

GABA_B receptor-mediated modulation of hypocretin/orexin neurones in mouse hypothalamus

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Hypocretin/orexin (Hcrt) is a critical neurotransmitter for the maintenance of wakefulness and has been implicated in several other functions, including energy metabolism and reward. Using whole-cell patch-clamp recordings from transgenic mice in which enhanced green fluorescent protein was linked to the Hcrt promoter, we investigated GABAergic control of the Hcrt neurones in hypothalamic slices. Bath application of GABA or muscimol caused an early hyperpolarization mediated by Cl⁻ and a late depolarization mediated by the efflux of bicarbonate. These GABA_A receptor-mediated responses were blocked by picrotoxin and bicuculline. Under the GABA_A blockade condition, GABA produced consistent hyperpolarization, decreased firing rate and input resistance. The selective GABA_B agonist (*R*)-baclofen caused a similar response with an EC₅₀ of 7.1 μM. The effects of (*R*)-baclofen were blocked by the GABA_B antagonist CGP 52432 but persisted in the presence of tetrodotoxin, suggesting direct postsynaptic effects. The existence of GABA_B modulation was supported by GABA_{B(1)} subunit immunoreactivity on Hcrt cells colabelled with antisera to the Hcrt-2 peptide. Furthermore, GABA_B receptor activation inhibited the presynaptic release of both glutamate and GABA. (*R*)-Baclofen depressed the amplitude of evoked excitatory postsynaptic currents (EPSCs) and inhibitory synaptic currents (IPSCs), and also decreased the frequency of both spontaneous and miniature EPSCs and IPSCs with a modest effect on their amplitudes. These data suggest that GABA_B receptors modulate Hcrt neuronal activity via both pre- and postsynaptic mechanisms, which may underlie the promotion of non-rapid eye movement sleep and have implications for the use of GABA_B agonists in the treatment of substance addiction through direct interaction with the Hcrt system.

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The control of sleep and wakefulness is thought to be mediated through an interaction between a distributed set of sleep-promoting and wake-promoting systems in the brain. The sleep-promoting system is primarily GABAergic in nature, with the relevant cell bodies located in the ventrolateral and median preoptic area. In contrast, the monoaminergic systems in the brainstem and the cholinergic systems in both the brainstem and basal forebrain have been recognized as wakefulness-promoting systems for some time (Brown *et al.* 2001; Saper *et al.* 2001; Jones, 2003; McGinty & Szymusiak, 2003). More recently, the hypocretin/orexin (Hcrt) system of the hypothalamus (De Lecea *et al.* 1998; Sakurai *et al.* 1998) has been identified as a powerful wakefulness-promoting system (Kilduff & Peyron, 2000), initially as a result of its dysfunction in the sleep disorder narcolepsy both in animals (Chemelli *et al.* 1999; Lin *et al.* 1999; Hara *et al.*

2001) and humans (Nishino *et al.* 2000; Peyron *et al.* 2000; Thannickal *et al.* 2000). The Hcrt neurones project to (Peyron *et al.* 1998; Date *et al.* 1999) and excite the monoaminergic (Horvath *et al.* 1999; Bayer *et al.* 2001; Brown *et al.* 2001; Eriksson *et al.* 2001; Yamanaka *et al.* 2002) and cholinergic (Eggermann *et al.* 2001; Burlet *et al.* 2002; Wu *et al.* 2004) nuclei. Moreover, the Hcrt neurones have been shown to directly excite thalamocortical synapses (Lambe & Aghajanian, 2003), a mechanism by which Hcrt neurones excite the prefrontal cortex and play a role in attention and alertness. In turn, the Hcrt neurones are thought to receive inhibitory GABAergic input from the preoptic area (Sakurai *et al.* 2005; Saper *et al.* 2005). Thus, the transition from wakefulness to sleep is likely mediated by a GABAergic inhibition of the wake-promoting Hcrt cells, which results in disfacilitation of the wake-promoting monoaminergic and cholinergic systems.

Given the central role of the Hcrt system in the regulation of wakefulness, the afferent inputs to the Hcrt cells and the response of these neurones to various neuroactive substances have been the focus of considerable recent research. Anatomical studies have indicated afferent inputs to the Hcrt neurones from the amygdala, the cholinergic neurones and GABAergic neurones in the basal forebrain, and serotonin (5-HT) neurones in the median/paramedian raphe nuclei (Sakurai *et al.* 2005; Yoshida *et al.* 2005). The creation of mice in which enhanced green fluorescent protein (EGFP) is under control of the *prepro-orexin* promoter (Yamanaka *et al.* 2003b) has allowed visualization of the Hcrt/EGFP cells and thus facilitated *in vitro* physiological studies of these neurones and identification of the neurotransmitters and neuromodulators to which these cells respond. Using these mice, the Hcrt cells have been shown to be excited by glutamate through both NMDA and AMPA receptors (Li *et al.* 2002; Yamanaka *et al.* 2003a), by ghrelin (Yamanaka *et al.* 2003b), by glucagon-like peptide 1 (Acuna-Goycolea & van den Pol, 2004), and by cholecystokinin (CCK) through the CCK-A receptor (Tsujino *et al.* 2005). In contrast, Hcrt cells can be inhibited by 5-HT acting through the 5-HT_{1A} receptor (Muraki *et al.* 2004), by noradrenaline through both α_1 and α_2 receptors (Li & van den Pol, 2005), by neuropeptide Y through the Y1 receptor (Fu *et al.* 2004), by leptin (Yamanaka *et al.* 2003b), by glucose (Yamanaka *et al.* 2003b; Burdakov *et al.* 2005), and by GABA through the GABA_A receptor (Li *et al.* 2002; Yamanaka *et al.* 2003b). Thus, the Hcrt cells seem to be under tight regulation by a combination of fast-acting neurotransmitters and slower-acting neuropeptides (Horvath & Gao, 2005).

In the present study, we have focused on GABAergic inputs to the Hcrt cells since the GABAergic system has both fast neurotransmission, mediated through the GABA_A receptors, and slower neurotransmission, mediated through GABA_B receptors. As indicated above, previous studies have examined GABA_A signalling in Hcrt cells, but the GABA_B system has not been investigated to this point. In the present study, we demonstrate that Hcrt cells are inhibited by both GABA_A and GABA_B receptor activation, anatomically document the presence of GABA_B receptors on Hcrt cells, and determine that GABA_B receptors modulate neurotransmission onto Hcrt cells through both presynaptic and postsynaptic mechanisms. In contrast to the transient inhibition mediated through GABA_A receptors, we suggest that the prolonged inhibition of Hcrt cells mediated by GABA_B signalling may underlie longer-term changes in behaviour and physiology in which the Hcrt cells are involved, such as the transition from wakefulness to sleep. A preliminary report of these data has appeared (Xie *et al.* 2004).

Methods

Animals

All experimental procedures involving animals were approved by the Animal Care and Use Committee at SRI International and were in accordance with NIH guidelines and the University of Tsukuba Animal Resource Center. All efforts were made to minimize animal suffering or discomfort and to reduce the number of animals used.

Slice preparation

Mouse brain slice preparation containing the lateral hypothalamic area (LHA) and the recording conditions followed the procedures previously described (Yamanaka *et al.* 2003a,b). Male and female Hcrt/EGFP mice, 3–6 weeks old, in which the human *prepro-orexin* promoter drives expression of EGFP were used for experiments. Mice were deeply anaesthetized with methoxyflurane and then decapitated. Brains were isolated in ice-cold oxygenated high sucrose solution containing (mM): sucrose 220, KCl 2.5, MgCl₂ 6, CaCl₂ 1, NaHCO₃ 26, NaH₂PO₄ 1.25, bubbled with 95% O₂–5% CO₂. Brain slices (250 μ m thickness) were cut coronally with a microtome (VT-1000S, Leica, Germany). Slices containing the LHA area were then transferred to an incubation chamber where they were superfused with physiological bicarbonate solution containing (mM): NaCl 130, KCl 5, CaCl₂ 2.4, MgSO₄ 1.3, NaHCO₃ 20, KH₂PO₄ 1.25, glucose 10, bubbled with 95% O₂–5% CO₂ at room temperature (RT; 22–24°C) for at least 1 h before recordings. The osmolarity of these external solutions was checked by a vapour pressure osmometer (Advanced Instruments, Norwood, MA, USA) and ranged between 290 and 310 mosmol l⁻¹.

