ABSENCE OF GABA TYPE A SIGNALING IN ADULT MEDIAL HABENULAR NEURONS

D.-G. WANG, a,b,c N. GONG,a,b B. LUOa AND T.-L. XUa,b*

*aDepartment of Neurobiology and Biophysics, School of Life Sciences, University of Science and Technology of China, Huang-Shan Road, Hefei 230027, Anhui, China
bInstitute of Neuroscience and Key Laboratory of Neurobiology, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, China
cDepartment of Human Anatomy, Xuzhou Medical College, 84 West Huai-Hai Road, Xuzhou 221002, Jiangsu, China

Abstract—Neural inhibition in the brain is mainly mediated by ionotropic GABA type A receptors. Apart from the GABA type A receptors, both K⁺-Cl⁻ cotransporter isoform 2 and the GABA-synthesizing enzyme, glutamic acid decarboxylase, are essential determinants for GABA type A receptor-mediated inhibition. By using immunofluorescent staining, we observed that K⁺-Cl⁻ cotransporter isoform 2 and the GABA type A receptor β2/3 subunits and a presynaptically localized glutamic acid decarboxylase isoform, glutamic acid decarboxylase 65, were all absent in adult Sprague-Dawley rat medial habenular nucleus, while immunopositive staining for glutamic acid decarboxylase 67, GABA and GABA type B receptor type 2 subunit were present in the medial habenular nucleus. Consistent with the lack of GABA type A signaling as detected by immunohistochemistry, GABA (100 μM) evoked no measurable currents in the medial habenular nucleus but induced bicuculline-sensitive currents in the lateral habenular nucleus and in the CA1 area of hippocampus. We also failed to record miniature inhibitory postsynaptic currents in medial habenular nucleus neurons. These results support the idea that GABAergic transmission in medial habenular nucleus is probably not mediated by any of the most common GABA type A receptor subtypes. Our data suggest that GABA type B receptor-mediated inhibition may play a role in balancing neuronal excitation in this special region. Further exploration for factors determining medial habenular nucleus neural inhibition will lead to a more complete understanding of control of synaptic balance in the CNS. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: central inhibition, synaptic balance, KCC2, GABA signaling, circadian rhythm, habenular nucleus.

*Correspondence to: T.-L. Xu, Institute of Neuroscience and Key Laboratory of Neurobiology, Chinese Academy of Sciences, 320 Yue-Yang Road, Shanghai 200031, China. Tel: +86-21-5492-1751; fax: +86-21-5492-1735. E-mail address: txu@ion.ac.cn (T.-L. Xu).

Abbreviations: ACSF, artificial cerebrospinal fluid; ChAT, choline acetyltransferase; CIC-2, voltage-gated chloride channel type 2; CNQX, 6-cyano-7-nitroquinolinoxaline-2,3-dione; d-AP5, (−)-2-amino-5-phosphonovaleric acid; GABA, glutamic acid decarboxylase; GABAAR, GABA type A receptor; GABAAR-Cl⁻ cotransporter isoform 2 and the potassium chloride cotransporter-2; Hb, the lateral habenular nucleus; Mhb, the medial habenular nucleus; mPSCs, miniature inhibitory postsynaptic currents; NDAE, Na⁺-dependent anion (Cl⁻-HCO₃⁻) exchanger; PBS, phosphate-buffered saline; V_m, holding potential.

Neural information processing is mediated by integration of excitatory and inhibitory synaptic inputs. Therefore, precise controls of the relative number of each type of synaptic inputs must exist to maintain an appropriate synaptic balance. Glutamate, which is released at excitatory glutamatergic synapses, is responsible for excitatory neurotransmission in the CNS. On the other hand, GABA is the main inhibitory neurotransmitter in the adult CNS. Upon release from presynaptic terminals, GABA binds to postsynaptic GABA type A receptor (GABAAR) and opens GABAAR-Cl⁻ channels. This leads to an influx of chloride ions into the postsynaptic cytoplasm. The resulting hyperpolarization raises the threshold for neuronal firing and thereby inhibits the postsynaptic neuron. Furthermore, the opening of GABAAR-Cl⁻ channels would decrease the input resistance of neurons, causing another type of shunting inhibition. Besides GABAAR, the action of GABA can also be mediated by GABAB receptor (GABABR). The GABABR is metabotropic G protein-coupled receptor and can locate at both pre- and postsynaptic sites. At GABAergic terminal, the activation of GABABR can cause presynaptic inhibition by suppressing calcium influx and reduce GABA release. The GABABR can also achieve postsynaptic inhibition by activating potassium currents that hyperpolarize the postsynaptic neuron (Misgeld et al., 1995; Bowery et al., 2002).

