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Effects of chosen chemokines on neuronal properties and synaptic transmission in central and basolateral complex of the rat amygdala

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Abstract

Streszczenie

Podziękowania

Abbreviations

ACSF	artificial cerebrospinal fluid
BLA	basolateral nucleus of the amygdala
CeA	central nucleus of the amygdala
CNS	central nervous system
DIC	differential interference contrast
ICM	intercalated cell masses
LHA	lateral hypothalamus area
mEPSC	miniature excibitory post-synaptic currents
mIPSC	miniature inhibitory post-synaptic currents
NDS	normal donkey serum
sEPSC	spontaneous excibitory post-synaptic currents
sIPSC	spontaneous inhibitory post-synaptic currents

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1 Introduction

1.1 Chemokines

1.1.1 Background

Chemokines (chemotactic cytokines) are a family of small (7-11 kDa) secreted proteins. So far, 53 human chemokines and 23 chemokine receptors have been cloned or characterized (http:// cytokine.medic.kumamoto-u.ac.jp/). They can be classified into CXC, CC, C or CX3C subfamilies based on the arrangement of the two cysteine residues near the N-terminus[1] 1.1. Chemokines exert their biological effects through cell surface receptors that belong to the superfamily of seven-membrane domain G-protein-coupled receptors (GPCRs) and are expressed in a wide variety of cells[2].

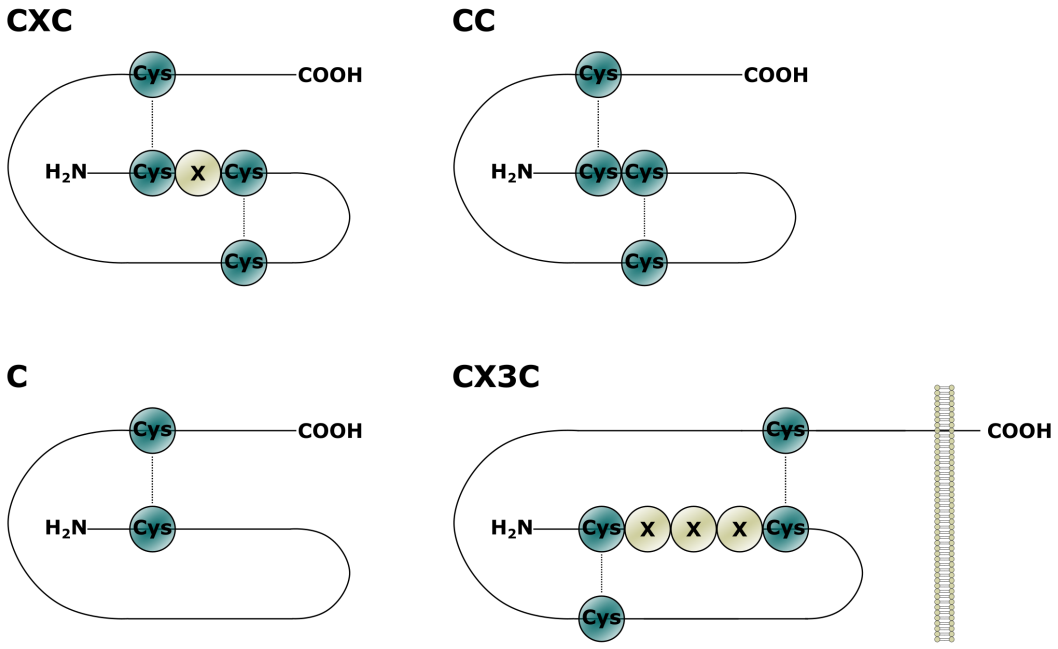

Figure 1.1: Chemokine families. Chemokines are classified in four distinct subclasses: C ( ), CC( ), CXC ( ), and CX3C( ) according to the number and spacing of their cysteine residues in their N-terminus. Cys – cysteine residue, X – amino acid residue, disulfide bridges are shown as dotted lines.

