoceptors and δ-opioid receptors require the simultaneous release of NE and endogenous opioids in the dorsal horn, which requires connections between NAergic neurons and opioid-releasing neurons, such as those located in the periaqueductal gray area (PAG) and rostroventromedial medulla (RVM). Since there are no NAergic neurons in the dorsal horn (Carlsson et al. 1964; Dahlstroem and Fuxe 1964), the interaction with the PAG and RVM must involve pontine NAergic neurons, such as the NAergic neurons of the A6 (also known as the locus coeruleus; LC) and A7 catecholamine cell groups. Morphological studies have proved the existence of synaptic connections between PAG/RVM neurons and NAergic A7 neurons (Bajic and Proudfit 1999; Bajic et al. 2001; Holden and Proudfit 1998). SubP appears to be the neurotransmitter for this synaptic transmission, because our previous physiological studies showed that SubP, acting at neurokinin 1 receptors, opens canonical transient receptor potential 6 channels to depolarize NAergic A7 neurons (Min et al. 2008b, 2009). Interestingly, axonal terminals from the RVM/PAG synapse not only on NAergic A7 neurons but also on local GABAergic interneurons (Bajic et al. 2001). Microinjection of bicuculline, a GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) antagonist, into the A7 area...
modulates nociceptive behavior, and this effect is abolished by intrathecal injection of adrenoceptor antagonists (Nuseir and Proudfit 2000). On the basis of these results, Nuseir and Proudfit (2000) proposed that NAergic A7 neurons are subject to tonic inhibition by local GABAergic interneurons, which are inhibited by inhibitory neurotransmitters released from axon terminals from the PAG/RVM, thus relieving the tonic inhibition of the NAergic A7 neurons and promoting NE release in the dorsal horn. However, there is no direct physiological evidence for the existence of tonic inhibition of NAergic A7 neurons. In the mammalian brain, there are two types of GABA receptor (GABA\(\alpha\)R), the GABA\(\alpha\)R and GABA\(\beta\) receptor (GABA\(\beta\)R). GABA\(\beta\)Rs are G protein-coupled receptors, the activation of which results in the release of G\(\beta\)\(\gamma\) subunits, which activate G protein-activated inward-rectifying K\(^+\) (GIRK) channels in the central nervous system (Cruz et al. 2004; Koyrakh et al. 2005; Luscher et al. 1997). Because of their metabolic coupling to K\(^+\) channels, activation of GABA\(\beta\)Rs can exert a stable and long-lasting inhibitory effect on target neurons. In this study, we report that NAergic A7 neurons are subject to tonic inhibition mediated by GABA\(\beta\)Rs through the control of GIRK channels. Preliminary results have been published in abstract form (Min et al. 2008a; Wu et al. 2008).

MATERIALS AND METHODS

Preparation of brain stem slices. The use of animals in this study was approved by the Ethics Committee for Animal Research of the National Taiwan University. Every effort was made to minimize the number of animals used and their suffering. Fifty-two Sprague-Dawley rat pups of both sexes, age 7–10 days, were used. They were decapitated and their brains rapidly exposed and chilled with ice-cold artificial cerebrospinal fluid (ACSF) consisting of (in mM) 119 NaCl, 2.5 KCl, 1.3 MgSO\(_4\), 26.2 NaHCO\(_3\), 1 NaH\(_2\)PO\(_4\), 2.5 CaCl\(_2\), and 11 glucose, oxygenated with 95% O\(_2\) and 5% CO\(_2\), pH 7.4. Sagittal brain stem slices (300 \(\mu\)m) containing the trigeminal motor nucleus (Mo5) and A7 area were cut using a vibroslicer (D.S.K. Super Microslicer Zero 1; Dosaka EM, Kyoto, Japan) (Min et al. 2003) and kept in an interface-type chamber at room temperature (24 –25°C) for at least 90 min to allow recovery.

Electrophysiology. Slices were transferred to an immersion-type recording chamber mounted on an upright microscope (BX51WI; Olympus Optical, Tokyo, Japan) and continuously perfused with oxygenated ACSF at 2–3 ml/min. Neurons were viewed using Nomarski optics; those located \(\sim\)200 \(\mu\)m rostral to the anterior border of Mo5 and having a large cell body (diameter \(\sim\)20–25 \(\mu\)m) were considered NAergic A7 neurons (Min et al. 2008b, 2009, 2010) and used for recordings (Fig. 1, A and B). The patch pipettes, pulled from borosilicate glass tubing (1.5-mm outer diameter, 0.32-mm wall thickness; Warner Instruments, Hamden, CT), had a resistance of \(\sim\)3–5 M\(\Omega\) when filled with internal solution consisting of (in mM) 131 K-glucuronate, 20 KCl, 10 HEPES, 2 EGTA, 8 NaCl, 2 ATP, and 0.3 GTP, with pH adjusted to 7.2 with KOH. To study synaptic transmission, we filled the patch pipettes with a solution in which K-glucuronate was replaced with either an equimolar amount of Cs-glucuronate, to record excitatory synaptic currents (EPSCs), or with an equimolar amount of CsCl, to record inhibitory synaptic currents (IPSCs).