GABA is produced mainly through enzymatic decarboxylation of glutamic acid by glutamic acid decarboxylase (GAD). GAD65/67 isoforms have been observed in the GABAAergic neurons that are distributed throughout the CNS (Kaufman et al., 1991; Esclapez et al., 1994). The GAD65 mainly contributes to GABA synthesis in axonal boutons while GAD67 synthesizes GABA in somata and dendrites. An efficient chloride extrusion mechanism to maintain low intracellular chloride concentrations in postsynaptic neurons is required for GABAAR-mediated inhibitory neurotransmission (Misgeld et al., 1986; Kaila, 1994). Chloride extrusion is accomplished primarily by the neuron-specific potassium chloride cotransporter-2 (KCC2) (Gillen et al., 1996; Payne, 1997; Mount et al., 1999; Rivera et al., 1999). The KCC2 is expressed throughout the adult CNS and it maintains the Cl⁻ equilibrium potential of mature neurons at values more negative than the resting membrane potential (Gillen et al., 1996; Payne, 1997; Mount et al., 1999; Rivera et al., 1999). Thus, abundant KCC2 expression is always linked to GABAAR-mediated inhibitory neurotransmission. For example, the developmental upregulation of KCC2 expression determines the switch of GABA from excitatory to inhibitory effect (Rivera et al., 1999). However, it is surprising that some populations of adult CNS...
neurons lack KCC2 mRNA. These include the neurons of olfactory bulb and suprachiasmatic nucleus, as well as those of the medial habenula (MHb) (Rivera et al., 1999; Kanaka et al., 2001). Olfactory bulb and suprachiasmatic nucleus neurons show excitatory responses to GABA (Desmannien et al., 1979; Rivera et al., 1999; Kanaka et al., 2001; Kaneko et al., 2004; Albus et al., 2005; Reisert et al., 2005), due to probably an impaired Cl⁻ extrusion resulting from the lack of KCC2 expression.

Mammalian MHb is part of the thalamus and is involved in the modulation of several fundamental functions including pain perception, sleep, neuroendocrine and stress responses (Sugama et al., 2002). Both glutamate and ATP act as excitatory neurotransmitters in the MHb (Khan et al., 2000; Kim and Chang, 2005). Normal neuronal function depends on a delicate balance between excitatory and inhibitory synaptic activity. However, the KCC2 mRNA signals were not detected in MHb (Rivera et al., 1999; Kanaka et al., 2001). It remains unknown whether GABA exerts excitatory effect in MHb, and if GABA is excitatory in MHb, then which mechanism might be involved in balancing neural excitation in this region. Therefore, in this study, we examined the elements related to GABA provoke excitation in the CNS. Because GABA itself promotes neuronal KCC2 expression via GABAAR activation (Ganguly et al., 2001), we also paid special attention to the expression of KCC2, GABA-synthesizing enzyme GAD65/67 and the GABAAR β2/3 subunits, which are the major GABAAR subunits which are involved in balancing neural excitation in this region. Therefore, in this study, we examined the elements related to GABA provoke excitation in the CNS. Because GABA itself promotes neuronal KCC2 expression via GABAAR activation (Ganguly et al., 2001), we also paid special attention to the expression of KCC2, GABA-synthesizing enzyme GAD65/67 and the GABAAR β2/3 subunits, which are the major GABAAR subunits which are involved in balancing neural excitation in this region.