They were originally identified as serving chemotactic function on immune cells; however, recent evidence has begun to elucidate novel, brain-specific functions of these

proteins, more relevant to ….. Of particular interest is the idea that central nervous system (CNS) chemokines are not only mediators of neuroinflammation, but also function as neuromodulators or neurotransmitters in the brain[3]. Like those systems, the chemokine system is widely but unevenly distributed in the brain and has both ligands and receptors expressed in neurons. For example, the chemokines SDF-1 /CXCL12 and fractalkine/CX3CL1 as well as their receptors CXCR4, CXCR7 and CX3CR1 are expressed constitutively, even in physiological conditions, throughout the CNS[4]. Why do cells (neurons, astrocytes, and microglia) in CNS express chemokines and chemokine receptors, and what is the neurophysiological relevance of chemokines in the CNS? The plausible explanation is that they may provide a bridge between the immune and nervous systems, which are classically viewed as two complex and distinct entities. In addition to the role of chemokines in the developing and diseased brain (for example in multiple sclerosis and Alzheimer's disease[7]), only a handful of studies have examined the basic neurophysiological correlates of chemokine signaling in the CNS. The effects of chemokines in the brain may be due to their ability to activate chemokine receptors localized on neurons and/or glia, thus leading to changes in neuronal membrane properties and/or synaptic transmission. Specifically, chemokines may alter synaptic transmitter release[9], modulate the functional properties of ionic channels[11] and promote the release of glutamate from astrocytes[11]. In addition, the chemokine system can alter the actions of neuronally active pharmacological agents such as opioids and the cannabinoid system[13]. Moreover, chemokine receptor knockout mice (CCR6, CCR7, CXCR5) have recently been shown to exhibit behavioral and neurobiological phenotypes of relevance to psychiatric disorders, which suggests their essential role in physiological conditions[14]. Yet, recognition and characterization of chemokine effects on neurophysiology is still lacking and such studies would provide a more complete understanding of the vital role played by these immune proteins in the nervous system, and may ultimately lead to novel therapies for neuro-immune diseases.

1.1.2 CX3CL1

Fractalkine, also known as CX3CL1, is a unique chemokine belonging to CX3C chemokine family that is present both as soluble and membrane-anchored form[17]. Fractalkine is the endogenous ligand for CX3CR1, a G-protein coupled receptor (GPCR) which specifically interacts with both forms of CX3CL1[18]. Recent reports confine the localization of CX3CL1 to neurons and astrocytes and CX3CR1 to

neurons and microglia in many brain regions including the hippocampus, amygdala, cerebral cortex, globus pallidus, striatum and thalamus[19]. In physiological conditions, CX3CL1/CX3CR1 signaling is involved in different neural functions both in development (participation in functional maturation of neuronal circuits driving cell positioning) and adulthood (regulation of synaptic plasticity and cognitive functions)[20]. Furthermore, fractalkine seems to play an essential role in a unique neuronal-glial interaction between neurons and CX3CR1-expressing microglia under normal and pathological states[21]. In fact, CX3CL1 is a confirmed inhibitor of basal glutamate synaptic activity in the hippocampus[23]. The ability of CX3CL1 to modulate glutamate function is thought to be responsible for some of its neuroprotective actions, and provides further support for the chemokine-neuromodulatory role in the CNS[3].

