Mechanisms of serotonergic neuromodulation in the olfactory cortex

Summary of the Ph.D.thesis

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2023, Szeged

The serotonergic system

Located at the brainstem raphé nuclei (RN), serotonergic neurons project to various forebrain areas and release serotonin (5-hydroxytryptamine, 5-HT) throughout the entire neuraxis. 5-HT is implicated in a variety of physiological functions, including the regulation of sensory and motor responses, brain states, learning and reward processing and social interactions. Dysfunctions of the serotonergic system are implicated in several neurological and psychiatric disorders, including depression and epilepsy. Several structural and functional aspects of the serotonergic system make it a likely contributor to the neuromodulation of sensory functions.

It has been implicated in the regulation of sensory functions at various stages of multiple modalities (eg. auditory, visual and somatosensory system). In a two alternative forced choice task, some dorsal RN (DRN) neurons show rapid, odor specific modulations of activity. Several studies examined the role of the 5-HT system in odor processing and demonstrated its complex modulatory effects on sensory input, but its cellular mechanisms and functions in the olfactory system have remained elusive.

The olfactory system

Multiple types of olfactory receptor neurons (ORNs) located in the sensory epithelium expressing a single unique odorant receptor (OR) protein (300 in humans, 1000 in rodents). ORNs distributed throughout the sensory epithelium converge to form a segregated, ordered spatial map in the olfactory bulb (OB) where each glomerulus receives projections from ORNs expressing the same receptor protein. The OB is thus a highly specialized extrathalamic relay station for olfactory information. The two types of projection neurons in the OB are the mitral cells (M) and tufted cells (T). The axons of M/T neurons form the lateral olfactory tract (LOT) that terminates in a variety of brain regions including the primary olfactory or "piriform" cortex (PirC), amygdala, hypothalamus and orbitofrontal cortex. Due to its phylogenetically old, relatively simple 3 layered structure and its spatially and pharmacologically distinct afferent and intracortical inputs, the primary olfactory cortex represents an ideal model system to study the serotonergic modulation of cortical circuits.

Specifically, the LOT inputs (afferent inputs) contact the distal dendrites of pyramidal and semilunar neurons, the main principal neurons of the PirC and also local interneurons in layer 1a, whereas synapses originating from intracortical sources (including other regions of the PirC, orbitofrontal cortex, amygdala and entorhinal cortex) are formed more proximally in layer 1b, layer 2 and layer 3.

Beyond sensory representations, the olfactory system is intimately linked to affective functions that are important for social interactions, including the regulation of mood and maternal behaviour, among others. Interestingly, several neuropsychiatric disorders are accompanied by impaired olfactory functions and reduced volume of the olfactory bulb. The link between depression and olfactory function seems particularly strong.

Because of its prominent projections targeting various olfactory areas including the OB and the PirC, 5-HT is ideally suited to influence olfaction, which may lead to changes in both sensory perception and regulation of higher brain functions. Several studies examined the role of the serotonergic system in odor processing and demonstrated its complex modulatory effects on sensory input. Selective stimulation of DRN 5-HT neurons resulted in the divisive suppression of spontaneous, but not odor-evoked spiking activity in the majority of neurons in the primary olfactory cortex and an increase in firing in a minority of neurons of anesthetized mice. However, odor-evoked activity was decreased and spontaneous activity remained unaltered during similar manipulation of 5-HT neurons, when monitoring the population Ca2+ dynamics of PirC principal neurons using fiber photometry in awake mice. The contrasting results might be due to different techniques used.

However, the mechanisms by which 5-HT shapes olfactory information processing remains to be elucidated. Testing the effect of 5-HT on the spontaneous (intracortical inputs) and odor-evoked activity (afferent inputs) of the anterior piriform cortex (aPC) has led to contrasting results.

AIMS

To reveal the cellular and network mechanisms of serotonergic neuromodulation and its effects on the function of the primary olfactory cortex we used a combination of *in vitro* and *in vivo* electrophysiology, optogenetics, pharmacology and immunohistochemistry.

Our specific aims were the following:

I. To explore the cellular effects of serotonin on the principal neurons of the primary olfactory cortex
II. To explore the cellular effects of serotonin on various interneurons of the primary olfactory cortex
III. To investigate the effect of serotonin on the afferent and intracortical synaptic inputs to the primary olfactory cortex
IV. To reveal the receptors involved in cortical serotonergic neuromodulation
V. To test whether the cortical effects of serotonergic stimulation are due to local 5-HT release or are the reflection of modulation at another station of the olfactory system

Viral injections

For the selective stimulation of DRN serotonergic neurons adult male heterozygous SERT-cre mice were injected with 0.5-1 µl of AAV2/1-Flex-ChR2-YFP (AV1-20298P, University of Pennsylvania, 1013 GC/mL) in the DRN [coordinates: anteroposterior (AP), -4.7 mm; dorsoventral (DV), 3.1-3.6 mm] leading to prominent and specific ChR2 expression in DRN 5-HT neurons and axons in the aPC. 8-16 weeks following the viral infections mice were used for electrophysiological experiments.