**EXPERIMENTAL PROCEDURES**

### Animals

The care and use of animals for these experiments followed the guidelines and protocols approved by the Institutional Animal Care and Use Committee of the Institute of Neuroscience, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences. Male adult Sprague–Dawley rats (250–280 g) were deeply anesthetized with pentobarbital sodium (60 mg/kg intraperitoneally) and transcardially perfused with 0.9% NaCl followed by ice-cold 40 g/L paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and post-fixed in the same fixing overnight before being transferred to a 30% sucrose (4 °C) for 2–3 days (in phosphate buffer). All rats were killed during daytime for immunohistochemical and brain slice experiments. All efforts were made to minimize the number of animals used and their suffering.

### Immunohistochemical staining

Male adult Sprague–Dawley rats (250–280 g) were deeply anesthetized with pentobarbital sodium (60 mg/kg intraperitoneally) and transected with 0.9% NaCl followed by ice-cold 40 g/L paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and post-fixed in the same fixative overnight before being transferred to a 30% sucrose (4 °C) for 2–3 days (in phosphate buffer). All brains were cut into 30 μm thick coronal sections on a freezing microtome (Leica SM 1900, Wetzlar, Germany). Sections were collected from each rat brain. Sections of rat brains containing both habenula and hippocampus (from bregma ~3.30 to –3.80 mm) were selected for immunofluorescence labeling and counterstaining with Cresyl Violet (red fluorescence). The free-floating method was used. After washing with 0.01 M phosphate-buffered saline (PBS), the sections were incubated with a blocking solution (10% normal goat serum, 0.1% Triton X-100 in PBS) for 30 min at 37 °C. The sections were divided into four groups, and then probed with 1) rabbit anti-KCC2 antibody (1:200, Upstate, Lake Placid, NY, USA) and mouse anti-GAD65 antibody (1:500, BD Biosciences, San Jose, CA, USA); 2) rabbit anti GABA (1:300, Sigma, St. Louis, MO, USA) and mouse anti-GABAAR β2/3 subunit (1:100, Chemicon, Temecula, CA, USA); 3) rabbit anti-choline acetyltransferase (Accurate, Westbury, NY, USA) and mouse anti-GAD67 antibody (1:300, Chemicon); and 4) guinea-pig anti-GABAAR subunit type 2 (1:100, Upstate) respectively at room temperature (20–25 °C) for 1 h, followed by overnight incubation at 4 °C. After rinsing in PBS, the sections were incubated with Cy5 goat anti-rabbit (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA, USA), donkey anti-mouse FITC-conjugated IgG (1:140, Rockland Immunocyticals, Gilbertsville, PA, USA), donkey anti-rabbit rhodamine-conjugated IgG (1:140, Rockland Immunocyticals). All sections were washed three times for 5 min each and then mounted on glass slides, covered with coverslips, sealed with nail polish oil and stored at 4 °C. Fluorescent images were captured by a laser-scanning confocal microscope (LSM510, Carl Zeiss, Jena, Germany). The LSM510-related software was used to control the microscope and its settings. Immunofluorescent controls for the specificity of the detection methods were carried out by omitting the incubation step with the primary antibodies. Slides not exposed to primary antibodies did not show specific labeling comparable to that obtained following primary antibody exposure.

