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Serotonin stimulates lateral habenula via activation of the post-synaptic serotonin 2/3 receptors and transient receptor potential channels

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Abstract

There is growing interest on the role of the lateral habenula (LHb) in depression, because it closely and bilaterally connects with the serotonergic raphe nuclei. The LHb sends glutamate efferents to the raphe nuclei, while it receives serotonergic afferents, and expresses a high density of serotonin (5-HT) receptors. Recent studies suggest that 5-HT receptors exist both in the presynaptic and postsynaptic sites of LHb neurons, and activation of these receptors may have different effects on the activity of LHb neurons. The current study focused on the effect of 5-HT on the postsynaptic membrane. We found that 5-HT initiated a depolarizing inward current ($I_{(5-HT)}$) and accelerated spontaneous firing in ~80% of LHb neurons in rat brain slices. $I_{(5-HT)}$ was also induced by the 5-HT uptake blocker citalopram, indicating activity of endogenous 5-HT. $I_{(5-HT)}$ was diminished by 5-HT_{2/3} receptor antagonists (ritanserin, SB-200646 or ondansetron), and activated by the selective 5-HT_{2/3} agonists 1-(3-Chlorophenyl) piperazine hydrochloride or 1-(3-Chlorophenyl) biguanide hydrochloride. Furthermore, $I_{(5-HT)}$ was attenuated by 2-Aminoethyl diphenylborinate, a blocker of transient receptor potential channels, and an IP3 receptor inhibitor, indicating the involvement of transient receptor potential channels. These results demonstrate that the reciprocal connection between the LHb and the 5-HT system highlights a key role for 5-HT stimulation of LHb neurons that may be important in the pathogenesis of depression.

Keywords

Lateral habenula; Depression; Serotonin receptors; Inward current; Dorsal raphe nuclei

1. Introduction

The lateral habenula (LHb), a phylogenetically conserved brain structure in the epithalamus, connects the forebrain with the monoamine neurons of the midbrain. Growing evidence

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indicates that LHb dysfunction is important in the pathophysiology of psychiatric disorders, such as major depression (Lecca et al., 2014; Proulx et al., 2014; Sachs et al., 2015). The LHb is one of the few brain structures to exhibit increased activity in animal models of stress and in human depression (Addolorato et al., 2012; Fowler and Kenny, 2014; Shumake et al., 2003). Deep brain stimulation of the habenula removes the depressive symptoms in both clinical (Sartorius et al., 2010) and preclinical settings (Addolorato et al., 2012; Meng et al., 2011). Lesion of the LHb can induce antidepressant-like effects, and pharmacological or electric inhibition of the LHb improves depressive-like behavior in rodents (Winter et al., 2011). Conversely, pharmacological activation of LHb neurons induces depressive-like responses in naive animals, and worsens the depressive-like behaviors in the hemi-parkinsonian rats (Han et al., 2015). Hence, the LHb is a reasonable and potential target for treating depression.

Since serotonin (5-HT) and the LHb have both been implicated to have significant roles in the onset of depression, it is important to better understand the effects of 5-HT on LHb neurons. Anatomical and functional evidence has shown that the LHb and the serotonergic midbrain raphe nuclei are closely interconnected (Bernard and Veh, 2012; Herkenham and Nauta, 1979). The LHb not only sends outputs to (Proulx et al., 2014), but also receives dense projections from the 5-HT system: the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) (Morin and Meyer-Bernstein, 1999). Additionally, the LHb expresses a high density of 5-HT_{2C} receptors (5-HT_{2C}Rs) (Pompeiano et al., 1994; Wagner et al., 2014) and other subtypes of 5-HT receptors (5-HTRs). Activation of 5-HT_{2C}Rs accelerates LHb neuronal firing of rats *in vivo* (Han et al., 2015), which may contribute to the pathophysiology of depression (Gurevich et al., 2002; Han et al., 2015; Martin et al., 2014). However, little is known regarding the mechanisms of 5-HT-induced excitation of LHb neurons.

In contrast to the excitatory action observed *in vivo*, a study in brain slices of mice reported that 1 μ M 5-HT suppresses the excitatory input from the basal ganglia to the LHb, but not the firing induced by a depolarizing current or the resting potential of LHb neurons (Shabel et al., 2012). In line with this, a study in brain slices of rats found that activation of 5-HT_{1B} receptors (5-HT_{1B}Rs) suppresses glutamate release on LHb neurons (Hwang and Chung, 2014). Conversely, a selective 5-HT reuptake inhibitor failed to reduce c-fos expression (a marker of neuronal activity) in the LHb in social defeat stress animals (Sachs et al., 2015). Together, these findings suggest that 5-HT may have different effects on the presynaptic and postsynaptic membrane of LHb neurons, both of which are important for LHb neuronal activity. However, evidence showing 5-HT modulation of LHb neuronal activity via post-synaptic mechanisms is lacking.