Recordings were made at room temperature (24–25°C), except for experiments shown in Fig. 7, which were performed at 34–35°C. Recordings were made in either current- or voltage-clamp mode with a patch amplifier (Multiclamp 700 A; Axon Instruments, Union City, CA). For current-clamp recording, the bridge was balanced and matched.
neurons were only accepted for further study if the membrane potential ($V_m$) was at least $-45$ mV without applying a holding current and the action potential (AP) was able to overshoot 0 mV. For the measurement of input resistance ($R_i$), a hyperpolarizing current pulse (40 pA, 1 s) was injected, the resultant change in membrane voltage was measured, and the $R_i$ calculated according to Ohm’s law. For voltage-clamp recordings, unless otherwise specified, the $V_m$ was clamped at $-70$ mV. A voltage step of 5 mV was applied at $-0.1$ Hz throughout the recording to monitor serial resistances, and the data were discarded if the values varied by more than 20% of the original value, which was usually <20 MΩ. Signals were low-pass filtered at a corner frequency of 2 kHz and digitized at 10 kHz using a Micro 1401 interface running Signal or Spike2 software (Cambridge Electronic Design, Cambridge, UK), respectively, for episode-based capture or continuous recording. To elicit synaptic activity, we delivered a constant-current pulse (50–250 μA; 100 μs) every 10 s through a bipolar stainless steel electrode (FHC, Bowdoinham, ME) locally positioned in the A7 area. To isolate IPSCs, we added 5 mM kynurenic acid to the ACSF, whereas 1 mM strychnine plus 100 μM picrotoxin (Ptx) were added to isolate EPSCs.

To confirm recordings were made from NAergic A7 neurons, the recorded neurons were labeled with biocytin from neurons showing DBH immunoreactivity (Fig. 1, D–E). All data shown hereafter were obtained under a fluorescence microscope (Axioplan 2; Zeiss, Oberkochen, Germany). The sections were examined for DBH and GABABR immunoreactivity on a confocal microscope (Leica TCS SP5) at the same settings for both immunolabels. The captured images were not corrected for contrast or background.

**Single-cell RT-PCR.** For single-cell reverse transcription polymerase chain reaction (RT-PCR) experiments, the patch pipettes were filled with 6–8 μl of autoclaved internal solution (KCl based, see *Electrophysiology* above). After whole cell recording (<10 min), the cell contents (including the nucleus) were aspirated as completely as possible into the patch pipette under visual control and then expelled immediately into 150 μl of lysis buffer (Stratagene, La Jolla, CA), and the total RNA from a single cell was isolated using an Absolutely RNA NanoPrep kit (Stratagene). The total RNA was reverse transcribed into cDNA using SuperScript III RT (Invitrogen, Carlsbad, CA), and the cDNA was used as a template in the multiplex PCR, which included primers for Girk1 (sense: 5'-GCAAGCTGCT-CAAAATTCGG-3', antisense: 5'-TCATCCCTGTGGTGTCCAG-3'), Girk2 (sense: 5'-CGGTGGGGTTTTTATCAG-3', antisense: 5'-TTATCTGGTCTAGGGATTTC-3'), Girk3 (sense: 5'-AGCAAAGACACCCCC-3', antisense: 5'-CTCCAGGTCTCCAGGTCGCC-3'), and DBH (sense: 5'-GACAGGCACCTACTTGGCGGA-3', antisense: 5'-ACCTGCTGTAGTCCGATAG-3'). The thermal cycling conditions for the multiplex PCR were 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 45°C for 1 min, and 72°C for 15 s. The PCR products were then used as the templates for nested PCR amplification using the following primers: Girk1 (sense: 5'-AGCTGCTGAAATCTTCGGAAGGAG-3', antisense: 5'-TCCCTGTTGTTTCCACAGATG-3'), Girk2 (sense: 5'-TGGGTTTTCTATCAAAGTGAATG-3', antisense: 5'-ATCGTGCTCTGGAGTTITCCAC-3'), Girk3 (sense: 5'-AGCAAGACACCCGGCAG-3', antisense: 5'-CAGGTGACGCAGTCCGCC-3'), and DBH (sense: 5'-CAGGACCTACTTGGCGGACG-3', antisense: 5'-ACCTGCTGTAGTCCGATAG-3'). The thermal cycling conditions for the nested PCR were the same as those for the multiplex PCR. After nested PCR amplification, the PCR products were analyzed by electrophoresis on an ethidium bromide-stained 2.5% agarose gel. The predicted sizes of the PCR-produced fragments were 231 bp for Girk1, 301 bp for Girk2, 315 bp for Girk3, and 261 bp for DBH. To avoid detection of genomic DNA, all primer pairs were designed to be intron spanning. As a positive control, midbrain tissue mRNA was extracted and subjected to single-cell RT-PCR amplification as described above. In the negative controls, the pipette touched, but did not impale, the neuron, and the pipette solution was treated in the same way as the test samples, resulting in no detectable band.

