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Original Article

5-Hydroxytryptamine inhibits neuronal high-voltage-activated calcium currents in the preoptic anterior hypothalamus via 5-hydroxytryptamine 1A and 7 receptors

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Abstract: 5-Hydroxytryptamine (5-HT) is involved in mammalian thermoregulation of the preoptic anterior hypothalamus (PO/AH), but the underlying molecular mechanisms are not fully understood. In this study, we investigated the influence of 5-HT and agonists and antagonists of the 5-HT1A, 5-HT2A, 5-HT3 and 5-HT7 receptors on voltage-gated calcium currents in the PO/AH slice with the whole-cell patch clamp technique. The results showed that 5-HT decreased high-voltage-activated (HVA) calcium currents in PO/AH neurons in a concentration-dependent manner, but it did not affect low-voltage-activated (LVA) calcium currents. The 5-HT-induced decrease in HVA calcium currents in PO/AH neurons was facilitated by both the 5-HT1A receptor agonist 8-hydroxy-2-(dipropylamino)-tetralin (8-OH-DPAT) and the 5-HT7 receptor agonist (2S)-(+)-5-(1,3,5-trimethylpyrazol-4-yl)-2-(dimethylamino)tetralin (AS 19), whereas it was inhibited by both the 5-HT1A antagonist N-{2-[4-(2-methoxyphenyl)-1-piperazinyl]-ethyl}-N-2-pyridinylcyclohexane-carboxamide (WAY 100635) and the 5-HT7 receptor antagonist 3-methyl*N*-[(1*R*)-1-methyl-3-(4-methyl-1-piperidinyl)propyl]-*N*-methylbenzenesulfonamide hydrochloride (SB 258719). Conversely, administration of agonists and antagonists of 5-HT2A and 5-HT3 receptors did not significantly influence HVA calcium currents in PO/AH neurons. In conclusion, our findings suggested that 5-HT1A and 5-HT7 receptors were involved in the 5-HT-induced decrease of HVA calcium currents in PO/AH neurons and might contribute to the hypothermic effects of thermoregulation.

Keywords: 5-hydroxytryptamine, calcium current, preoptic anterior hypothalamus, 5-HT receptor, whole-cell patch clamp

Introduction

The preoptic anterior hypothalamus (PO/AH) plays a vital role in thermoregulation, regulating body temperature by heat production and heat loss responses. Electrophysiological studies have revealed that some neurons in the PO/AH have thermosensitivity properties, but the mechanisms remain controversial. Several ion channels expressed in PO/AH neurons are considered to be involved in neuronal thermosensitivity, including voltage-gated calcium channels. These channels are divided into low-voltage-activated (LVA) and high-voltage-activated (HVA) calcium channels, both of which contribute to neuronal thermosensitivity [1-3]. However, how calcium currents through these ion channels take part in thermoregulation is poorly understood.

5-Hydroxytryptamine (5-HT) is a neurotransmitter widely distributed in the central nervous system, where it binds 5-HT receptors to exert a variety of physiological functions including temperature regulation. At least fourteen 5-HT receptor subtypes have been identified, and their functions are gradually being elucidated. Among these receptors, 5-HT1A [4], 5-HT2A [5], 5-HT3 [6], and 5-HT7 receptors [7] are reportedly involved in thermoregulation. 5-HT1A,

5-HT3, and 5-HT7 receptors are considered to be involved in heat loss, while the 5-HT2A receptor is related to heat production. Precisely how these receptors participate in thermoregulation is unknown. Ion channels are the foundation of neuronal excitability, but it is not clear whether 5-HT is involved in regulating calcium channels in PO/AH neurons. The present study was performed to investigate voltage-gated calcium channels in PO/AH neurons and explore the effects of 5-HT to elucidate the mechanisms underlying 5-HT-mediated thermoregulation.

Materials and methods

Ethics statement

All experimental procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee of Peking University First Hospital (approval number: J201242). Experiments were performed on 80 female Sprague Dawley rats weighing 30-50 g on postnatal days 14-18 (Experimental Animal Center, Academy of Military Medical Sciences, Beijing, China). Rats were housed under a natural light-dark cycle conditions (12-/12-h light/dark) with free access to food and water until sacrifice. Every effort was made to minimize both animal suffering and the number of animals used.

Slice preparation

The rat was anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg) before being quickly sacrificed by decapitation. The brain was then rapidly removed and placed in an ice-cold cutting solution (in mM): 90 sucrose, 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 0.5 $CaCl_2$, 25 $NaHCO_3$, and 10 glucose (pH 7.4, saturated with 95% O_2 and 5% CO_2) for 1 min [8, 9]. Coronal brain slices (300-μm thick) containing the PO/AH were prepared using a vibratome (LEICA VT1000S, Wetzlar, Germany) in ice-cold cutting solution aerated with 95% O_2 and 5% CO_2. Brain slices were then incubated in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, and 10 glucose (pH 7.4 adjusted with HCl). Brain slices were recovered at 33°C for 45 min before electrophysiological recording [8, 9]. Slices were then individually transferred to a recording chamber that was continuously perfused with ACSF saturated with 95% O_2 and 5% CO_2 at a rate of 1-1.5 ml/min. To isolate calcium currents, the ACSF composition was as follows (in mM): 95 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 0.001 tetrodotoxin (TTX), 2 4-aminopyridine (4-AP), and 30 tetraethylammonium chloride (TEA-Cl).