The LHb consists of medial (LHbm) and lateral (LHbl) subdivisions that have different connectivities. Whereas the LHbl receives inputs mainly from the basal ganglia (Shabel et al., 2014) and sends outputs through the rostromedial tegmental nuclei (RMTg) to the dopamine system and DRN (Proulx et al., 2014; Sego et al., 2014); the LHbm receives inputs from the limbic areas and sends outputs mainly to interneurons in the DRN and MRN (Kim, 2009; Varga et al., 2003). Hence, changes in the activity of LHb neurons in these sub-

regions could lead to different reactions on the target brain areas. However, it is unknown whether 5-HT actions on neurons from these two sub-regions are the same.

To address these questions, we performed electrophysiological recording from neurons in the LHb in brain slices of rats, with a focus on the postsynaptic membrane. We found that 5-HT induced a depolarizing inward current via the activation of 5-HT_{2/3} receptors in neurons in both the LHbm and LHbl. Our results support a key role for 5-HT stimulation of LHb neurons that may be important in the pathogenesis of depression.

2. MATERIALS AND METHODS

2.1. Animals

All procedures were approved by the Animal Care and Utilization Committee of Rutgers, the State University of New Jersey, in accordance with National Institutes of Health guidelines minimizing the number of animals used and their suffering. Animals were housed under standard conditions at 22–24 °C, 50–60% humidity, and a 12 h light/dark cycle. Food and water were available to all rats *ad libitum*. We performed electrophysiological recordings on neurons from about 130 Sprague-Dawley rats at postnatal days 25–35 of both sexes. Since the data obtained from juvenile male rats did not significantly differ significantly from female rats, the data were pooled together. Every effort was made to minimize both the number of animals used and their suffering.

2.2. Brain Slice Preparation

Brain slices were prepared as previously described (Ye et al., 2006; Zuo et al., 2013; Zuo et al., 2015). Briefly, animals were anesthetized with ketamine/xylazine (80 mg/5 mg/kg i.p.) and then euthanized by decapitation. Coronal epthalamic slices (250–300 µm) were cut with a Compresstome VF-200 slicer (Precisionary Instruments Inc., Greenville, NC, USA) in ice-cold glycerol-based artificial cerebrospinal fluid (GaCSF) containing (in mM): 252 glycerol, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂, 25 NaHCO₃, 1 L-ascorbate, and 11 glucose, and oxygenated with 95% O₂/5% CO₂ (carbogen). Slices were then incubated at least 1-hr at 24–25°C in carbogenated aCSF of similar composition as GaCSF, but with 126 mM NaCl replacing glycerol.

2.3. Electrophysiological Recording

Electrophysiological recordings were obtained with an Axon 200B amplifier, a Digidata 1440A A/D converter, and Clampfit 10.3 software (Molecular Devices Co., Union City, CA, USA). Data were filtered at 2 kHz and sampled at 5 kHz with an Axon Digidata 1440A interface. The experiments were done at 33°C, maintained by an automatic temperature controller (Warner Instruments, Hamden, CT, USA). aCSF was perfused at a rate of 1.5–2 ml/min. Patch pipettes were pulled from glass capillaries and had a resistance of 6–8 MΩ when filled with internal solutions containing (in mM) 140 cesium methanesulfonate, 5 KCl, 2 MgCl₂, 10 HEPES, 2 MgATP, 0.2 GTP for recordings under voltage clamp; and 140 potassium gluconate, 5 KCl, 2 MgCl₂, 10 HEPES, 2 MgATP, and 0.2 GTP under current clamp. The pH of these solutions was adjusted to 7.2 with Tris-base and the osmolality to 310 mOsmol/L with sucrose. LHb neurons were visually identified with a microscope

Fig. 1B–D). In 1/10 LHbm and 1/8 LHbl neurons, 5-HT induced a small hyperpolarization (data not shown).

