SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

INVESTIGATION OF THE ENDOCANNABINOID AND CHOLINERGIC SIGNALING ON THE ASTROCYTES AND NEURONS IN THE PEDUNCULOPONTINE NUCLEUS OF MICE

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The Examination takes place at the Library of Department of Physiology, Faculty of Medicine, University of Debrecen, at 10:30 am, 30th November, 2015.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1 pm, 30th November, 2015.

Introduction

The pedunculopontine nucleus (PPN) is takes place in the midbrain, in the pontomesenchephalicus tegmentum, and boarded by lateral lemniscus and superior cerebellar peduncle. PPN forms cholinergic and non-cholinergic synapses with thalamus, basal forebrain, basal ganglia and nuclei and auditory pathway. The PPN is the cholinergic part of the reticular activating system (RAS). This structure has increased activity in wakefullness and REM-seep and injury of this nucleus can lead to hallucinations.

Neurons of the PPN can be grouped by their functional characteristics (I-III). In group I, the neurons have low threshold calcium spikes and able to fire bursts. These neurons mainly are non-cholinergic cells. In group II, the neurons have low threshold calcium spikes and A-type potassium current. The majority of these neurons are cholinergic. Group III neurons possess A-type potassium current and less than half of them is cholinergic. The non-cholinergic cells have quiescent, irregular or regular firing groups ones and have different relationships with cortical activity. PPN have a lot of afferent and efferent projections.

PPN have a lot of afferent projections from nucleus subthalamicus, cerebellar cortex, globus pallidus, substantia nigra and spinal cord. Furthermore there are many efferent projections to the globus pallidus, cortex, thalamus, nucleus caudatus, spinal cord and brainstem.

There have numerous function of this region, for example coordination of the movement, waking-sleep cycle, sensory function. The cholinergic neurons can coexpression many transmitters like glutamate, GABA, nitric-oxide, neuropeptide, and substance-P. Cells of the PPN have several receptors like NMDA, M2, M3, M4, 5HT2, noradrenerg, and serotoninerg receptor. Cholinergic, endocannabinoid, serotonergic neuromodulatory actions, as well as orexin or ghrelin are known to act on the excitability of the nucleus.

Cannabinoids

Endocannabinoids have multiple effects on brain functions via modulating several signaling processes and synaptic connections. Neurons and astrocytes can activated with canabinoid and muscarinic agonists. The first receptor which was discovered, was the CB1 receptor, and this receptor can activated by exogenous and endogenous cannabinoids. The

mainly endogenous cannabinoids the anadamide and 2-AG (arachidonyl-glycerol). The endogenous cannabinoids activated other receptors too, for example CB2, GPR55, GPR119, TRPV1, 5-HT3, nAch, NMDA, opoid receptor. When the CB1 receptor activated, this receptor inhibite the adenylate-ciklase and these activation results blocks of the P/Q-, N- and L-type calcium channels. Among several other effects, CB1 receptor stimulation is able to modify the sleep–wake cycles. Early studies indicated that REM sleep duration and the eye movement activity are affected in marijuana smokers.

Presynaptic CB1 receptor

Several studies demonstrated the presynaptic localization of the CB1 receptor. The endocannabinoid signaling created by retrograde signaling, which have two type DSI (depolarization-induced suppression of inhibition) and DSE (depolarization-induced suppression of excitation). The CB1 receptor inhibite the voltage-dependent calcium channels and it's blocks the neurotransmitter release.

Furthermore severeal studies demonstrated, that CB1 receptors expressed on the astrocytes. When this receptor activated, form the astrocytes release Ca2+ and glutamate. Finally these glutamate can activate the NMDA receptor, and it's cause slow-inward current (SIC). Demonstrated that from the astrocytes can release endocannabinoids too, and it's can activate the surrounding neuron sor astrocytes.

Astrocytes

These cells are non-excitability because haven't got enough voltage-dependent ion channels than the neurons. Astrocytes membrane expressed too potassium and sodium channels, but can't be formed action potential. On the other hand the calcium concentration in the astrocytes can be change and it' s called „calcium excitability".

