Dual GABAergic Synaptic Response of Fast Excitation and Slow Inhibition in the Medial Habenula of Rat Epithalamus

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Submitted 22 May 2007; accepted in final form 29 June 2007

Kim U, Chung L-Y. Dual GABAergic synaptic response of fast excitation and slow inhibition in the medial habenula of rat epithalamus. J Neurophysiol 98: 1323–1332, 2007. First published July 5, 2007; doi:10.1152/jn.00575.2007. We report here a novel action of GABAergic synapses in regulating tonic firing in the mammalian brain. By using gramicidin-perforated patch recording in rat brain slices, we show that cells of the medial habenula of the epithalamus generate tonic firing in basal conditions. The GABAergic input onto these cells at postnatal days 18–25 generates a combinatorial activation of fast excitation and slow inhibition. The fast excitation, mediated by γ-aminobutyric acid type A receptors (GABAARs), is alone capable of triggering robust action potentials to increase cell firing. This excitatory influence of GABAergic input results from the Cl\(^{-}\) homeostasis that maintains intracellular Cl\(^{-}\) at high levels. The GABAAR excitation is often followed by a slow inhibition mediated by GABABRs that suppresses tonic firing. Interestingly, in a subpopulation of the cells, the GABAB inhibition exhibits a remarkably low threshold for synaptic activation in that low-strength GABAergic input often activates selectively the GABAAR slow inhibition, whereas the GABAAR excitation requires further increases in stimulus strength. Our study demonstrates that the dual activation of GABAergic excitation and inhibition through GABAAR and GABABRs generates distinct temporal patterns of cell firing that alter the cellular output in an activity-dependent manner.

INTRODUCTION

In the adult mammalian brain, GABAergic synapses typically inhibit postsynaptic target cells by activating mainly γ-aminobutyric acid type A and type B receptors (GABAARs and GABABRs) (Curtis et al. 1970; Dutar and Nicoll 1988a; Hill and Bowery 1981). The GABAARs generate a fast hyperpolarization of postsynaptic cells by increasing Cl\(^{-}\) conductance (Allen et al. 1977; Curtis et al. 1970; Gray and Johnston 1985). This GABAAR-mediated inhibition (GABAAR inhibition) composes the fundamental—and oftentimes the sole—form of GABAergic inhibition in response to low-strength activation of GABAergic input. The GABAARs, on the other hand, are generally activated in response to either high-frequency or large-intensity stimulation of GABAergic input (Buhl et al. 1994; Isaacson et al. 1993; Miles and Wong 1984). The activation of GABAARs produces a slowly rising and longer-lasting membrane hyperpolarization through increases in K\(^{+}\) conductance (Dutar and Nicoll 1988b; Gahwiler and Brown 1985; Hill and Bowery 1981; Newberry and Nicoll 1984). Such differences in the activation threshold and time-scale of the two types of GABAergic inhibition diversify the neurotransmitter action of GABA in regulating neural network activity. Moreover, the differential activation of GABAARs and GABABRs in an activity-dependent manner often determines the pattern of neural network oscillations as shown, for instance, in the thalamus (Kim and McCormick 1988a,b; Kim et al. 1995, 1997).

In contrast to the typical GABAergic inhibition just cited, a question has been raised recently as whether GABAergic input under any physiological condition is also capable of generating an excitation of postsynaptic cells. Relevant to this question, it is noteworthy that certain electrical properties of the postsynaptic membrane were shown to exploit the otherwise typical GABAergic inhibition to promote action potential generation. For example, as most vividly illustrated in the thalamus and the inferior olive (Crunelli and Leresche 1991; Deschenes et al. 1984; Jahnson and Llinas 1984; Llinas and Yarom 1981), the membrane hyperpolarization by GABAergic inhibition can lead to a rebound activation of low-threshold Ca\(^{2+}\) current. This Ca\(^{2+}\) current, in turn, generates a transient membrane depolarization that induces a burst of high-frequency action potentials. More recently, a different kind of GABAergic excitation was demonstrated in layer V cells of the cerebral cortex (Gulledge and Stuart 2003). The resting membrane potential of these cells is markedly hyperpolarized and negative to the GABAAR reversal potential. Therefore synaptic activation of GABAARs in these cells results in membrane depolarization toward the GABAAR reversal potential. However, because the GABAAR reversal potential in these cells is still 10–15 mV more negative than spike threshold, this membrane depolarization by GABAARs alone is incapable of triggering action potentials, even though it can boost the excitatory influence of glutamatergic input that is concurrently active.

Yet, the question still persists whether activation of GABAergic input alone—without relying on such distinct electrical properties of the postsynaptic membrane as mentioned earlier—can still exert an excitatory influence on the postsynaptic cell under physiological conditions in the mature mammalian brain. Here, we provide evidence in the habenula at postnatal days 18–25 supporting the GABAergic excitation that alone triggers robust action potentials.