Whole-cell patch clamp recordings

Patch pipettes were prepared from borosilicate capillary glass (GC150-10, Harvard Apparatus, Holliston, MA, USA) with a micropipette puller (P-97, Sutter Instruments, Novato, CA, USA). The pipettes were routinely filled with a KCl-internal solution containing (mM): KCl 145, MgCl₂ 1, EGTA-Na₃ 1.1, Hepes 10, Na₂ATP 2, Na₂GTP 0.5, adjusted to pH 7.2 with KOH. Some experiments were conducted using a potassium methanesulphonate (K-MeSO₄) internal solution containing (mM): K-MeSO₄ 144, Hepes 20, EGTA 0.2, NaCl 2.8, Na₂ATP 2, Na₂GTP 0.5, pH 7.2–7.4. Osmolarity of these internal solutions was between 280 and 290 mosmol l⁻¹. Pipette resistance measured in the external solution was 3–10 M Ω . The series resistance during recording was 10–25 M Ω and was compensated only in current-clamp mode. The reference electrode was an Ag–AgCl pellet immersed in the bath solution.

For electrophysiological recording, a single slice was transferred to a recording chamber (RC-271, Warner Instruments LLC, Hamden, CT, USA) and was superfused with the physiological bicarbonate solution at RT. Some experiments were conducted in a Hepes external solution containing (mM): NaCl 150, KCl 2.5, CaCl₂ 2, MgCl₂ 2, NaH₂PO₄ 1.3, Hepes 10, and glucose 10, pH 7.4 with NaOH. The liquid junction potential of the patch pipette with the KCl solution and perfused Hepes external solution was estimated to be 3–5 mV and was not corrected for in the data analysis.

Hcrt/EGFP neurones were visualized under an upright microscope (Leica DM LFSA, Leica Instruments) using both infrared-differential interference contrast (IR-DIC) microscopy and fluorescence microscopy. Infrared images were acquired via a charge-coupled device (CCD) camera optimized for infrared wavelengths (DAGE-MITI, Michigan City, IN, USA); fluorescence images were acquired using a digitizer system (Leica LEI-750D, Leica Instruments). Recording pipettes were advanced towards individual fluorescent cells in the slice under positive pressure and, on contact, tight seals between the pipette and the cell membrane on the order of 0.5–1.0 GΩ were made by negative pressure. The membrane patch was then ruptured by suction and membrane potential or current was monitored using an Axopatch 1D patch clamp amplifier (Molecular Devices, formerly Axon Instruments, Union City, CA, USA). Only cells in which the resting membrane potential was –40 mV or more negative and had an input resistance greater than 200 MΩ were included in this study.

In current-clamp mode, hyperpolarizing or depolarizing current pulses (20–30 pA, 800 ms durations) were applied to cells at 5 s intervals from the resting membrane potential of 60 mV adjusted with direct current (DC), unless specified otherwise. The membrane input resistance was estimated by dividing the electronic hyperpolarizing potential by the injected current.

Evoked excitatory and inhibitory postsynaptic currents (eEPSCs) and (eIPSCs) were recorded from Hcrt/EGFP neurones under voltage-clamp at –60 mV using a KCl internal solution. Under this recording condition, both eEPSCs and eIPSCs were recorded as inward currents. Electrical stimuli (100–200 μA, 0.1 ms, 0.1 Hz) were generated using bipolar stimulation electrodes (TM33A05, World Precision Instruments, Sarasota, FL, USA) placed within the LHA. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded using the KCl internal solution supplemented with the sodium channel blocker QX-314 (1 mM) to inhibit action potentials in the neurone and with bicuculline (BIC; 20 μM) and picrotoxin (PTX; 100 μM) added to the physiological bicarbonate solution to block GABA_A receptor-mediated neurotransmission. Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded

using the same pipette solution but in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) and DL-2-amino-5-phosphono-pentanoic acid (AP-5, 50 μM) to block AMPA and NMDA receptor-mediated neurotransmission. Miniature EPSCs were recorded using KCl internal solution in the presence of 1 μM TTX and BIC (30 μM) and PTX (100 μM). Miniature IPSCs were recorded using a KCl internal solution in the presence of 1 μM TTX, 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 50 μM AP-5. Most drug applications were via bath perfusion. In some experiments, local drug applications were employed through a thin polyethylene tube positioned near the cells recorded.

The output voltage signal was low pass filtered at 5 kHz and digitized at 10 kHz, while the output current signal was low pass filtered at 1 kHz and digitized at 5 kHz. Data were recorded on a computer through a Digidata 1322A A/D converter using pCLAMP software version 9.2 (Molecular Devices, formerly Axon Instruments, Union City, CA, USA). Frequencies and amplitudes of spontaneous and miniature synaptic events were measured using Mini Analysis Program version 6.0 (Synaptosoft, Inc., Decatur, GA, USA) from records before, during and after washout of the drug. Only those events with amplitudes > 10 pA were used. Electrophysiological traces were processed for presentation using Origin 7.5 (OriginLab Corp., Northampton, MA, USA) and Canvas 9.0 software (Deneba Systems, Miami, FL, USA).

Drugs

GABA, baclofen, tetrodotoxin (TTX), *N*-2,6-dimethylphenylcarbamoylmethyl-triethylammonium bromide (QX-314), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 6,7-dinitroquinoxaline-2,3-dione (DNQX), DL-2-amino-5-phosphono-pentanoic acid (AP-5), BIC and PTX, and most laboratory reagents were purchased from Sigma (St Louis, MO, USA), and CGP 52432 was purchased from Tocris (UK). In the electrophysiological experiments, all drugs were made in stock solution (10–100 mM), freshly dissolved in external solution and applied either by bath application or by local application.

Immunohistochemistry

Adult male mice C57BL/6J (20–25 g, Charles-River, San Diego, CA, USA) were anaesthetized with sodium pentobarbital (50 mg kg⁻¹, i.p.) and perfused sequentially with 10 ml chilled saline and 30 ml of chilled 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were removed and immersed in the same fixative solution for 4 h and were then transferred to 30% sucrose solution for 2 days at 4°C. The brains were quickly frozen in embedding solution with O.C.T. compound (Sakura Finetechnical Co. Ltd, Tokyo, Japan). To visualize

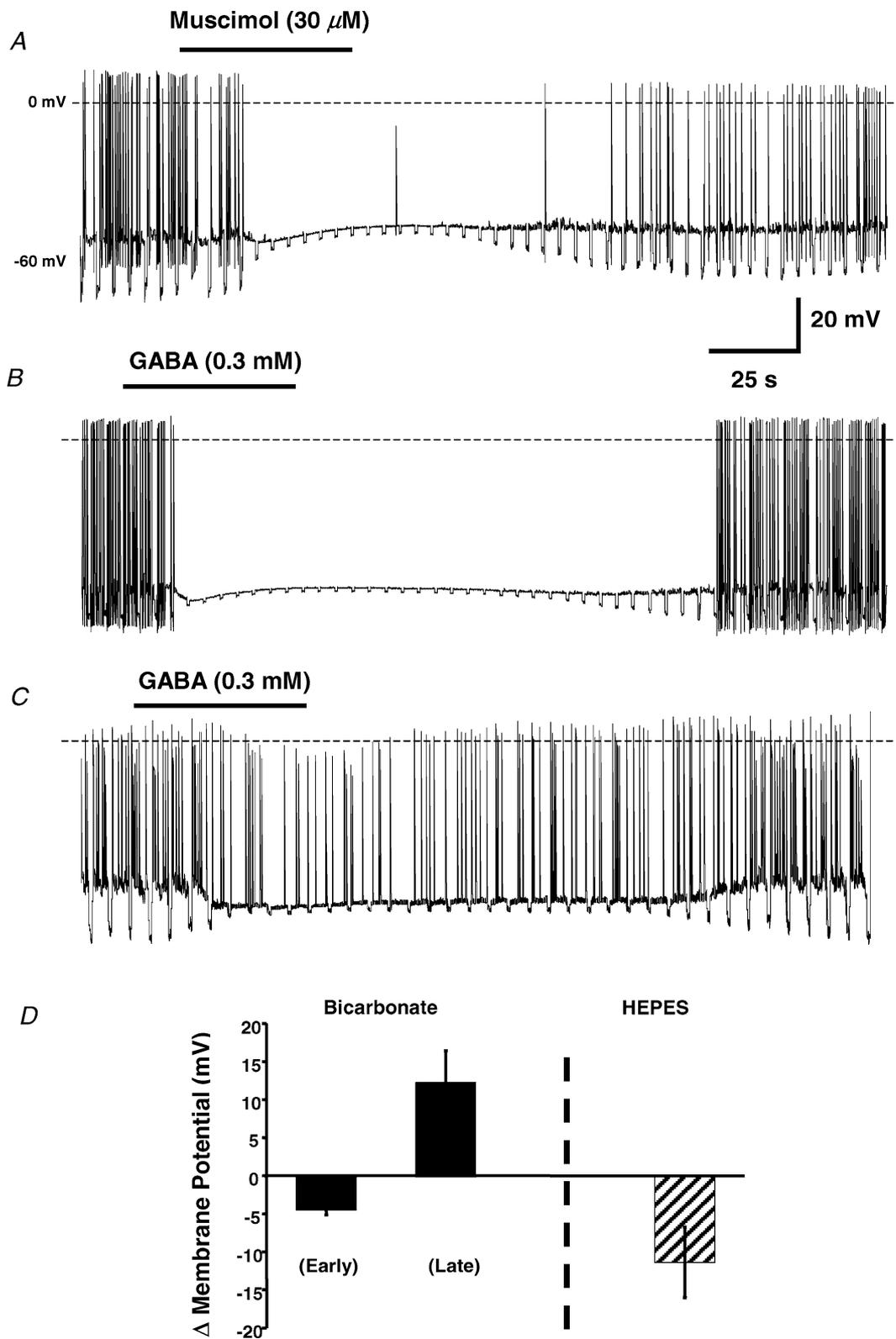


Figure 1. Effects of GABA on membrane potential and firing of action potentials of Hcrt/EGFP neurones in mouse hypothalamic slices