1.1.3 CXCL12

SDF-1 (CXCL12) is another chemokine which has recently attracted much attention due to its extensive distribution in the CNS as well as the fact that it can be produced not only by glial cells but also by neurons[26]. This chemokine has been shown to play an essential role in brain plasticity during development but also in regulating the activity of various brain circuits in normal and pathological conditions[29]. Furthermore, recent studies have shown that there are several cross-talks between the CXCL12 and with its receptor CXCR4 and other neurotransmitter systems in the brain (e.g. GABA, glutamate and endocannabinoids)[27]. The neuromodulatory actions of CXCL12 have been observed in various neuronal populations, including the hippocampus[30], cerebellum[9], MCH neurons of the lateral hypothalamus area[31] (LHA), vasopressinergic neurons of the hypothalamus[32], dopaminergic neurons of the substantia nigra[33], the dorsal raphe nucleus[27] and recently the amygdala[34]. What is particularly interesting, CXCL12 has often similar effects in these various circuits (increase in glutamate and/or GABA synaptic activity), however these effects occur via different mechanisms, even within one structure. For example, in the dorsal raphe nucleus CXCL12 increases the frequency of recorded spontaneous inhibitory post-synaptic currents (sIPSC) in serotonergic neurons through a pre-synaptic mechanism, but decreases sIPSC amplitude via a post-synaptic mechanism in non-serotonergic neurons[27]. Moreover, CXCL12 often appears to exert opposite effects on neuronal function depending on its concentration, e.g. in MCH-expressing neurons of the LHA, where the action potential discharge is different depending on the concentration[31]. Of particular interest is the observation that

pro-inflammatory cytokine stimulation leads to elevated levels of CXCL12 and other chemokines by activation of glial or endothelial cells which in turn release chemokines[35]. The chemokines released bind to CXCR4 present on neurons and induce changes in excitability that could induce an adaptive response to inflammation, leading to "sickness behavior," characterized by decreased mood, anorexia, and fatigue[36]. Sickness behavior is considered to be the physiological and psychological effect of immune activation during the course of infection, which is primarily mediated by the central action of peripherally released proinflammatory cytokines. Given the abundance of chemokines and their receptors in the CNS, it is not surprising that changes of cytokine/chemokine levels during inflammation are causing multiple physiological and behavioral perturbations. For example, effects of CXCL12 on dorsal raphe neurons could underlie depressive symptoms frequently observed with inflammation[38], as dysfunction of the serotoninergic systems is implicated in depression. Similarly, the effects of CXCL12 on MCH neurons which are part of the feeding behavior and metabolism control circuit[39] could explain anorexia. These symptoms of sickness behavior are usually reversible when inflammation stops. Overall, this chemokine system is one of the key players in the neuro-immune interface that participates in shaping CNS responses to changes in the environment.

1.2 Neuro-glial interactions

1.2.1 Neuron-microglia signaling

1.3 Amygdala

The amygdala, a limbic region in the medial temporal lobe. Amygdala activity is critical for mood regulation and the central and peripheral stress response as well as attaching emotional valence to perceived stimuli[40]. The various nuclei of the amygdaloid complex can be grouped in to three major functionally different domains by their different connectivity, immunohistochemical and cytoarchitectural profiles: the basolateral complex of the amygdala (BLA), the central amygdala (CeA) and intercalated cell masses (ICM). The amygdala is very densely interconnected.

1.3.1 Basolateral amygdala

The BLA is considered to be the main point of entry for cortical and subcortical sensory inputs into the amygdala[40]. Within the BLA, which developmentally and structurally is a cortical-like structure, approximately 80% of all neurons are glutamatergic pyramidal-like principal cells and the remaining 20% belong to a heterogeneous group of interneurons[42]. The pyramidal-like cells follow the electrophysiological profile of their cortical counterparts, having either a regular spiking or intrinsically bursting phenotype, with pronounced spike frequency adaptation[43]. There are at least five distinct types of interneurons, which differ in their neurochemistry and electrophysiological properties and morphology, however most of them are of the fast-spiking variety, with no apparent adaptation[42]. In the BLA, principal cells form local synapses with inhibitory interneurons. However, they also have numerous longer distance collaterals synapsing onto other principal cells[40]. Principal cells in the BLA send their axons to the CeA, either directly or via intercalated GABAergic cell clusters in the intermediate capsule[44].