**RESULTS**

**NAAergic A7 neurons express functional GABA$_B$ receptors.** To confirm recordings were made from NAAergic A7 neurons, the recorded neurons were labeled with biocytin-avidin-AMCA (Fig. 1C) and stained with antibody against DBH (Fig. 1D). All data shown hereafter were obtained from neurons showing DBH immunoreactivity (Fig. 1, C–E), which also exhibited previously reported physiological properties of NAAergic A7 neurons, including not showing voltage sag and rebound APs on injection of a hyperpolarizing current pulse and showing a prominent voltage-dependent delay in initiation of the first AP on injection of depolarizing current pulses (Min et al. 2008b, 2009, 2010).
In the first series of experiments, we investigated whether NAergic A7 neurons expressed functional GABA B receptors. Recordings were made using patch pipettes filled with K-gluconate-based internal solution, and 5 mM kynurenic acid, 100 μM Ptx, and 1 μM strychnine were added to the bath medium to block glutamatergic, GABAergic, and glycinegic synaptic transmission. The recorded NAergic A7 neurons were injected with a depolarizing current to control their firing frequency at ~1 Hz, although these neurons can fire spontaneously at ~0.5 Hz under resting conditions (Min et al. 2008b). Local perfusion of 1 mM baclofen, a GABA B agonist, through a pipette electrode using air pressure (20 ms, 10–20 psi) resulted in robust hyperpolarization of the Vm and completely prevented firing of NAergic A7 neurons (n = 5 cells) (Fig. 2, A and B). This inhibitory effect of baclofen on NAergic A7 neurons was blocked by addition of 20 μM CGP 54626 to the ACSF, showing that the effect was mediated by baclofen acting at postsynaptic GABA B Rs (Fig. 2, A and B). Consistent with these observations, IHC staining showed that DBH-immunoreactive (ir) A7 neurons (Fig. 2, C1 and C2) were strongly stained with antibodies against the GABA B-R (Fig. 2, C3 and C4).
2, C3 and C4). In addition, some axonal fibers with swollen varicosity-like structures in the A7 area were also found to be GABA<sub>B</sub>-ir (arrows in Fig. 2C3). These staining patterns were consistently observed in all three animals examined. Together, these physiological and morphological results show that functional GABA<sub>B</sub>Rs are present on NAergic neurons and axonal terminals in the A7 area.

To quantify the effects of baclofen, in the next series of experiments, 100 μM baclofen was bath applied for 5 min to reach a stable concentration in the slice (Fig. 3A) and was found to hyperpolarize the V<sub>m</sub> by 27 ± 2 mV (Fig. 3, A and C, V<sub>m</sub> at rest = −46.2 ± 0.8 mV; P < 0.05, paired t-test) and completely abolish spontaneous firing (firing rate at rest: 5.8 ± 2.7 spikes/10 s) in the five NAergic A7 neurons tested (Fig. 3, A and D). The hyperpolarizing effect of baclofen on NAergic A7 neurons was associated with a significant 30 ± 3% reduction in the R<sub>n</sub> (P < 0.05, paired t-test) (Fig. 3, B and E). These results show that the hyperpolarization of NAergic A7 neurons by baclofen was caused by GABA<sub>B</sub>R activation.

**Activation of GABA<sub>B</sub> receptors opens GIRK channels.** We next investigated which K<sup>+</sup> channels were coupled to GABA<sub>B</sub>R activation. In voltage-clamp recording mode, bath application of 100 μM baclofen in 2.5 mM K<sup>+</sup> ACSF evoked an outward current (I<sub>Bac</sub>) in the six NAergic A7 neurons tested (mean amplitude: 43.4 ± 6.8 pA). Since the recordings were made in conditions in which synaptic transmission was blocked, the I<sub>Bac</sub> resulted from direct activation of GABA<sub>B</sub>Rs on NAergic A7 neurons. Voltage ramps (from −120 to −20 mV; duration 1 s) were then applied during the baseline recording, during the I<sub>Bac</sub> peak, and during washout of baclofen (Fig. 4C), and the current-voltage (I-V) relationship for the I<sub>Bac</sub> was obtained by subtracting the membrane currents evoked by the voltage ramp during baseline from that during baclofen application. As shown in Fig. 4D, the I-V relationship for the I<sub>Bac</sub> showed prominent inward rectification and that the I<sub>Bac</sub> was reversed at a V<sub>m</sub> of approximately −99 mV, a value near the theoretical K<sup>+</sup> equilibrium potential (E<sub>K</sub> = −105 mV; Fig. 4E).