The Ca2+ signaling is different of the excitability and non-excitability cells. In the excitability cells the Ca2+ signal come from on the one hand voltage-dependent Ca2+ channels or on the other hand from the intracellular stores. In the non-excitability cells the calcium comes from intracellular stores. The calcium signal can blocked by thapsigargin, or blocked of the IP3 receptor.

These cells important in the synaptic neurotransmission, because they can connect with neurons and can created the „tripartite synapse". On the astrocytes expressed the

neurotransmitter receptors for many neurotransmitters for example glutamate, adenosine, norepinephrin, GABA, histamine, and acetylcholine.

Objectives

- Examination of the effects of CB1 receptor and muscarinic acetylcholine receptor agonists on the membrane potential and action potential firing frequency with patch clamp technique.
- The pharmacological characterization of the CB1 receptor stimulation on neurons, and mapping of the other receptors.
- Examination of the correlation between neuronal subpopulations and CB1 receptor stimulation effect.
- Investigation of the activation of astrocytes by cannabinoid stimulation, and testing the possible neuron-astrocyte interactions in the endocannabinoid signaling.
- Determination of the localization of the CB1 receptor by immunohistochemistry.
- Comparison of the effects of cannabinoid and cholinergic stimulation on the same PPN neurons.

Materials and methods

Solutions and chemicals

Experiments were performed in an artificial cerebrospinal fluid (aCSF). For the slice preparation, a modified aCSF (low Na aCSF) was used where 95 mM NaCl was replaced by sucrose and glycerol.

Animals, preparation

For our experiments was used 8- to 15-day-old C3H mice as well as mice-expressing tdTomato fluorescent proteins in a GAD2- or ChAT-dependent way. The floxed-stop-tdTomato, GAD2-cre and ChAT-cre mouse lines were purchased from Jackson Laboratories. In other experiments CB1 receptor knockout and wild-type littermates were also used. After decapitation of the animal and removal of the brain, 200 μm-thick coronal midbrain slices were prepared in ice-cold low Na aCSF and for the slicing was used a vibratome. The slices were incubated in normal aCSF for 60 min on 37^{o}C.

Electrophysiology

Brain slices were visualized with a Zeiss Axioskop microscope. Patch pipettes had 5 MΩ pipette resistance, and was filled with a K-gluconate pipette solution. We did whole-cell patch-clamp recordings and were using an Axopatch 200A amplifier. All recordings were performed at room temperature. For the data acquisition was achieved using the Clampex 10.0 software, while data analysis was performed using the Clampfit 10.0 or MiniAnalysis programs.

Membrane potential of the neurons was measured with current-clamp mode of the patch clamp technique. For comparing the membrane potentials, two 120-s-long trace segments were selected. Histograms of the membrane potential values were made from these periods, and the value corresponding to the largest bin was considered as resting membrane potential. In those experiments where membrane potential changes were monitored continuously to demonstrate the time course of the drug effect, the above-mentioned procedure was performed at every 30 s of the trace.

Pharmacology

We used for our experiments CB1 receptor agonists (ACEA and WIN55,212-2). The ACEA was administered at a concentration of 5 μM, whereas WIN55,212-2 was used in 1 μM. The muscarinic agonist carbamylcholine chloride (carbachol) was used in 50 μM concentration. The anandamide membrane transport inhibitor, UCM707, was applied in 10 μM concentration. For other experiments, slices were perfused with 10 μM NBQX, 50 μM DAP5, 1 μM strychnine and 10 μM bicuculline, which can blocked the ionotropic glutamatergic, glycinergic and GABAergic neurotransmissions. In further experiments, 100 μM CPCCOEt and 10 μM MPEP were used to block group I metabotropic glutamate receptors (mGluR I); while 10 μM LY341495 was used to block group II mGluRs (mGluR II). Beside of these for the spontaneous action potential firing we used 1 μM tetrodotoxin (TTX).