1.3.2 Central amygdala

Contrary to the BLA, CeA is considered as a main output nuclei. Most ot the CeA neurons are GABAergic in nature, morphologically reminiscent of striatal medium spiny neurons. Three electrophysiological subtypes of CeA neurons have been described: regular spiking, low threshold bursting and late firing[45]. The CeA network then receives signals from the BLA and computes the inhibitory GABAergic output to various brain areas.

1.3.3 Amygdala and neuroinflammation

Amygdala is considered to be an essential component of the brain circuitry that forms the neural response to systemic immune activation[46] and it is involved in sickness behavior[47]. Regarding neuroinflammation circuit, pro-inflammatory cytokines signal the brain mainly through the activation of vagal afferent fibers projecting to the nucleus of the solitary tract (NTS) and then to amygdala[50]. Therefore, it is important to get better insights into brain activity during acute and chronic immune responses.

Evidence from many animal studies indicates that LPS significantly increases c-fos expression in the central nucleus of the amygdala (CeA) and, to a lesser extent, in the basolateral nucleus of the amygdala (BLA)[52]. Moreover, functional and neuroanatomical studies indicate that peripheral immune activation may also affect amygdala function in humans. For example, depressive-like symptoms and anxiety can be transiently elicited in healthy human subjects by peripheral administration of LPS[53]. These findings suggest that in humans, similar to rodents, the amygdala is involved in the integration of behavioral and immune responses. A recent study documented that LPS-induced anxiety in mice as well as a pronounced increase in sEPSC frequency resulting in the hyperexcitability of BLA neurons were mediated through the CXCL12/CXCR4 interaction[34]. This suggests that alterations of CXCL12 signaling in this area play critical roles in the development of anxiety induced by systemic inflammation, and that CXCR4 may be a potential therapeutic target for inflammation-induced anxiety, a key component of a sickness behavior. Several studies have indicated the relative abundance of CX3CL1 receptors within the amygdala complex, including the BLA and the CeA[56], suggesting a possible influence on amygdala function. Although CX3CL1 also modulate synaptic activity and has been shown to modulate glutamate and GABA release in the hippocampus[57] and dorsal raphe nucleus[58], its neuromodulatory role in other brain centers remains unknown. Surprisingly, so far there have been no neurophysiological studies of CX3CL1 effects in the amygdala.

2 Aim of the study

Considering described evidence and proposed hypotheses the present studies were focused on two separate but physiologically and functionally related subjects:

- Investigation of …
- Investigatation of …

To address these issues several research techniques were employed: whole-cell patch-clamp and extracellular recordings ex vivo, immunofluorescent and immunohistochemical staining and …

Detailed research objectives addressed in each study, as well as techniques used are listed below:

3 Materials and methods

3.1 General

3.1.1 Ethic approval

All procedures were conducted in accordance with the directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Procedures in immunostaining and patch-clamp experiments were additionally conducted in accordance with the Polish Act on the Protection of Animals Used for Scientific or Educational Purposes of 15 January 2015, and approved by the 2nd Local Ethics Commissions on Animal Research (Krakow, Poland). All efforts were made to minimize suffering and to reduce the number of animals used.

3.1.2 Animals

Male Wistar rats (50-180g on arrival) were purchased from Charles River Laboratories (Research Models and Services; Germany). Four to five animals were housed per polypropylene cage (55x35x20 cm) in controlled environment (22 ± 1°C, 45 ± 5 % relative humidity, 12:12 h light/dark cycle, lights on at 7:00 a.m.) with commercial food and fresh water *ad libitum*. Animals were acclimated to the housing environment for at least 4 days before the beginning of the experiments. Rats were weighing 180-300g during the tissue preparation / 5-10 weeks of age. Animals at this age are considered as young adults.