When the extracellular K<sup>+</sup> concentration ([K<sup>+</sup>]<sub>i</sub>) in the ACSF was increased to 7.5 mM (prepared by equimolar substitution of K<sup>+</sup> for Na<sup>+</sup>) and the V<sub>m</sub> clamped at −80 mV, application of baclofen induced inward currents (Fig. 4B) that were reversed at a V<sub>m</sub> of approximately −71 mV (Fig. 4D), again near the theoretical E<sub>K</sub> (−77 mV) for a [K<sup>+</sup>]<sub>i</sub> of 7.5 mM (Fig. 4E). These physiological features suggested that the I<sub>Bac</sub> was mediated by GIRK channels. This argument was supported by the results of experiments showing that the amplitude of the I<sub>Bac</sub> was significantly reduced by either of two GIRK channels.
channel blockers, Ba2+ or Tertiapin-Q (Fig. 5, A and B), whereas its reversal potential (E_r) was not affected (Fig. 5B). The peak amplitude of the \( I_{\text{Bac}} \) recorded in the presence of 1 mM Ba2+ or 500 nM Tertiapin-Q was 13.4 ± 2.7 or 22.9 ± 2.4 pA, respectively, significantly lower than the control value (P < 0.001, ANOVA). We also used single-cell RT-PCR to examine the expression profile of GIRK channels in NAergic A7 neurons. Since the most abundant GIRK subtypes in mammalian brain are GIRK1, GIRK2, and GIRK3 (Stanfield et al. 2002), these three subtypes were analyzed. The cytoplasm were harvested from 33 neurons in the A7 area, and DBH mRNA was detected in 27, which were presumed to be NAergic A7 neurons. In these 27 NAergic neurons, GIRK channel mRNAs were detected in 12, 6 of which contained only GIRK1 mRNA, 1 only GIRK3 mRNA, 4 both GIRK1 and GIRK3 mRNAs, and 1 all three GIRK mRNAs (Fig. 5, C and D). These results suggest that heterotetrameric GIRK1/3 channels might dominate functional GIRK channels in NAergic A7 neurons.

**Tonic activation of GABA_ARs on NAergic A7 neurons by ambient GABA.** In current-clamp recording mode, CGP 54626 application not only blocked the effect of baclofen but also increased the baseline firing rate (see Fig. 2, A and B), suggesting there might be GABA_Ar-mediated tonic inhibition of NAergic A7 neurons. To test this possibility, we examined the effect of CGP 54626 application on whole cell currents in NAergic A7 neurons and found that it induced a small, but significant, inward current with an amplitude of 7.5 ± 0.9 pA (n = 8 cells; P < 0.01, paired t-test); similar results were obtained when 20 \( \mu \)M SCH 50911, a competitive GABA_Ar blocker, was used (7.7 ± 0.3 pA, n = 6 cells; P < 0.01, paired t-test) (Fig. 6, A and C). Pooling the CGP 54626 and SCH 50911 data together showed that there was a GABA_Ar-mediated standing current of 7.5 ± 0.5 pA in NAergic A7 neurons. Addition of 10 \( \mu \)M (S)-SNAP 5114 plus 50 \( \mu \)M NNC 711 to the bath medium to block GABA transporters (GAT1 and GAT2) resulted in a significant outward current of 11 ± 0.9 pA.
increase, showing the dependence of the GABABR-mediated
current on the ambient GABA concentration (Fig. 6). We next estimated the ambient GABA concentration in the A7 area in the resting condition by using a protocol modified from Herman and Jhar (2007) that was designed to estimate the ambient glutamate concentration in a hippocampal slice. We first constructed the dose-dependent curve for the whole cell current induced in NAergic A7 neurons by baclofen, a selective GABABR agonist not taken up by GABA transporters. As shown in Fig. 6, D and E1, application of various concentrations of baclofen induced a maximum whole cell current of 47 ± 4.2 pA, with an EC50 of 30 μM. Similarly, application of various concentrations of GABA resulted in a maximum whole cell current of 42 ± 5 pA, with an EC50 of 33 μM. Superimposition of the two curves shows great consistency between the dose-dependent effects of baclofen and GABA (Fig. 6E2). This observation is in line with the similar binding affinities of baclofen and GABA for the GABABR (Bowery 1993). The standing current of 7.5 pA accounted for 0.8% of the baseline value (Fig. 6F). This observation is in line with the similar binding affinities of baclofen and GABA for the GABABR (Bowery 1993). The standing current on the ambient GABA concentration (Fig. 6).