The habenula is located on the posterior medial surface of the caudal thalamus. It serves a pivotal role in linking various limbic forebrain areas with the midbrain neuromodulatory centers such as the interpeduncular nucleus, substantia nigra...
pars compacta, ventral tegmental area, and dorsal raphe nucleus (Herkenham and Nauta 1977, 1979). This habenular link has been implicated in a variety of behaviors including reward and motivation, pain, nutrition, anxiety and learned helplessness, reproduction, and sleep-waking cycle (Ellison 1994; Klemm 2004; Lecourtier and Kelly 2007; Sutherland 1982). The habenula is generally divided into the medial and lateral nucleus (Andres et al. 1999; Geisler et al. 2003; Kim and Chang 2005). Each nucleus can be further divided into a number of subnuclei (Andres et al. 1999; Geisler et al. 2003), raising the possibility for the presence of multiple channels of information flow from the limbic forebrain to the midbrain via the habenula. As a step toward understanding how the information flow from the limbic forebrain to the midbrain is regulated, we first examined how GABAergic synapses control the habenular output from the medial nucleus. Our study showed that these GABAergic synapses produce a fast excitation of postsynaptic cells that is often followed by a slow inhibition. The excitation and inhibition are mediated by GABAA Rs and GABAB Rs, respectively, and produce a distinct regulation of tonic firings of habenular cells.

METHODS

Slice preparation

Sprague–Dawley rats (18- to 25 days old) were used (Charles Rivers Laboratories, Wilmington, MA). All experimental procedures were approved by the Animal Care and Use Committee at the Pennsylvania State University College of Medicine, in accordance with the National Institutes of Health Guide for the Use and Care of Laboratory Animals. Animals were deeply anesthetized with an intraperitoneal injection of a lethal dose of sodium barbiturate. Brain slices (400 μm thick) in the coronal plane were generated from the habenula of the epithalamus. The slices were preincubated before experiments at room temperature for 2 h in artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 1.2 MgSO4, 2 CaCl2, 15 dextrose, saturated with 95% O2-5% CO2 (pH 7.4). When needed for recording, individual slices were transferred to a submerged-type recording chamber that was fixed to the stage of a BX50WI microscope (Olympus, Tokyo, Japan) and was superfused at 3 ml/min with aCSF.

Electrophysiological recordings

All recordings were performed at 34–35°C. Whole cell and gramicidin-perforated patch recordings were obtained using visual control using a ×40 water-immersion objective lens with an infrared differential interference contrast filter. Recording pipettes were pulled from borosilicate glass capillaries (OD 1.5 mm, ID 0.84 mm). For whole cell patch recording, the pipette was filled with internal solution composed of (in mM): 130 K-glucuronate, 10 HEPES, 1 EGTA, 2 MgCl2, 0.1 CaCl2, 4 Na2-ATP, 0.3 GTP (pH 7.2, 280 mMol/kg). Typical electrode resistance was 3–5 MΩ, with access resistance in the range of 5–10 MΩ. The liquid junction potential was calculated (15 mV) and the membrane potential corrected accordingly.

For perforated patch recording, gramicidin (Sigma, St. Louis) was dissolved in dimethylsulfoxide (DMSO, 10 mg/ml). The gramicidin solution was then diluted to a final concentration of 5–10 μg/ml with internal solution composed of 140 mM potassium gluconate, 4 mM KCl, and 10 mM HEPES (pH 7.2, 280 mMol). When bumetanide—the sulfamoylbenzoic acid “loop” diuretic that inhibits the Na-K-Cl cotransporter—was tested for its effect on the GABAAR reversal potential, KCl in the internal solution was increased to 40 mM, whereas potassium gluconate was decreased to 104 mM. The liquid junction potential was calculated (15 and 12 mV, respectively, with the 4 and 40 mM KCl internal solutions) and Vm corrected accordingly. Perforated patches developed steadily for 20–30 min after gigaseal formation. Typical series resistance was 20–30 MΩ. Whole cell and perforated patch recordings were made using an Axoclamp 2B amplifier (Axon Instruments, Forest City, CA).

For extracellular recording, the patch pipette was filled with aCSF (3–5 MΩ). Single-unit extracellular signals were recorded with an Xcell-3 amplifier (FHC, Bowdoinham, ME), sampled at 20 kHz.

Synaptic stimulation

Stimulating electrode (tip diameter 10–15 μm) was fabricated from a theta glass capillary and filled with aCSF. To evoke synaptic potentials, a train of stimuli at 50–200 μA was delivered at 1–20 Hz every 60 s. Each stimulus was 100 μs in duration. To isolate the GABAergic response, all synaptic potentials were recorded in the presence of 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 100 μM d-2-amino-5-phosphonovaleric acid (o-APV) [selective blockers of α-aminoo-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and N-methyl-D-aspartate (NMDA)—subtype glutamate receptors, respectively], 1 μM LY 341495 (agonist of group II metabotropic glutamate receptors), and 2 μM mecamylamine (antagonist of nicotinic cholinergic receptors).

Data analysis

Data were collected and analyzed using pClamp8 software by an A/D converter (Digidata 1320, Axon Instruments) and are presented as means ± SD. All differences stated in the text are statistically significant (paired t-test, P < 0.05).