3.1.3 Reagents

3.2 Whole-cell patch-clamp technique in acute brain slices

3.2.1 Tissue Preparation

Male Wistar (4-7-week-old) rats were anesthetised with isoflurane (AErrane, Baxter, Poland) and decapitated between 07:00 and 09:00 a.m. Brains were collected in ice-cold, low-sodium, high-magnesium ACSF (artifical cerebrospinal–fluid), containing (in mM):

65 sucrose, 76 NaCl, 25 $NaHCO_3$, 1.4 NaH_2PO_4, 25 glucose, 2.5 KCl, 7 $MgCl_2$, 0.4 Na-ascorbate, and 2 Na-pyruvate (bubbled with 95% O_2/5% CO_2), pH 7.4; osmolality 290–300 mOsmol kg^{-1}) and cut into 300 µm thick coronal sections on a Leica VT 1000 vibrating microtome (Leica Instruments, Germany). After slicing, brain slices containing amygdala were transfered and held in a an incubation chamber filled with carbogenated ACSF, containing (in mM): 92 NaCl, 30 $NaHCO_3$, 2.5 KCl, 1.2 NaH_2PO_4, 2 $CaCl_2$, 20 HEPES, 2 $MgSO_4$ and 25 glucose, 5 Na-ascorbate, 2 thiourea, 3 Na-pyruvate, pH = 7.35; osmolality 290–300 mOsmol kg^{-1}). Slices were incubated at 31–33 °C for 20 min and then allowed to equilibrate for at least 1h at room temperature (RT). After this recovery period, individual slices were transferred to a recording chamber placed on a fixed stage of an Zeiss Axioskop 2 (Zeiss, Germany) upright microscope, where the tissue was perfused (1-2 ml/min) with carbogenated, warm (32°C) ACSF containing (in mM): 124 NaCl, 30 $NaHCO_3$, 2.5 KCl, 1.2 NaH_2PO_4, 2 $CaCl_2$, 5 HEPES, 2 $MgSO_4$ and 10 glucose, pH = 7.35; osmolality 290–300 mOsmol kg^{-1}).

3.2.2 Whole-cell patch-clamp recordings and data acquisition

Neurons were identified based on their location and somatic morphological characteristics under infrared differential interference contrast (DIC). The image from the microscope was enhanced using a CCD camera and displayed on a computer monitor. The border of basolateral or central amygdala were identified based on visual control by low magnification objective (2.5 x) and referenced to the Paxinos Atlas[59], corresponding to -1.92 mm -3.12 caudal to bregma 3.1. Neurons with a healthy appearance presented a smooth surface, and the cell body and parts of the dendrites could be clearly seen. Neurons were approached under visual control with a patch pipette using a three-dimensional micromanipulator (uMp micromanipulator system, Sensapex, Finland). Recording micropipettes were fabricated from borosilicate glass capillaries (3–6 MΩ; Sutter Instruments, USA) using horizontal puller (Sutter Instruments P–97) and filled with following solutions.

In experiment measuring excitatory synaptic transmission and excitability, the intrapippete solution contained (in mM): 130 potassium gluconate, 5 NaCl, 0.3 $CaCl_2$, 2 $MgCl_2$, 5 Na_2ATP, 0.4 Na_3GTP, 1 EGTA, 10 HEPES, pH 7.2, osmolality 290-300 mOsmol kg^{-1}) and biocytin (0.1%, for subsequent immunofluorescent identification of recorded neurons). In experiment measuring inhibitory synaptic transmission, the