### Electrophysiological recording

The P14-P15 Sprague–Dawley rats were decapitated under ether vapor anesthesia. The brains were quickly isolated and mounted in 4% agarose in an oxygenated artificial cerebrospinal fluid (ACSF). Transverse slices (400 μm) of brain containing habenular nucleus and hippocampus were made by a Leica VT1000S vibratome. They were then incubated in ACSF for at least 1 h at room temperature (20–25 °C) before electrophysiological recording. The ACSF had the following composition (mM): 126 NaCl, 3 KCl, 2 CaCl₂, 2 MgSO₄, 24 NaHCO₃, 1.25 NaNH₂PO₄, 2 CaCl₂, 10 glucose. The ACSF had a pH of 7.4 when saturated with 95% O₂–5% CO₂, and an osmolarity of 305–310 mOsm kg⁻¹. Whole-cell recordings were performed by a patch clamp amplifier (EPC-9, HEKA Electronics, Lambrecht, Germany). For recording miniature inhibitory postsynaptic currents (mIPSCs), (α-) 2-aminophosphonovoric acid (d-AP5, 25 μM) and 6-cyano-7-nitroquinazoline-2,3-dione (CNQX, 10 μM) were added to the ACSF to block glutamatergic synaptic input, and TTX (1 μM) was added to block action potential. To record GABAAR-, GABAAR- and glycine receptor-mediated currents, the agonists (100 μM GABA, 10 μM baclofen and 500 μM glycine, respectively) and the antagonists (10 μM bicuculline, 500 μM phaclofen and 2 μM strychnine, respectively) were used through bath application. Recording electrodes were fabricated from 1.5-mm outer diameter glass microcapillaries, and they had a resistance of 4–6 MΩ. Whole-cell voltage-clamp recordings were obtained with pipettes containing (in mM): 120 KCl, 30 NaCl, 0.5 CaCl₂, 1 MgCl₂, 5 EGTA, 10 HEPES, and 2 NaATP, pH 7.2. For recording GABAAR-mediated currents, 2 mM MgATP was added into the intracellular pipette solution. The liquid junction potential was measured and corrected. Signals were filtered (1–5 kHz) and sampled at 50–200 μs intervals with a 12 bit A/D converter connected to a personal computer, and were stored on a hard disk. After recordings, we analyzed only the neurons whose resting potential were below ~50 mV. All drugs were purchased from Sigma.

### Data analysis

Data were analyzed using SigmaStat and shown as the mean±S.E.M., with statistical significance assessed by one-way analysis of variance (ANOVA).
Fig. 1. Identification of the habenular complex. (A) The sketch of MHb and LHb in habenular complex. (B) Nissl staining of habenular complex neurons. Note the tightly packed neurons in MHb, while neurons in LHb are sparse. (C) ChAT immunostaining in habenular complex. Positive stainings were seen in both MHb and LHb. Scale bar = 500 μm. 3V, the third ventricle. In this and subsequent figures, the dotted circle indicates the boundary of the MHb.

Fig. 2. MHb neurons lack KCC2, GABA\textsubscript{A}R β2/3 and GAD65 immunostaining. (A–D) Representative sections stained with KCC2 antibody. KCC2 immunostaining was absent in MHb neurons (A and C), while present in the LHb (A and B) and in the CA1 region of hippocampus (Hipp). Note that the KCC2 immunoreactivity was localized in somatic and dendritic membranes of CA1 pyramidal neurons (D). (E–H) Representative sections stained with GABA\textsubscript{A}R β2/3 antibody. GABA\textsubscript{A}R β2/3 immunostaining was absent in MHb neurons (E and G), but present markedly in LHb (E and F) and hippocampal neurons (H). (I–L) Representative sections stained with GAD65 antibody. The immunoreactivity of the GAD65 is similar to that of KCC2. MHb neurons lacked GAD65 immunostaining while neurons of the LHb and Hipp had abundant GAD65 staining. Scale bar = 500 μm in A, E and I, and 50 μm in the other panels.
Identification of the habenular complex

In coronal sections stained with Cresyl Violet, the borders between the MHb and lateral habenular nuclei (LHb) and between the habenular complex and neighboring dorsal thalamus are clearly delineated (Fig. 1B). The MHb is characterized by a remarkable high density of cells. Due to the tight packing of cells, the MHb stained much more densely than the LHb, where cells dispersed more loosely. Consistent with the previous report (McCormick and Prince, 1987), we also found positive immunostaining with anti-choline acetyltransferase (ChAT) antibody. The features of the borders revealed by Nissl staining were used in the following studies to identify the immunostained cells in the habenular complex.

The MHb neurons lacked KCC2 expression

By using immunofluorescence staining, we observed that KCC2 immunoreactive staining was absent in the plasma membranes of the MHb neurons (Fig. 2A, 2C). However, there was abundant KCC2 immunoreactive staining in the hippocampal CA1 region (Fig. 2D), and the LHb which is lateral to the MHb (Fig. 2A, 2B). The plasma membranes of neuronal somata and dendrites were clearly stained by KCC2 antibody in the CA1 region and the LHb (Fig. 2A, 2B). The positive staining we observed in the CA1 region is consistent with a previous report (Gulyas et al., 2001), indicating that the MHb neurons lacking KCC2 expression cannot be attributed to artificial negative staining. A possible effect of environmental lighting cycles on KCC2 expression in the MHb neurons was also excluded because the MHb neurons from rats killed during nighttime did not show KCC2 staining either (data not shown).