Drugs

For bath application of LY 341495, CGP 55845 (Tocris, St. Louis), and bumetanide (Sigma), they were initially dissolved in DMSO and then added to aCSF. The final concentration of DMSO was 0.01%. The bath infusion of 0.01% DMSO alone in aCSF did not alter the electrical and synaptic property of habenular cells. CNQX, APV, bicuculline, and mecamylamine (Sigma) were dissolved directly in aCSF for bath application.

GABA immunohistochemistry

Animals were perfused through the heart with 2% glutaraldehyde for 20 min. Brains were cut into 50-μm-thick sections with a Vibratome 1000plus (Vibratome, St. Louis). The sections were preincubated with 10% normal goat serum in PBS for 1 h to block nonspecific staining. Sections were then incubated overnight at room temperature in the primary GABA antibody (raised in rabbit; Chemicon, Temecula, CA) diluted 1:2,000–4,000 in PBS containing 1% normal goat serum in PBS for 1 h. After rinse, sections were incubated in biotinylated anti-rabbit IgG (raised in goat) 1:2,000–4,000 in PBS containing 1% normal goat serum. After rinse, GABA antibody (raised in rabbit; Chemicon) diluted to a final 1:1,000–2,000 in PBS were incubated in the avidin and biotinylated HRP solution (Vectastain elite ABC kit) and were then reacted with 0.05% 3,3-diaminobenzidine (DAB) in 0.1 M phosphate buffer (pH 7.2) in the presence of 0.01% hydrogen peroxide.

GABAAR and KCC2 immunohistochemistry

Animals were perfused with 4% paraformaldehyde for 40 min. Brain sections (50 μm thick) were preincubated with 10% normal goat serum in PBS for 1 h. The sections were incubated overnight in either the primary GABAAR antibody (raised in guinea pig; Chemicon) diluted 1:1,000–2,000 in PBS containing 1% normal goat serum or KCC2 antibody (raised in rabbit; Chemicon) diluted to a final...
concentration of 20 μg/mL in PBS containing 1% normal goat serum. After rinse, sections for GABA$_A$R immunohistochemistry were incubated for 1 h in biotinylated anti-guinea pig IgG (raised in goat) diluted 1:200. For KCC2 immunohistochemistry, the sections were incubated in biotinylated anti-rabbit IgG (raised in goat) diluted 1:200 (Vector) for 1 h. After rinse, sections were incubated in the avidin and biotinylated HRP solution (Vectastatin elite kit) and were then reacted with DAB.

RESULTS

Tonic firing of cells in the medial habenula

All recordings were made from neurons in the ventral half of the medial habenula. By using extracellular and whole cell patch recordings, we first examined the electrical property of the cells. Medial habenular cells at rest generate tonic firing at frequencies of 0.5 to 12 Hz (5.0 ± 2.1 Hz; n = 30 cells) (Fig. 1A). The tonic firing continued in the presence of 20 μM CNQX and 100 μM d-APV, the antagonists of AMPA/kainate- and NMDA-type glutamate receptors, respectively. This indicates that the tonic firing results from the intrinsic property of the cell membrane. To measure cell input resistance, we injected steady negative current at minimum amplitudes (5–10 pA) that suppressed tonic firing. Under this condition, a negative current pulse (10–20 pA, 300 ms) was superimposed to elicit a transient membrane hyperpolarization. The cell input resistance calculated from such voltage response was 1.2 ± 0.4 GΩ (n = 20 cells). The detailed description of the intrinsic and morphological properties of habenular cells was provided in our recent papers (Chang and Kim 2004; Kim and Chang 2005).

Dual GABAergic response of fast excitation and slow inhibition

For recording the GABAergic synaptic response, we used gramicidin-perforated patch recording. This recording technique prevents the dialysis of the cytoplasm by the patch pipette solution, thus preserving the intracellular Cl$^-$ concentration and its physiological change in response to synaptic activation of GABA$_A$Rs. To isolate GABAergic transmission, we blocked ionotropic glutamate receptors by including in the bathing medium 20 μM CNQX and 100 μM d-APV. A stimulating electrode was placed in the medial habenula 100–150 μm dorsal to the cells recorded.

In 10 cells recorded, a train of stimuli at 1–10 Hz (90–130 μA) produced both fast excitatory postsynaptic potentials (EPSPs) and a slowly rising inhibitory postsynaptic potential (IPSP). These two nonglutamatergic synaptic potentials exert a distinct effect on cell firing. The representative example is shown in Fig. 1B. In this cell, synaptic stimulation at 4 Hz generated fast EPSPs, which were successful only sporadically to trigger action potentials. This sporadic action potential generation was due to the counterbalance of the EPSPs by a slow IPSP, as subsequently detailed in Fig. 4. When stimulation rate was increased to 8 Hz, the EPSPs became more potent in triggering action potentials: all of the initial four to five EPSPs consistently activated action potentials (n = 6 trials of stimulation), thus briefly increasing cell firing to 8 Hz. The increase in stimulation rate also resulted in an increase in the amplitude of the slow IPSP. This IPSP appeared to prevent some of the EPSPs late in the sequence from reaching spike threshold. On termination of stimulation, the IPSP in response to 8- to 10-Hz stimulation decayed for the next 1–3 s and suppressed tonic firing.