3.2. 5-HT initiates an inward current in LHb neurons

Under voltage clamp at a holding potential of -70 mV and in the presence of TTX (0.5 μ M), strychnine (1 μ M), gabazine (10 μ M), AP5 (50 μ M) and DNQX (20 μ M), which block action potentials, glycine, GABA_A, NMDA and AMPA receptors, bath application of 10 μ M 5-HT produced an inward current in the majority of the neurons, in the LHbm (22/34, 64.7 %) and LHbl (32/42, 76.2 %) (Fig. 1E–F, I). A second 5-HT application to the same cell after more than 15 min washout of the first application, induced a current with the same amplitude of that induced by the first application (Fig. 1G). Two way ANOVA revealed that the amplitude of the inward current induced by 5-HT in LHbl neurons was significantly greater than that in LHbm neurons (main effect of subregions, $F_{1,166} = 8.0$, $p = 0.005$). The curves in Fig. 1H were fitted to the average current amplitude at each 5-HT concentration by using the logistic equation (main effect of doses, $F_{5,166} = 24.5$, $p < 0.001$). *Post-hoc* tests showed that the current amplitude was significantly increased from baseline following the application of a moderate dose of 5-HT (LHbm 10 μ M, LHbl 3 μ M). The current amplitudes induced by 10 μ M ($p = 0.005$) and 30 μ M ($p < 0.001$) 5-HT were significantly greater in LHbl neurons than LHbm neurons. Best-fit curves revealed that the EC₅₀s for 5-HT were 7.8 ± 2.4 μ M for LHbm cells and 6.8 ± 1.2 μ M for LHbl cells. However, there was no significant effect on the interaction between subregions and doses ($F_{5,166} = 1.4$, $p = 0.25$).

In a few LHb neurons (LHbm: 3/34; LHbl: 4/42), 10 μ M 5-HT induced a weak outward current, which was almost completely abolished by the 5-HT_{1A} receptor inhibitor WAY₁₀₀₆₃₅ (1 μ M, from 6.5 ± 0.9 pA to 2.1 ± 0.7 pA, $n = 7$, $t = 3.7$, $p < 0.01$). 10 μ M 5-HT did not induce an observable response in the rest of the LHb neurons (LHbm: 9/34; LHbl: 6/42; Fig. 1I). These data indicate that 5-HT exposure would result in a greater depolarization/inward current of neurons in the LHbl than that in LHbm.

3.3. 5-HT accelerates spontaneous action potential firing of LHb neurons

In accordance with the depolarizing effect, 5-HT significantly accelerated firing in the majority of neurons in the LHbm (65/83, Fig. 2A) and LHbl (47/58, Fig. 2B) in the absence of neurotransmitter receptor antagonists (main effect of dose, $F_{5,100} = 7.4$, $p < 0.001$), and the magnitude of the increase in spontaneous firing was the same in both subregions ($F_{1,100} = 0.92$, $p = 0.34$, or subregions $\times$ doses interaction, $F_{5,100} = 0.12$, $p = 0.98$; Two-way ANOVA). The dose-response curves in Fig. 2C were fit to the averaged percent increase in firing rate at each 5-HT concentration. Best-fit curves revealed that the EC₅₀s for 5-HT were 1.3 ± 0.02 μ M for LHbm and 1.7 ± 0.6 μ M for LHbl cells. The second 5-HT application to the same neuron produced acceleration similar to that of the first application (Fig. 2D). Conversely, 10 μ M 5-HT lowered the firing rate of some (6/29) neurons in the LHbm by $70.8 \pm 10.2\%$ ($p < 0.001$); and some (3/18) neurons in LHbl by $50.2 \pm 13.1\%$ ($p < 0.05$). There was no significant difference between these groups ($t = 1.2$, $p = 0.27$, LHbm vs. LHbl, Fig. 2E–F). These data indicate that 5-HT similarly affects the activity of LHbm and LHbl neurons.

Additionally, we examined the effect of 5-HT on LHb neuronal firing in the presence of neurotransmitter receptor antagonists (strychnine, gabazine, AP5 and DNQX). Under these experimental conditions, 10 μ M 5-HT increased the firing rate of LHbl neurons by $93.9 \pm 17.5\%$ ($n = 15$, $p < 0.001$) that was significantly greater than that of LHbm neurons, by $50.9 \pm 10.3\%$, $n = 14$, $p < 0.001$ ($t = 2.7$, $p = 0.047$, LHbm vs. LHbl; Fig. 2G, H). Remarkably, 5-HT increased firing similarly in the absence and presence of these antagonists (LHbm: $t = 0.88$, $p = 0.39$ vs. without antagonists; LHbl: $t = 0.87$, $p = 0.39$ vs. without antagonists; Fig. 2H).