Calcium imaging

For the measure of the intracelullar calcium concentration we used calcium imaging. We preincubated the slices with the Oregon Green 488 BAPTA 1 (OGB) fluorescent dye. The slices was put on the cell culture dishes which contain nACSF. The slices were visualized with a Zeiss Axioskop microscope. The microscope was equipped with a fluorescent imaging system containing a Polychrome V light source, a CCD camera, an imaging control unit (ICU), and the Till Vision software. The excitation wavelength was set to 488 nm. The fluorescent filter set was composed of a dichroic mirror and an emission filter. Throughout the calcium imaging, frames with 344 x 260 pixel resolution were employed with a frame rate of 10 Hz.

In parallel with these experiments, we used patch-clamp experiments too. In order to confirm glial origin of the slow calcium waves, whole-cell patch-clamp recordings were performed on cells displaying slow calcium waves. Cells with slow calcium waves showed characteristic morphological and functional signs of astrocytes when we loaded with Alexa Fluor 594 dye. Finally we used loose-patch recordings on cells and it' s demonstrated the passive membrane properties.

Visualization of the labeled neurons

The neurons were filled with biocytin during the electrophysiological recordings. The slices accommodating the filled neurons were fixed overnight. Permeabilization was achieved in Tris-buffered saline supplemented with 0.1 % Triton X-100 and 10 % bovine serum. The slices were incubated in phosphate buffer-containing streptavidin-conjugated Alexa488 for 90 min. In samples without tdTomato expression, post hoc ChAT–immunohistochemistry was performed following the biocytin recovery. The cells were visualized using a Zeiss LSM 510 confocal microscope.

Immunohistochemistry

For these experiments were used mice-expressing tdTomato in ChAT-dependent manner and additionally on wild-type C3H mice. Animals were deeply anesthetized with sodium pentobarbital and transcardially perfused with Tyrode's solution, followed by perfusion with fixative containing 4 % paraformaldehyde dissolved in 0.1-M phosphate buffer. After the transcardial fixation, the mesencephalon was removed, postfixed in the original fixative for 4 h, and immersed into 10 and 20 % sucrose dissolved in 0.1-M PB until they sank. In order to enhance reagent penetration, the removed mesencephalons were freeze-thawed. Fifty-micrometer-thick transverse sections were cut on a vibratome, and the sections were extensively washed in 0.1-M PB. We used single immunostaining protocol was performed to study the cellular distribution of CB1-R in the PPN. Freefloating sections were first incubated in rabbit anti-CB1 receptor antibody for 48 h at 4^{o}C, and then were transferred into anti-rabbit IgG conjugated with Alexa Fluor 647. Before the antibody treatments, the sections were kept in 10 % normal goat serum for 50 min. Antibodies were diluted in 10-mM TPBS containing 1 % normal goat serum. Sections were mounted on glass slides and covered with Vectashield. Beside of this we used double immunostaining protocols were performed to study the co-localization of CB1-R immunoreactivity with glial fibrillary acidic protein (GFAP), a marker of astrocytes. Free-floating sections were first incubated with a mixture of antibodies that contained rabbit anti-CB1 receptor, mouse anti-GFAP. The sections were incubated in the primary antibody solutions for 2 days at 4^{o}C and were transferred for an overnight treatment into the appropriate mixtures of secondary antibodies that contained goat anti-rabbit IgG conjugated with Alexa Fluor 647 and goat anti-mouse IgG conjugated with Alexa Fluor 488. Before the antibody treatments, the sections were kept in 10 % normal goat

serum for 50 min. Antibodies were diluted in PBS containing 1 % normal goat serum. Sections were mounted on glass slides and covered with Vectashield.

Series of 1-µm-thick optical sections with 500 nm separation in the z-axis were scanned with an Olympus FV1000 confocal microscope equipped with 609 PlanApo N oil-immersion objective and FV10-ASW software. The specificity of the primary antibody against CB1receptor has extensively been characterized previously, using knockout control.

To test the specificity of the immunostaining protocol, free-floating sections were incubated according to the immunostaining protocol described above with primary antibodies omitted or replaced with 1 % normal goat serum. For quantitative analysis of co-localization of CB1 receptor- and GFAP-immunopositivity, free-floating sections were first incubated with a mixture of antibodies that contained rabbit anti-CB1 receptor, goat anti- ChAT and mouse anti-GFAP.

