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Serotonin modulates outward potassium currents in mouse olfactory receptor neurons

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Summary

Monoaminergic neurotransmitter 5-hydroxytryptamine (5-HT), also known as serotonin, plays important roles in modulating the function of the olfactory system. However, thus far, the knowledge about 5-HT and its receptors in olfactory receptor neurons (ORNs) and their physiological role have not been fully characterized. In the present study,

reverse transcription - polymerase chain reaction (RT-PCR) analysis revealed the presence of 5-HT_{1A} and 1B receptor subtypes in mouse olfactory epithelium at the mRNA level. With subtype selective antibodies and standard immunohistochemical techniques, both receptor subtypes were found to be positively labeled. To further elucidate the molecular mechanisms of 5-HT act on the peripheral olfactory transduction, the whole-cell patch clamp techniques were used on freshly isolated ORNs. We found that 5-HT decreased the magnitude of outward K⁺ current in a dose-dependent manner and these inhibitory effects were markedly attenuated by the 5-HT_{1A} receptor blocker WAY-100635 and the 5-HT_{1B} receptor antagonist GR55562. These data suggested that 5-HT may play a role in the modulation of peripheral olfactory signals by regulating outward potassium currents, both 5-HT_{1A} and 5-HT_{1B} receptors were involved in this regulation.

Key words

Serotonin, ORNs, whole-cell patch clamp, K⁺ channel

Introduction

Olfactory receptor neurons (ORNs) which typically respond to the presence of odorant with an increase in the frequency of firing of action potentials (Getchell 1986) are the key element of the olfactory pathway. The detection and discrimination of different odorant molecules are correlated with the electrophysiology of the olfactory receptor neurons. 5-HT exerts multiple biological functions including olfactory processing, by binding to cell surface receptors which can be classified into seven distinct families (5-HT₁ to 5-HT₇). With the exception of the 5-HT₃ receptors, which are ligand-gated ion channel, all of others are known to be seven transmembrane spanning

G-protein coupled receptors (Pierce *et al.* 2002). The central olfactory system which includes the olfactory tubercle, the anterior olfactory nucleus, the piriform cortex, and the entorhinal cortex (Hamada *et al.* 1998), has been shown to contain significant amount of serotonergic neurons and 5-HT receptor subtypes (Takeuchi *et al.* 1982; McLean and Shipley 1987; Won *et al.* 1998; Bauman and Amaral 2005). The electrophysiological function of 5-HT was examined in olfactory system. For example, via 5-HT_{1A} receptors, 5-HT was reported to inhibit the neuronal excitability of the entorhinal cortex in the superficial layers by activating the TWIK-1 type of the two-pore domain K⁺ channels (Deng *et al.* 2007). It also has been demonstrated that 5-HT causes reversible reduction of rapidly activating A-type K⁺ current and delays rectifier-type K⁺ conductance and voltage-activated Ca²⁺ influx in the antennal lobe cells of moths (Mercer *et al.* 1995). In addition, 5-HT depolarizes the juxtglomerular cells by activating 5-HT_{1C} receptors in the olfactory bulb, and the action of 5-HT in mitral cells of olfactory bulb contains two types of responses, hyperpolarized or depolarized (Hardy *et al.* 2005). These data indicate that 5-HT and its receptors may act a role in the olfactory system. However, the knowledge about 5-HT and its receptors in the peripheral olfactory system is limited.

As one of the major 5-HT receptor families, 5-HT₁ receptors were reported to be widely expressed in the olfactory regions of the brain where olfactory nerve projects to. 5-HT_{1A} receptors immunoreactivity has been observed in anterior olfactory nucleus, piriform cortex, entorhinal cortex and olfactory bulb (Pompeiano *et al.* 1994; Suehiro *et al.* 1998; Palchaudhuri and Flugge 2005; Muller *et al.* 2007). It was also reported that 5-HT_{1B} receptors were expressed high in olfactory tubercle (Bonaventure *et al.* 1998) and olfactory bulb (Pompeiano *et al.* 1994). However, there was no report concerning about

the expression and distribution of 5-HT₁ receptors in mouse ORNs, although it was shown that the 5-HT_{3A} receptors were expressed predominantly in the basal layer of olfactory epithelium but not in the olfactory nerve (Johnson and Heinemann 1995).