A, when superfused with the physiological bicarbonate solution, bath application of muscimol (30 μ M) induced an early small membrane hyperpolarization followed by a later depolarization associated with a dramatic decrease in the input resistance. Consequently, spontaneous firing of action potentials was blocked in the Hcrt neurone.

hypocretin-2 (Hcrt-2) labelling, cryostat sections of the lateral hypothalamus (20 μm) were incubated overnight at 4°C in primary antisera (goat anti-orexin B, 1 : 5000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by a 60 min incubation in a blocking solution (3% normal donkey serum in phosphate-buffered saline (PBS) with 0.3% Triton X-100 (3% NDST)), rinsed 3 times using a 1% NDST solution, and visualized following a 2-h incubation with a secondary antibody containing a red fluorophore (donkey antigoat Alexa 488, Molecular Probes, 1 : 750 in PBS). To determine GABA_{B(1)} subunit expression, the same sections were again incubated overnight at 4°C in primary antisera, this time directed against GABA_{B(1)} subunit (guinea pig anti-GABA_{B(1)}, 1 : 2500; Chemicon, Temecula, CA, USA), followed by a 60-min incubation in a blocking solution (3% normal goat serum in PBS with 0.3% Triton X-100 (3% NGST)) and three rinses with a 1% NGST solution. GABA_{B(1)} subunit expression was visualized following a 2 h incubation in secondary antisera conjugated against a green fluorophore (Alexa 546 goat antiguinea pig, Molecular Probes, 1 : 750 in PBS). Slides were air dried and cover-slipped using Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Sections were observed using a light microscope (Leica DM5000B) equipped for fluorescence and with a CCD video camera operating with a computer-based anatomical mapping and image-analysis system (StereoInvestigator, Microbrightfield, Inc., Williston, VT, USA). Specificity of both antibodies was confirmed using a preadsorption with each respective peptide and observing no signal.

Statistical analysis

Data are presented as means \pm s.e.m. and were analysed by two-way ANOVA followed by *post hoc* analysis of significance by Fisher's protected least significant difference test using the StatView 4.5 software package (Abacus Concepts, Berkeley, CA, USA). Probability (*P*) values less than 0.05 were considered statistically significant.

Results

The Hcrt/EGFP neurones were visually identified under fluorescence illumination and patch recordings of the

same neurones were made using IR-DIC. Under control conditions in current clamp mode, Hcrt/EGFP neurones displayed spontaneous firing of action potentials at 0.5–3 Hz when the slices were perfused with the physiological bicarbonate external solution and recordings were made using a KMeSO₄ internal solution. When recordings were made using a KCl internal solution, the spontaneous firing of action potentials was slightly increased (1–5 Hz), as some action potentials could be triggered by large spontaneous depolarizing synaptic events mediated by GABA_A receptors. Action potential overshoots were usually greater than 40 mV and resting membrane potentials were slightly depolarized (-51.3 ± 3 mV; $n = 58$). These results are consistent with previously reported electrophysiological properties for Hcrt neurones (Li *et al.* 2002; Eggermann *et al.* 2003; Yamanaka *et al.* 2003a).

GABA_A receptor-mediated modulation of Hcrt neuronal activity

To characterize inhibitory regulation of Hcrt neurones, our initial studies examined the effect of the major inhibitory neurotransmitter GABA on Hcrt neuronal firing and membrane potential in current clamp mode. The physiological bicarbonate external solution and KMeSO₄ internal solution were initially used. The membrane potential of Hcrt neurones was held at -60 mV with DC injection and the input resistance was monitored with hyperpolarizing current pulses (-0.3 nA, 800 ms) delivered every 5 s. The GABA_A selective agonist muscimol or GABA was then bath applied to the slices. The bath application of muscimol (30 μM) produced a biphasic response in the Hcrt neurones. The response consisted of an initial, short hyperpolarization (-4.5 ± 0.6 mV, $n = 3$) followed by a long depolarization (12.4 ± 4 mV) during the 1 min drug application (Fig. 1A). Associated with the biphasic membrane potential change, muscimol decreased input resistance by 85% and subsequently the firing of action potentials ceased. Similarly, 0.3 mM GABA also induced a biphasic membrane potential response: a decrease in input resistance and a decrease in the firing of action potentials (Figs 1B, $n = 4$). These GABA-induced effects rapidly reversed upon removal of GABA from the external

Calibration in A also applies to B and C. B, bath application of GABA (0.3 mM) also caused a biphasic change in membrane potential, decreased the input resistance and blocked all action potentials. C, when the external solution was changed to Hepes, bath application of GABA (0.3 mM) caused a monophasic hyperpolarization, decreased the input resistance and reduced action potentials. Three Hcrt/EGFP neurons were held at -60 mV with DC injection and hyperpolarizing current pulses (0.3 nA, 800 ms) were applied every 5 s throughout the experiment. The position of 0 mV is indicated by the dotted line and most single spike amplitudes were truncated due to limited temporal resolution. Recordings of these three cells used K-MeSO₄ internal solution. D, bar graph summarizing the effect of 300 μM GABA on the membrane potential of Hcrt neurones when applied in the presence and absence of HCO₃⁻ in the external solution. 'Early' and 'late' refer to initial and subsequent responses induced by the bath application of GABA. Data are presented as means \pm s.e.m. ($n = 4$ in bicarbonate and $n = 3$ in Hepes experiments).