Patch clamp recording

The PO/AH was located between the anterior commissure and optic chiasm using an upright microscope (BX51WI, Olympus, Japan) with differential interference contrast. For the whole-cell recording, patch microelectrodes were pulled by a Flaming-Brown puller (Model P-97; Sutter Instruments, Novato, CA, USA). The composition of the internal solution was (in mM) 122.5 $CsMeHSO_3$, 17.5 CsCl, 10 HEPES, 0.5 EGTA, 4 ATP-Na, and 0.3 GTP-Na (pH 7.2 with CsOH). When filled with the internal solution, pipettes had a resistance of 4-7 MΩ.

All electrophysiological recordings were performed using the conventional whole-cell patch clamp recording model. After obtaining a tight seal (>1 GΩ) by applying negative pressure, the whole-cell configuration was achieved after disrupting the cell membrane. Currents were low-pass filtered at 2 kHz and digitized at a rate of 10 kHz. All responses were recorded with an EPC10 amplifier and Pulse v8.80 software (HEKA, Lambrecht, Germany). Series resistance and membrane capacitance were compensated to the greatest extent possible.

Drug application

Drugs used in the present study included: TEA-Cl, Nickel chloride ($NiCl_2$), and serotonin hydrochloride from Sigma (St. Louis, MO, USA); tetrodotoxin (TTX) and 4-AP from Alomone Laboratories (Jerusalem, Israel); mibefradil dihydrochloride, 8-OH-DPAT, WAY100635, (4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide (TCB-2), 4-(4-Fluorobenzoyl)-1-(4-phenylbutyl) piperidine oxalate (4F 4PP), m-Chlorophenylbiguanide hydrochloride (m-CPBG), tropanyl 3,5-dichlorobenzoate (MDL 722-22), AS 19, and SB 258719 from Tocris Bioscience (Bristol, UK). All drugs were made up as stock solutions in distilled water or dimethyl sulfoxide and were kept in frozen aliquots.

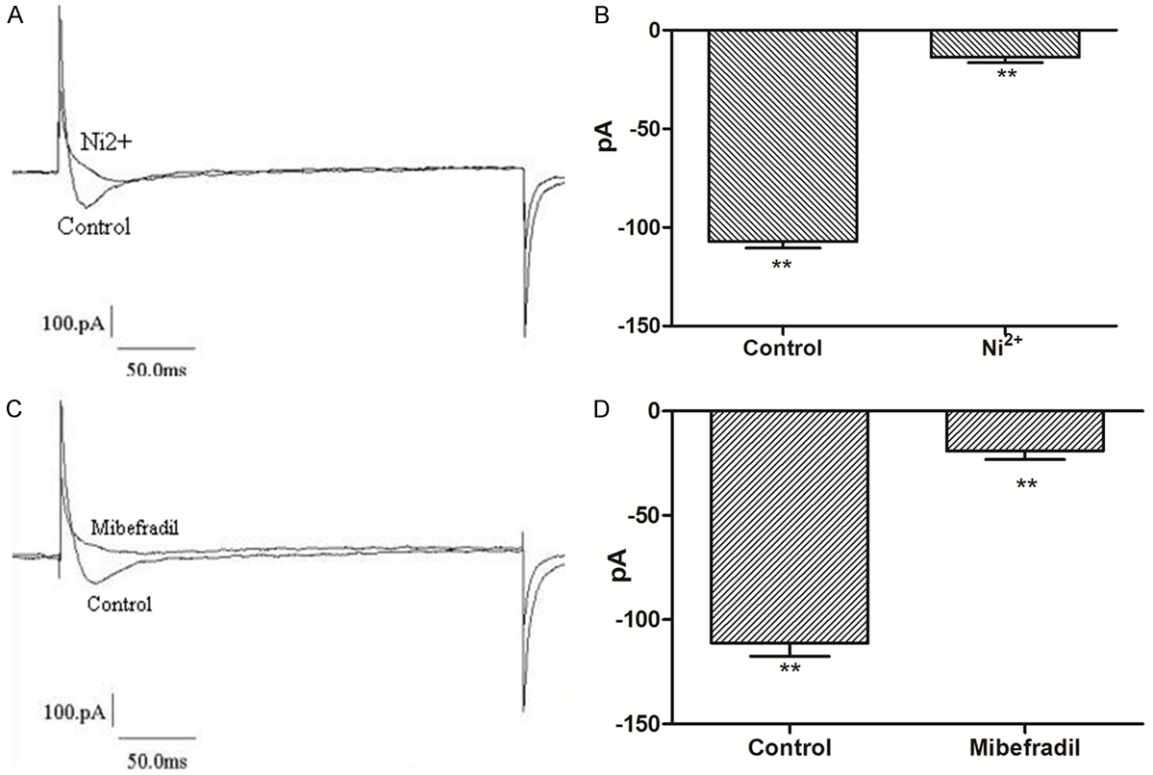

Figure 1. Identification of the LVA calcium current in PO/AH neurons. A: A typical LVA calcium current trace, and was blocked by 200 μM $NiCl_2$. B: Histogram showing the inhibitory effect of $NiCl_2$ on the LVA calcium current. C: The inhibitory effect of mibefradil (10 μM) on the LVA calcium current. D: Histogram showing the effect of mibefradil on the LVA calcium current (**p<0.01, paired-sample t test).