Interestingly, when we induced the rupture of the perforated membrane seal by applying gentle suction that converted the recording configuration to conventional whole cell patch, the EPSPs were abolished rapidly within 5–15 s (Fig. 1Bb, n = 10 cells). The abolition of the EPSPs uncovered the slow IPSP in full that had counterbalanced the fast EPSPs and suppressed tonic firing. It is to be noted that the patch pipette solution for these experiments contained 4 mM Cl$^-$, whereas Na$^+$ and K$^+$ in the pipette solution were 8 and 135 mM, respectively. Along with the consideration that ionotropic glutamate receptors were blocked in our preparation, such rapid abolition of EPSPs on the intracellular dialysis by the low Cl$^-$ pipette solution led us...
to speculate that the EPSPs might have resulted from a Cl⁻ movement across the cell membrane. Our hypothesis was that intracellular Cl⁻ in medial habenular cells is maintained at high levels in basal conditions and that synaptic activation of GABAARs, therefore, results in Cl⁻ efflux, thus generating EPSPs to trigger action potentials. Indeed, in 14 cells tested, the block of GABAARs resulted in an abolition of the EPSPs. The representative case is illustrated in Fig. 2. In this cell, a train of stimulation generated a sequence of EPSPs and a slow IPSP. The effect of these antagonistic synaptic potentials on cell firing again varied with the frequency and strength of stimulation. For example, 10-Hz stimulation at 70 μA generated EPSPs of moderate size that only sporadically triggered action potentials, probably as a result of the counterbalance by the slow IPSP (Fig. 2A). However, when stimulus intensity was increased to 90 μA, most stimuli generated large enough EPSPs that triggered action potentials, thus increasing firing rate to around 10 Hz. In addition, as shown in Fig. 2B, the increase in stimulation rate to 20 Hz further potentiated every EPSP to trigger the action potential. These EPSPs were blocked 20–60 s after bath infusion of 20 μM bicuculline, the selective antagonist of GABAARs (Fig. 2C) (n = 14 cells). Blocking of the EPSPs uncovered the slow IPSP that suppressed tonic firing. The slow IPSP was blocked by bath infusion of 1 μM CGP 55845, the selective antagonist of GABAARs (Fig. 2D) (n = 14 cells). Taken altogether, these observations indicate that the EPSPs were mediated by GABAARs and the slow IPSP by GABAARs.

In five cells analyzed, individual GABAAR EPSPs reached their peak amplitude within 3–10 ms (6.7 ± 2.1, n = 50) after onset and exhibited a duration of 54.9 ± 19.5 ms. In response to five stimuli at 10–20 Hz, the GABAAR IPSP showed a rise time of 264 ± 30 ms after onset with duration of 988 ± 282 ms (n = 30).

Cellular mechanism of GABAAR excitation

To characterize the mechanism underlying the fast excitation mediated by GABAARs, we measured the reversal potential of the GABAAR-mediated synaptic response (GABAAR reversal potential) (n = 10 cells)

FIG. 2. Nonglutamatergic fast excitation is mediated by GABAergic input. A: stimulation at 10 Hz in the current intensity of 70 μA generates both fast EPSPs and slow IPSP (top). Action potential generation by the EPSPs is enhanced with increasing stimulus intensity to 90 μA (bottom). First 3 EPSPs and the action potentials are expanded in timescale (inset). Action potentials are truncated in this and all of the following figures. B: with increases in stimulus frequency to 20 Hz, every stimulus generates the EPSP large enough to trigger the action potential. C: EPSPs are blocked by bicuculline. D: slow IPSP is blocked by CGP 55845.

FIG. 3. γ-Aminobutyric acid type A (GABAAR) reversal potential is more positive than spike threshold. Aa: in the voltage-clamp recording mode, activation of GABAergic input generates both fast and slow currents. Fast currents are reversed in polarity at a potential greater than 45 mV. Ab: inward currents are blocked by bicuculline. Ac: slow outward current is blocked by CGP 55845. Ad: plot of the fast synaptic current elicited by the first stimulus of the train against the holding potential. B: hyperpolarizing shift of the GABAAR reversal potential by 20 μM bumetanide in 4 different cells.
fast synaptic currents followed by a slow outward current (Fig. 3Aa). The fast synaptic currents were inward in the entire subthreshold voltage range and were blocked by 20 μM bicuculline, indicating its production by GABAARs (Fig. 3Ab). The slow outward synaptic current was blocked by 1 μM CGP 55845, suggesting its generation by GABABRs (Fig. 3Ac). In four cells analyzed, the rise time of individual GABA EPSCs was 4.8 ± 0.9 ms with a duration of 32.2 ± 5.4 ms (n = 40).

To measure the GABA reversal potential, we plotted the fast GABA synaptic current generated in response to the first stimulus of the train as a function of the holding potential (Fig. 3Ad). The GABA reversal potentials measured in this manner (n = 3 cells) were not statistically different from those measured after GABAARs were selectively blocked by bath infusion of CGP (n = 7 cells), so they were pulled together. The GABA reversal potential was in the range of −48 to −25 mV (−40 ± 7 mV; n = 10 cells). In these cells, the spike threshold estimated as the onset of the regenerative phase of the action potential was −48 ± 2 mV, suggesting that the GABA reversal potential is more positive than spike threshold.