The aim of the present study was to detect the expression and distribution of 5-HT_{1A} and 5-HT_{1B} receptor subtypes in olfactory epithelium. Moreover, we investigated the effect of exogenous 5-HT on the profile of the K⁺ channel in acutely isolated ORNs, and studied the involvement of 5-HT_{1A} and 1B receptors in such modulation.

Materials and methods

Animals and reagents

The Kun-Ming (KM) mice, between 7- 14 days old, were sacrificed in this study. Our experiment was approved by the animal care and use committee at the Huaxi Medical School of Sichuan University, and our research was carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) in order to minimize the number of animals used and their suffering.

Trizol RNA isolation reagent was obtained from Roche (Switzerland). RT-PCR Kit was purchased from MBI (Lithuania). Taq enzyme, 5-HT, dNTPs were purchased from Sigma (St. Louis, USA). DNA Marker was from TianweiEra (Beijing, China). Anti-NF-L, anti-serotonin_{1A} (5-HT_{1A})-receptor, anti-serotonin_{1B} (5-HT_{1B})-receptor and biotin-labeled anti-rabbit IgG were purchased from Santa Cruz. SP Kit 9003 were obtained from Zhongshanjinqiao Biotechnology (Beijing, China). The DAB developer was from Pierce. Biotin-labeled anti-goat IgG was obtained from Jinmai Biotechnology (Tianjin, China). Pancreatic enzyme, 5-HT, WAY-100635 and GR55562 were from

Sigma (St. Louis, USA).

Extraction of total RNA and RT-PCR

The mice were deeply anesthetized with Nembutal (50mg/kg, i.p.) and then decapitated. Total RNA was extracted from the freshly isolated olfactory epithelium using Trizol reagent. RT-PCR was carried out as we previously described (Long et al., 2008) using the following primer pairs: 5-HT_{1A} receptors (Genbank number: NM_008308), forward primer 5'-CAGCTACCAAGTGATCACCTCT-3' and reverse primer 5'-CTCATGGTATCAGTGCTGGTC-3'; 5-HT_{1B} receptors (Genbank number: NM_010482), forward primer 5'-ACCAAGTCAAAGTGCGAGTCT -3' and reverse primer 5'-ATCTGCAAGGATGCCTGCTG -3'. All primers were synthesized by Shanghai SAGON Company.

In this study, ascertained 5-HT_{1A} receptor positive cortex of frontal lobe tissues (Verge et al. 1986) and 5-HT_{1B} positive striatum tissues (Monsma *et al.* 1993) were used as positive controls. Ultra pure water (PCR grade, RNase-free) was as the negative controls.

Immunohistochemistry

The KM mice were anesthetized and decapitated rapidly. The nasal cavity was removed and then fixed in 4% neutral formalin for 48 hours, and decalcified 48-72 hours using EDTA decalcifying fluid. After rinsing, the nasal cavity was embedded in paraffin. Tissues were cut consecutively into 3-5 μ m slices and deparaffinized.

Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 15-20 minutes. After antigen retrieval in boiling autoclave for 2 minutes, the sections were treated with 1% Triton X-100 at 4°C for 10 minutes, then rinsed with PBS

for three times. To avoid nonspecific binding with primary antibodies, tissue sections were exposed to normal goat serum (1: 20) for 10 minutes, then lay in hatch base for 20 minutes at 37°C. Consequently, sections were incubated with primary antibodies, anti-neurofilament light chain (NF-L) antibody (rabbit anti- mouse IgG, 1: 200), anti-5-HT_{1A} receptor (goat anti-mouse IgG, 1: 200), and anti-5-HT_{1B} receptor (goat anti-mouse IgG, 1: 200) respectively, then at 37°C in hatch base for 30 minutes, followed by incubation at 4°C moist chamber overnight. After rinsed for 5 minutes for three times with PBS, the sections were incubated at 37°C for 40 minutes with biotinylated anti-rabbit IgG (1: 200), or biotinylated anti-goat IgG (1: 200). The sections were then rinsed in PBS three times, followed by adding streptavidin-biotin-peroxidase complex at 37°C for 20 min, rinsed with PBS four times. Finally the sections were stained with DAB solution, followed by counterstaining with hematoxylin and monitored under a microscope (Olympus, Japan) equipped with a camera connected to a computer. As negative controls, immunostaining of tissue sections was processed without primary antibodies.