Both GAD65 and GABA<sub>α</sub>R β2/3 subunits were absent in the MHb neurons

In addition to KCC2, GABA<sub>α</sub>R and GABA are also required for GABA<sub>α</sub>R-mediated inhibition in adult CNS. Our immunofluorescence staining indicates that GABA<sub>α</sub>R β2/3 subunits were absent in the MHb (Fig. 2E, 2F) but present in the LHb (Fig. 2E, 2F) and hippocampal CA1 region (Fig. 2H). Similarly, GAD65 was also absent in MHb neurons (Fig. 2I, 2K) but present in LHb (Fig. 2I, 2J) and hippocampal CA1 neurons (Fig. 2L).

Exogenous GABA induced no measurable currents in MHb neurons

To explore the direct effect of exogenously applied GABA, we performed electrophysiological recordings in brain slices from habenular nuclei and CA1 area of hippocampus. At a holding potential ($V_{h}$) of $-50$ mV, $100 \mu M$ GABA evoked no detectable currents in MHb neurons ($n=11$), but induced large inward currents, which were completely blocked by $10 \mu M$ bicuculline, the selective GABA<sub>α</sub>R antagonist, in either LHb ($n=6$) or hippocampal neurons ($n=7$) (Fig. 3). We also failed to observe any measurable currents induced by glycine ($500 \mu M$), another fast inhibitory neurotransmitter, in MHb neurons; but $500 \mu M$ glycine induced an inward current, which was completely blocked by the selective glycine receptor antagonist strychnine ($2 \mu M$), in the LHb and hippocampal neurons (Fig. 4).

![Fig. 3. GABA induces no currents in MHb neurons. (A) Representative current traces induced by 100 µM GABA in the absence or presence of 10 µM bicuculline. GABA (100 µM) evoked bicuculline-sensitive currents in both LHb and hippocampal neurons, but not in MHb neurons. (B) Statistical data showing the averaged peak amplitude of $I_{GABA}$ induced by 100 µM GABA in the MHb, LHb and hippocampal neurons. * P<0.05, compared with $I_{GABA}$ in MHb group. Each column represents mean±S.E.M (n=6–11). $V_{h}=-50$ mV.](image)

![Fig. 4. Glycine induces no currents in MHb neurons. Representative current traces showing 500 µM glycine-induced currents in the absence or presence of 2 µM strychnine. Glycine evoked strychnine-sensitive currents in both LHb and hippocampal neurons but not in MHb. Similar results were obtained from another four neurons. $V_{h}=-50$ mV.](image)
endogenous ligand for activating the GABABR because however, local GABAergic interneurons may produce given the finding that GAD65 was absent in the MHb. This result was somewhat unexpected mIPSCs were evident (Fig. 5).

Another four neurons. VH blocked by 10^{-7} M, CNQX (10^{-5} M) and TTX (1 \mu M) to block action potential and fast excitatory synaptic inputs. At a V_h of −70 mV, mIPSCs could be detected in LHb neurons as in many other CNS neurons, which were completely abolished by 10 \mu M bicuculline. Similar results were obtained from another four neurons. V_h = −70 mV.

These results confirm that both functional GABA_A R and glycine receptors were absent in the MHb neurons.

To further confirm the absence of the fast inhibitory neurotransmission to the MHb, we recorded the miniature inhibitory postsynaptic currents (mIPSCs) in the presence of d-AP5 (25 \mu M), CNQX (10 \mu M) and TTX (1 \mu M) to block action potential and fast excitatory synaptic inputs. At a V_h of −70 mV, mIPSCs could be detected in LHb neurons as in many other CNS neurons, which were completely abolished by 10 \mu M bicuculline. However, in MHb neurons, no mIPSCs were evident (Fig. 5).