One possible explanation for such depolarized GABA reversal potential is Cl− homeostasis that maintains intracellular Cl− at high levels. To test for this possibility, we examined how the block of the Na+,K+,Cl−-cotransporter (NKCC) affects the GABA reversal potential. The NKCC is known to import and accumulate Cl− in the cytoplasm of neurons (Jang et al. 2001; Kakazu et al. 1999; Russell 2000). In four cells tested, bath infusion of 20 μM bumetanide, the selective blocker of NKCC, resulted in a hyperpolarizing shift of the GABA reversal potential by 5 to 9 mV (7 ± 2, P < 0.02) (Fig. 3B).

**GABA inhibition counterbalancing GABA excitation**

Our data so far showed that GABAergic input onto medial habenular cells generates a dual synaptic response of excitation and inhibition mediated by GABAARs and GABABRs, respectively. We show below that the relative strength of the GABA excitation versus GABA inhibition varies widely among different cells.

As illustrated earlier (Figs. 1B and 2), in 59% of cells analyzed (16 of 27 cells), at least three of the initial five stimuli at 5–20 Hz produced GABA EPSPs that are potent enough to trigger action potentials. In the other 41% of cells (11 of 27), however, GABA IPSP production was so prominent that it appreciably hyperpolarized the membrane near to the K+ equilibrium potential (−100 mV). In this manner, the GABA IPSP effectively prevented all GABA EPSPs except the first from reaching spike threshold. The representative case is shown in Fig. 4A. In this cell, five stimuli were applied at 6 Hz (Fig. 4Aa). The first stimulus activated the EPSP that triggered the action potential, which was evident from the spike interval that was much shorter than those between spontaneous action potentials. The first stimulus also activated a prominent, slow IPSP that hyperpolarized the membrane to −70–−75 mV. The slow IPSP by the next stimuli further hyperpolarized the

![Fig. 4. Counterbalance of GABA excitation by GABA inhibition. Aa: stimulation at 6 Hz generates a prominent membrane hyperpolarization by the slow IPSP that prevents all EPSPs except the first from reaching spike threshold. Ab: EPSPs are blocked by bicuculline, uncovering the slow IPSP. Ac: slow IPSP is blocked by CGP 55845. Bb: in this cell, selective block of the slow IPSP by CGP 55845 results in robust action potentials in response to stimulation (Bc). Bc: robust action potential generation is blocked by bicuculline.](http://jn.physiology.org/doi/pdf/10.1152/jn.00562.2007)
membrane to the peak (−95 to −100 mV), suppressing not only tonic firing but also countervailing the EPSPs. Bath infusion of 20 μM bicuculline selectively blocked the EPSPs, uncovering the slow IPSP in full (Fig. 4Ab). The slow IPSP was blocked by further infusion of 1 μM CGP 55845 (Fig. 4Ac).

The GABA_B slow IPSP, to counteract the action potential generation by GABA_A EPSPs, is more vividly demonstrated by selectively blocking GABA_BRs (n = 5 cells). The representative example is presented in Fig. 4B. In this cell, a train of three stimuli at 7 Hz generated the slow IPSP that hyperpolarized the cell membrane to −100 mV, suppressing tonic firing (Fig. 4Ba). When the GABA_B IPSP was blocked by 1 μM CGP 55845, the stimulation produced robust action potentials (Fig. 4Bb). This robust action potential generation was blocked by 20 μM bicuculline (Fig. 4Bc).

Interestingly, in five of those 11 cells that generated prominent membrane hyperpolarization by the GABA_B IPSP, the train of stimulation at near-minimal strengths resulted in a selective activation of the GABA_B IPSP, whereas activation of GABA_A EPSPs required further increases in stimulus strength. The representative case is shown in Fig. 5. In this cell, when a train of 15 stimuli was applied at 6 Hz in the current amplitude of 53 µA, the slow IPSP in response to the first five stimuli hyperpolarized the membrane to around −80 mV at peak (Fig. 5Aa). This peak hyperpolarization was maintained by the rest of the stimulation. No EPSP was activated in response to this low-strength stimulation. When stimulus intensity was raised to 73 µA, it took only the first three stimuli to produce the slow IPSP that hyperpolarized the membrane to the peak at around −95 mV (Fig. 5Ab). This peak hyperpolarization was maintained by the remaining stimulation. In contrast to the stimulation at 53 µA, each stimulus at this increased intensity generated an EPSP. The bath infusion of 20 μM bicuculline and 1 μM CGP 55845 blocked both EPSPs and slow IPSP, indicative of their generation by GABA_ARs and GABA_BRs, respectively. This feature of selective activation of GABA_B IPSP is further illustrated by the plot of how the amplitudes of GABA_A EPSP and GABA_B IPSP change depending on stimulus strength (Fig. 5B).