3.4. 5-HT subtypes and transient receptor potential channels mediate 5-HT's actions on LHb neurons

We next identified the receptor subtypes that mediate 5-HT-induced inward current. We took advantage of the observation that the second application of 5-HT to the same neurons induced a current with the same amplitude to that of the first application (Fig. 1G). We first applied 10 μ M 5-HT for 10 min, following the complete recovery and a washout period of $>15\sim 20$ min, we applied an antagonist for 10 min before the second 5-HT application. We then compared the peak amplitudes-induced by 5-HT in the absence and presence of the antagonists. Inward currents were significantly reduced by the 5-HT_{2A/2C}R antagonist, RIT (10 μ M) (LHbm: $t = 5.2$, $p < 0.001$; LHbl: $t = 3.4$, $p = 0.014$; Fig. 3A), the 5-HT_{2B/2C}R antagonist, SB200646 (4 μ M) (LHbm: $t = 2.9$, $p = 0.021$; LHbl: $t = 3.5$, $p = 0.008$; Fig. 3B), or the 5-HT₃R antagonist, OND (5 μ M) (LHbm: $t = 2.9$, $p = 0.014$; LHbl: $t = 3.6$, $p = 0.004$; Fig. 3C).

Transient receptor potential (TRP) channels that are involved in mediating nociceptive and stressful processes in the nervous system are expressed in the LHb (Geppetti and Trevisani, 2004; Kastner et al., 2013). 5-HT modulates nonselective cation currents in several brain areas, such as the lateral amygdala (Yamamoto et al., 2014) and hypothalamic arcuate nucleus (Sohn et al., 2011). To determine a potential role of TRP channels in the 5-HT-induced current in the LHb, we examined the effects of 2-APB, a broad-spectrum blocker of TRP channels and IP₃ receptors. As expected, the inward current-induced by 5-HT was greatly attenuated ($F_{4, 74} = 7.5$, $p < 0.001$; Fig. 3D–E) by 2-APB (100 μ M, $p = 0.047$). Additionally, 5-HT-induced inward current was greatly reduced by CdCl₂ (100 μ M), a non-selective voltage gated calcium channel blocker ($p = 0.007$) and by the omission of extracellular Ca²⁺ ($p = 0.006$). 5-HT (10 μ M) failed to induce an inward current when the recording electrode was loaded with BAPTA (50 μ M), which chelates intracellular Ca²⁺ ($p < 0.001$). These pharmacological and ion substitution experiments suggest that 5-HT-induced the opening of cationic channels that are permeable to Na⁺, K⁺, and/or Ca²⁺ in the LHb neurons as previously reported in the amygdala neurons (Yamamoto et al., 2014).

We then compared 5-HT-induced acceleration of firing of LHb neurons in the absence and presence of 5-HT antagonists as well as the TRP channel blocker. As the second 5-HT application produced a similar effect in the same neurons (Fig. 2D), we were able to study the actions of 5-HT antagonists in the same cells. The 5-HT-induced acceleration of firing was greatly attenuated by RIT (LHbm: $t = 4.4$, $p = 0.005$; LHbl: $t = 8.3$, $p < 0.001$; Fig. 4A, D), SB-200646 (LHbm: $t = 7.8$, $p < 0.001$; LHbl: $t = 4.4$, $p = 0.007$; Fig. 4B, D), or OND

(LHbm: $t = 4.6$, $p < 0.001$; LHbl: $t = 11.6$, $p < 0.001$; Fig. 4C, D), in the LHbm and the LHbl (all $p > 0.1$). Moreover, 5-HT-induced acceleration was significantly attenuated by 2-APB ($n = 8$, $t = 8.6$, $p < 0.001$). Together, these data indicate that 5-HT activated LHbm and LHbl neurons via 5-HT_{2/3} receptors and TRP channels.

3.5. The agonists of 5-HT_{2/3} receptors mimic 5-HT's effects on LHb neurons

We next investigated the effects of mCPP, a 5-HT₂R agonist that preferentially activates 5-HT_{2C}R; and mCPBG, a selective 5-HT₃R agonist, on the inward current and the ongoing discharges of LHb neurons. Similar to the effects of 5-HT, 10 μ M mCPP produced strong inward currents in 7/12 LHbm neurons ($t = 4.1$, $p = 0.001$; Fig. 5A left panel) and in 8/11 LHbl neurons ($t = 6.9$, $p < 0.001$; Fig. 5B left panel), and significantly accelerated the firing in 7/9 LHbm neurons ($t = 4.1$, $p = 0.002$; Fig. 5A right panel) and in 6/7 LHbl neurons ($t = 5.2$, $p < 0.001$; Fig. 5B right panel). Furthermore, 25 μ M mCPBG induced an inward current in 10/23 LHbm neurons ($t = 6.9$, $p < 0.001$) and in 5/12 LHbl neurons ($t = 4.3$, $p = 0.003$; Fig. 5C–E) and accelerated the firing in 7/10 LHbm and 6/9 LHbl neurons (LHbm: $t = 3.9$, $p < 0.001$; LHbl: $t = 3.1$, $p = 0.006$; Fig. 5C–F). There was no significant difference regarding the effects of mCPP and of mCPBG between LHbm and LHbl subregions (all $p > 0.5$, unpaired t -test). These data provide further evidence that functional 5-HT_{2/3}R are expressed in the LHb, and their activation produces an inward current and increases activity of LHb neurons.