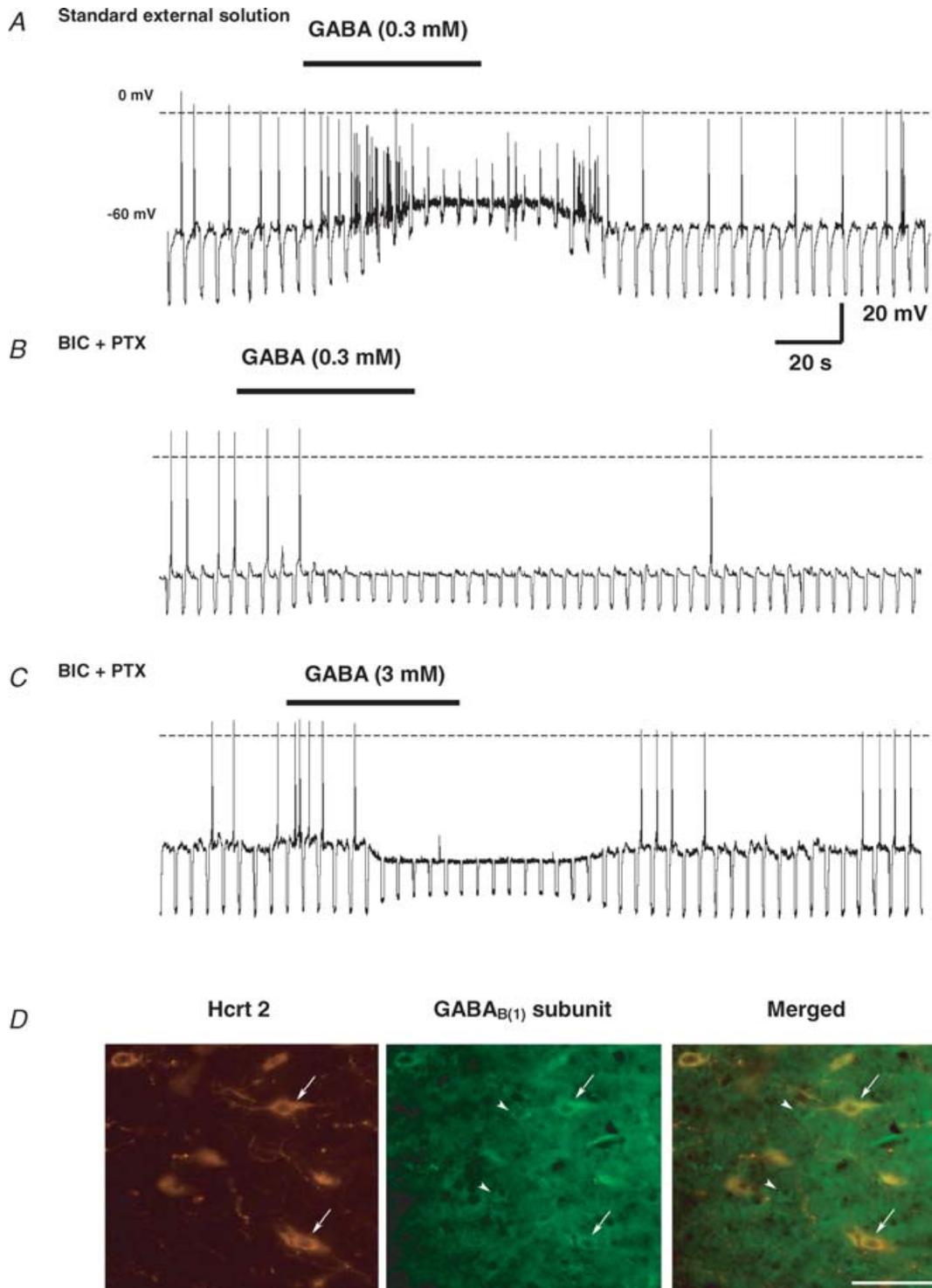


Figure 2. GABA_B receptor-mediated responses and protein expression in Hcrt neurones

A, bath application of GABA (0.3 mM) caused consistent monophasic depolarization when recorded using KCl internal solution and standard external solution. *B*, in the presence of bicuculline (BIC, 40 μ M) and picrotoxin (PTX, 500 μ M), GABA (0.3 mM) did not obviously alter the membrane potential but decreased the input resistance (by 28%) in the same neurone as in *A*. *C*, under the same recording conditions as in *B*, a higher concentration of GABA (3 mM) produced hyperpolarization (-6 mV), decreased input resistance (by 32%) and blocked firing of action potentials in another Hcrt neurone. Three Hcrt neurones were all held at -60 mV with DC injection and hyperpolarizing current pulses (0.3 nA, 800 ms) were applied every 5 s throughout the experiment. Calibration in *A* also applies to *B* and *C*. The position of 0 mV is indicated by the dotted line and most single spike amplitudes

solution and were completely blocked by the combination of the GABA_A receptor antagonists, BIC (50 μM) and PTX (200 μM ; data not shown).

Similar biphasic responses to GABA have been observed in other brain regions in which the depolarization component is thought to be dependent on HCO_3^- (Staley & Proctor, 1999; Isomura *et al.* 2003). Therefore, we removed HCO_3^- from the external solution and replaced it with Hepes. Under these conditions, bath-application of 0.3 mM GABA produced a consistent hyperpolarization (-11.4 ± 4.6 mV, $n = 3$) of the Hcrt neurones (Fig. 1C). These results are summarized in Fig. 1D.

GABA_B receptor-mediated modulation of Hcrt neuronal activity

To distinguish GABA_B receptor-mediated responses induced by GABA from GABA_A receptor-mediated responses, a KCl internal solution and the physiological bicarbonate external solution were used. Under these conditions, bath application of 0.3 mM GABA produced a large depolarization of the Hcrt neurones (31.8 ± 4.6 mV, $n = 3$; Fig. 2A). However, in the presence of saturating concentrations of the GABA_A receptor antagonists BIC (40 μM) and PTX (500 μM), bath application of 0.3 mM GABA produced little membrane potential change, though the input resistance was decreased by approximately 30% and firing of action potentials was completely blocked (Fig. 2B). When the GABA concentration was increased to 3 mM, a clear hyperpolarization (-4.7 ± 2 mV, $n = 3$) and a further decrease in the input resistance ($50 \pm 6\%$ of control) was observed (Fig. 2C). The GABA-induced responses were blocked by the GABA_B receptor antagonist CGP 52432 (data not shown). The above experiments were suggestive of the presence of functional GABA_B receptors on Hcrt neurones.

Since GABA_B receptors had not been previously identified on mouse Hcrt neurones, we also conducted neuroanatomical studies to confirm the presence of these proteins in Hcrt cells. Using double immunofluorescence, we labelled LHA cells with antibodies targeting the Hcrt-2 peptide and the GABA_{B(1)} subunit. We found many cells in the LHA to be GABA_{B(1)}-immunoreactive (GABA_{B(1)}-ir) and several neurones in the LHA that were both Hcrt-2-ir and GABA_{B(1)}-ir (Fig. 2D). It appeared that every cell in the LHA that expressed Hcrt-2 was GABA_{B(1)} positive, but not all GABA_{B(1)}-ir cells were Hcrt-2-containing neurones (Fig. 2D). These results support the presence of a GABA_B receptor on Hcrt cells.

To further characterize GABA_B receptor-mediated modulation on Hcrt neuronal activity, we tested the effect of the GABA_B selective agonist (*R*)-baclofen on Hcrt firing rate and membrane potential. Similar to the results observed when GABA was applied in the presence of GABA_A receptor antagonists, bath application of 10 μM (*R*)-baclofen hyperpolarized Hcrt neurones (-5.6 ± 2 mV, $n = 8$) and decreased input resistance by $28 \pm 5\%$, and consequently blocked the spontaneous firing of action potentials. In addition (*R*)-baclofen reduced membrane excitability in response to a depolarizing current injection (Fig. 3A, $n = 4$). The effect of (*R*)-baclofen on Hcrt membrane potential was concentration-dependent with an estimated half-maximal effective concentration (EC_{50}) of 7.1 μM (Fig. 3B). This GABA_B-mediated effect was reversible upon washout of (*R*)-baclofen. The ability of (*R*)-baclofen to reduce input resistance and firing rate of Hcrt neurones could be reversed by the addition of CGP 52432 (Fig. 3C). Lastly, in the presence of tetrodotoxin (TTX, 0.5 μM), spontaneous action potentials in Hcrt neurones were completely blocked, but local application of (*R*)-baclofen still produced hyperpolarization and decreased input resistance (Fig. 3D). After washout and recovery from the drug action, the patch recording was switched to voltage-clamp mode. When membrane potential was held at -60 mV (*R*)-baclofen (10 μM) induced an outward current in the same neurone. Both current- and voltage-clamp recordings in the presence of TTX indicate direct postsynaptic effects of baclofen on Hcrt neurones (Fig. 3D). The current-voltage relationship of neuronal responses in the presence and absence of baclofen indicates the reversal potential of baclofen-induced response is around -116 ± 6 mV, which is close to the K^+ equilibrium potential (approximately -110 mV) under our experimental conditions (Fig. 3E). Taken together, these data suggest that the hyperpolarizing effect of GABA on Hcrt neurones observed in the presence of GABA_A receptor blockade was due to a direct postsynaptic activation of GABA_B receptors, presumably mediated by an inward rectifying potassium current.

Presynaptic GABA_B receptor function in the hypothalamus

In most brain regions, GABA_B receptors are also found presynaptically where they inhibit neurotransmitter release (Bowery *et al.* 2002; Bettler *et al.* 2004). To

were truncated due to limited temporal resolution. *D*, the GABA_{B(1)} subunit is expressed in all Hcrt-2 (Hcrt)-positive cells, as shown by double-label immunofluorescence in the LHA. Left panel: Hcrt-2 immunoreactivity (red). Middle panel: GABA_{B(1)}-immunoreactivity (green). Right panel: merged image. Arrows show GABA_{B(1)}-immunoreactive cells which are positive for Hcrt-2 immunoreactivity; arrowheads indicate GABA_{B(1)}-immunoreactive cells that do not colabel with Hcrt-2. Scale bar, 50 μm .

determine whether GABA_B receptors also modulate neurotransmission onto Hcrt cells, we evaluated the effect of the GABA_B agonist (*R*)-baclofen on evoked and spontaneous synaptic currents in voltage clamp mode.

