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Direct presynaptic and indirect astrocyte-mediated mechanisms both contribute to endocannabinoid signaling in the pedunculopontine nucleus of mice

Running title: Astrocyte-mediated endocannabinoid signaling in the PPN

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Abstract

The pedunculopontine nucleus (PPN), a cholinergic nucleus of the reticular activating system, is known to be involved in the regulation of sleep and wakefulness. Endogenous and exogenous cannabinoids, either by systemic or local administration to the pedunculopontine nucleus can both influence sleep. We previously demonstrated that activation of astrocytes by cannabinoid type 1 (CB1) receptor agonists was able to modulate the membrane potential of PPN neurons, even in the presence of blockers of fast synaptic neurotransmission. In the present work we provide evidence that synaptic inputs of PPN neurons are also affected by activation of presynaptic and astrocytic CB1 receptors.

Using slice electrophysiology combined with calcium imaging, optogenetics and immunohistochemistry, we revealed a direct presynaptic inhibitory action on inhibitory postsynaptic currents, along with a mild increase of excitatory postsynaptic currents during CB1 receptor stimulation. Besides inhibition of excitatory and inhibitory neurotransmission through stimulation of presynaptic CB1 receptors, astrocyte- and mGluR-dependent tonic inhibition and excitation also developed. The mild stimulatory action of CB1 receptor activation on excitatory neurotransmission is the combination of astrocyte-dependent tonic excitation on excitatory neurons and the canonical presynaptic CB1 receptor activation and consequential inhibition of excitatory synaptic neurotransmission, whereas the astrocyte-dependent stimulatory action was not observed on inhibitory neurotransmission within the PPN.

Our findings demonstrate that endocannabinoids act in the PPN via a dual pathway, consisting of a direct presynaptic and an indirect, astrocyte-mediated component, regulating synaptic strength and neuronal activity via independent mechanisms.

Introduction

The pedunculopontine nucleus is known as one of the mesencephalic cholinergic nuclei, which provides cholinergic fibers for several subcortical locations (i.e. the thalamus, basal ganglia or pontine structures). Besides its contribution to motor regulation, it modulates transitions between sleep and wakefulness and between slow wave- and REM-sleep (Garcia-Rill 1991; Garcia-Rill et al, 2011; Reese et al, 1995; Petzold et al, 2015).

Endocannabinoids, besides their generally recognized actions, are also sleep-promoting substances. Not only the sleep disturbances of marijuana smokers has been known for a long time (e.g. Feinberg et al, 1975), but physiological roles of endocannabinoids in sleep have also been demonstrated. As a part of the homeostatic drive of sleep ("process S"), increased anandamide and oleamide concentrations were detected in the liquor in case of sleep deprivation (Guan et al, 1997; Herrera-Solís et al, 2010). Furthermore, direct injection of anandamide to the PPN induced sleep and prolonged the duration of both REM- and slow wave sleeps (Murillo-Rodriguez, 2008; Murillo-Rodriguez et al, 2008).

In our previous study we demonstrated that CB1 receptor agonists hyperpolarized or depolarized both cholinergic and non-cholinergic neurons of the PPN. Membrane potential changes of the neurons were preceded by a frequency increase of calcium waves in neighboring astrocytes, suggesting that the excitability of the PPN neurons is regulated by endocannabinoids, at least partly, in a non-neuronal way; possibly via changes of the ambient glutamate level by astrocytes (Kőszeghy et al, 2015).

However, it is widely accepted that endocannabinoids exert their neuronal effects via presynaptic CB1 receptors and inhibit both excitatory and inhibitory neurotransmissions (e.g. see Lovinger, 2008; Kano, 2014; Diana et al, 2002; Hoffman and Lupica, 2000; Diana and Marty, 2004; Katona and Freund, 2012). Depolarization of the postsynaptic neuron leads to endocannabinoid release and these molecules inhibit excitatory and inhibitory inputs (depolarization-induced suppression of excitation and inhibition, DSE, DSI;

respectively; Llano et al, 1991; Vincent et al, 1992; Kreitzer and Regehr, 2001; Ohno-Shosaku et al, 2002). Presynaptic actions of endocannabinoids were recently demonstrated in the laterodorsal tegmental nucleus (LDT), another cholinergic nucleus neighboring the PPN, where both postsynaptic depolarization and CB1 receptor activation by agonists decreased the frequency of spontaneous and miniature inhibitory postsynaptic currents (sIPSCs; mIPSCs; Soni et al, 2014; Soni and Kohlmeier, 2015).