Slice preparation

Mice were deeply anesthetized with ketamine and xylazine (80 and 10 mg/kg, respectively), and perfused through the heart with a solution containing (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 N-acetyl-L-cysteine, 5 Na-ascorbate, 3 Na-pyruvate, 10 $MgSO_4$, and 0.5 $CaCl_2$. The same solution was used to cut 320 µm coronal slices containing the aPC at 4°C and for the initial storage of slices (32°C-34°C for 12 min) following which the slices were stored in a solution containing the following (in mM): 30 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.3 $NaHCO_3$, 20 HEPES, 25 glucose, 5 N-acetyl-L-cysteine, 5 Na-ascorbate, 3 Na-pyruvate, 3 $CaCl_2$, and 1.5 $MgSO_4$.

In vitro electrophysiology

For recording, slices were submerged in a chamber perfused with a warmed (34°C) continuously oxygenated (95% O2, 5% CO2) ACSF containing the following (in mM): 130 NaCl, 3.5 KCl, 1 KH_2PO_4, 24 $NaHCO_3$, 1.5 $MgSO_4$, 3 $CaCl_2$, and 10 glucose. Whole-cell patch clamp recordings were performed in either current-clamp or voltage clamp mode using 4-6 MOhm pipettes. For whole-cell current clamp the pipettes contained (in mM): 126 K-gluconate, 4 KCl, 4 ATP-Mg, 0.3 GTP-Na_2, 10HEPES, 10 creatine-phosphate, and 8 Biocytin, pH 7.25; osmolarity, 275 mOsm. For whole-cell voltage clamp the pipettes contained (in mM): 130 Cs-gluconate, 5 NaCl, 3 ATP-Mg, 0.3 GTP-Na_2, 10 EGTA, 10 HEPES, 12 creatine-phosphate, and 8 Biocytin, pH 7.25; osmolarity, 275 mOsm. Neurons were visualized using DIC imaging on an Olympus BX51WI microscope (Tokyo, Japan). Membrane potentials and currents were recorded using a Multiclamp 700B amplifier (Molecular Devices, USA). The liquid junction potential (-13 mV) was compensated for. Series resistance was continuously monitored and compensated (80%) during the course of the experiments; recordings were discarded if the series resistance changed more than 25%.

For synaptic stimulation two concentric bipolar stimulating electrodes (FHC, Germany) were positioned in the lateral olfactory tract (LOT) and layer 2 (L_2) for afferent, and associational fiber stimulation, respectively. A recording pipette filled with ACSF (resistance: 4 MΩ)

was then positioned above L_2. Stimulation consisted of brief (0.1 ms) current pulses (10-100 μA). Afferent and associational stimulation was separated by 0.5 seconds. After obtaining a baseline of field excitatory postsynaptic potentials (fEPSPs), EPSCs or EPSPs serotonin was applied to the recording chamber. ChR2 expressing axons in the aPC were photostimulated through the microscope objective using the epifluorescent illumination via a LED light source (Thorlabs, Germany). Light intensity was set to 0.5 mW. Photostimulation consisted of a 3 second train of 10 ms pulses at 10 Hz. Control and photostimulation trials were intermingled. For the focal application of 5-HT patch pipettes were loaded with 100 μM 5-HT dissolved in ACSF, positioned near (40-60 μm) the soma of the neuron recorded and connected to a Picospritzer III (Parker Hannifin, USA). 5-HT was ejected using a 2 s long (~200 mbar) pulse.

In vivo electrophysiology

For the selective stimulation of DRN 5-HT neurons *in vivo*, SERT-cre mice previously (4-8 weeks) injected with 0.5–1 μl of AAV2/1–Flex–ChR2–YFP (AV-1-20298P, University of Pennsylvania, 1013 GC/mL) in the DRN were anesthetized with Urethane (1.2 g/kg), mounted in a stereotaxic frame and small holes drilled above the target areas (OB: AP, +6.0-7.0 mm; lateral, 2.2 mm; DV, 2.5–4.0 mm, aPC: AP, +2.3 mm; lateral, 2.5 mm; DV, 2.9–3.6 mm) and recording microelectrodes lowered into the OB and aPC, respectively. The DRN

was photostimulated using an optical fiber (200 µm diameter; numerical aperture 0.38, positioned at a 32° angle at the following coordinates: AP, −4.7 mm; DV, 3.0–3.6 mm) coupled to a 470 nm laser (Laserglow Technologies). OB and aPC neurons were recorded simultaneously with glass electrodes (impedance: 8-20 MOhm) filled with saline and connected to a DC amplifier (Axoclamp 2B, Axon Instruments, USA). Electrophysiological data were acquired using a Power 1401 and Spike2 software (Cambridge Electronic Design, UK) at 30 kHz sampling rate and stored on a personal computer for offline analysis. Spike sorting was performed using Spike2 software (Cambridge Electronic Design, UK). The 5-HT1B receptor antagonist GR127935 (3 mg/kg, dissolved in saline) was administered intraperitoneally.