Since GABA transporters might not be fully operational at room temperature, it might be argued that the ambient GABA concentration is much lower and that GABABR-mediated tonic inhibition may not exist at a physiological temperature. To address this argument, we performed current-clamp recording with the recording temperature raised to 34°C. As shown in Fig. 7A, bath application of 100 (i) or 200 μM (ii) GABA hyperpolarized the Vm and suppressed the spontaneous firing of NAergic A7 neurons. This inhibitory effect of GABA was also blocked by CGP 54626 (Fig. 7A), showing that NAergic A7 neurons were subject to tonic inhibition mediated by GABABR. Interestingly, addition of CGP 54626 to the bath not only blocked the inhibitory effect of GABA but also enhanced the spontaneous firing of NAergic A7 neurons (Fig. 7A and B), showing that NAergic A7 neurons were subject to tonic inhibition mediated by GABABR at physiological temperature. In all 10 NAergic A7 neurons tested, the spontaneous firing rate was increased in the presence of CGP 54626 to 126.9 ± 6.5% of the baseline value (P < 0.0001, paired t-test) (Fig. 7B).

GABA receptors exert presynaptic inhibition on NAergic A7 neurons. Since the IHC results showed the presence of GABABR-ir axonal terminals in the A7 area, we examined whether synaptic transmission onto NAergic A7 neurons was subject to presynaptic modulation by GABABR. A bipolar stainless steel stimulating electrode was locally placed in the
A7 area to evoke synaptic activity in NAergic A7 neurons, recordings were made using pipettes filled with Cs-gluconate-based solution, and Ptx and strychnine were added to the ACSF to isolate glutamatergic synaptic transmission. As shown in Fig. 8A, the glutamate receptor dl-/H9251-amin-3-hydroxy-5-methylisoxazole propionic acid subtype (AMPAR)-mediated EPSC showed a linear I-V relationship when recording was made with the addition of 50 /H9262 M APV, a blocker of the glutamate receptor N-methyl-D-aspartate subtype (NMDAR), to the bath (Fig. 8, A1 and A2) and was completely blocked by DNQX, a blocker of the AMPAR, as shown in Fig. 8A3, in which the Vm was clamped at -70 mV. In another series of experiments, in which DNQX was added to the bath to block AMPARs, extracellular stimulation evoked an outward synaptic current that was voltage dependent (Fig. 8, B1 and B2), and this activity was completely blocked by application of APV, as shown in Fig. 8B3, in which the Vm was clamped at +50 mV. These results show that the EPSCs evoked in NAergic A7 neurons are mediated by both NMDARs and AMPARs. Application of baclofen significantly reduced the amplitude of the EPSCs to 62.7 /H11006 7.5% of control levels (n = 10; P < 0.01, paired t-test) (Fig. 8C). Moreover, this inhibitory effect of baclofen was associated with an increase in the paired-pulse ratio (PPR; control: 1.03 ± 0.08, baclofen: 1.34 ± 0.09; P < 0.05, paired t-test) (Fig. 8D), a measure of presynaptic modulation (Zucker and Regehr 2002), showing presynaptic modulation of EPSCs by GABABRs. Coapplication of CGP 54626 antagonized the effect of baclofen on the EPSC amplitude and PPR (Fig. 8, C and D). These results suggest the presence of functional GABABRs on the excitatory terminals on the NAergic A7 neurons.
neurons. Although addition of CGP 54626 to the ACSF blocked the effect of baclofen, it did not enhance basal excitatory synaptic transmission to any significant extent (Fig. 8, C and D), showing that GABABR-mediated tonic inhibition of excitatory synaptic transmission in NAergic A7 neurons did not occur.

To explore a possible physiological role of GABABRs in excitatory synaptic transmission, we delivered tetanus stimulus consisting of 5 pulses at 50 Hz or 10 pulses at 100 Hz, followed by a test pulse 30 ms later. We hypothesized that this tetanus stimulus might recruit more GABAergic terminals neighboring the activated glutamatergic terminals and result in release and spillover of sufficient GABA to activate GABABRs located on glutamatergic terminals, thereby inhibiting the EPSCs evoked by the subsequent test pulse. Figure 8E shows the results of a representative experiment and demonstrates that the amplitude of the EPSCs evoked by the test pulse was significantly smaller than that evoked by the first pulse of the tetanus stimulation, an effect that could possibly be ascribed to either depletion of synaptic vesicles by tetanus stimulation or inhibition by GABA released from neighboring GABAergic terminals. However, application of CGP 54626 did not affect the amplitude of the EPSCs evoked by the test pulse (Fig. 8E), showing that it was not inhibited by GABABRs. No significant effect of CGP 54626 on the test pulse EPSC amplitude was observed in any of six NAergic A7 neurons (paired t-test).