Before recordings, they were diluted to the final concentrations in the bath solution.

Data analysis

All data were acquired with Pulse v8.80 software and are expressed as mean ± standard error of the mean (SEM). Student's paired t-test or one-way analysis of variance (ANOVA) was used to analyze differences between groups. Analyses were carried out with SPSS 21.0 software (IBM Corp Armonk, NY, USA). *P<0.05 and **P<0.01 were considered as statistically significant and highly statistically significant, respectively.

Results

Identification of the LVA calcium current in PO/AH neurons

A transient calcium current was evoked by a series of depolarizing pulses (from -70 to -30 mV, in 10 mV increments, 200 ms duration) every 10 s with a holding potential (V_h) of -90 mV. HVA calcium currents (e.g., L-, P/Q-, R-, and N-types) were observed when voltages were greater than -30 mV. To avoid contamination of these calcium currents, we limited the maximum command pulse to -30 mV. This transient calcium current reached its peak at a voltage of -30 mV and was blocked by 200 μM Ni^{2+} and 10 μM mibefradil (**Figure 1**, n=6, **p<0.01, paired-sample t test), suggesting that this transient calcium currentl was the LVA calcium current (T-type).

Effects of 5-HT on the T-type calcium current in PO/AH neurons

Treatment with 10 μM 5-HT had no significant effects on the LVA calcium current recorded in PO/AH neurons (**Figure 2A**, n=6, p>0.05). Even higher concentrations (20 and 50 μM) in the bath failed to alter the LVA calcium current (**Figure 2B**; n=6, p>0.05, one-way ANOVA).

Identification of the HVA calcium current in PO/AH neurons

An inward current was elicited in PO/AH neurons by delivering a series of test potentials

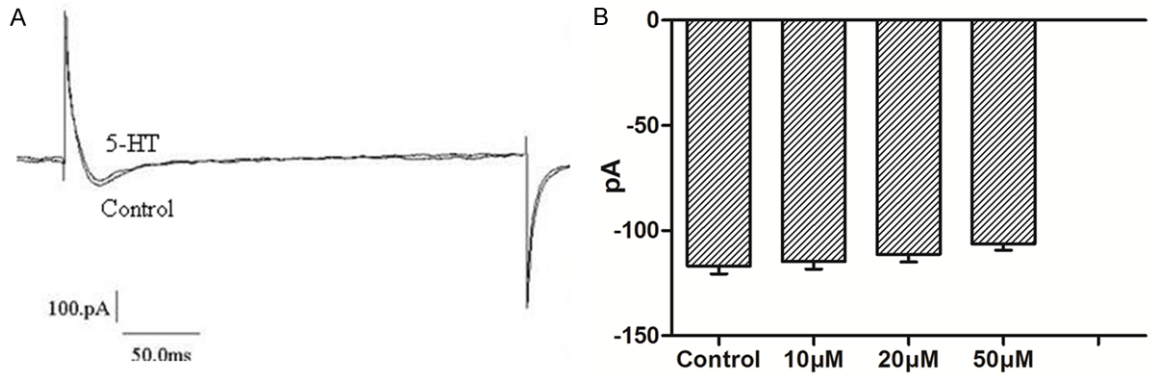

Figure 2. 5-HT does not affect the LVA calcium current in PO/AH neurons. A: 5-HT (10 μM) did not significantly inhibit the LVA calcium current in PO/AH neurons. B: Histogram depicting effects of different concentrations (10, 20, and 50 μM) of 5-HT on the LVA calcium current in PO/AH neurons ($p>0.05$, one-way ANOVA).

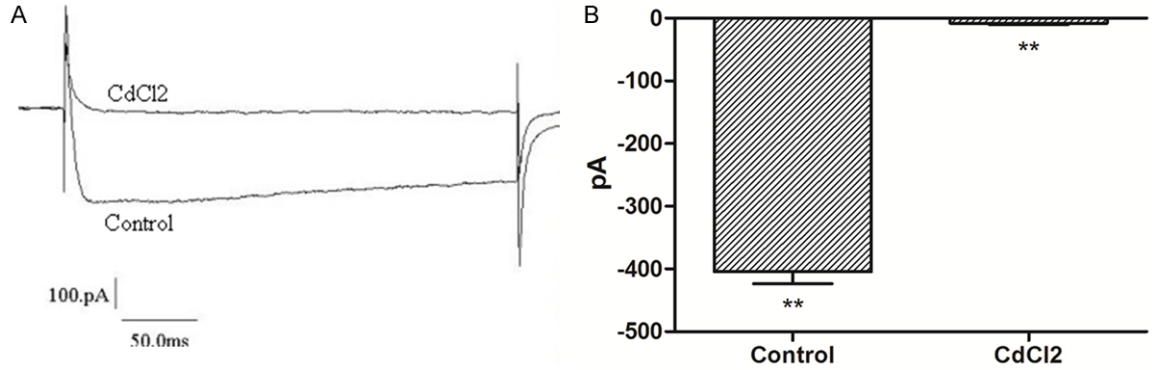

Figure 3. Identification of the HVA calcium current in PO/AH neurons. A: An HVA calcium current trace evoked from a holding potential of -60 mV to a test potential of 0 mV and the effect of 200 μM $CdCl_2$ on it. B: Showed the histogram of the inhibitory effect of $CdCl_2$ on the HVA calcium current (**$p<0.01$, paired-sample t test).