Dissection and identification of ORNs

The dissection and identification of olfactory receptor neurons were conducted as described by Ni MM et al (Ni *et al.* 2008). The olfactory receptor neurons are bipolar neurons with an apical, ciliated dendritic region, a somata and a basal unmyelinated axon under an inverted microscope (Olympus, Japan) (Lucero and Chen 1997). They could create a 2 ~ 5 GΩ seal in the whole-cell mode.

Whole-cell patch-clamp recordings

The whole-cell patch-clamp technique was used to record membrane currents in

olfactory receptor neurons. Cells were placed on the stage of an inverted Olympus IX70 microscope. Electrodes with resistances of 4 ~ 6 M Ω were pulled from borosilicate glass (outer diameter, 1.2 mm) using a Narishige PC-10 electrode puller (Tokyo, Japan) and were filled with the electrode solution (in mmol/l): 145 KCl, 4 MgCl₂, 5.0 EGTA, 10 HEPES, 1 ATP and 0.1 GTP (PH 7.4 with KOH). The extracellular solution contained (in mmol/l): 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 10 glucose and 1 sodium pyruvate (PH 7.4 with NaOH). The seal resistance was usually 1 to 10 G Ω . Currents were monitored using an Axopatch 200B voltage amplifier (Axon Instruments, Union City, CA, USA), low pass filtered at 2 KHz, digitized with a Digidata 1200Axon A/D-D/A interface, acquired and analyzed with the aid of pCLAMP10.0 software (all from Axon Instruments). All experiments were performed at room temperature. Series resistances were compensated by 80%. For the patch-clamp recording, a neuron with smooth membrane, good refraction, and without intracellular Brownian motion was chosen. The morphological condition of the neuron was closely monitored during the course of experiment to ensure it remained healthy.

Data analysis

The effects of drugs were analyzed with the current density. Data were analyzed using Clampfit (version 10.0, Axon Instruments), SPSS13.0, and Excel (Microsoft). Data are expressed as the mean \pm standard error. The paired Student's *t*-test was used to determine the effects of drugs. Increases or decreases in outward potassium currents were considered to be statistically significant at a value of $p < 0.05$. The *n* represents cell number.

Results

mRNA expression of 5-HT_{1A} and 5-HT_{1B} receptors in olfactory epithelium

As shown in Fig. 1, strong mRNA expression of 5-HT_{1A} and 5-HT_{1B} receptors was found in positive controls. On the contrary, nothing was amplified from the blank controls. The mRNA for 5-HT_{1A} (a 172 bp PCR product) and 1B receptor (a 169 bp PCR product) subtypes was strongly amplified in olfactory epithelium. The transcript of a housekeeping gene, a 600-bp glyceraldehydes-3-phosphate dehydrogenase (GAPDH), served as internal control.

Distribution of 5-HT_{1A} and 5-HT_{1B} receptors in the olfactory epithelium

5-HT receptor subtypes 1A and 1B immunoreactivities were shown in the olfactory epithelium (Fig.2). In the negative control in which the first antibodies were omitted (Fig.2A), no staining was detected. Cell nuclei were counterstained with haematoxylin. The olfactory epithelium is pseudostratified and can roughly be divided into three layers: sustentacular cell layer (SCL), nerve cell layer (NCL), and basal cell layer (BCL). The apical wide sublayer is primarily made up by the cell bodies of sustentacular cells. NF-L, a low molecular weight of silk protein, is specifically present in neuronal cells. NF-L-expressing cells was intensively stained, which can be considered as an indication for NCL. On the other side, basal cells are situated at the base of the epithelium (Fig.2B). As shown in Fig.2C and 2D, 5-HT_{1A} receptors were distinctly localized to the cell bodies presumably belong to the neurons zone (Fig.2C), while 5-HT_{1B} receptors were mainly on the neural dendrites and dendrite terminals of olfactory receptor neuron cells (Fig.2D).