3.7. Endogenous serotonergic tone in the LHb neurons

The LHb neurons are innervated by 5-HT afferents. To determine the activity of the endogenous 5-HT, we examined the effect of citalopram, a highly selective 5-HT reuptake blocker (Dufour et al., 1987). Bath application of 10 μ M citalopram for 10 min generated a small inward current in 5/10 LHbm neurons (8.2 ± 2.6 pA, $t = 3.2$, $p = 0.013$; Fig. 6A, E) and 6/11 LHbl neurons (10.1 ± 3.7 pA, $t = 2.7$, $p = 0.02$; Fig. 6B, E). This current was significantly attenuated by either RIT ($n = 4$, $p = 0.046$) or SB200646 ($n = 5$, $p = 0.038$). In the remaining neurons tested, 10 μ M citalopram induced either a weak outward current or no apparent changes. Citalopram also reversibly increased the ongoing firing rate in 7/14 LHbm neurons ($t = 3.7$, $p = 0.003$; Fig. 6C, F) and in 7/13 LHbl neurons ($t = 5.6$, $p < 0.001$; Fig. 6D, F). Citalopram (10 μ M) lowered the firing rate in 5/14 LHbm neurons (to $81.5 \pm 5.4\%$ of control, $n = 5$, $t = 3.4$, $p = 0.01$) and in 4/13 LHbl neurons (to $70.3 \pm 10.1\%$ of control, $n = 4$, $t = 2.9$, $p = 0.026$). There was no significant difference in citalopram-induced changes in inward currents or spontaneous firings between LHbm and LHbl subregions (all $p > 0.05$, unpaired t -test; Fig. 6E, F). These results imply that endogenous 5-HT could activate 5-HT₂R in the LHb.

4. Discussion

We reported here the first electrophysiological evidence that 5-HT directly depolarized and increased the activity of LHb neurons, and 5-HT₂ receptors, 5-HT₃ receptors and TRP channels mediated this effect. Importantly, the 5-HT reuptake blocker mimicked the action of 5-HT, indicating that LHb neurons are modulated by endogenous serotonergic activity.

4.1. 5-HT induced membrane depolarization/inward current and excitation of LHb neurons involving TPR channels

We demonstrated that 5-HT induced a depolarizing inward current and accelerated firing in the majority of LHb neurons. The inward current was significant following the application of a moderate dose of 5-HT ($3 \mu\text{M}$), and increased with concentration with an EC_{50} of $\sim 7 \mu\text{M}$. Notably, the application of low concentration 5-HT ($1 \mu\text{M}$) failed to induce an apparent inward current. This is consistent with a previous study showing that $1 \mu\text{M}$ 5-HT failed to significantly affect the intrinsic excitability of LHb neurons (Shabel et al., 2012).

5-HT-induced depolarization leading to neuronal excitation has been reported in several brain regions (Blomeley and Bracci, 2009; Yamamoto et al., 2014), and this effect of 5-HT involves several ionic mechanisms, including an increase of the cationic conductance. Transient receptor potential channels (TRPs) are a group of ion channels (Nilius and Owsianik, 2011) that are mostly non-selective cation channels; only few are highly Ca^{2+} selective. TRPs are implicated in diverse physiological and pathological functions, such as psychiatric disorders (Marsch et al., 2007; Riccio et al., 2009) and pain (Chahl, 2011). Dense expression of TRPC1/4/5 (Fowler et al., 2007; Von Niederhausern et al., 2013), TRPV1 (Aguiar et al., 2014; Mezey et al., 2000) and TRPM3 (Kastenhuber et al., 2013) have been found in the LHb. We reported that 5-HT-induced inward current and excitation of LHb neurons was significantly attenuated by 2-APB, a broad-spectrum blocker of TRP channels and IP3 receptors. In addition, 5-HT-induced current was markedly diminished in Ca^{2+} -free aCSF or by the calcium channel blocker CdCl_2 , and was almost completely abolished by loading BAPTA, a Ca^{2+} chelator into the recording electrode. Thus, we propose that 5-HT_{2/3}R activation induces TRP-like current, and that some of the 5-HT excitatory effects depend on extracellular and intracellular Ca^{2+} .