The selective activation of GABA_B inhibition was also demonstrated by recording synaptic currents in the voltage-clamp mode (n = 3 cells). The representative case is shown in Fig. 5C. This cell was voltage-clamped at −70 mV. Two stimuli at 7 Hz in the current intensity of 60 µA produced only slow outward currents (Fig. 5Ca), whereas the increase in stimulus intensity to 80 µA produced both fast inward and slow outward currents (Fig. 5Cb). Bath infusion of 20 µM bicuculline and 1 μM CGP 55845 abolished altogether the inward and outward currents (Fig. 5Cc), confirming their generation by GABA_ARs and GABA_BRs, respectively. The individual GABA_B IPSCs reached the peak within 64 to 113 ms (89 ± 24, n = 4) after onset and decayed for the next 236 to 438 ms (312 ± 67).

**FIG. 5.** Low-strength GABAergic input activates selectively GABA_B inhibition. *Aa:* 6 Hz stimulation at 53 µA generated only the slow IPSP. *Ab:* increase in stimulus intensity to 73 µA generated both EPSPs and the slow IPSP. *Ac:* EPSPs and slow IPSP were blocked by bicuculline and CGP 55845. *B:* response amplitude curves for GABA_A EPSP and GABA_B IPSP in 3 different cells. In each plot, data represented are mean ± 1 SD from 3 to 6 trials. In each trial, 10–15 stimuli were applied at 10 Hz. Because the cells spontaneously generated action potentials and did not show a stable baseline, the amplitude of GABA_B IPSP was measured from spike threshold. To measure the amplitude of GABA_B IPSP, individual EPSPs by the last 5 stimuli of the train in all the trials were pulled together and averaged. It is noteworthy that the lowest stimulus intensity activated only the GABA_B IPSP. *Ca:* cell is voltage-clamped at −70 mV. Stimulation at 60 µA generates only slow outward currents. *Cb:* increase in stimulus intensity to 80 µA generates both fast inward and slow outward currents. *Cc:* these fast and slow currents are blocked by bicuculline and CGP.
Morphological correlates of GABAergic transmission in the habenula

Our Nissl staining with cresyl violet showed that the medial habenula is densely packed with cells (Fig. 6A). The strategic role of the habenula that connects the limbic forebrain to the midbrain neuromodulatory centers is manifested in the two conspicuous fiber bundles in the diencephalon: the stria medullaris and the fascicles retroflexus (Fig. 6B). The stria medullaris forms the afferent pathway to the habenula from the limbic forebrain, whereas the fascicles retroflexus forms the efferent pathway from the habenula to the midbrain (Herkenham and Nauta 1977, 1979).

Our immunohistochemistry with GABA antibody revealed a high density of GABAergic terminals in the medial habenula (Fig. 6C, a and b). These terminals surrounded the somata and were also scattered in areas between the somata, possibly synapsing onto the dendrites (Fig. 6Cc). In contrast, we did not observe any cell body immunostained, suggesting that the GABAergic terminals originate from outside the medial habenula.

Our staining with GABA_bR antibody revealed a strikingly high density of GABA_bRs in the medial habenula (Fig. 6Da). The expression density was much higher than that in any other brain areas including the lateral habenula, thalamus, hippocampus, and cerebral cortex. On the subcellular level, GABA_bR immunolabels were observed outlining the somatic membrane of medial habenular cells and in the periphery of the cytoplasm (Fig. 6D, b and c).

Our immunohistochemistry with antibody to the type II K^+,-Cl^-cotransporter (KCC2), the most abundant isoform of KCC in the mammalian brain, revealed the lack of its expression in the medial habenula (Fig. 6E). The KCC2 is known to extrude Cl^- and function to maintain Cl^- concentration low in the cytoplasm of neurons (Fiumelli et al. 2005; Rivera et al. 1999, 2004; Woodin et al. 2003).

DISCUSSION

Our study demonstrated that GABAergic synapses in the medial habenula at postnatal days 18–25 generate a dual synaptic response of fast excitation and slow inhibition through GABA_ARs and GABA_BRs, respectively.

GABA_A excitation

In contrast to the typical production of fast inhibition in numerous brain areas, our study showed that GABA_ARs in the medial habenula generate a fast excitation of postsynaptic cells. This GABAergic excitation is produced in the entire subthreshold voltage range and capable of triggering robust action potentials (Figs. 1 and 2). The GABAergic excitation results from the GABA_A reversal potential that is more positive than spike threshold (Fig. 3). In consequence, synaptic activation of GABA_ARs produces an inward current that depolarizes the membrane past spike threshold toward the GABA_A reversal potential, thus triggering action potentials. This depolarized GABA_A reversal potential appears to be generated, at least in part, by intracellular Cl^- homeostasis, based on two observations. First, the block of NKCC resulted in a hyperpolarizing shift of the GABA_A reversal potential (Fig. 3B), suggesting...
that Cl\(^-\) import by NKCC contributes to the formation of the depolarized GABA\(_A\) reversal potential. Second, our immunohistochemistry disclosed the lack of KCC2 expression in the mature medial habenula. The KCC2 is known to extrude Cl\(^-\), thus decreasing the intracellular Cl\(^-\) concentration (Fiumelli et al. 2005; Rivera et al. 1999, 2004). It is reasonable to expect that the lack of KCC2 expression would help to maintain intracellular Cl\(^-\) at high levels in medial habenular cells, thus contributing to the formation of the GABA\(_A\) reversal potential more positive than spike threshold. Consistent with our immunohistochemistry, a previous study showed the lack of KCC2 mRNA expression—albeit a significant density of NKCC mRNAs—in the medial habenula of the adult rat brain (Kanaka et al. 2001).