Isolation of outward potassium currents

The olfactory receptor neurons were held at a holding potential of -100 mV, and depolarized between -70 and +70 mV (10 mV steps). To isolate voltage-gated K⁺ from

Na⁺ currents and Ca²⁺ currents, tetrodotoxin (0.1μmol/l) and CdCl₂ (0.5μmol/l) were used in the extracellular solution (Fig.3B). As shown in Fig. 3D, the outward currents were strongly reduced by 15mM tetraethylammonium, a well known blocker of K⁺ channels (Fig.3C was the control). In the following experiments, cells were bathed in extracellular solution containing tetrodotoxin (0.1μmol/l) and CdCl₂ (0.5μmol/l) and with a normal pipette solution.

Effect of 5-HT on the outward potassium currents of mouse olfactory receptor neurons

Mammalian ORNs respond to cell depolarization with an outward K⁺ at least comprised two different currents, a fast transient component (I_A), as well as a slowly activating delayed rectifier K⁺ currents component (I_K). As shown in Fig. 4A and 4B, the amplitude of voltage-gated K⁺ channels, contained both two currents were reduced by application of 5-HT (10nmol/l). Previous studies demonstrated that the fast transient component, but not the delayed rectifier K⁺ currents component, could be abolished by setting the holding potential at -40mV. To isolate the delayed rectifier K⁺ currents component from the fast transient component, the olfactory receptor neurons were clamped at a holding potential of -40 mV, depolarized by voltage steps from -20 to +80 mV in 10 mV increments (Fig.4C). The membrane capacitance of olfactory receptor neurons was 2.03 ± 0.28 pF (n=43 cells).

As shown in Fig.4D, at a holding potential of -40 mV, the delayed rectifier K⁺ currents component was inhibited by 5-HT (10nmol/l). 5-HT reduced the magnitude of the delayed rectifier K⁺ currents component from 1801.12 ± 165.86 pA/pF to 1111 ± 110.73 pA/pF (n = 5, p<0.05). The effect of 5-HT on the fast transient component was

determined by subtracting the delayed rectifier K⁺ currents component elicited at a holding potential of -40 mV after application of 5-HT, from the currents recorded at the holding potential of -100 mV (Fig.4B). By subtracting the delayed rectifier K⁺ currents component from the currents, the calculation showed that 5-HT (10nmol/l) reduced the amplitude of the fast transient component from 323.22 ± 53.35 to 177.49 ± 50.05 pA/pF (n=6, p<0.05).

At the holding potential of -100 mV, 5-HT (10nmol/l, 100nmol/l, 1 μ mol/l) reduced the magnitude of potassium in ORNs by (38.55 ± 4.40) % (p<0.05, n=5), (45.75 ± 10.44) % (p<0.05, n=7) and (56.43 ± 6.01) % (p<0.05, n=10), respectively, in a dose-dependent manner (Fig.5A).

Effect of 5-HT_{1A} receptor antagonist on 5-HT induced inhibition of the potassium currents

To test the involvement of 5-HT_{1A} receptor in 5-HT-mediated blockage of the outward potassium currents, a selective 5-HT_{1A} receptor antagonist, WAY-100635 (5nmol/l), was applied to the bath solution, and then 5-HT was added. The potassium currents were reduced from 326.47 ± 89.07 pA/pF to 258.44 ± 18.08 pA/pF at the holding potential of -80 mV and the stimulating potential of +60mV. The inhibitory effect of 5-HT (10nmol/l) on potassium currents decreased from (38.55 ± 4.40) % to (26.00 ± 6.67) % with the treatment of WAY-100635 (n=7, p<0.05) (Fig.5B). This result suggested that selective 5-HT_{1A} receptor antagonist WAY-100635 could antagonize the inhibitory effect of 5-HT on olfactory receptor neuron outward potassium currents.