Immunohistochemistry

Following *in vitro* recordings, the slices were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4°C overnight. Slices were cryoprotected using 20% sucrose in 0.1 M PB, freeze-thawed with liquid nitrogen, washed thoroughly with PB and re-sectioned to 50 µm thickness. After blocking with 10 % NHS in TBS, slices were incubated with the primary antibody Rb-α-5-HT (Immunostar, Hudson, WI, United States, Rb-α-5-HT polyclonal, 1:1000) overnight. Following several TBS washes, the slices were incubated with the secondary antibody Alexa488-conjugated Donkey-

α-Rb (1:400) for 2 hours and mounted in Vectashield-DAPI medium for microscopy.

Depolarizing current pulses employed during in vitro electrophysiological recordings resulted in an adequate filling of neurons by Biocytin. Following recordings, the slices were placed between two Millipore filters to avoid deformations and were immersed in a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH=7.4) at 4°C for at least 12 h. After several washes in 0.1 M PB, slices were cryoprotected in 10%, then 20 % sucrose in 0.1 M PB. Slices were freeze-thawed in liquid nitrogen, then embedded in 10 % gelatine. 320 µm thick slices embedded in gelatine blocks were resectioned at 60 µm thicknesses and washed in PB, then in triton X-100 (0.4%) in tris buffered saline (1X TBS). The sections were incubated with streptavidin-conjugated Alexa 488 (1:500) and triton X-100 (0.4%) for 2 hrs at room temperature to identify the Neurobiotin-labeled neuron by fluorescent microscopy. Fluorescent images were acquired with a confocal microscope (Olympus FV1000).

Data analysis

Data were analyzed using Spike2 (Cambridge Electronic Design), Clampfit (Molecular Devices) and Origin Pro (Microcal) software. Data are presented as mean ± s.e.m. Statistical significance was considered at p values below 0.05.

RESULTS

In order to provide comprehensive insights into the effects of 5-HT on the neurons of the aPC, we conducted a series of experiments utilizing *in vitro* and *in vivo* electrophysiological approaches combined with, optogenetic, pharmacological, and immunohistochemical techniques. Our findings collectively demonstrate the following key observations:

(i) To characterize the extent of serotonergic innervation of the aPC we performed immunohistochemical experiments. The results revealed dense 5-HT fibers in the aPC with subregion and layer specific features. Specifically, the aPC contained relatively dense 5-HT fibers in all its layers, most fibers were observed in layer 1 and 3.

(ii) By performing simultaneous OB and aPC recording during 5-HT PS we excluded the possibility that the prominent suppression of aPC neuronal activity upon 5-HT PS is originating from the OB as the neuronal activity in the two regions showed a differential effect: increased activity in OB neurons and suppression of most aPC neurons.

(iii) We identified that aPC interneurons, including perisomatic inhibitory fast-spiking interneurons, are excited by 5-HT, whereas principal neurons are inhibited. This differential

response highlights the specific modulation of distinct neuronal populations within the aPC by 5-HT.

(iv) Additionally, we observed that 5-HT can exert differential effects on synaptic inputs to the aPC. Specifically, it suppresses intracortical inputs while increasing afferent inputs. This suggests a dual role of 5-HT in modulating synaptic transmission within the aPC, with distinct effects on local circuitry and incoming sensory information.

(v) Further investigation into the mechanisms underlying the suppression of feedback inputs revealed that 5-HT primarily acts through a 5-HT1B receptor-dependent pathway to reduce glutamate release. This specific receptor-mediated modulation provides mechanistic insight into the observed synaptic alterations induced by 5-HT.

(vi) *In vivo* experiments employing targeted stimulation of 5-HT neurons originating from the DRN and subsequent systemic application of 5-HT1B receptor antagonists revealed that the suppression of baseline aPC neuronal activity induced by DRN 5-HT stimulation can be effectively blocked. This highlights the significance of 5-HT1B receptors in mediating the suppressive effects of 5-HT on aPC neuronal activity in a broader physiological context.

These comprehensive results have important implications for understanding the network mechanisms underlying cortical neuromodulation, particularly in the context of olfactory coding within the central nervous system. By elucidating the specific effects of 5-HT on different neuronal populations and synaptic inputs in the aPC, our results contribute to a more detailed understanding of how neuromodulation influences sensory processing and information flow within cortical circuits.

LIST OF PUBLICATIONS

Publications related to the subject of the thesis:

1. Ildikó Piszár and Magor L. Lőrincz (2022) Differential serotonergic modulation of principal neurons and interneurons in the anterior piriform cortex. Front Neuroanat 16:821695. DOI: 10.3389/fnana.2022.821695 IF: 3.543
2. Ildikó Piszár and Magor L. Lőrincz (2023) Differential serotonergic modulation of synaptic inputs to the olfactory cortex. International Journal of Molecular Sciences 24(3):1950. DOI: 10.3390/ijms24031950 IF: 5.6