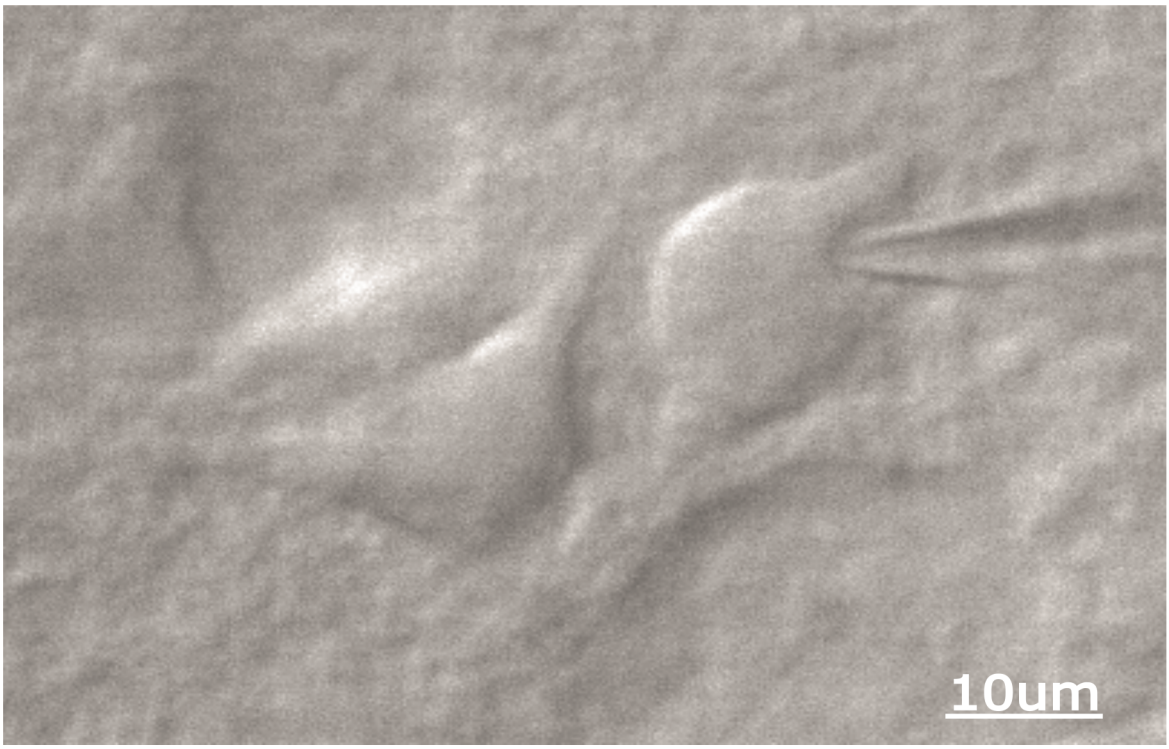

Figure 3.1: Figure caption

solution contained ….. The pipette was positively pressurized by applying a mouth suction to help prevent contamination of the tip and to blow aside surrounding tissue and lowered to the vicinity of the membrane keeping a positive pressure. After forming a high-resistance seal (> 1 GΩ, cell-atached configuration) between the recording pipette and cell membrane by applying negative pressure, a second pulse of negative pressure was used to break the patch of membrane enclosed with the pipette's tip and access inside of the neuron. An electrical contact between the cytoplasm and a patch electrode was established (whole-cell configuration). After break-in, cells stabilized for 5 min, during which intracellular solution slowly diffuses from the pipette into cytoplasm. Ground electrode, which was chloride-coated silver wire in chamber filled with bath solution, kept the electrical potential of the outer side of cell membrane at 0 mV. Electrical potential of the inner side of cell membrane was controlled through an patch electrode filled with intrapipette solution with chlorided silver wire connected to headstage () and amplifier (). Signals were low-pass filtered at 3 kHz, digitized at 20kHz and sampled through an analogue-to-digital converter (). Data were stored on a computer disk and analyzed offline using Clampfit 10.2 (Molecular Devices) software.

Voltages were corrected for the liquid-junction potential (estimated as ~12 mV). The access resistance was monitored throughout each experiment. Only recordings with stable access resistance lower than 30 MΩ were considered acceptable for analysis. All peptides and drugs were delivered via a bath perfusion system.

3.2.3 Electrophysiological identification and classification of chosen neurons

Whole-cell patch-clamp is capable of detecting transmembrane currents under voltage-clamp configuration, or measuring membrane voltages under current-clamp configuration. Once the whole-cell recording was obtained, cell characteristics were recorded using current-clamp techniques. The input-output relationship was obtained for each neuron using hyper- and depolarizing current pulses (500 ms). The frequency-current (F/I) relationship was determined by analyzing the number of action potentials evoked by current steps of increasing amplitude.