It has also been reported that, 5-HT could reduce potassium currents (Blomeley and Bracci, 2009; Bonsi et al., 2007; Yamamoto et al., 2014), open hyperpolarization-activated and cyclic nucleotide-gated nonselective ion channels (Lecourtier and Kelly, 2007) and activate the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger (Eriksson et al., 2001). Although we did not investigate these mechanisms, potassium conductance (Weiss and Veh, 2011) as well as HCN_{2/3/4} subunits (Poller et al., 2011) are distributed in a subpopulation of LHb neurons. Therefore, this conductance may also contribute to 5-HT-induced depolarization and inward current in the LHb.

4.2. Receptor subtypes involved in 5-HT-induced excitation

5-HT exerts differential effects through multiple receptor subtypes (5-HT₁₋₇). The 5-HT₂R family consists of 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} subtypes (Barnes and Sharp, 1999). In the current study, to identify the subtypes of 5-HTRs involved, we examined the effect of 5-HT₂ agonist mCPP, which has higher affinity for 5-HT_{2C} than 5-HT_{2A/2B} receptors. mCPP mimicked the action of 5-HT, producing an inward current and enhancing the firing rate in the majority of LHb neurons. We also showed that the selective 5-HT_{2A/2C} or 5-HT_{2B/2C} antagonists substantially attenuated the inward current and the acceleration of firing induced by 5-HT. These data are in line with previous immunohistochemical studies showing that dense 5-HT_{2C}Rs (Aizawa et al., 2012; Wagner et al., 2014) and very high level of 5-HT_{2C}

binding sites in the LHb (Pompeiano et al., 1994), as well as some 5-HT_{2A}Rs (Bull et al., 2006) in the LHb.

Among all 5-HTRs, only 5-HT₃R is a ligand-gated ion channel, whereas the others belong to the G-protein coupled receptor superfamily. 5HT₃R agonist mCPBG initiated a postsynaptic current and stimulated LHb neurons in some LHb neurons. In addition, 5HT₃R blocker partly inhibited the action of 5-HT, suggesting functional 5-HT₃Rs are present in the LHb (Mitchell and Pratt, 1991). The other 5-HTRs, including 5-HT_{1A} (Cornide-Petronio et al., 2013), 5-HT_{1B} (Hwang and Chung, 2014) and 5-HT_{5B} (Wagner et al., 2014), but not 5-HT_{1C} (Mengod et al., 1990), were also found in the LHb. Consistent with these findings, we found that the 5HT_{1A}R antagonist blocked 5-HT-induced hyperpolarization/outward currents in the minority of LHb neurons. These data suggest that some 5-HT_{1A}Rs are expressed on LHb neurons. Collectively, these data reveal a regional specificity in the pattern of expression and function of 5-HTR subtypes in the LHb: activation of 5-HT₂Rs and/or 5-HT₃Rs resulted in depolarization and increased neuronal activity in the majority of the neurons, whereas activation of 5-HT_{1A}Rs resulted in hyperpolarization and decreased excitability in the minority of neurons.

4.3. The difference in 5-HT-induced response between medial and lateral LHb

In the presence of neurotransmitter antagonists, 5-HT induced a greater inward current and a greater acceleration of spontaneous firings in LHbl than LHbm neurons. This may due to a higher expression of 5-HTRs in the LHbl than LHbm, or to the different distribution of 5-HTR subtypes in these two subregions. This possibility is supported by a previous study showing different distribution of 5-HT_{2C}Rs in the LHbm and in the LHbl (Aizawa et al., 2012). Another possibility is due to the difference in coupling efficiency of 5-HTRs in these subregions. However, our finding that 5-HT_{2/3} antagonists similarly inhibited LHbm and LHbl neurons does support this possibility.

Interestingly, in the absence of neurotransmitter antagonists, 5-HT-induced acceleration of firing of LHbm neurons was similar to that of LHbl neurons. 5-HT could affect LHb neuronal activities through both pre- and post-synaptic mechanisms. A previous study has shown that 5-HT reduces glutamate release from the basal ganglia to the LHbl (Shabel et al., 2012), which could counteract 5-HT's excitatory effect on the postsynaptic membrane. Given that LHbm and LHbl neurons are innervated by distinct inputs, if 5-HT exerts a stronger inhibition on the excitatory transmission on LHbl than on LHbm neurons, or 5-HT displays distinct modulations on the other neurotransmitters, such as GABA or various neuropeptides between these two subregions, in the balance of excitation and inhibition, the net outcome of neuronal activities may be similar.