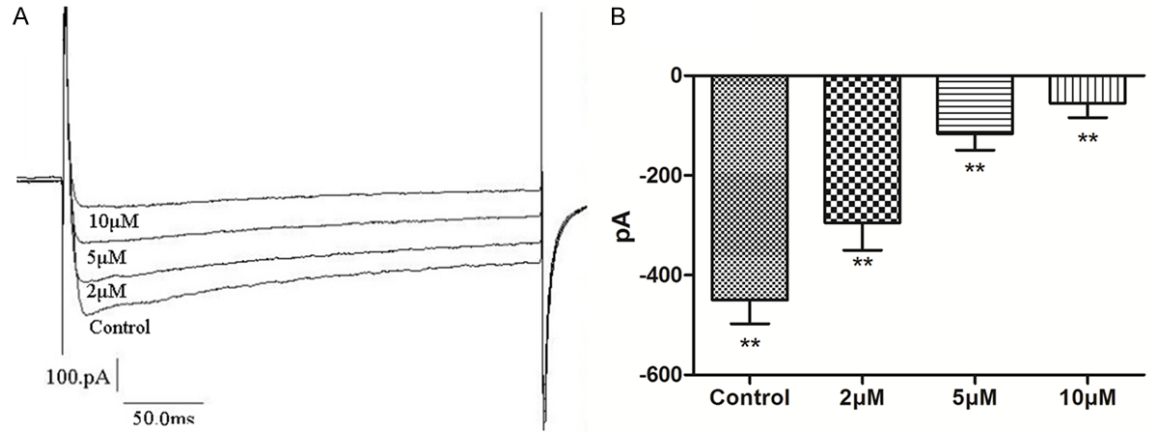

Figure 4. The effect of 5-HT on the HVA calcium current in PO/AH neurons. A: A typical HVA calcium current trace was evoked by a step pulse from a holding potential of -60 mV to a test potential of 0 mV, and the traces after application of 2, 5, and 10 μM 5-HT were shown. They showed that the HVA calcium current decreased in a concentration-dependent manner. B: Histogram of the average amplitude of the HVA calcium currentbefore (control) and after application of different concentrations of 5-HT. Significant differences were indicated by **$p<0.01$ (one-way ANOVA).

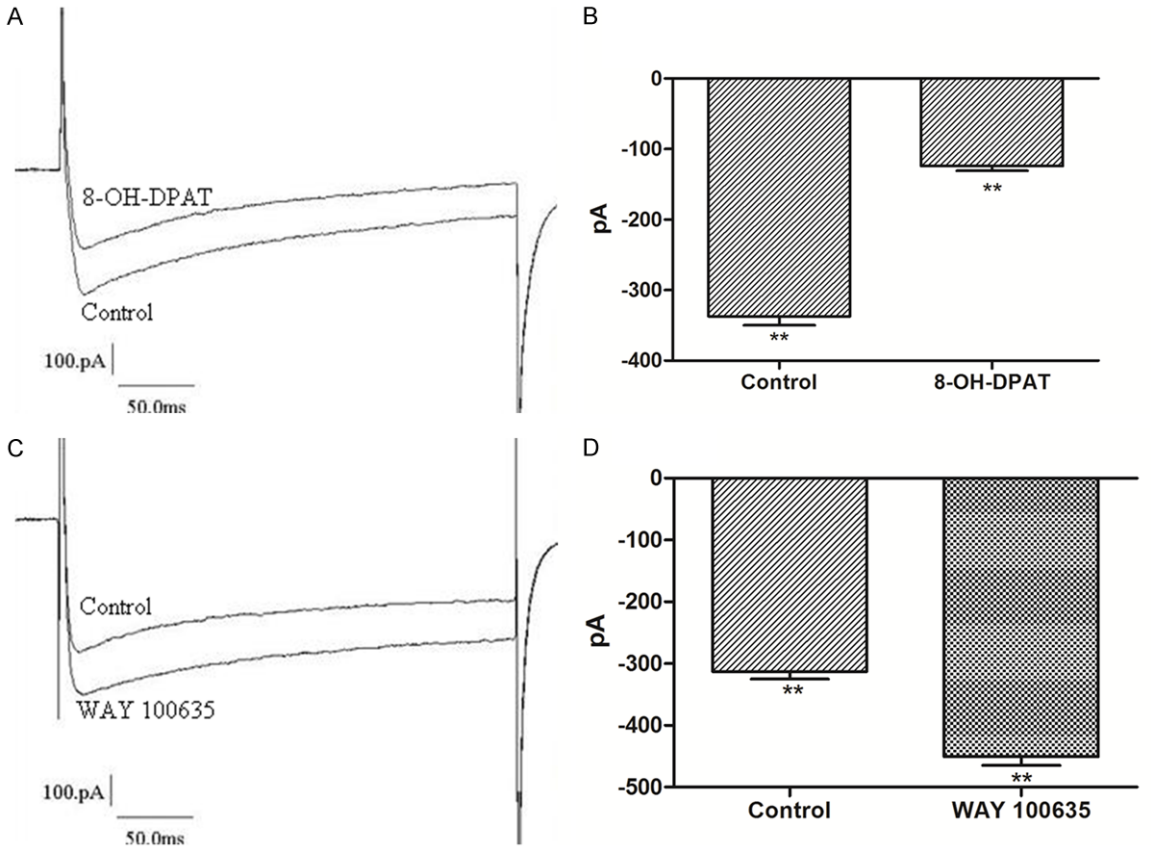

Figure 5. The effect of the 5-HT1A receptor agonist and antagonist on the HVA calcium current in PO/AH neurons. A, C. Showed HVA calcium current traces before (control) and after administration of 10 μM 8-OH-DPAT and WAY100635, demonstrating that 8-OH-DPAT decreased the HVA calcium current while WAY 100635 increased the HVA calcium current. B, D. Showed histograms of the effects of 8-OH-DPAT and WAY100635 on the HVA calcium current. Significant differences were indicated by **p<0.01 (paired-sample t test).