The co-localization of CB1 receptor immunoreactive puncta with astrocytic (GFAP-IR) profiles was quantitatively analyzed in the multiple stained sections. CB1 receptor immunoreactive profiles over the edges of the standard grid were selected and examined whether they were also immunoreactive for the axonal or glial markers.

Statistical analysis

All data are represented as mean ± SEM. To compare the central tendency (mean or median) of two independent datasets, either Student's t test or the Mann–Whitney U test was used. For comparisons involving multiple datasets, either ANOVA or Kruskal–Wallis ANOVA on Ranks was performed, followed by an appropriate post hoc test to test for pairwise differences. For the comparison of before and after values, either the paired form of Student's t test or the signed rank test was used. Parametric and non-parametric tests were used as appropriate for the given dataset. For all tests, p values below 0.05 were accepted as significant. Calculations were done using the statistical module SigmaPlot 12 and GraphPad Prism 6.

Results

CB1 receptor activation evokes heterogeneous response in PPN neurons

For the observation of the CB1 receptor we used whole-cell patch-clamp recordings and we can demonstrated the membrane potential and the firing pattern of the PPN neurons. The location of the cells was confirmed, and their morphological characteristics were revealed with biocytin labeling. In some cases, neurons were identified by tdTomato fluorescent protein expressed in a ChAT- or GAD65-dependent way. Cells responded to 5 μM ACEA in different ways: some depolarized and increased action potential firing frequency; others hyperpolarized, reduced firing frequency or stopped completely. The remaining cells showed minimal or no response. PPN neurons from CB1 receptor-deficient mice also showed significantly smaller membrane potential alterations compared to wild type animals.

We used another cannabinoid receptor agonist, WIN55,212-2 and the anandamide membrane transport inhibitor UCM707 and it's resulted a similar effect. There was no significant differences between the effects of the three applied CB1 receptor agonists on the pattern of resting membrane potential or firing frequency changes.

In order to compare changes elicited by ACEA on different neuronal types, tdTomato-expressing cholinergic and GABAergic neurons were patched. Both cell groups exhibited all three types of responses to ACEA application. However, the proportions of the responses were different. Cholinergic cells were depolarized, hyperpolarized or lacked response in similar proportions, whereas the majority of the GABAergic neurons were depolarized.

Preincubation with TTX or inhibition of fast synaptic neurotransmission by a cocktail composed of NBQX, D-AP5, strychnine and bicuculline did not change the effects of ACEA on resting membrane potential significantly. The blocking cocktail, however, completely abolished spontaneous and evoked postsynaptic currents.

In the next experiments were preincubated with group I or II mGluR antagonist. Intristingly, after preincubation with group I mGluR blockers neurons were either depolarized or uneffected by ACEA, and the hyperpolarization was almost fully abolished. In contrast to mGluRI blockers when we used mGluR II blockers resulted minimal depolarization but hyperpolarization was dominated. And when we used together the fast synaptic neurotransmission blockers and the mGluR blockers application of ACEA in no apparent change of the resting membrane potential or firing frequency in the recording neurons.

Activation of CB1 receptors alters astrocyte calcium wave frequency

In the following experiments the neuronal and glial activity was monitored simultaneously. When we blocked of the fast synaptic transmission and action potential firing it's did not abolish the cannabinoid-induced neuronal membrane potential changes; we tested whether activation of astrocytes via CB1 receptors may be influencing the neuronal activity. Slices were loaded with OGB calcium sensitive dye, and changes of the fluorescence intensity were detected. Which cells had slow calcium waves, was loaded with Alexa 594 fluorescent dye, and these cells had characteristic morphological and functional signs. In addition, the slow calcium waves were detectable in the presence of TTX but disappeared when applying thapsigargin.

In the next step was that a neuron was patched in the OGB-loaded slice in current clamp configuration, and calcium wave changes were detected. Patched neuron respomded to ACEA in different ways, and slightly before or parallel with these changes the astrocytes produced slow calcium waves.