4.4. Function of the LHb

The LHb controls monoaminergic systems in the midbrain, and may act as a center for the etiology of psychiatric disorders. The LHb has been implicated in depression: *in vivo* activation of LHb neurons induces depressive-like behaviors (Han et al., 2015), while inactivation can correct depression symptoms (Addolorato et al., 2012; Fowler and Kenny, 2014; Sachs et al., 2015; Sartorius et al., 2010; Winter et al., 2011; Yang et al., 2008). Based

on our *in vitro* study, we propose that 5-HT excites LHb neurons via the binding to the 5-HT_{2/3}Rs, and the activation of TRP channels. This effect of 5-HT may promote aversion and induce depressive-like behaviors, probably by inhibiting DRN serotonergic neurons through the RMTg or the DRN interneurons.

Chronic use of serotonin-based antidepressant drugs suppress LHb neuronal activity ((Freo et al., 2000; Shabel et al., 2014) but see (Sachs et al., 2015)) and relieve depression symptoms in humans (Trivedi et al., 2006). We showed here that an elevation of extracellular 5-HT levels through the inhibition of 5-HT reuptake resulted in 5-HTR activation and modulation of activity of some LHb neurons. These data are perhaps unexpected and counterintuitive, since from a clinical perspective, selective serotonin reuptake inhibitors, including citalopram, which are widely used for depression treatment, should inhibit the majority of LHb neurons. Remarkably, the LHb is activated by the omission of an expected reward, the expectation of punishment and the aversive stimulus (pain, fear). Conversely, 5-HT has been implicated in the prediction of aversive events and in behavioral inhibition. There is evidence that in the acute phase of selective serotonin reuptake inhibitor administration, fear response is enhanced, depending on a 5-HT_{2C}R-mediated mechanism (Burghardt et al., 2007; Burghardt et al., 2004). Other adverse effects, including nausea, nervousness, dizziness, insomnia, are also observed with citalopram administration. We speculate that the side effects of citalopram may result from stimulating LHb neurons, and the therapeutic effect from inhibiting LHb neurons.

5. Conclusion

In the present *in vitro* study, we provided a cellular mechanism for 5-HT to activate LHb neurons. By activating functional 5-HT_{2/3}Rs and TRP channels in the postsynaptic membrane of LHb neurons, 5-HT induced depolarization and accelerated firing. By activating LHb neurons, 5-HT may provide a negative feedback to RN neurons. This may be important for the homeostatic balance underlying the neural circuit between the LHb and RN.

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Highlights

- 5-HT induced an inward current ($I_{(5-HTi)}$) and increased firing in ~80% LHB cells
- A 5-HT uptake blocker induced $I_{(5-HTi)}$, indicating endogenous 5-HT activity
- $I_{(5-HTi)}$ was blocked/activated by 5-HT_{2/3} antagonists/agonists
- A transient receptor potential channel blocker inhibited $I_{(5-HTi)}$

Fig. 1. 5-HT induces depolarization/inward current in LHbm and LHbl neurons. (A) A scheme of the delineation of individual LHb subnuclei (modified from Fig. 3E of Gonçalves et al. (2012)). Under current clamp and in the presence of 0.5 μ M TTX, 10 μ M 5-HT induced depolarization in an LHbm (B) and an LHbl (C) neuron. For this and the other figures, horizontal bar above the voltage (or current) trace signals the application of the indicated drug. (D) Summary of 5-HT-induced depolarization in the LHbm and LHbl neurons. Numbers of cells are indicated. $###p < 0.001$, Student's paired t -test was used for drug application vs. pre-drug control. NS means no significant difference between LHbm and LHbl neurons, using an unpaired t -test. Under voltage clamp and at a holding potential of -70 mV, bath application of 30 μ M 5-HT induces an inward current in an LHbm (E) and an LHbl (F) neuron in the presence of a cocktail of blockers: 0.5 μ M TTX, 50 μ M AP5, 20 μ M DNQX, 10 μ M gabazine and 1 μ M strychnine. (G) Summarized time course of the inward currents induced by two applications of 5-HT ($n = 5$): The peak amplitudes of the two inward currents were similar. (H) Significant different dose-response relations of 5-HT-induced inward currents in LHbm (Δ) and LHbl ($\bullet$) neurons. The smooth curve is the best fit of the data to the logistic equation. $*p < 0.05$, $***p < 0.001$, two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used, 5-HT (0.3-30 μ M) vs. 0.1 μ M 5-HT. $\&\&p < 0.01$ indicating significant difference between LHbm and LHbl

subregions. (I) Percent distribution of responses induced by 10 μ M 5-HT in LHbm and LHbl neurons (inward current are from 22/34 LHbm neurons and 32/42 LHbl neurons).

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Fig. 2.