ranging from -50 to 50 mV (10 mV increments, 300 ms duration) from a holding potential of -60 mV. The current was activated at about -50 mV and reached the peak amplitude at about 0 mV. $CdCl_2$ (200 μM) almost completely inhibited this inward current (**Figure 3**, n=6, **p<0.01, paired-sample t test), suggesting that it was the HVA calcium current.

5-HT inhibited the HVA calcium current in PO/AH neurons

Application of 2, 5, or 10 μM 5-HT dramatically inhibited the HVA calcium current from -449.75±47.69 to -294.57±55.09 pA, -116.24±33.06 pA, and -55.32±28.73 pA, respectively (**Figure 4**, n=8, **p<0.01, one-way ANOVA). We completed the recording in 10 minutes to avoid the influence of the rundown of the calcium current on the effect of 5-HT.

Effects of the 5-HT1A receptor agonist 8-OH-DPAT and antagonist WAY 100635 on the HVA calcium current in PO/AH neurons

In the presence of 1 μM 5-HT, application of 10 μM 8-OH-DPAT significantly inhibited the HVA calcium current from -337.30±12.64 pA to -125.96±8.91 pA (**Figure 5A** and **5B**, n=10, **p<0.01, paired-sample t test), whereas application of 10 μM WAY 100 635 increased the HVA calcium current from -312.70±12.40 pA to -450.82±14.04 pA (**Figure 5C** and **5D**, n=10, **p<0.01, paired-sample t test).

Influences of 5-HT2A and 5-HT3 receptor agonists and antagonists on the HVA calcium current in PO/AH neurons

The 5-HT2A receptor agonist TCB-2, the 5-HT2A receptor antagonist 4F 4PP, the 5-HT3 receptor agonist m-CPBG, and the 5-HT3 receptor

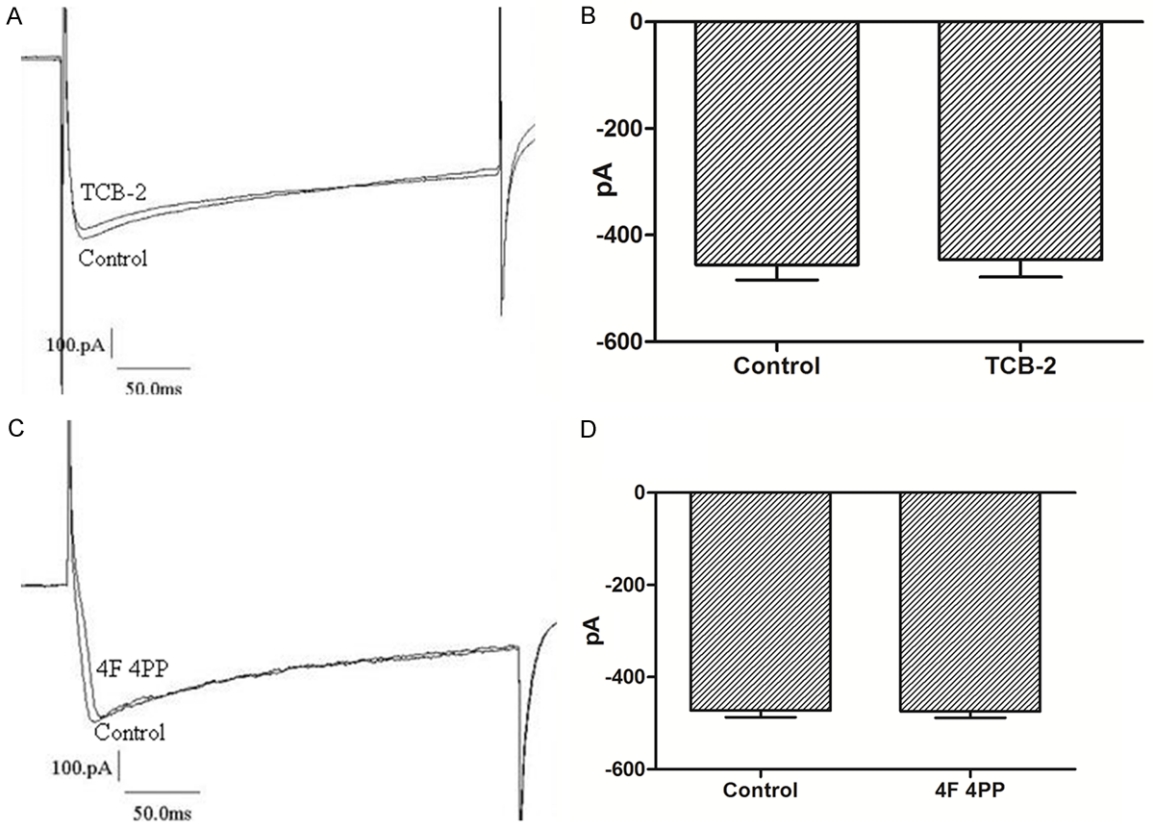

Figure 6. Effects of the 5-HT2A agonist (TCB-2) and antagonist (4F 4PP) on the HVA calcium current. Traces (A and C) and histograms (B and D) showed that neither TCB-2 nor 4F 4PP influence the amplitude of the HVA calcium current. (n=6, p>0.05, paired-sample t test).

antagonist MDL 72222 (all at 10 μM in the recording solution to fully activated or inhibited 5-HT receptors) did not significantly affect HVA calcium current amplitude (**Figures 6** and **7**, n=6, p>0.05,paired-sample t test).