We first examined whether (*R*)-baclofen could alter currents elicited by the stimulation of neurotransmitter release. The neurones were voltage-clamped at -60 mV and synaptic currents were elicited every 10 s with local

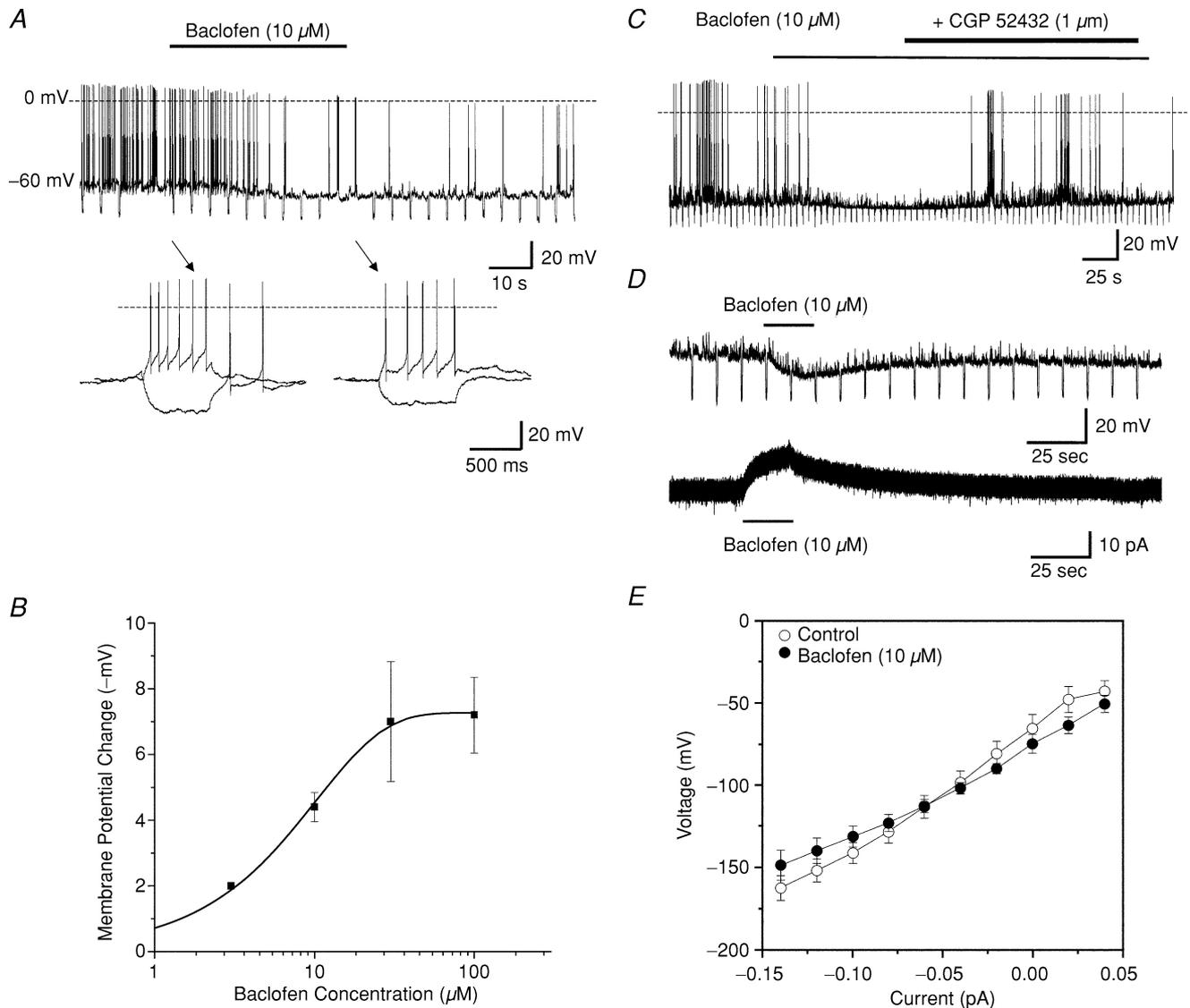


Figure 3. Modulation of Hcrt/EGFP neuronal activity by the GABA_BR selective agonist (*R*)-baclofen

A, bath application of (*R*)-baclofen ($10 \mu\text{M}$) hyperpolarized the Hcrt/EGFP neurones, decreased the input resistance, and suppressed the spontaneous firing of action potentials. Baclofen also depressed membrane excitability in response to depolarizing current injection (lower traces). *B*, (*R*)-baclofen ($3\text{--}100 \mu\text{M}$) caused membrane hyperpolarization in a concentration-dependent manner. The data were fitted using a dose-response curve fitting model (OriginPro 7.5) and yielded an estimated half-maximal effective concentration (EC_{50}) of $7.1 \mu\text{M}$. Values are means \pm S.E.M. ($n = 3\text{--}8$). *C*, the baclofen-induced responses were blocked by the selective GABA_B antagonist CGP 52432 ($1 \mu\text{M}$). The position of 0 mV is indicated by the dashed line and most single spike amplitudes were truncated due to limited temporal resolution. *D*, in the presence of TTX ($0.5 \mu\text{M}$), all spontaneous action potentials were blocked and local application of baclofen ($10 \mu\text{M}$) caused a hyperpolarization and a decrease in input resistance. After washout and recovery from the drug action under voltage-clamp mode (*R*)-baclofen ($10 \mu\text{M}$) induced an outward current in the same neurone at a holding potential of -60 mV. *E*, the current-voltage relationship of neuronal responses in the presence and absence of baclofen shows the reversal potential for baclofen-induced response around -115 mV, which is close to K⁺ equilibrium potential (calculated value of 108 mV) under our specific experimental conditions in which the external solution had 2 mM K⁺ and internal solution 145 mM K⁺ ($n = 7$).

stimulation from a bipolar stimulating electrode placed within the LHA slice. EPSCs were isolated by pretreating the slices with PTX (100 μ M). Under these conditions, bath application of (*R*)-baclofen (10 μ M) reversibly depressed the evoked EPSC (eEPSC) amplitude to $18 \pm 4\%$ of control values ($n = 5$) (Fig. 4A). Similarly, when evoked inhibitory postsynaptic currents (eIPSCs) were pharmacologically isolated using CNQX (20 μ M) and AP-5 (40 μ M) to block AMPA/KA and NMDA receptors, respectively, (*R*)-baclofen (10 μ M) reduced the amplitude of eIPSCs to $16 \pm 5\%$ of control values (Fig. 4B). These effects could be due to a decrease in the postsynaptic response to the neurotransmitters and/or to a change in the amount of neurotransmitter being released.

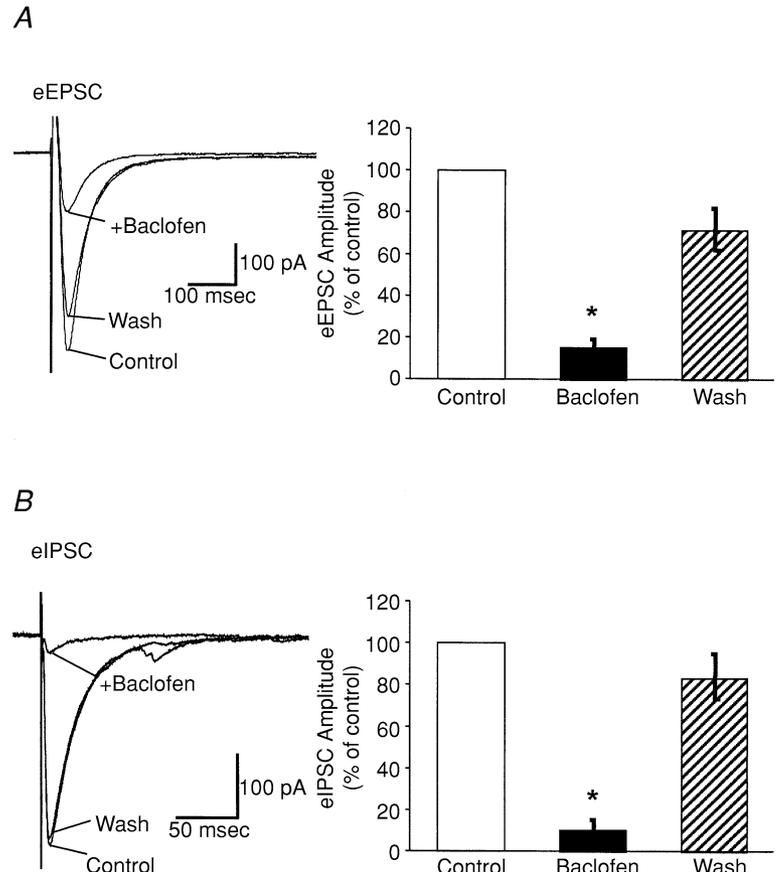
To further examine the possibility of a presynaptic function for GABA_B receptors in modulating excitatory and inhibitory synaptic input to Hcrt neurones, we examined the effect of (*R*)-baclofen on spontaneous EPSCs and IPSCs. sEPSCs and sIPSCs were recorded in voltage clamp at a holding potential of -60 mV. Spontaneous excitatory events were recorded in the presence of BIC (20 μ M) and PTX (100 μ M). Under voltage clamp at a holding current of -60 mV, the mean frequency of sEPSCs was 3.8 ± 1.9 Hz ($n = 7$). Subsequent bath application of (*R*)-baclofen (10 μ M) decreased the frequency of sEPSCs to $26 \pm 6\%$ of control values ($n = 7$). The decrease in