GABAB receptors play a role in autoregulation of GABA release from inhibitory terminals making contact with NAergic A7 neurons. When kynurenic acid was added to block glutamatergic transmission and recordings were made using patch pipettes filled with CsCl-based solution, IPSCs were evoked in NAergic A7 neurons, with a reversal potential of ~0 mV (Fig. 9A), close to the theoretical $E_{Cl}$ (~0.5 mV) under our experimental conditions. These IPSCs were partially blocked
by application of strychnine, a glycine receptor (GlyR) blocker, and were completely blocked by subsequent application of strychnine and Ptx, a GABAAR blocker (Fig. 9B), and similar results were obtained when Ptx was applied first, followed by Ptx plus strychnine (data not shown), showing that IPSCs in NAergic A7 neurons are mediated by both GABAARs and GlyRs. The relative contributions of GlyR- and GABAAR-mediated components to the total IPSC amplitude were estimated as 27/731 and 69/7315%, respectively (Fig. 9B).

Application of 10 μM baclofen dramatically reduced the amplitude of the IPSCs to 36.4 ± 7.6% of baseline (n = 5 cells; P < 0.05, paired t-test) and increased the PPR (control vs. baclofen: 1.11 ± 0.10 vs. 1.37 ± 0.14; P < 0.01, paired t-test) (Fig. 9D), and both effects were antagonized by addition of CGP 54626 to the ACSF. Consistent with the PPR results, 10 μM baclofen significantly increased the interevent interval of GABAergic mIPSC by 67.8 ± 21.6% (n = 5 cells; P < 0.05, paired t-test) but unexpectedly caused a 17.7 ± 10.8% decrease in the amplitude, although this was nonsignificant (P = 0.194, paired t-test) (Fig. 9, E and F). Interestingly, baclofen did not affect either the amplitude or the interevent interval of glycinergic mIPSC, the respective values for the baseline and during baclofen application being 13.6 ± 1.2 and 11.9 ± 0.7 pA for the amplitude and 5.6 ± 1.7 and 5.9 ± 0.9 s for the interevent interval (Fig. 9, E and F). These results show that functional GABABRs are present on the GABAergic terminals, but not the glycinergic terminals, that synapse on NAergic A7 neurons. We adopted a similar approach to that described above to explore a possible physiological role of GABABRs on inhibitory terminals. Tetanus stimulus consisting of 10 pulses at 10 Hz was delivered, and the amplitudes of the IPSCs evoked by the tenth pulse in the control conditions...
and in the presence of CGP 54626 were compared. As shown in Fig. 9G, under control conditions, the amplitude of the IPSCs evoked by the tenth pulse was significantly smaller than that evoked by the first pulse of tetanus stimulation, which again might possibly be ascribed to either depletion of synaptic vesicles by tetanus stimulation or inhibition by GABA released upon tetanus stimulation. Addition of CGP 54626 significantly increased the amplitude of the IPSC evoked by the tenth pulse to 162/25% of the value under control conditions (n = 6 cells; \(P < 0.001\), paired t-test) (Fig. 9G), showing that the IPSCs induced by the test pulse were inhibited by GABA acting at GABA\(_B\)Rs.

**DISCUSSION**

In this study, we found that NAergic A7 neurons expressed functional GABA\(_B\)Rs and GIRK1 and GIRK3 channel proteins and that activation of GABA\(_B\)Rs opened these channels to suppress the excitability of NAergic A7 neurons. We also report an ambient GABA concentration of 2.8 \(\mu M\) in the A7 area, which continuously activated GABA\(_B\)Rs to exert post-synaptic tonic inhibition on NAergic A7 neurons. In addition, GABA\(_B\)Rs were shown to be expressed on excitatory and inhibitory terminals on NAergic A7 neurons. However, although they exerted autoregulation of GABA release from inhibitory terminals, the physiological role of GABA\(_B\)Rs located on excitatory terminals making contact with NAergic A7 neurons is not clear.