Effect of 5-HT_{1B} receptor antagonist on 5-HT induced inhibition of the potassium currents

To determine whether the 5-HT_{1B} receptor also mediates the inhibition of 5-HT on outward potassium currents, we applied 5-HT_{1B} receptor antagonist GR55562 (100nmol/l) to the bath, then added 5-HT. The current density of potassium was reduced from 504.75 ± 96.72 pA/pF to 344.58 ± 58.18 pA/pF. The inhibitory effect of 5-HT was significantly reduced from (38.55 ± 4.40) % to (26.52 ± 7.87) % with 100nmol/l GR55562 pre-treatment (n=9, p<0.05) (Fig.5B). This result suggested that the 5-HT_{1B} receptor might also mediate the inhibition caused by 5-HT in olfactory receptor neurons.

Discussion

Types of currents in mouse olfactory receptor neurons have been described previously (Lagostena and Menini 2003). The I_A appeared in a huge amount of neurons and muscle, play critical role in determining the excitability of neurons (Han and Lucero 2006). The I_K helps determine the spike width and post-spike hyperpolarization to sharp the maximal spike frequency of neurons (Gruhn *et al.* 2005). In the present study, we demonstrated that exogenous 5-HT reduced the magnitude of the outward potassium currents of mouse ORNs, both the amplitude of I_K and I_A were decreased. Although the physiological and pathological significance of the activation of potassium currents by serotonin is still unclear in the present investigation, the decrease in potassium currents appears to be responsible for the increased excitability in olfactory sensory neurons following application of 5-HT.

It was reported that 5-HT_{1A} receptors localized pre-synaptically on the cell bodies and dendrites of serotonergic neurons in the raphe nuclei and post-synaptically on many nonserotonergic neurons (Olivier and van Oorschot 2005). The post-synaptic 5-HT_{1A} receptors have been reported to mediate decreases of neuronal firing in the pyramidal

cells of the hippocampus and prefrontal cortex (Riad *et al.* 2000). Previous findings have provided evidence that the contributions of pre- and post-synaptic 5-HT_{1A} receptors to behavior can be dissociated (Ogren *et al.* 2008). In this study, the 5-HT_{1A} receptors were localized to the cell bodies presumably belong to the neurons zone. Furthermore, preprocessed by 5-HT_{1A} receptor-selective antagonist WAY100635 before application of serotonin abolished the decrease effect on the outward potassium currents. This result suggest that response to serotonin of ORNs was functionally mediated by 5-HT_{1A} receptors. 5-HT_{1B} receptors are also G protein-coupled receptor which are localized pre- and post-synaptically. In our present study, we demonstrated that 5-HT_{1B} receptors were mainly on the neural dendrites and dendrite terminals of ORNs, where the influence of cytoplasm washing out is delayed and limited. However, at the existence of GR55562, a 5-HT_{1B} receptor blocking drug, cells inhibition of K⁺ currents by 5-HT was reduced. It is suggested that the 5-HT_{1B} receptor subtype may participate in the inhibitory effect of 5-HT on olfactory receptor neurons potassium currents. Serotonin translates information about behavioral context into changes in sensory processing via a diverse array of receptors, these receptors may interact to shape sensory encoding. These two receptors are G-protein coupled receptor and their activation is through a G-protein-mediated mechanism (Adayev *et al.* 2005), the precise mechanisms of serotonin and 5-HT_{1A} and 5-HT_{1B} receptors interaction in ORNs are still unclear. Whether it is through the same signal pathway requires to be clarified by further experiment.

In conclusion, this is the first study to characterize the regulation of 5-HT and its two receptor subtypes on the outward potassium current in the mouse ORNs. This study is an essential first step towards further understanding the cellular effects of 5-HT

modulation of the periphery olfactory system. Further investigation of the regulatory molecules and intracellular signalling systems are imperative to be explored at a variety of levels biophysically and electrophysiologically .

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Figure legends

Fig. 1 A, 5-HT_{1A} receptor mRNA expression. Lanes: M, 100 bp marker; 1, cortex of frontal lobe tissues (positive control); 2, no cDNA template (negative control). 3, olfactory epithelium. B, 5-HT_{1B} receptor mRNA. Lanes: M, 100 bp marker; 1, striatum tissues (positive control); 2, no cDNA template (negative control); 3, olfactory epithelium.