Effects of the 5-HT7 receptor agonist AS 19 and the antagonist SB 258719 on the HVA calcium current in PO/AH neurons

The 5-HT7 receptor agonist AS 19 (10 μM) reduced the amplitude of the HVA calcium current from -744.36±81.32 pA to -606.55±119.24 pA whereas application of 10 μM SB 258719 increased the HVA calcium current from -642.93±52.73 pA to -717.72±51.71 pA (**Figure 8**, n=8, **p<0.01, paired-sample t test).

Discussion

Thermoregulation is a complex physiological process that relies on various ion channel types. It is likely that neurons in the PO/AH express the same ion channels, but variation in the proportions of expressed channels determines electrophysiological characteristics and whether a neuron is sensitive to temperature according to a Hodgkin-Huxley-like model [1, 10]. Studying thermosensitive channels contributes to a better understanding of neuronal thermosensitivity. Identified thermosensitive channels include voltage-gated calcium channels [11], two-pore potassium leak channels [12], $K_V4.1$ channels (transient potassium A-type currents) [13, 14], hyperpolarization-activated cyclic nucleotide-gated (HCN) channels [14], and transient receptor potential channels (TRPs) [15]. Their functions vary with temperature.

Neurotransmitters including 5-HT, norepinephrine, and dopamine have all been implicated in thermoregulation via their receptors. The 5-HT1A, 5-HT2A, 5-HT3, and 5-HT7 receptors have been connected to thermoregulation [4-7]. However, how these receptors participate

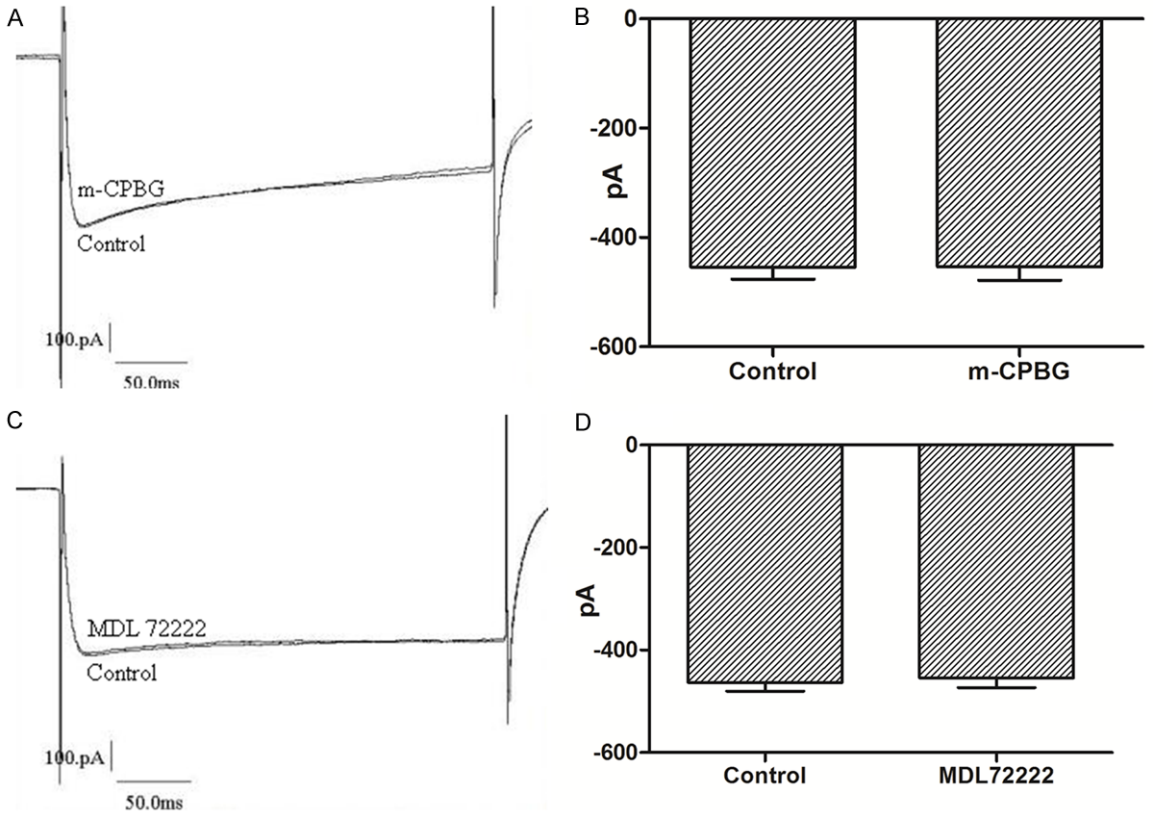

Figure 7. Effects of the agonist (m-CPBG) and antagonist (MDL 72222) of the 5-HT3 receptor on the HVA calcium. Traces (A and C) and histograms (B and D) showed that the amplitude of the HVA calcium current was not significantly changed after application of 10 μM m-CPBG and MDL 72222 (n=6, p>0.05, paired-sample t test).