Astrocytic CB1 receptor activation is responsible for neuronal membrane potential changes upon ACEA application

In the following experiments in order to prove that CB1 receptors are located on astrocytes, we performed immunohistochemistry. Co-localization of CB1 receptor and glial fibrillary acidic protein (GFAP), a marker for astrocytes was also demonstrated. In order to prove that CB1 agonists were activating astrocytes directly through these CB1 receptors, we repeated the combined Ca-imaging and patch-clamp experiments on CB1 knockout mice. Compared to wild type animals, loaded astrocytes showed spontaneous calcium waves at a significantly lower initial frequency. In the next step when ACEA was applied, the frequency did not increase significantly.

On the other hand astrocytic activation might also occur via indirect mechanisms and after activated by altered neuronal activity. For this test we used tetrodotoxin (TTX) and it could suspend the neuronal action potential firing in slices. Furthermore in the presence of TTX, the calcium wave frequency was significantly lower than in control conditions. In turn, when we used ACEA, the calcium wave frequency was significantly increased.

Next, we wanted to test that silencing of astrocytes with thapsigargin can prevent neuronal membrane potential changes. We set the intracellular calcium concentration with the

pipette solution. After preincubation with thapsigargin, there wasn't astrocytic slow calcium waves and beside of this the neuronal responses to ACEA were largely diminished.

Effects of of the cholinergic and endocannabinoid agonists is very similar on the PPN neurons

For these experiments we used whole-cell patch-clamp recordings and we observation the membrane potential and the firing pattern of the PPN neurons. Cells responded to 50 μM carbachol in different ways: 45% were depolarized with an increase in action potential firing frequency and 32% were hyperpolarized and decreased firing frequency or completely stopped activity. The rest of the cells 23% did not respond to carbachol.

Because of these overlaps between responses elicited by ACEA and carbachol, we next investigated together these agonists. With whole-cell patch-clamp experiments were demonstrated, where carbachol was first applied and, after washed out the carbachol from the solution, and finally 5 μM ACEA, was applied. In seven cases, carbachol caused depolarization. On these neurons, application of ACEA resulted in depolarization as well. In four cases, carbachol did not result in any change; on these cells, and after ACEA application was ineffective in the same manner. Five neurons were hyperpolarized by carbachol and on these cells, application of ACEA showing a significant difference from the effect of carbachol: one of these cells was depolarized, one was hyperpolarized, and three did not respond to ACEA. Changes in the firing frequency showed similar tendencies.

Discussion

Activation of astrocytic CB1 receptors leads to modulation of neuronal activity in the PPN

Astrocytes are able to modulate neuronal functions. They express a lot of functional neurotransmitter receptors through which they can be activated in a specific way and release several gliotransmitters for example glutamate, ATP, adenosine, TNFα or peptides. The best known investigated gliotransmitter, the glutamate, which can released from astrocytes in multiple ways: calcium-dependent exocytosis, reversal of uptake by plasma membrane glutamate transporters, cystine–glutamate antiport, functional unpaired connexon/pannexon ‘‘hemichannels’’, ionotropic purinergic receptors, organic anionic transporters and cell swelling-induced anion channel opening were also reported.

The CB2 receptor on astrocytes is also demonstrated, but the contribution of this receptor to the effects observed by us is not likely for two reasons. On the other hand the ACEA is highly selective to CB1 receptor, and the effects of ACEA and WIN55,212-2 were absent from the CB1 receptor knockout mice. In these regions, the increased neuronal activity leads to endocannabinoid release and these endocannabinoids activates the astrocytes, and from these cells releasing the glutamate. Glutamate can either potentiate glutamatergic synapses or initiate long-term depression in NMDA receptor or presynaptic mGluR-dependent ways. We demonstrated that activation of neurons can activate the astrocytes, via CB1 receptor and from the astrocytes release glutamate, which acts on metabotropic glutamate receptors.