Fig. 2 A, Control (no primary antibody). B, Immunostaining for NF-L, a neuronal cell marker, used here to be considered as an indication for NCL. C, Immunohistostaining for 5-HT_{1A} receptor reveals an intracellular localization of the cell presumably belong to the neurons zone ($\times 200$). D, Immunohistostaining for 5-HT_{1B} receptor shows the immunoreactivities mainly on the neural dendrite and dendrite terminals of ORNs ($\times 200$).

Fig. 3 Whole-cell voltage-gated currents in a mouse olfactory receptor neuron at a holding potential of -100mV , stimulating voltage from -70mV to $+70\text{mV}$ in 10mV steps for 300ms . A, Voltage-gated currents were elicited in a mouse olfactory receptor neuron as described in the Methods. There were transient inward currents followed by outward currents. B, Voltage-gated K^+ channels were isolated by blocking Na^+ currents with $0.1\ \mu\text{M}$ tetrodotoxin and Ca^{2+} currents with CdCl_2 ($0.5\ \mu\text{M}$). C and D, The outward currents were strongly reduced when $15\ \text{mM}$ tetraethylammonium, a well known blocker of K^+ channels, was applied to the bath, suggesting that the outward currents were potassium currents.

Fig. 4 A, B, The holding potential was $-100\ \text{mV}$ and the stimulating voltage was from -70mV to $+70\text{mV}$ in 10mV steps for 300ms . A, The outward potassium currents were activated before application of 5-HT. B, Both the slowly activating delayed rectifier K^+

currents component and the fast transient component magnitudes were decreased following bath application of 5-HT. C, The outward potassium currents contain a fast transient component, as well as a delayed rectifier K⁺ currents component. The delayed rectifier K⁺ currents component was isolated by holding cells at -40 mV to various voltages between -20 and +80 mV in steps of 10 mV for 300ms in the same cell. D, The magnitude of delayed rectifier K⁺ currents component was reduced after the application of 5-HT to the bath solution.

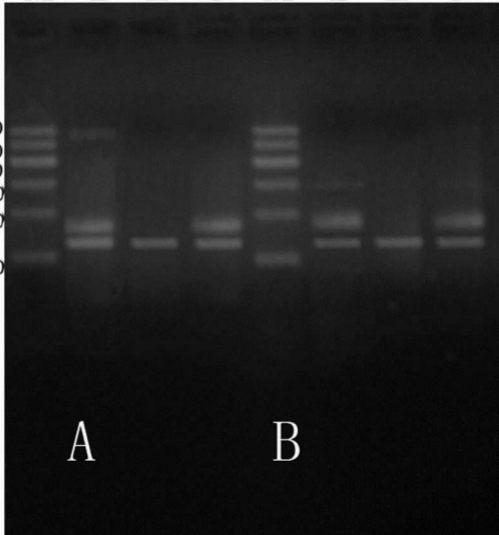
Fig. 5 A, B, The holding potential was -100 mV and the stimulating voltage was +70mV for 300ms. A, Application of 5-HT reduced the magnitude of outward potassium currents in a dose-dependent manner. B, The percentage reduction of K⁺ currents by 5-HT treatment in the presence of GR55562 or WAY-100635. The inhibition by 5-HT was attenuated from (38.55 ± 4.40) % to (26.00 ± 6.67) % with the addition of 100nm GR55562 (n=9, p<0.05). The inhibitory effect of 5-HT (10nm/l) on potassium was reduced from (38.55 ± 4.40) % to (20.82 ± 7.86) % in the presence of WAY-100635 (n=7, p<0.05).