Along with KCC2, three other isoforms of KCC (KCC1, -3, and -4) are known to be present in the mammalian brain (Clayton et al. 1998; Kanaka et al. 2001; Karadsheh et al. 2004; Payne et al. 1996; Pearson et al. 2001). In comparison to KCC2, these isoforms are expressed either at a much lower density or only in restricted areas of the rat brain. For example, KCC1 mRNA expression was shown to be generally low at all developmental stages, whereas the moderate density is restricted only to the olfactory bulb, hippocampus, cerebellum, and choroid plexus epithelial cells (Clayton et al. 1998; Kanaka et al. 2001). KCC3 is expressed at high levels only in the myelinated white matter tracts such as the corpus callosum, the dorsal column of the spinal cord, and Purkinje neurons and their axons in the cerebellum (Pearson et al. 2001). KCC4 is perhaps the least abundant because only the minimal expression level was detected in the forebrain, whereas high expression levels were restricted to the spinal cord and peripheral nerves (Karadsheh et al. 2004). Unlike the brain-specific expression of KCC2, the three other isoforms just cited are expressed in other body tissues as well. KCC1 and KCC4 were shown to be activated by cell swelling, whereas the brain-specific KCC2 is not responsive but is uniquely active under basal isotonic conditions (Gillen and Forbush 1999; Mercado et al. 2000; Payne 1997; Song et al. 2002). For these reasons, KCC2 is considered to play a major role in regulating Cl\(^-\) in neurons, whereas the other isoforms may be more important in cell volume regulation. However, it remains to be seen whether and, if so, to what extent these other KCCs are involved in Cl\(^-\) homeostasis and thus in GABAergic transmission in the brain.

Our finding that GABA\(_A\) excitation in the medial habenula arises, at least in part, from intracellular Cl\(^-\) homeostasis is similar to what was observed in the neonatal rat brain: until postnatal days 3–5, activation of GABA\(_A\)Rs in response to exogenous agonist generates a transient membrane depolarization in numerous areas (Ben-Ari 2002; Chen et al. 1996; Cherubini et al. 1991; Payne et al. 2003). This GABA\(_A\) depolarization in the infant brain seems to arise from the maintenance of intracellular Cl\(^-\) at high levels as the result of a much higher density of NKCC expression than KCC2. After postnatal days 3–5, however, KCC2 expression increases dramatically to maintain intracellular Cl\(^-\) at low levels (Lu et al. 1999; Yamada et al. 2004). As a result, GABA\(_A\)Rs begin to generate membrane hyperpolarization that inhibits neuronal activity.

More recently, a GABAergic excitation similar to what we report here was demonstrated in a limited domain of mature neurons (Szabados et al. 2006). In layer 2/3 pyramidal cells of the cerebral cortex at postnatal days 20–35, synaptic activation of GABA\(_A\)Rs in the membrane of the axon initial segment produced a depolarizing postsynaptic potential that triggered the action potential; in contrast, activation of GABA\(_B\)Rs in the somatodendritic membrane of these cells generated the typical IPSP. In these cells, the expression density of KCC2 in the membrane of the axon initial segment was shown to be much lower than that in the somatodendritic membrane. However, the GABA\(_B\) reversal potential was not measured in this study and it remains to be seen whether the low-density expression of KCC2 indeed underlies the GABA\(_A\) excitation in the axon initial segment by virtue of the formation of the GABA\(_A\) reversal potential more positive than spike threshold. In addition, the GABA\(_A\) excitation in the axon initial segment was not countervailed by GABA\(_B\) slow inhibition.

The GABA\(_A\) excitation in the medial habenula is different from that shown in mature hippocampal pyramidal cells (Michelle and Wong 1991; Staley et al. 1995). In these cells, tetanic stimulation of GABAergic synapses produces a biphasic sequence of fast hyperpolarization and depolarization mediated solely by GABA\(_A\)Rs. The early hyperpolarizing response is mediated by the typical Cl\(^-\) influx through the open GABA\(_A\)R channels. The sustained Cl\(^-\) influx, however, results in dissipation of the transmembrane Cl\(^-\) electrochemical gradient, whereas HCO\(_3^-\) continues to move out of the cell through the open GABA\(_A\)R channels and thus generates the late depolarizing response. In contrast, the GABA\(_A\) excitation in the medial habenula is not preceded by the fast hyperpolarization and does not require tetanic stimulation of GABAergic input. In fact, this GABA\(_A\) excitation can be generated in response to a single GABAergic input as indicated by the membrane depolarization and the action potential generation in response to the first GABAergic input in the train of stimulation (Figs. 1B, 2, and 4).