in thermoregulation is largely unknown. Most studies measured body temperature in thermoregulation-deficient animals after injecting agonists and antagonists of these receptors. In our study, we used the whole-cell patch clamp technique in brain slices to investigate the relationship between 5-HT and one of the thermoregulation-related voltage-gated calcium channels, with the ultimate goal of verifying the mechanism of 5-HT-mediated thermoregulation by 5-HT receptors.

Voltage-gated calcium channels include LVA and HVA channels with varying activation voltages. T-type calcium channels belong to the LVA channel family, which activate with small depolarizations and are known to regulate neuronal excitability. T-type calcium channels are critical determinants of rebound depolarization, a neuronal firing behavior [16, 17]. These channels also take part in spontaneous firing of PO/AH neurons. Additionally, T-type calcium channels participate in a low threshold spike (LTS) upon termination of the hyperpolarizing pulse in PO/AH neurons [1]. However, we neither observed any effect on the T-type calcium current after 5-HT addition nor did we see any following effects after application of agonists and antagonists, suggesting that 5-HT did not affect T-type calcium current in the process of thermoregulation. This conclusion is in accordance with previously reported results [18].

As was the case with T-type calcium channels, HVA calcium channels (including L-, N-, P/Q and R-type calcium channels) require stronger depolarization for their activation and contribute to calcium influx, meaning that they are involved in numerous physiological functions. 5-HT can modulate neuronal excitability by affecting calcium channels in various neuron subtypes such as in spinal neurons [19], trigeminal motoneurons [18], and hippocampal neurons [20]. These studies all achieved the same conclusion that 5-HT could decrease the HVA calcium current. We also observed the

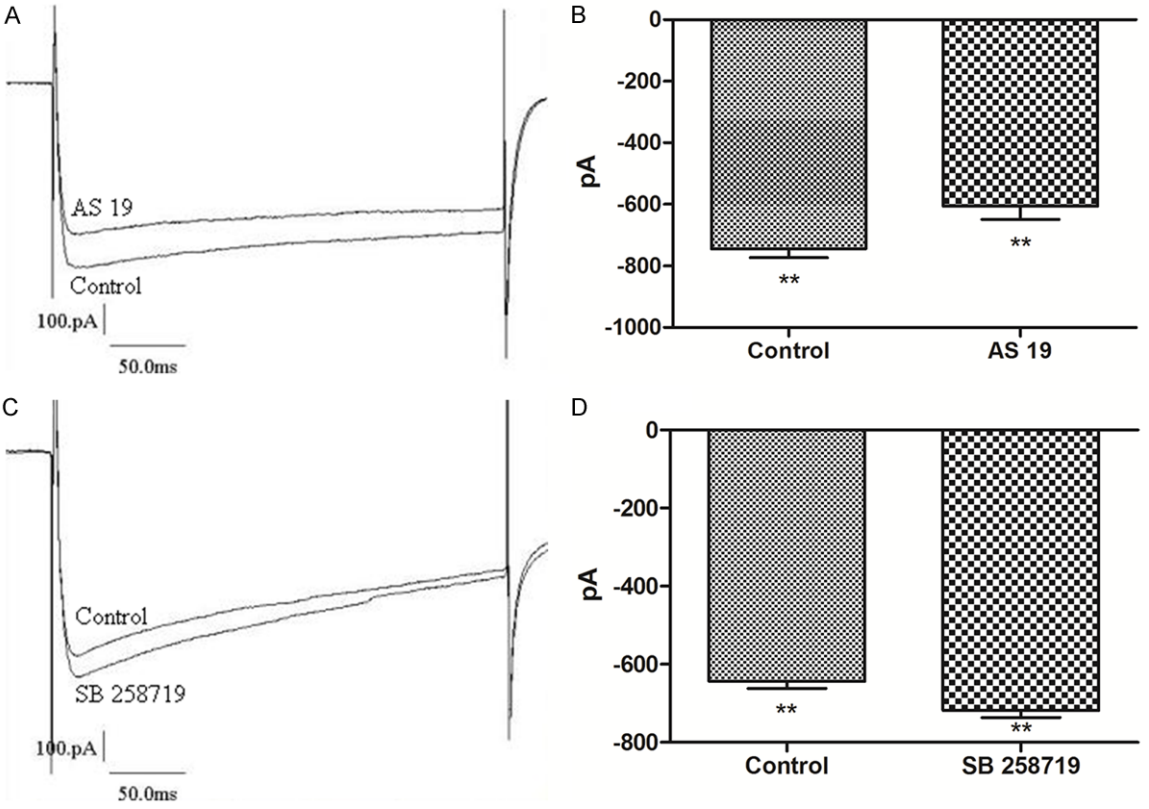

Figure 8. Effects of the 5-HT7 receptor agonist and antagonist on the HVA calcium current (traces and histograms). A and C: HVA calcium current traces before (control) and after application of 10 μM AS 19 and 10 μM SB258719, respectively. B and D: Histograms of effects of AS 19 and SB258719 on the HVA calcium current. Significant differences were indicated by **p<0.01 (paired-sample t test).

inhibitory effect of 5-HT on the HVA calcium current in PO/AH neurons. Inhibition of the HVA calcium current and additional inhibition of PO/AH neuronal excitability might explain the finding that 5-HT administration lead to hypothermia in rats, mice, guinea pigs, and rabbits [21]. The effects of 5-HT on thermoregulation are bidirectional. The 5-HT1A, 5-HT3 and 5-HT7 receptors are involved in heat loss, whereas the 5-HT2A receptor is related to heat production [4-7].