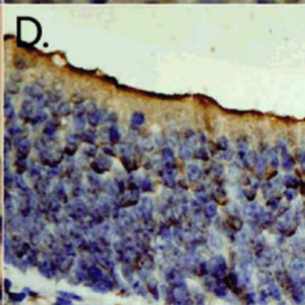
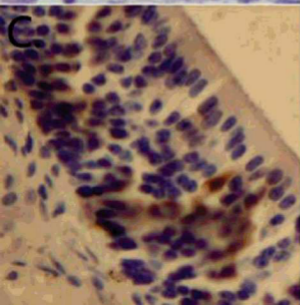
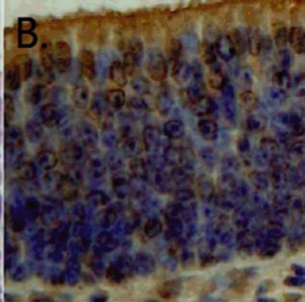
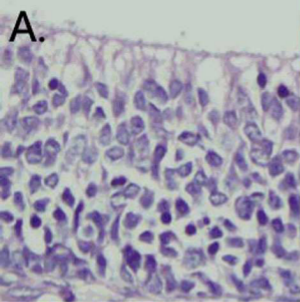
M 1 2 3 M 1 2 3

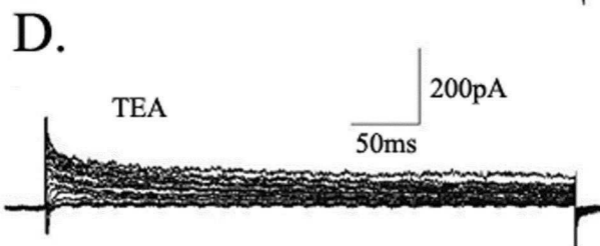
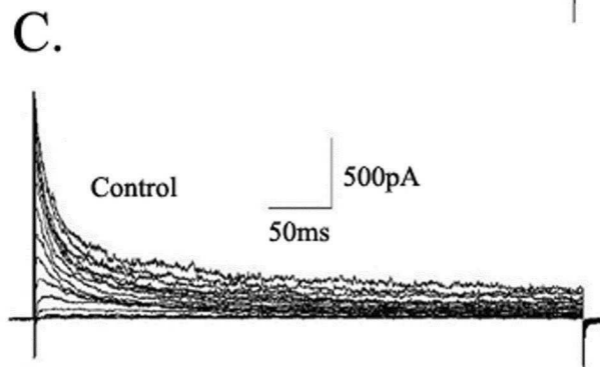
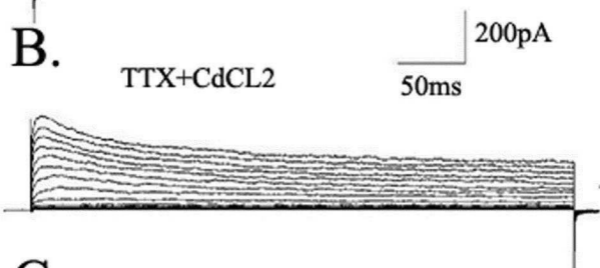
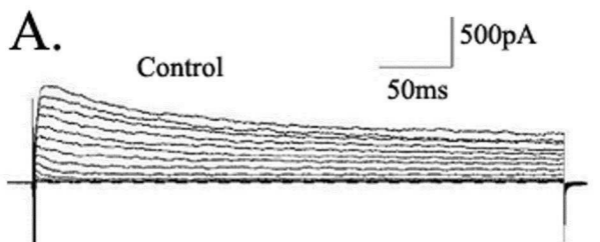
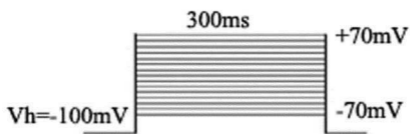
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500bp
400bp
300bp
200bp
100bp