Our argument for a tonic inhibition of NAergic A7 neurons is based on the recording of the GABA\(_B\)R-mediated standing current, the amplitude of which depended on GABA uptake, a crucial determination of the ambient GABA concentration. This tonic inhibition of NAergic A7 neuron was physiological,
since when recorded at a temperature (34–35°C) close to physiological, the neuronal firing rate was still significantly increased when GABA<sub>B</sub>Rs were blocked by CGP 54626. The peak synaptic cleft GABA concentration has been estimated to be >500 μM (Draguhn and Heinemann 1996; Isaacson et al. 1993; Roepstorff and Lambert 1994), and, since we found that 300 μM GABA completely blocked the spontaneous firing of NAergic A7 neurons (data not shown), GABA<sub>B</sub>Rs that mediated tonic inhibition should be located outside the synaptic cleft, where the ambient GABA concentration is low but sufficient for tonic GABA<sub>B</sub>R activation. Using a procedure modified from that described by Herman and Jhar (2007), we estimated an ambient GABA concentration of 2.8 μM in the A7 area, which is consistent with the value reported in the hippocampus using a microdialysis probe (Lerma et al. 1986). Since this ambient GABA concentration did not significantly affect the amplitude and shape of GABAergic mIPSC, any desensitization of the GABA<sub>A</sub> receptors that were located at synaptic sites and mediated phasic transmission was minor.

Activation of postsynaptic GABA<sub>B</sub>Rs was coupled to GIRK channels, since baclofen induced an outward current that exhibited inward rectification and was reversed at a V<sub>m</sub> near the theoretical E<sub>K</sub> when tested at two different [K<sup>+</sup>]o values and was blocked by the GIRK channel blockers Ba<sup>2+</sup>- and Tertiapin-Q. The pore-forming α-subunit of the GIRK channel family consists of four subtypes, GIRK1, GIRK2, GIRK3, and GIRK4, with the most abundant subtype in the mammalian brain being GIRK1, GIRK2, and GIRK3, which assemble into a functional GIRK channel made up of homotetramers (GIRK2) or heterotetramers (GIRK1/2, GIRK1/3, and GIRK2/3) (Hedin et al. 1996; Kofuji et al. 1995; Kravpivinsky et al. 1995; Stanfield et al. 2002). Although it is widely believed that most neuronal GIRK channels contain GIRK1 and GIRK2 (Karschin et al. 1996; Lucher et al. 1997; Signorini et al. 1997), our single-cell RT-PCR results suggested that GIRK1/3 (rate detected: 4 of 27 cells) might play a more important role than GIRK1/2/3 (1 of 27 cells) does in NAergic A7 neurons. The existence of functional GIRK channels lacking GIRK2 has been reported in many types of central nervous system neuron, such as NAergic LC neurons (Torrecilla et al. 2002), basket and stellate cells in the cerebellum (Aguado et al. 2008), and Bergman glia (Fernandez-Alacid et al. 2009). Furthermore, there is evidence for oligomerization between GABA<sub>B</sub>R and GIRK1/3 channels (Ciruela et al. 2010). All of these findings suggest the existence of functional GIRK1/3 channels in some types of neurons. In our study, the most frequently detected type of GIRK mRNA in NAergic A7 neurons was GIRK1 mRNA alone (6 of 27 cells). GIRK1 homotetramers have been shown not to be functional (Hedin et al. 1996; Kofuji et al. 1995; Kravpivinsky et al. 1995) because of their lack of a forward endoplasmic reticulum trafficking signal, which prevents their trafficking to the plasma membrane. However, GIRK1 channels can be trafficked to the plasma membrane once they assemble with GIRK2, which has various trafficking signals in its intracellular NH<sub>2</sub>- and COOH-terminal domains (Kravpivinsky et al. 1995; Liao et al. 1996; see Lujan et al. 2009 for review), or with GIRK3, which has been proposed to regulate GIRK channel trafficking by delivering channels to lysosomes (Ma et al. 2002) or through interactions with nexin 27 protein (Lunn et al. 2007; Ma et al. 2002). It is therefore unusual that GIRK1 mRNA alone was the most frequently detected expression pattern. However, the success rate of harvesting mRNAs for proteins of interest depends on the relative number of their mRNA copies in the cytoplasm. GIRK1 mRNA was detected in 11, GIRK3 mRNA in 5, and GIRK2 mRNA in 1 of the 27 cells analyzed, but this might reflect the order of the number of mRNA copies, with GIRK1 > GIRK3 > GIRK2. Since the only combinations of mRNAs seen in our study were GIRK1/2/3 and GIRK1/3, it is possible that in the six cases in which GIRK1 alone was detected, GIRK3 and/or GIRK2 might also have been expressed but not detected. However, we cannot rule out the possibility that there might be an as yet unidentified mechanism for the trafficking of GIRK1 homotetramers in NAergic A7 neurons.