The 5-HT1A receptor is a G-protein coupled receptor (GPCR) that activates $G_{i/o}$ proteins and inhibits adenylate cyclase to downregulate cAMP formation. In neurons, this may lead to membrane depolarization and inhibition of firing [22]. Accordingly, applying the 5-HT1A receptor agonist 8-OH-DPAT could significantly decrease core temperature and attenuate both shivering and peripheral vasoconstriction [4], and its effects could be prevented by the 5-HT1A antagonist WAY 100635, suggesting that the 5-HT1A receptor was involved in heat loss [21, 23, 24]. Our results demonstrated that the 5-HT1A receptor agonist 8-OH-DPAT could significantly decrease the HVA calcium current in PO/AH neurons, whereas the 5-HT1A antagonist WAY 100635 inhibited the effect of 8-OH-DPAT, suggesting that these effects further influence the activities of PO/AH neurons that participate in heat loss and production. This receptor pathway may be one of the ion channel mechanisms whereby 5-HT takes part in thermoregulation. This conclusion was in line with that of previous studies [18, 25, 26].

The 5-HT2A receptor is also a GPCR that couples to $G_{q/11}$ to increase inositol phosphate and cytosolic Ca^{2+} levels by activating phospholipase C to inhibit K^+ currents. The 5-HT2A receptor mediated heat production through a process different from that of the 5-HT1A receptor; as a result the balance between 5-HT1A and 5-HT2A receptor levels might contribute to thermoregulation [5, 27, 28]. However, we did

not find any effect of the 5-HT2A receptor agonist and antagonist on the HVA calcium current, suggesting that the 5-HT2A receptor exerted its thermoregulatory effect via other ion channel mechanisms. To our knowledge, there are no other reports showing a relationship between the 5-HT2A receptor and the HVA calcium current.

Unlike other 5-HT receptors, the 5-HT3 receptor belongs to the ligand-gated ion channel receptor superfamily. They trigger rapid depolarization due to the opening of non-selective cation channels (Na^+, Ca^{2+} influx, K^+ efflux) [22, 29]. The 5-HT3 receptor also participates in thermoregulation. The 5-HT3 receptor agonist m-CPBG elicited a long-lasting and dose-dependent hypothermic response that was stronger than that of the 5-HT1A receptor [6, 30]. We found that the 5-HT3 receptor agonist m-CPBG and the antagonist MDL 72222 did not alter the amplitude of the HVA calcium current, revealing that the 5-HT3 receptor regulated temperature by other unknown mechanisms.

The 5-HT7 receptor is another important player in thermoregulation and is involved in the process of heat loss. It couples to G_s and activates adenylate cyclase, leading to increased cAMP production, which resembles the function of the 5-HT1A receptor [31]. Substantial evidence supported the hypothesis that both the 5-HT1A and 5-HT7 receptor regulated temperature; for instance, 8-OH-DPAT-induced hypothermia was fully inhibited by the 5-HT7 receptor antagonist SB 269970. Low dose 8-OH-DPAT dereased body temperature in 5-$HT7^{+/+}$ mice but not in 5-$HT7^{-/-}$ mice, whereas a higer dose 8-OH-DPAT induced hypothermia both in 5-$HT7^{+/+}$ and 5-$HT7^{-/-}$ mice. These findings demonstrated that the 5-HT1A and 5-HT7 receptors independently regulated body temperature. In addition, the 5-HT7 receptor seemed to be more effective at low agonist concentration, while the 5-HT1A receptor came into play at higher concentrations [7, 17, 31-33]. We found that the 5-HT7 receptor agonist AS 19 diminished the HVA calcium current, whereas the 5-HT7 receptor SB 258719 augmented it. These findings suggested that the 5-HT7 receptor exerted its hypothermic effect by inhibiting the HVA calcium current in PO/AH neurons.

Ion channel mechanisms underlying the influences of 5-HT receptors on thermoregulation are poorly understood. In this study, we demonstrated the effect of 5-HT and agonists and antagonists of specific 5-HT receptor subtypes on HVA calcium currents to elucidate the involvement of the 5-HT1A and 5-HT7 receptors. However, the ion channel mechanisms underlying thermoregulation by other 5-HT receptors (especially potassium currents) remain unknown and should be further explored.

In conclusion, we found that 5-HT did not affect the LVA calcium current but decrease the HVA calcium current in PO/AH neurons, which was related to the 5-HT1A and 5-HT7 receptors rather than the 5-HT2A and 5-HT3 receptors. HVA calcium currents likely represent one of the ion channel mechanisms underlying thermoregulation through the 5-HT1A and 5-HT7 receptors. Effects of 5-HT2A and 5-HT3 receptors on thermoregulation might have other ion channel mechanisms.

Acknowledgements

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Disclosure of conflict of interest

None.

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