The role of mGluRs in glia–neuron signaling

For this experiments we used mGluR I and II blockers and the hyperpolarization is due to the activation of postsynaptic mGluR I, whereas depolarization depends on mGluR II. In this study demonstrated the presence and function of mGluR I and II and activation of astrocytes leading to stimulation of presynaptic mGluRs, and help to the long-term potentiation. This study is the first that indicates indirect stimulation of different classes of postsynaptic mGluRs by activation of astrocytic CB1 receptors in the midbrain. In the central nervous system there are many metabotropic glutamate receptors; and it’s located on both pre-

and postsynaptic locations. Astrocytes possess both group I and II mGluRs, and it' s help the glutamate release from the astrocytes. The glutamate and the group I and II mGluR agonists elicited calcium signals from the astrocytes, and it's inducing ''glutamate-induced glutamate release''. Several studies demonstrated that activation of postsynaptic mGluR I and II leads to changes of the neuronal membrane potential.

Activation of postsynaptic mGluR I causes depolarization and mGluR II stimulation leads to hyperpolarization. The mGluR I activation depolarized neurons via activation of L-type calcium channel or a non-selective cationic conductance. In contrast to mGluR I receptor when activate the mGluR II receptor it's either suppresses L-type calcium channel or activates potassium channels, thus hyperpolarizing the neurons expressing them.

The endocannabinoid and muscarinic effects is overlap

We demonstrated that PPN neurons depolarized by the muscarinic agonist carbachol are also depolarized by ACEA. Furthermore which neurons lack response to muscarinic stimulation also did not respond to ACEA. In contrast to these observed, neurons which hyperpolarized by carbachol respond to ACEA in a heterogenous way: they can be depolarized, hyperpolarized or lack response.

Demonstrated that activation of muscarinic receptors can stimulate endocannabinoid synthesis. Activation of the M1 receptor increases endocannabinoid synthesis in the neurons and, in turn, this endocannabinoid acts on astrocytes CB1 receptors. Neurons lacking response to carbachol did not respond to ACEA as well. It was shown that non-responding cells remain silent because of the increased endocannabinoid tone surrounding them. Finally the hyperpolarization by carbachol does not completely overlap with the hyperpolarization by ACEA because these cells hyperpolarized by carbachol respond in a heterogeneous manner to ACEA. The neurons hyperpolarized by carbachol have M2 or M4 receptors, and activation of these receptors might overwrite the effect of endocannabinoids synthesized in an M1-dependent manner. So the neurons expressing different muscarinic receptors and mGluRs, which can also explain our findings.

In order to summarize our finding we propose the following model. A neuron activated by excitatory input or having intrinsically high action potential firing frequency releases endocannabinoids.

The endocannabinoids activate the surrounding astrocytes via CB1 receptor. Finally from the astrocytes release glutamate which acts on either group I or group II mGluR and cause depolarization or hyperpolarization of the neighbouring neurons.

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Subject: Ph.D. List of Publications

Candidate: Adrienn Kovács
Neptun ID: OS6FE9
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Kovács, A.**, Bordás, C., Pál, B.: Cholinergic and endocannabinoid neuromodulatory effects overlap on neurons of the pedunculopontine nucleus of mice.
Neuroreport. 26 (5), 273-278, 2015.
DOI: http://dx.doi.org/doi: 10.1097/WNR.0000000000000342
IF:1.52 (2014)

2. Kőszeghy, Á., **Kovács, A.**, Bíró, T., Szűcs, P., Vincze, J., Hegyi, Z., Antal, M., Pál, B.: Endocannabinoid signaling modulates neurons of the pedunculopontine nucleus (PPN) via astrocytes..
Brain. Struct. Funct. 220 (5), 3023-3041, 2015.
DOI: http://dx.doi.org/DOI 10.1007/s00429-014-0842-5
IF:5.618 (2014)

List of other publications

3. Bordás, C., **Kovács, A.**, Pál, B.: The M-current contributes to high threshold membrane potential oscillations in a cell type-specific way in the pedunculopontine nucleus of mice.
Front. Cell. Neurosci. ,9 Article ID 121, 2015.
DOI: http://dx.doi.org/10.3389/fncel.2015.00121
IF:4.289 (2014)

Total IF of journals (all publications): 11,427
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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