sEPSC frequency was maintained throughout the duration of the (*R*)-baclofen application and fully recovered to baseline after washout of (*R*)-baclofen (Fig. 5A and B). The application of (*R*)-baclofen did not significantly inhibit the amplitude of these events (Fig. 5C). To isolate sIPSCs, the slices were pretreated with saturating concentrations of both the AMPA/KA and NMDA receptor antagonists CNQX (20 μ M) and AP-5 (40 μ M), respectively. Under these conditions, the mean frequency of sIPSCs recorded from Hcrt neurones was 4.5 ± 1.2 Hz ($n = 7$). Subsequent bath application of (*R*)-baclofen also significantly decreased the frequency of the inhibitory events to $20 \pm 6\%$ of control in all cells tested ($n = 7$, Fig. 5D and E). The effect of (*R*)-baclofen on sIPSC frequency was maintained during the (*R*)-baclofen application and returned to baseline upon washout of (*R*)-baclofen. The application of (*R*)-baclofen did not significantly inhibit the amplitude of these events (Fig. 5F). Taken together, these results suggest that GABA_B receptors may act on presynaptic neurones to decrease the release of both excitatory and inhibitory neurotransmitters in the LHA.

To determine if (*R*)-baclofen was acting directly at presynaptic terminals to decrease the probability of glutamate and GABA release, we further examined the effects of GABA_B receptor activation on the frequency and amplitude of miniature EPSCs (mEPSCs) and IPSCs

Figure 4. Modulation of evoked synaptic currents in Hcrt/EGFP neurones by (*R*)-baclofen

A, eEPSCs were pharmacologically isolated by adding picrotoxin (100 μ M) to the external solution to block inhibitory synaptic currents. Bath application of (*R*)-baclofen (10 μ M) reversibly inhibited the amplitude of eEPSCs. The left panel shows average of five traces for an individual neuron under control conditions, in the presence of (*R*)-baclofen, and following washout of (*R*)-baclofen. The right panel shows a bar graph summarizing the effect of (*R*)-baclofen on eEPSC amplitude. The amplitude of eEPSCs was depressed to $18 \pm 4\%$ of control ($n = 5$, $P < 0.001$). All eEPSCs were recorded using KGlu as the pipette internal solution. **B**, eIPSCs were recorded in the presence of CNQX (20 μ M) and AP-5 (40 μ M) to block excitatory synaptic transmission. Bath application of (*R*)-baclofen (10 μ M) also reversibly inhibited the amplitude of eIPSCs. The left panel shows average of five traces for an individual neuron under control conditions in the presence of (*R*)-baclofen and following washout of (*R*)-baclofen. The right panel shows a bar graph summarizing the effect of (*R*)-baclofen on eIPSC amplitude. The amplitude of eIPSCs was depressed to $16 \pm 5\%$ of control ($n = 5$, $P < 0.001$). All eIPSCs were recorded using KCl as the pipette internal solution. All cells recorded were voltage clamped at a holding potential of -60 mV and the bipolar stimulation electrode used to evoke synaptic currents was placed close to the recorded Hcrt/EGFP neuron in the LHA slice.



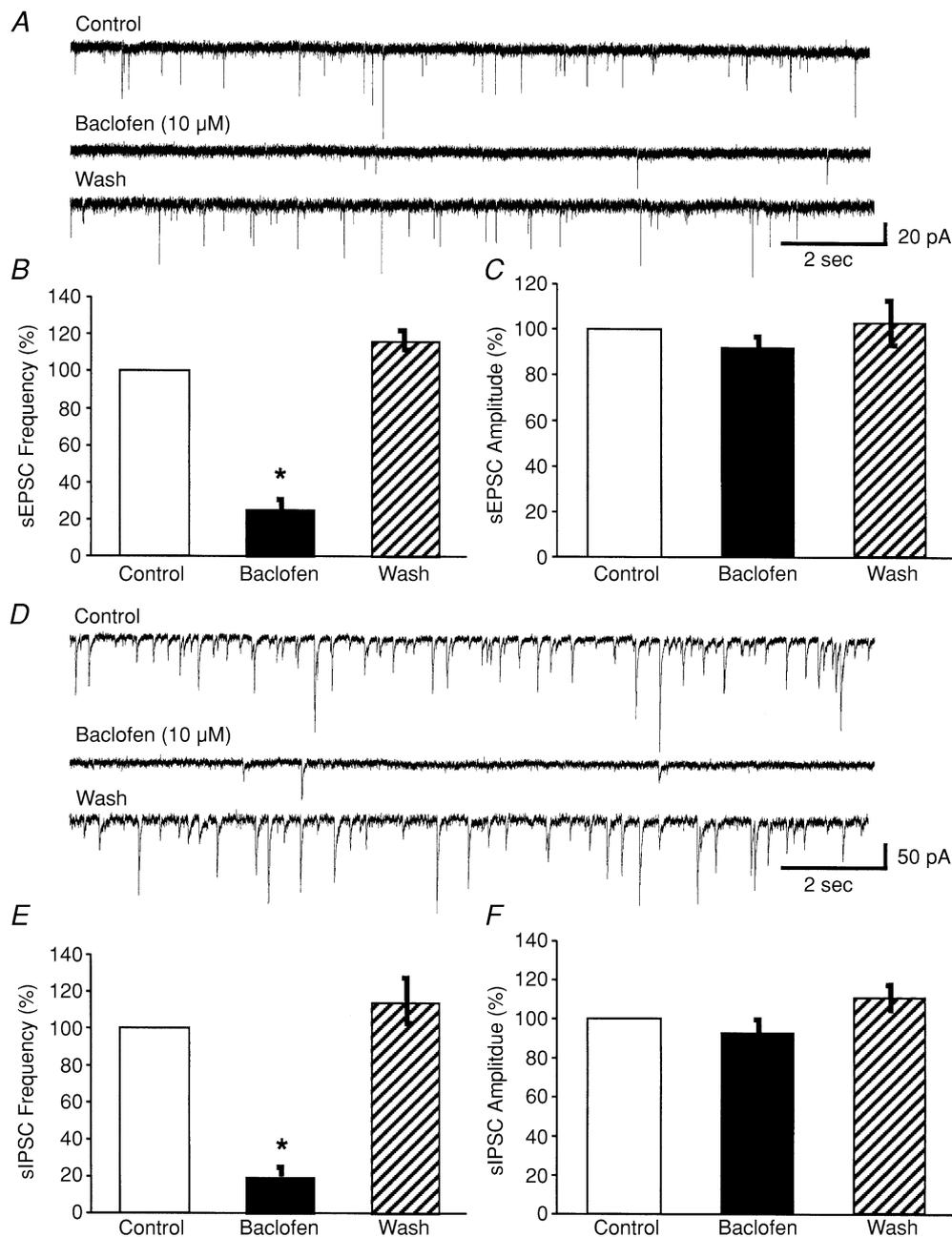


Figure 5. Modulation of spontaneous EPSCs and IPSCs by GABA_B receptor activation

A, representative traces showing sEPSCs in control conditions, in the presence of (*R*)-baclofen, and following washout of (*R*)-baclofen. (*R*)-Baclofen reversibly decreased the frequency but not the amplitude of sEPSCs. *B*, bar graph summarizing the effect of (*R*)-baclofen on the frequency of sEPSCs. The mean frequency of sEPSCs was reversibly inhibited to $26 \pm 6\%$ of control ($n = 7$, $P < 0.001$). *C*, bar graph summarizing the effect of (*R*)-baclofen on the amplitude of sEPSCs. The mean amplitude of sEPSCs was not significantly altered ($79.9 \pm 15.4\%$ of control; $n = 4$, $P = 0.13$). All sEPSCs were recorded in voltage clamp at a holding potential of -60 mV in the presence of bicuculline ($20 \mu\text{M}$) and picrotoxin ($100 \mu\text{M}$). *D*, representative traces showing sIPSCs in control conditions, in the presence of (*R*)-baclofen, and following washout of (*R*)-baclofen. (*R*)-Baclofen ($10 \mu\text{M}$) reversibly decreased the frequency but not the amplitude of sIPSCs. *E*, bar graph summarizing the effect of (*R*)-baclofen on the frequency of sIPSCs. The mean frequency of sIPSCs was reversibly inhibited to $20 \pm 6\%$ of control ($n = 7$, $P < 0.001$). *F*, bar graph summarizing the effect of (*R*)-baclofen on the amplitude of sIPSCs. The mean amplitude of sIPSCs was not significantly altered ($79.8 \pm 12.9\%$ of control; $n = 4$, $P = 0.17$). sIPSCs were recorded under voltage clamp at a holding potential of -60 mV in the presence of CNQX ($20 \mu\text{M}$) and AP-5 ($40 \mu\text{M}$).