In addition to DBH-ir neurons, axonal fibers with varicosity-like structures in the A7 area also showed GABA<sub>B</sub>R immunoreactivity. This staining pattern indicates that the GABA<sub>B</sub>R might be located at presynaptic terminals and modulate synaptic transmission onto NAergic A7 neurons. Indeed, bath application of baclofen dramatically reduced the amplitude of EPSCs evoked in NAergic A7 neurons and increased their PPR, an indicator of presynaptic modulation (Zucker and Regehr 2002). Surprisingly, although it blocked the effects of baclofen, application of CGP 54626 itself did not have any detectable effect on the amplitude and PPR of EPSCs. It also did not affect the amplitude of EPSCs preceded by conditioned stimulation of 5–10 pulses at 100 Hz, which is designed to result in more GABA release. It could be that the ambient GABA concentration around the excitatory terminals is too low to activate presynaptic GABA<sub>B</sub>Rs. As in the brain stem and spinal cord (Jonas et al. 1998; Russier et al. 2002; Yang et al. 1997), the IPSCs evoked in NAergic A7 neurons consisted of GABA<sub>A</sub>-R- and GlyR-mediated components. An interesting finding was that baclofen affected GABAergic mIPSC, but not glycinegic mIPSC, which might be ascribed to the facts that GABA and glycine are released from different terminals and that GABA<sub>B</sub>Rs are only located on GABAergic terminals. Baclofen also dramatically reduced the amplitude and increased the PPR of the evoked IPSCs. Application of CGP 54626 blocked the effects of baclofen but did not further enhance the amplitude and PPR of the IPSCs. However, it did increase the amplitude of the IPSCs evoked by a train of stimulation of 10 pulses at 10 Hz. Clearly, the ambient GABA concentration around the GABAergic terminals is not sufficient under resting conditions to continuously activate presynaptic GABA<sub>B</sub>Rs; however, during the repeated release of GABA by high-frequency stimulation, ambient GABA around inhibitory terminals can accumulate to a sufficient level to activate presynaptic GABA<sub>B</sub>Rs, which, in turn, exert an autoregulatory effect on GABA release. Together, our results show that it is postsynaptic, but not presynaptic, GABA<sub>B</sub>Rs that are activated by ambient GABA. This might be attributed to the fact that postsynaptic GABA<sub>B</sub>Rs are bathed in a higher ambient GABA concentration than presynaptic GABA<sub>B</sub>Rs, due to the differences in the distance between GABA-releasing sites and GABA<sub>B</sub>Rs in the different locations, in the sensitivity of pre- and postsynaptic signal transduction mechanisms, and/or in the GABA reuptake mechanisms that may not be homogeneously distributed.

In conclusion, we have shown that presynaptic GABA<sub>B</sub>Rs on inhibitory axonal terminals, which might be coupled to G<sub>G</sub>A
protein, exert autoregulation of GABA release through the downregulation of voltage-dependent calcium channels. We also have shown that the postsynaptic GABA\(_{B}\)Rs, possibly coupled to \(G_\beta\) protein, are continuously activated by ambient GABA to mediate tonic inhibition of NAergic A7 neurons through the opening of GIRK1/3 channels. Although we did not observe any physiological role for presynaptic GABA\(_{B}\)Rs on excitatory axonal terminals, it should be borne in mind that the ambient GABA concentration would be considerably lower in slice preparations than in the living brain, since the boundary layer of GABA diffusing from the synaptic cleft is removed by continuous perfusion of the slice with medium. Thus, although no physiological roles of presynaptic GABA\(_{B}\)Rs were observed in the slices, this might not be the situation in the A7 area in the living brain. This also means that tonic inhibition by postsynaptic GABA\(_{B}\)Rs might be greater in the living brain than in brain slices. The source of the GABAergic axonal terminals that synapse on NAergic A7 neurons has not yet been identified. In the LC (NAergic A6 neurons), a few GABAergic interneurons are found around the pericereular dendritic zone of NAergic A6 neurons, and inhibitory synaptic connections between these GABAergic and NAergic neurons have been demonstrated (Aston-Jones et al. 2004). In the A7 area, non-NAergic interneurons are scattered among the NAergic neurons and make synaptic contacts with them (Min et al. 2008b). These non-NAergic neurons are presumed to be GABAergic (Bajic et al. 2001; Holden and Proudfoot 1998; Nuseir and Proudfoot 2000) and are proposed to be subject to inhibitory control by enkephalin released from neurons in the RVM and ventrolateral PAG. Activation of these nuclei of descending analgesic pathways would relieve the tonic inhibition of NAergic neurons in the A7 area, which, in turn, would increase NA release in the dorsal spinal cord (Bajic et al. 2001; Holden et al. 1999; Nuseir and Proudfoot 2000). Since the RVM and PAG also release endogenous opioids into the dorsal spinal cord, the release of NAergic A7 neurons from tonic inhibition through the inhibitory inputs from the RVM and PAG on GABAergic interneurons in the A7 area might ensure the simultaneous release of NA and endogenous opioids and thus the synergistic activation of \(\alpha_2A\)-adrenoceptors and \(\mu\)-opioid receptors in the dorsal spinal cord. As a result, effective analgesia can be produced by very low concentrations of endogenous opioids in physiological conditions.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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