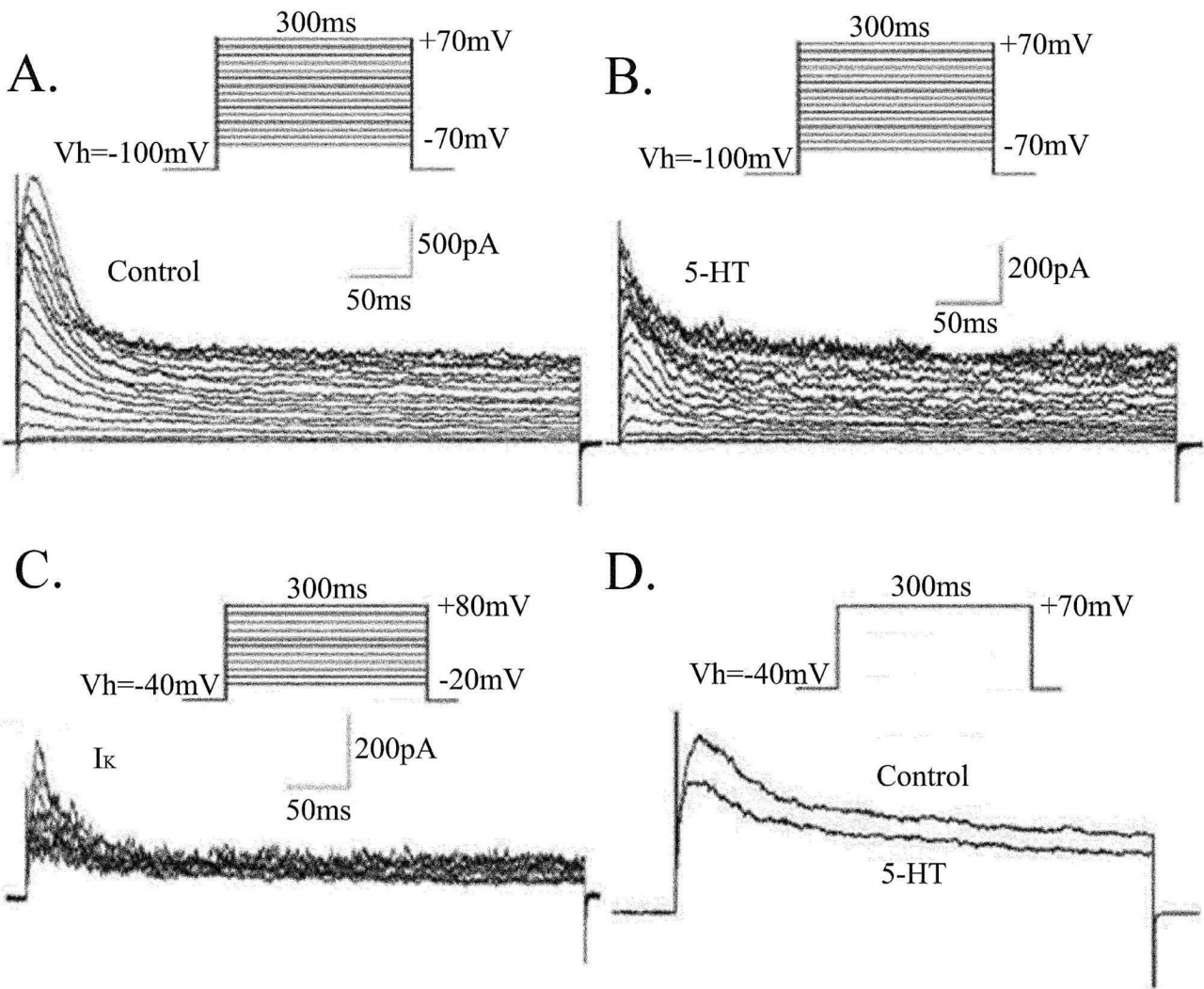
A

B



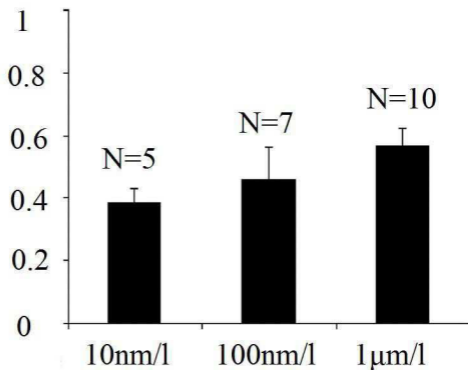






A.

5-HT-induced blockade of potassium currents(%)

**B.**

5-HT-induced blockade of potassium currents(%)