(mEPSCs). mEPSCs and mIPSCs were recorded in voltage clamp at a holding potential of -60 mV. mEPSCs were pharmacologically isolated by adding BIC ($20 \mu\text{M}$), PTX ($100 \mu\text{M}$) and TTX ($1 \mu\text{M}$) to the external solution. Under these conditions, the mean frequency of mEPSCs was 3.5 ± 0.4 Hz ($n = 4$). Bath application of (*R*)-baclofen ($10 \mu\text{M}$) significantly decreased the frequency of mEPSCs to $53.4 \pm 4.6\%$ of control (Fig. 6A and B). The amplitude of these events was not significantly altered by GABA_B receptor activation (Fig. 6C). mIPSCs were isolated by adding DNQX ($40 \mu\text{M}$), AP-5 ($40 \mu\text{M}$) and TTX ($1 \mu\text{M}$) to the external solution. Under these conditions, the mean frequency of mIPSCs was 0.4 ± 0.1 Hz ($n = 4$). Bath application of (*R*)-baclofen ($10 \mu\text{M}$) decreased the frequency of mIPSCs to $49.3 \pm 3\%$ of control (Fig. 6D and E). Activation of GABA_B receptors also caused a modest reduction in the amplitude of these events (Fig. 6F). These results suggest that GABA_B receptors can presynaptically decrease the release of both glutamate and GABA onto Hcrt neurones in the LHA.

Discussion

Hcrt neuron activity is modulated by both GABA_A and GABA_B receptors

Previous studies have demonstrated that LHA neurones in the developing nervous system can be depolarized by GABA (Obrietan & van den Pol, 1995). In the present study, we extend these observations by showing that GABA_A receptor activation by GABA or muscimol produces a biphasic membrane potential change in Hcrt cells. Depolarizing responses to GABA are frequently reported in the developing nervous system (Obrietan & van den Pol, 1995; Chen *et al.* 1996; Wang *et al.* 2001) and, in the adult brain, in the presence of exogenous zinc or after intense stimulation (Xie & Smart, 1993; Isomura *et al.* 2003; Stein & Nicoll, 2003). The mechanisms proposed for GABA-mediated depolarization involve two GABA_A channel permeant anions, Cl⁻ and HCO₃⁻. During development, the Cl⁻ electrochemical equilibrium potential (E_{Cl}) is positive relative to resting membrane potential (V_{rest}) and activation of GABA_A receptors results in a net efflux of Cl⁻ and membrane depolarization (Lee *et al.* 2005). As development proceeds, a decrease in intracellular Cl⁻ occurs (likely to be due to up-regulation of the neurone-specific potassium chloride cotransporter, KCC2; Lee *et al.* 2005), E_{Cl} becomes negative relative to V_{rest} , and GABAergic stimulation results in a net influx of Cl⁻ and, thus, hyperpolarization. In contrast, in the mature brain, previous studies have shown that activation of dendritic GABA_A receptors in CA1 hippocampal neurones causes a depolarization while somatic GABA_A receptor activation causes a hyperpolarization (Stein & Nicoll, 2003). Although the mechanism underlying the

depolarization is not fully understood, it is likely to involve permeability of GABA_A channels to HCO₃⁻. In the hippocampus, strong activation of dendritic GABA_A receptors is proposed to cause the collapse of the hyperpolarizing chloride gradient, thereby unmasking the depolarizing HCO₃⁻ drive. Moreover, the depolarizing currents observed in the hippocampus *in vitro* have also been demonstrated *in vivo*, suggesting that they may play a role in physiological responses to GABA_A receptor activation (Mercuri *et al.* 1991; Fan *et al.* 2005). In the present study, which was conducted on hypothalamic slices from postnatal 3- to 6-week-old mice that may not be completely developmentally mature, HCO₃⁻ flux appears to be the primary contributor to GABA-mediated depolarization (Fig. 1). Whether these depolarizing responses play a role in the response of Hcrt neurones to GABA *in vivo* is yet to be determined. The previous studies using local application of muscimol to the recorded cell body (Li *et al.* 2002; Eggermann *et al.* 2003; Yamanaka *et al.* 2003a) may preferentially activate somatic receptors, while the current study using bath application of GABA or muscimol would more likely activate both the somatic and dendritic GABA_A receptors. In contrast, bath or local application of baclofen consistently induced hyperpolarization of Hcrt neurones.

Several lines of evidence indicate that GABAergic neurotransmission to the Hcrt neurones occurs through GABA_B as well as through GABA_A receptors. In the presence of the GABA_A receptor blockers BIC and PTX, bath application of GABA hyperpolarized the Hcrt neurones and decreased the firing rate and the input resistance (Fig. 2). Similar results were obtained using the GABA_B receptor agonist (*R*)-baclofen, and the effects of (*R*)-baclofen could be blocked by the GABA_B agonist CGP 52432 (Fig. 3). Furthermore, the magnitude of membrane hyperpolarization was dependent upon the baclofen concentration (Fig. 3C). In support of these physiological observations, neurones in the LHA that were immunoreactive for Hcrt-2 also stained for the GABA_{B(1)} subunit (Fig. 2C). GABA_{B(1)}-ir and Hcrt-2-ir have also been reported to colocalize in the rat LHA (Backberg *et al.* 2003). In contrast to GABA_A neurotransmission, which can be depolarizing under certain conditions as discussed above, GABA_B receptor-mediated neurotransmission is consistently hyperpolarizing.

In addition to evidence for direct GABA_B-mediated effects on Hcrt neurones, we also found evidence for GABA_B-mediated modulation of both excitatory and inhibitory inputs to the Hcrt cells. (*R*)-Baclofen reduced the amplitude of evoked EPSCs and IPSCs (Fig. 4) and also decreased the frequency of spontaneous and miniature excitatory and inhibitory events, suggesting that activation of GABA_B receptors at presynaptic terminals inhibits the release of both glutamate and GABA onto the Hcrt cells (Figs 5 and 6). These effects are similar to the role of GABA_B