The MHb neurons expressed GABA, GAD67 and GABA_B R type 2 subunit

GABA mediates inhibition by activating either GABA_A R or GABA_B R receptor (GABA_B R). To investigate whether GABA_B R is expressed in the MHb, we detected the expression of GABA_B R type 2 subunit in the MHb. We found a high density of GABA_B R type 2 subunit in the MHb (Fig. 6I, 6K). This result was somewhat unexpected given the finding that GAD65 was absent in the MHb. However, local GABAergic interneurons may produce endogenous ligand for activating the GABA_B R because GAD65 staining may represent the presynaptically localized GABA derived from GABAergic projections outside the MHb. To test this possibility, we immunostained GABA, and GAD67, which is responsible for synthesizing GABA in somata and dendrites. A moderate GAD67 immunostaining was found in MHb (Fig. 6A, 6C), LHB (Fig. 6A, 6B) and hippocampal neurons (Fig. 6D). There was also GABA-positive immunostaining in the MHb (Fig. 6E, 6G), LHB (Fig. 6E, 6F) and in the hippocampus (Fig. 6H). Thus, it is very likely that GABA_B R signaling may be present in the MHb.

Baclofen induced GABA_B R-mediated currents in MHb neurons

To confirm the presence of GABA_B R signaling in MHb, the specific GABA_B R agonist baclofen was used to record GABA_B R-mediated currents. Consistent with the previous studies in many CNS regions, we found that 10 \mu M baclofen activated an obvious outward current in both MHb and LHb at a V_h of −50 mV (Fig. 7A, 7B). These currents could be abolished in the presence of a specific GABA_B R antagonist phaclofen (500 \mu M) (Fig. 7C, 7D). This result suggests that GABA_B R signaling is present in MHb.

DISCUSSION

From its fundamental involvement in neuronal excitability, spike-timing, and synaptic plasticity, GABA_A R-mediated neural inhibition is assumed to be present in most, if not all, neurons. In this study, we have provided direct evidence that GABA_A R signaling is absent in the adult MHb neurons. Although the reasons for the absence of GABA_A R signaling in MHb are unknown, such absence may explain many distinct electrophysiological and functional properties between the MHb and the LHb. For example, it is well known that GABA_A R signaling is crucially involved in circadian rhythms generation in the suprachiasmatic nuclei (Albus et al., 2005). A recent study reported that LHb rather than MHb is also involved in circadian rhythms regulation (Zhao and Rusak, 2005). It would be very interesting to know whether the absence of GABA_A R signaling in the MHb may explain the inability of MHb in generating circadian rhythms. Moreover, the MHb neurons generate tonic trains of action potentials, whereas LHb neurons are capable of producing action potentials in burst mode (Kim and Chang, 2005). This difference may also attribute to the lack of GABA_A R signaling in MHb neurons.

Lack of expression of KCC2, GABA_A R and GAD accounts for the absence of GABA_A R signaling

Efficient chloride extrusion is required for GABA_A R-mediated inhibitory neurotransmissions (Kaila, 1994; Misgeld et al., 1995). KCC2 is critical for extruding Cl^- and maintaining postsynaptic chloride homeostasis (Thompson and Gahwiler, 1989; Gillen et al., 1996; Payne et al., 1996; Hiki et al., 1999; Mount et al., 1999; Rivera et al., 1999; Payne et al., 2003). It keeps the Cl^- equilibrium potential (E_Cl^-) at values more negative than the resting membrane potential in adult neurons (Gillen et al., 1996; Payne et al., 1996; Hiki et al., 1999; Mount et al., 1999). Employing a widely-used KCC2 antibody (Lu et al., 1999; Mount et al., 1999; Rivera et al., 1999; Gulyas et al., 2001), we found that KCC2 expression was completely absent in MHb neurons. The data thus extend the previous finding that MHb neurons lack KCC2 mRNA (Kanaka et al., 2001).