One question that still remains is whether and, if so, how this GABAergic excitation in the medial habenula changes as the brain becomes fully mature beyond the juvenile postnatal days 18–25. We were not able to address this question because our gramicidin-perforated recording from habenular cells at postnatal days 35–60 was highly unstable because the membrane seal spontaneously ruptured at the very early stage of perforation.

**GABA\(_B\) inhibition in the medial habenula**

The GABA\(_A\) excitation in the medial habenula is counterbalanced by the slow inhibition mediated by GABA\(_B\)Rs. The relative strength of the GABA\(_A\) excitation versus GABA\(_B\) inhibition appears to vary widely among different cells. In those cells in which the GABA\(_B\) inhibition was most prominent, the GABA\(_B\) IPSP produced a large membrane hyperpolarization that prevented most of the GABA\(_A\) EPSPs from triggering action potentials (Figs. 4 and 5).

Such prominent activation of GABA\(_A\) inhibition may result from the high density of GABA\(_A\)R expression in the medial habenula. Previous in situ hybridization studies revealed that the medial habenula is among the brain areas that express the highest density of GABA\(_A\)R mRNAs (Jones et al. 1998; Kaupmann et al. 1998; Künner et al. 1999). Consistent with these studies, our GABA\(_B\)R immunohistochemistry revealed a remarkably high density of GABA\(_B\)Rs in the somatodendritic membrane of medial habenular cells. Yet, it is unclear how the
low-strength stimulation of GABAergic input selectively activates GABA\(_{\text{A}}\) inhibition as shown in a subpopulation of medial habenular cells (Fig. 5). Considering the prevailing view that GABA\(_{\text{A}}\)Rs are localized mostly in the extrasynaptic membrane (Kulik et al. 2003; Mody 1994; Scanziani 2000), one possibility is that the GABA uptake system in the medial habenula is exceptionally poor in its efficiency. Accordingly, GABA would diffuse out of the synaptic cleft relatively easily and reach the GABA\(_{\text{A}}\)Rs localized in the extrasynaptic membrane of neighboring cells, thus generating only the GABA\(_{\text{B}}\) IPSP in these cells. An alternative possibility, considering the high-density expression of GABA\(_{\text{A}}\)Rs in the somatodendritic membrane of medial habenular cells, is that some of these receptors are colocalized with GABA\(_{\text{A}}\)Rs in the postsynaptic membrane apposing GABAergic terminals. The affinity of GABA\(_{\text{A}}\)Rs for GABA is known to be much higher than that of GABA\(_{\text{B}}\)Rs (Sodickson and Bean 1996). Therefore minimum release of GABA in the postsynaptic membrane, whereas increases in GABA release by increasing stimulus intensity result in GABA binding to both GABA\(_{\text{A}}\)Rs and GABA\(_{\text{B}}\)Rs. It also cannot be ruled out that some GABAergic synapses in the medial habenula contain only GABA\(_{\text{A}}\)Rs in the active zone of the postsynaptic membrane and our low-strength stimulation activated only these synapses. It remains to be seen whether any of the above-cited possibilities indeed underlies the selective activation of GABA\(_{\text{B}}\) inhibition in the medial habenula. It is noteworthy that the selective activation of GABA\(_{\text{B}}\) slow inhibition was reported previously in a few brain areas such as the ventral tegmental area, lateral amygdala nucleus, CA1 hippocampal pyramidial neurons, and layer V pyramidal neurons of somatosensory cortex (Benardo 1994; Nurse and Lacaille 1997; Sugita et al. 1992; Williams and Lacaille 1992). In these areas, activation of local GABAergic interneurons often generated either the GABA\(_{\text{A}}\) fast inhibition or GABA\(_{\text{B}}\) slow inhibition alone in the postsynaptic cell, raising the possibility that synaptic inputs to GABA\(_{\text{A}}\)Rs and GABA\(_{\text{B}}\)Rs may originate from separate populations of interneurons.

In recent years, it has been indicated that the synaptic response mediated by GABA\(_{\text{A}}\)Rs and GABA\(_{\text{B}}\)Rs can be regulated to a differential degree by intracellular signaling through various kinase systems (Dutar and Nicoll 1988a; Hahner et al. 1991; Kano et al. 1992; Kuramoto et al. 2007; Nusser et al. 1999; Poisbeau et al. 1999; Song and Messing 2005). Such differential regulation, if occurring in the medial habenula, would alter the relative size of the GABA\(_{\text{A}}\) excitation versus GABA\(_{\text{B}}\) slow inhibition, thus varying the scale of the GABAergic excitation under different neuromodulatory conditions. It is an interesting possibility that the difference in the relative strength of the GABA\(_{\text{A}}\) excitation versus GABA\(_{\text{B}}\) inhibition among different medial habenular cells in our study may be due, at least in part, to the difference in the basal activity of those kinase systems among these cells.

In conclusion, our study demonstrated that the GABAergic regulation of the cellular output can be more versatile than hitherto considered in the mature mammalian brain and produces a distinct temporal pattern of cell firing.

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J Neurophysiol • VOL 98 • SEPTEMBER 2007 • www.jn.org