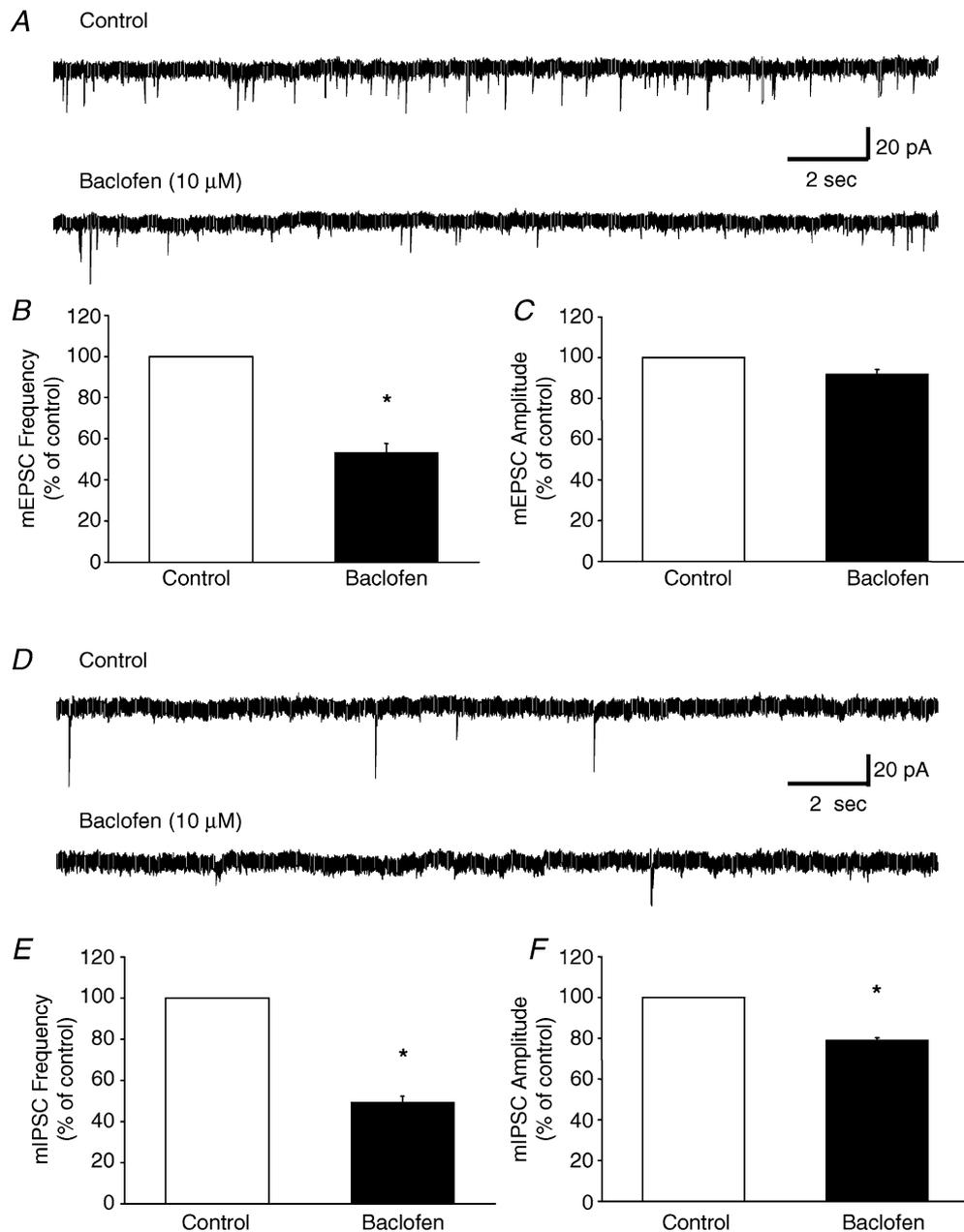


Figure 6. Modulation of miniature EPSCs and IPSCs by GABA_B receptor activation

A, representative traces showing mEPSCs in control conditions and in the presence of (*R*)-baclofen. (*R*)-Baclofen (10 μ M) decreased the frequency but not the amplitude of mEPSCs. *B*, bar graph summarizing the effect of (*R*)-baclofen on the frequency of mEPSCs. The mean frequency of mEPSCs was inhibited to $53.4 \pm 4.6\%$ of control ($n = 4$, $P < 0.05$). *C*, bar graph summarizing the effect of (*R*)-baclofen on the amplitude of mEPSCs. The mean amplitude of mEPSCs was not significantly altered ($94.1 \pm 1.3\%$ of control; $n = 4$, $P = 0.21$). All mEPSCs were recorded in the presence of BIC (20 μ M), PTX (100 μ M) and TTX (1 μ M) in voltage clamp at a holding potential of -60 mV. *D*, representative traces showing miPSCs in control conditions and in the presence of (*R*)-baclofen. (*R*)-Baclofen (10 μ M) decreased the frequency and amplitude of miPSCs. *E*, bar graph summarizing the effect of (*R*)-baclofen on the frequency of miPSCs. The mean frequency of miPSCs was inhibited to $49.3 \pm 3.0\%$ of control ($n = 4$, $P < 0.01$). *F*, bar graph summarizing the effect of (*R*)-baclofen on the amplitude of miPSCs. The mean amplitude of miPSCs was decreased in the presence of (*R*)-baclofen ($79.1 \pm 1.18\%$ of control; $n = 4$, $P < 0.05$). miPSCs were recorded in the presence of DNQX (40 μ M), AP-5 (20 μ M) and TTX (1 μ M). All miniature synaptic currents were recorded under voltage clamp at a holding potential of -60 mV.

receptors demonstrated in other brain regions (Doze *et al.* 1995; Vogt & Nicoll, 1999; Porter & Nieves, 2004).

As previously reported (Horvath & Gao, 2005), the frequency of miniature EPSCs was almost 10-fold higher than the frequency of miniature IPSCs, further indicating that there are fewer inhibitory synaptic inputs to Hcrt neurones than excitatory inputs. In the absence of TTX, the frequency of spontaneous IPSCs was almost equal to the frequency of spontaneous EPSCs, which suggests that the activity of presynaptic GABAergic inputs might compensate for the lower number of inhibitory synapses onto the Hcrt neurones. However, it appears that basal GABAergic tone does not significantly influence the spontaneous firing of Hcrt neurons *in vitro* since GABA receptor antagonists did not alter the firing rate of these neurones (data not shown). Thus, it is more likely that GABAergic inputs from remote brain regions influence the activity of Hcrt neurons.

Implications for control of sleep and wakefulness

The LHA both contains local GABAergic neurones and receives GABAergic input from other brain areas, including regions of the preoptic area that have been implicated in sleep promotion (Abrahamson *et al.* 2001; Gong *et al.* 2004; Sakurai *et al.* 2005). The $\alpha 3$ (Moragues *et al.* 2003; Backberg *et al.* 2004; Sergeeva *et al.* 2005) and ϵ (Moragues *et al.* 2003; Backberg *et al.* 2004; Sergeeva *et al.* 2005) GABA_A receptor subunits have been localized on Hcrt neurones. In addition, GABA_{B(1)} subunits have been previously identified in the rat LHA (Backberg *et al.* 2003) and are shown to be colocalized with Hcrt in the current study. Microdialysis delivery of the GABA_A receptor antagonist, bicuculline, into the LHA of spontaneously sleeping rats resulted in a dose-dependent decrease in non-REM and REM sleep time, an increase in time awake, and an increased number of Fos-ir Hcrt neurones adjacent to the microdialysis probe (Alam *et al.* 2005). These results support the hypothesis that Hcrt neurones are subject to endogenous GABAergic inhibition mediated through GABA_A receptors during sleep, but the role of GABA_B receptors in sleep remains unclear.

Both clinical and basic studies suggest a role for GABA_B receptors in the regulation of behavioural state. The GABA_B agonist baclofen (25 mg) administered before sleep in a clinical study significantly prolonged total sleep time and reduced time spent awake after sleep onset (Finnimore *et al.* 1995). In aged rats, the GABA_B antagonist CGP 35348 increased the duration of non-REM, REM and total sleep duration compared to saline-injected controls (Puigcerver *et al.* 1996). Blockade of GABA_B receptors in the thalamus by microdialysis of CGP 35348 or another GABA_B antagonist, 2-OH-saclofen, did not affect wakefulness or total sleep time in freely moving cats, but deep slow wave sleep and the mean power of

slow waves (< 10 Hz) decreased while light slow wave sleep was increased (Juhász *et al.* 1994). Microinjection of either the GABA_A agonist muscimol or the GABA_B agonist baclofen into the basal forebrain induced an increase in slow-wave sleep and an inhibition of wakefulness, but only muscimol caused a decrease in desynchronized sleep parameters (Manfridi *et al.* 2001). Microinjection of baclofen into the pedunculopontine tegmentum (PPT) suppressed spontaneous REM sleep in a dose-dependent manner (Ulloor *et al.* 2004). GABA_B receptors have been implicated in presynaptic control of GABA release onto the histaminergic cells of the tuberomammillary nucleus (Stevens *et al.* 1999), which may provide a neural substrate for the effects observed *in vivo*. γ -Hydroxybutyrate (GHB) has long been known to increase slow wave sleep (stage 3 and 4) and total sleep time in both narcoleptic patients (Mamelak *et al.* 1977; Scharf *et al.* 1985; Scrima *et al.* 1989; Scrima *et al.* 1990) and healthy subjects (Van Cauter *et al.* 1997). Many investigations have concluded that the effects of exogenously applied GHB are mediated by GABA_B receptors (Xie & Smart, 1992; Schuler *et al.* 2001; Kaupmann *et al.* 2003; Crunelli *et al.* 2005; Snead & Gibson, 2005). Together, these results point to an under-appreciated role for GABA_B receptors in sleep. Given the central role of Hcrt cells in maintenance of wakefulness, GABA_B-mediated modulation of Hcrt neuron activity may also play a role.

Hcrt, GABA_B receptors and other physiological functions

In addition to sleep/wakefulness, the Hcrt neurones have been implicated in neuroendocrine control (Van Den Pol *et al.* 1998; Samson *et al.* 2005), energy regulation (Hara *et al.* 2001; Yamanaka *et al.* 2003b; Sakurai, 2005) and various aspects of autonomic function (Samson *et al.* 2005). In some of these systems, Hcrt and GABAergic interactions have been well established. For example, Hcrt modulates release of both GABA and glutamate in the arcuate nucleus (van den Pol *et al.* 1998) and modulates GABAergic inputs to the dorsal medial nucleus of the vagus (Davis *et al.* 2003). A number of recent studies have implicated the Hcrt system in addictive behaviours for substances such as morphine and cocaine (Georgescu *et al.* 2003; Boutrel *et al.* 2005; Harris *et al.* 2005; Borgland *et al.* 2006). The apparent resistance of human narcoleptics, in whom the Hcrt neurons degenerate, to develop dependence on medications such as amphetamines has been known for some time (Guilleminault *et al.* 1974). In animal models, the Hcrt ligand knockout mouse fails to develop conditioned place preference to morphine (Narita *et al.* 2006). Since GABA_B receptor agonists have shown potential for treatment of addictive disorders (Roberts, 2005), GABA_B receptor-mediated modulation of Hcrt neuronal activity and synaptic inputs may be important in modifying addictive behaviours.

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