There are other Cl^- extruding mechanisms that may contribute to the intracellular chloride regulation in MHb. For example, Na^-dependent anion (Cl^-/HCO_3^-) exchanger (NDAE) is capable of causing Cl^- extrusion (Romero et al., 2000; Grichtchenko et al., 2001). However, because the activity of NDAE depends largely on intracel-
lular pH, it is much less effective at Cl\(^{-}\)/HCO\(_3\) extrusion than KCC2 (Kaila, 1994; Schwiening and Boron, 1994). In addition to Cl\(^{-}\)/HCO\(_3\) transporters, the inwardly rectifying voltage-gated chloride channel type 2 (ClC-2), is likely to play a role in the clearance of intracellular chloride (Staley, 1994; Smith et al., 1995). ClC-2 is expressed in retinal rod bipolar cells (Enz et al., 1999), hippocampal pyramidal cells (Miguel et al., 1986; Staley, 1994; Smith et al., 1995; Sik et al., 2000) and neurons of substantia nigra (Gulacsi et al., 2003). But their expression does not explain the hyperpolarizing GABA\(_{\lambda}\) responses observed in these cells (Gulacsi et al., 2003). Apparently, further studies are required for understanding the intracellular chloride regulation in MHB neurons without KCC2 expression.

In addition to KCC2, we also studied the expression of GABA\(_{\lambda}\)R and GABA-synthesizing enzymes, which are essential elements of GABAergic transmission. GABA is produced mainly through enzymatic decarboxylation of glutamic acid by GAD. GAD65/67 isoforms have been observed in the same GABAergic neurons with distinct cellular distribution (Kaufman et al., 1991; Esclapez et al., 1994). We did not detect GAD65-positive staining in the MHB by using immunofluorescent staining, suggesting the lack of GABA in presynaptic terminals. The GABA\(_{\lambda}\)R \(\beta2/3\) subunits were also undetectable in the MHB. Furthermore, our electrophysiological recordings indicate that mIPSCs were absent in the MHB, and exogenously applied GABA did not induce any detectable currents in MHB neurons but evoked GABA\(_{\lambda}\) responses in LHb and hippocampal neurons. In our experiment, with the high concentration of Cl\(^{-}\} in the pipette, the Cl\(^{-}\) reverse potential was approximately 0 mV, while the \(V_{\lambda}\) was −50 mV for recording GABA- and glycine-induced currents and −70 mV for recording mIPSCs. Thus, the absence of GABA-induced currents and mIPSCs was not due to the lack of Cl\(^{-}\) driving force. Therefore, our results provide direct evidence that GABA\(_{\lambda}\) signaling is absent in the adult rat MHB.

![Fig. 6. MHB neurons express GAD67, GABA and GABA\(_{\lambda}\)R2. (A–D) Representative sections stained with GAD67 antibody. Compared with GAD65 (Fig. 3I–L), GAD67 was less abundant in the habenular complex and hippocampus (A, B and D). But moderate staining was also observed in the MHB (C). (E–H) Representative sections stained with GABA antibody. There are diffusive GABA positive neurons in the MHB (E and G), LHb (E and F) and hippocampus (H). (I–L) Representative sections stained with GABA\(_{\lambda}\)R subunit 2 (GABA\(_{\lambda}\)R2) antibody. The MHB neurons were strongly stained (I and K). There were moderate positive immunostainings in the LHb (I and J) and hippocampus (L). Scale bar=500 \(\mu\)m in A, E, and I, 50 \(\mu\)m in the other panels.](image-url)
However, it should be noted that, like the olfactory bulb, MHb often shows the expression of some unusual receptor or channel subtypes. For example, the \( \alpha_4 \) subunit of the nicotinic receptor has been shown to express in MHb specifically (Duvoisin et al., 1989). The small conductance calcium-activated potassium channel type 3 is particularly strong here (Tacconi et al., 2001). Furthermore, an orphan G protein-coupled receptor GPCR-2037 also shows specific expression in MHb (Berthold et al., 2003). Therefore, in our study, we cannot exclude the expression of some unusual GABA\(_A\)R subtypes in MHb. It is also possible that there exist other unidentified GABA\(_A\)R-mediated currents that cannot be detected in our experimental conditions. Nonetheless, our immunohistochemical and electrophysiological data indicate that the most common GABA\(_A\)R subtypes do probably not mediate GABAergic transmission in MHb.