5-HT accelerates ongoing discharges of LHbm and LHbl neurons. 5-HT accelerated firing in an LHbm (A) and an LHbl (B) neuron recorded in the cell-attached mode. (C) Similar dose-response relations of 5-HT-induced % increase in firing rate of LHbm (Δ) and LHbl ($\bullet$) neurons. $**p < 0.01$, $***p < 0.001$, two-way ANOVA followed by Tukey's multiple comparisons test was used, 5-HT (0.3–30 μ M) vs. 0.1 μ M 5-HT. (D) There was no significant difference between the acceleration induced by two applications of 10 μ M 5-HT. (E) 10 μ M 5-HT inhibited firing in a minority of LHbm (Δ) and LHbl ($\bullet$) neurons. (F) 5-HT-induced inhibition was not significantly different between LHbm and LHbl. (G) Mean time course of 10 μ M 5-HT-induced acceleration of firing in LHbm (Δ) and LHbl ($\bullet$) neurons in the presence of neurotransmitter receptor antagonists (strychnine, gabazine, AP5).

and DNQX). (H) Summary of 10 μ M 5-HT-induced increase in firing rate in the absence (aCSF) and presence of neurotransmitter antagonists. $^{\$}p < 0.05$, Student's unpaired *t*-test.

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Fig. 3. 5-HT-induced inward current is mediated by 5-HT_{2/3} receptors and involved in TRP channels. (A–C) In these experiments, 10 μM 5-HT was first applied for 10 min, and then washed out (not illustrated). When the currents recovered to baseline levels, the 5-HT antagonists were applied for 10 min before the second 10 min application of 10 μM 5-HT. Mean time course of inward currents generated by 10 μM 5-HT on the LHbm (left panels) and LHbl (middle panels) neurons in the absence (○) and presence of 5-HT antagonists (■). 5-HT-induced inward current was partly blocked by RIT (5-HT_{2A} antagonist, 10 μM), SB200646 (5-HT_{2B/2C} antagonist, 4 μM) or OND (5-HT₃ antagonist, 5 μM). Summary of 10 μM 5-HT-induced inward currents in the absence (5-HT) and presence of 5-HT_{2/3} antagonists (right panels). Numbers of cells are indicated. #*p* < 0.05, ##*p* < 0.01, ###*p* <

0.001, Student's paired *t*-test for 5-HT vs. 5-HT plus antagonists. (D) Mean time course of inward currents induced by 10 μ M 5-HT in LHb neurons maintained in Ca^{2+} free aCSF, or CdCl_2 (100 μ M), 2-APB (100 μ M) was added in bath solution or BAPTA (50 μ M) was added in the pipette solution. (E) Summarized peak effects in D (see methods). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA followed by Tukey's post-test.

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Fig. 4. 5-HT receptor subtypes mediate 5-HT-induced acceleration of ongoing firing of LHb neurons. (A–C) 5-HT-induced acceleration of ongoing discharges was partly blocked by the antagonists of 5-HT_{2A}Rs, 5-HT_{2B/2C}Rs and 5-HT₃Rs in LHbm (left panels) and LHbl (right panels) neurons. (D) Summary of 5-HT antagonists induced inhibition on the acceleration induced by 5-HT in LHbm and LHbl neurons. ## $p < 0.01$, ### $p < 0.001$, Student's paired t -test for antagonist application vs. pre-antagonist control was used.

Fig. 5.

5-HT_{2/3} receptor agonists mimic the effect of 5-HT on inward current and firing in LHbm and LHbl neurons. Mean time course of inward currents induced by the agonists of 5-HT_{2R} (10 μ M mCPP) and 5-HT_{3R} (25 μ M mCPBG) in LHbm (A, C left panels) and LHbl (B, D left panels) neurons. Mean time course of mCPP- or mCPBG-induced acceleration of firing in LHbm (A, C right panels) and LHbl (B, D right panels) neurons. Mean inward currents (E) and mean changes of firing rates (F) generated in LHbm and LHbl neurons by mCPP and mCPBG. $^{\#}p < 0.05$, $^{##}p < 0.01$, $^{###}p < 0.001$, paired t -test for agonists application vs. pre- agonists control.

Fig. 6.

Endogenous 5-HT generates inward currents and accelerates firing in LHb neurons. Summarized time course of inward currents induced by the 5-HT transport inhibitor citalopram (10 μ M) in LHbm (A) and LHbl (B) neurons. Summarized time course of citalopram-induced acceleration of firing in LHbm (C) and LHbl (D) neurons. The effects of citalopram on inward currents (C) and the changes of firing rates (D) in LHbm and LHbl neurons. # $p < 0.05$, ### $p < 0.001$, paired t -test for citalopram application vs. pre-citalopram control was used. There is no significant difference between the effects of citalopram on LHbm and LHbl neurons, unpaired t -test (E, F).