3.2.3.1 Electrophysiological identification of principal cells in the BLA

Principal neurons were identified based on their unique electrophysiological properties.

3.2.3.2 Electrophysiological identification of late-firing cells in the CeA

3.2.3.3 Electrophysiological identification of regular-firing cells in the CeA

3.2.4 Recording protocols and data analysis

The frequency-current (F/I) relationship was determined by analyzing the number of action potentials evoked by current steps of increasing amplitude and fitting a linear regression model. The gain (slope) and rheobase (minimal current necessary to fire the first action potential) parameters will be determined for further analysis.

3.2.5 Post-recording immunostaining

After recording slices were fixed overnight with a 4% formaldehyde at 4°C and stained according to following protocol in order to examine neurochemical content of recorded neurons. In brief, free-floating sections were blocked and permeabilised (10% normalised donkey serum (NDS), 0.1% Triton X100 in PBS) at 4°C overnight and incubated with a primary antibodies solution supplemented with an ExtrAvidin-Cy3 conjugate: mouse anti-OXT (1:1000 or 1:10000), rabbit anti-AVP (1:500 or 1:5000), ExtrAvidin-Cy3 (1:200), 2% NDS, 0.3% Tx100 in PBS, for 72 hours at 4°C. Finally, sections were incubated with a secondary antibody mixture for 24 h at 4°C (2% NDS in PBS solution containing donkey anti-mouse Alexa Fluor 647-conjugated antibody (1:400) and donkey anti-rabbit Cy3-conjugated antibody (1:400)). Each step was followed by a PBS wash. Stained sections were mounted and coverslipped with Vectashield, and imaged with an Axio Observer Z1 (Zeiss) confocal laser microscope or Imager.M2 (Zeiss) fluorescence microscope.

3.3 Extracellular recording and LTP

3.4 Immunohistochemistry

A subset of brains were stained for NeuN to visualize neuronal nuclei. After intracardial perfusion with 4% paraformaldehyde in PBS, brains were postfixed overnight and transferred to 30% sucrose in PBS. After the brains sank, sagittal slices (40 µ m) were prepared using a cryostat and mounted onto glass slides. Slices were washed in PBS (3 × 10 min each) and then blocked for 1 h (10% normal goat serum, 0.1% BSA, 0.05% Triton X-100 in PBS). Primary antibody (rabbit anti-NeuN, diluted 1:1,000; Millipore ABN78) was dissolved in carrier solution (1% normal goat serum, 0.1% BSA, 0.05% Triton X-100 in PBS) and applied overnight. Slices were then washed in PBS (3 × 10 min each) and incubated for 2 h in secondary antibody solution (Alexa Fluor-568 goat anti-rabbit; 1:500 dilution; Invitrogen A-11011). Sections were washed in PBS three more times before the slides were cover slipped with Vectashield with DAPI.

4 Results

5 Discussion

6 Conclusions

7 Appendix

7.1 PhD thesis founding

This PhD thesis and candidate were supported by:

- **National Science Centre Poland doctoral scholarship, ETIUDA**: Wpływ wybranych chemokin na właściwości komórek nerwowych i przekaźnictwo synaptyczne w obszarze kompleksu ciała migdałowatego szczura [Effects of chosen chemokines on neuronal properties and synaptic transmission in central and basolateral complex of the rat amygdala], UMO-2019/32/T/NZ4/00592 - grant holder 2019-2020
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7.2 PhD candidate's scientific activity

7.2.1 Concerning PhD thesis

7.2.1.1 Publications

7.2.1.2 Oral presentations

7.2.1.3 Posters

7.2.2 Other

7.2.2.1 Publications

7.2.2.2 Oral presentations

7.2.2.3 Posters

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