Factors affecting MHb neural inhibition

The fast excitatory synaptic transmission in the rat MHb is mediated by ATP and glutamate (Edwards et al., 1992; Robertson et al., 1999; Price et al., 2003). There are alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, metabotropic glutamate receptors, and NMDA receptor NR2B subunits in the MHb (Khan et al., 2000; Kim and Chang, 2005). In addition, acetylcholine also plays an excitatory role in the MHb (McCormick and Prince, 1987). Since we have shown that GABA\(_A\) signaling is absent in the MHb, what factors or mechanisms determine the MHb neural inhibition that would be crucial in controlling synaptic balance? Though glycine is also an inhibitory neurotransmitter, glycnergic synapses are mainly located in the spinal cord, cerebellum and brain stem (Aragon and Lopez-Corcuera, 2003; Eulenburg et al., 2005). In the present study, exogenous glycine (500 \( \mu M \)) induced no detectable currents in MHb neurons, suggesting that glycine receptor-mediated inhibition may not play a role in the MHb.

On the other hand, it is well accepted that GABA exerts its inhibitory effects through both GABA\(_A\)R and postsynaptic GABA\(_B\)R. In support of this notion, we observed an abundant expression of GABA\(_A\)R type 2 subunit and a marked baclofen-induced current in the MHb. Since we observed that GAD67 and GABA were positively immunostained in the MHb, suggesting the presence of endogenous GABA\(_A\)R ligand, we assumed that GABA\(_A\)R-mediated inhibition might play a role in the MHb. This assumption is supported by a previous observation that Kir3.2 potassium channels, which are tightly coupled with GABA\(_A\)Rs in CNS neurons, were abundantly expressed in the MHb (Geisler et al., 2003). Additionally, there is a high density of \( \mu \)-receptors in the MHb (Cherubini and North, 1985). Endogenous opioid and exogenous morphine may inhibit neuronal excitation via the postsynaptic \( \mu \)-receptors, and also suppress the release of neurotransmitters and or neuromodulators from axon terminals through the presynaptic \( \mu \)-receptors (Ding et al., 1996). Endogenous adenosine can also inhibit the release of glutamate or ATP through adenosine receptors and exert its inhibitory role (Robertson and Edwards, 1998). Finally, other mechanisms such as the small conductance calcium-activated potassium channel SK3 (Tacconi et al., 2001), the orphan G protein-coupled receptors GPCR-2037 (Berthold et al., 2003) and GPRg1 (Matsuo et al., 2005), which are all densely distributed in the MHb, have to be considered.

Is the lack of KCC2 expression a result of the absence of GABA\(_A\) signaling?

In early development, GABA is excitatory and involved in many functions including neuronal development and mat-
uration (Ben-Ari, 2002). Because GABA itself promotes neuronal KCC2 expression via GABA\(_A\)R activation and subsequent calcium influx through voltage-dependent Ca\(^{2+}\) channels (Ganguly et al., 2001), it would be very interesting to know whether the lack of KCC2 expression in MHB is a result of the absence of GABA\(_A\) signaling. On the other hand, developmental increase of KCC2 expression shifts the function of GABA from excitatory to inhibitory (Lu et al., 1999; Rivera et al., 1999; Lee et al., 2005). The adult neurons lacking KCC2 expression always generate excitation in response to GABA (Kanaka et al., 2001). Interestingly, the effect of GABA was shifted from inhibition to excitation under certain pathological conditions in which KCC2 expression was down-regulated (Nabekura et al., 1999; Rivera et al., 1999; Lee et al., 2005). The adult neurons lacking KCC2 expression always generate excitation in response to GABA (Kanaka et al., 2001). Interestingly, the effect of GABA was shifted from inhibition to excitation under certain pathological conditions in which KCC2 expression was down-regulated (Nabekura et al., 1999; Rivera et al., 1999; Lee et al., 2005). This missing pathway may explain many distinct properties of the MHB neurons including their inability to generate circadian rhythms. Without this pathway, GABA\(_A\)R- and/or other G protein-coupled receptor-mediated inhibition may play a role in balancing excitatory synaptic activities.

**CONCLUSION**

In summary, our study demonstrates the absence of GABA\(_A\) signaling in the adult MHB. This missing pathway may explain many distinct properties of the MHB neurons including their inability to generate circadian rhythms. Without this pathway, GABA\(_A\)R- and/or other G protein-coupled receptor-mediated inhibition may play a role in balancing excitatory synaptic activities.

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