The effect of endocannabinoids on excitatory neurotransmission, however, seems to be far more complex. In the hippocampus, endocannabinoids released from neurons do not only activate presynaptic CB1 receptors causing presynaptic inhibition but also stimulate neighboring astrocytes possessing CB1 receptor. Activation of this receptor, in turn, leads to generation of calcium waves on astrocytes and consequential glutamate release, resulting in activation of presynaptic mGluRs and potentiation of certain excitatory synapses (Navarrete and Araque, 2010).

In the present paper we show that, simultaneously with the previously reported tonic excitation and inhibition (Kőszeghy et al., 2015), spontaneous and miniature EPSCs and IPSCs also displayed characteristic changes in PPN neurons upon stimulation of CB1 receptors. CB1 receptor agonists slightly increased the frequency of the spontaneous excitatory postsynaptic currents (sEPSCs), while reduced the frequency of sIPSCs. When EPSCs were elicited by stimulation of presynaptic excitatory fibers, depolarization of the postsynaptic neuron or application of CB1 receptor agonists exerted an inhibitory effect on the neurotransmission in the majority of the cases. Optogenetic stimulation of astrocytes did not exert any presynaptic effect, but elicited, as anticipated, tonic currents on neurons. Our findings indicate that endocannabinoids exert dual action in the PPN, including a direct presynaptic inhibition of both excitatory and inhibitory inputs, and an indirect, astrocyte-dependent modulation of neuronal membrane potential and excitability.

Materials and methods

Solutions, chemicals

All experiments were performed in an artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl, 120; KCl, 2.5; $NaHCO_3$, 26; glucose, 10; NaH_2PO_4, 1.25; $CaCl_2$, 2; $MgCl_2$, 1; myo-inositol, 3; ascorbic acid, 0.5; and sodium-pyruvate, 2; pH 7.2. A different aCSF (low Na aCSF) was used for preparation, where 95 mM NaCl was replaced by sucrose (130 mM) and glycerol (60 mM). All chemicals were purchased from Sigma (St. Louis, MO, USA), unless stated otherwise.

Animals, preparation

Animal experiments were conducted in accordance with the appropriate international and Hungarian laws and institutional guidelines on the care of research animals. The experimental protocols were approved by the Committee of Animal Research of the University of Debrecen. 8-15 days old wild type C3H (n = 16) and Bl6 (n = 7) mice as well as mice expressing channelrhodopsin-2 (ChR2) in a glial fibrillary acidic protein (GFAP)-dependent way, from both sexes (n = 15), were used for the functional experiments. The homozygous floxed-stop-channelrhodopsin-2 (B6;129S-*Gt(ROSA)26Sor*$^{tm32.1(CAG-COP4*H134R/EYFP)Hze}$/J) and GFAP-cre (B6.Cg-Tg(Gfap-cre)73.12Mvs/J) lines were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were crossed in the animal house of the Department of Physiology. No obvious differences in endocannabinoid actions were found between different mouse strains (Kőszeghy et al, 2015). In some experiments, CB1 receptor knockout mice (n = 9; a kind gift from A. Zimmer - Bonn, Germany) were also used (Zimmer et al., 1999).

After decapitation of the animal and removal of the brain, 200 μm-thick coronal midbrain slices were prepared in ice-cold low Na aCSF using a Microm HM 650V vibratome (Microm International GmbH, Walldorf, Germany). The slices were incubated in normal aCSF for 60 minutes on 37°C prior to recording.

Electrophysiology

Coronal midbrain slices were visualized with a Zeiss Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany). Patch pipettes with 5 MΩ pipette resistance were pulled, and filled with a solution containing (in mM): K-gluconate, 120; NaCl, 5; 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES), 10; EGTA, 2; $CaCl_2$, 0.1; Mg-ATP, 5; Na3-GTP, 0.3; Na2- phosphocreatinine, 10; biocytin, 8; pH 7.3. In experiments when sIPSCs and mIPSCs were investigated, K-gluconate was replaced with equimolar KCl. Whole-cell patch-clamp recordings were performed at room temperature using an Axopatch 200A amplifier (Molecular Devices, Union City, CA, USA). Data acquisition was achieved by Clampex 10.0 software (Molecular Devices, Union City, CA, USA), while data analysis was performed using Clampfit 10.0 (Molecular Devices) and MiniAnalysis (Synaptosoft, Decatur, GA, USA) softwares.

For recording tonic currents, spontaneous and miniature EPSCs and IPSCs, voltage-clamp traces were recorded at a holding potential of -60 mV before and after drug application or optogenetic manipulation (5 minutes in each condition). Changes of the tonic currents were assessed by constructing histograms of the recorded current values from the last minute of the given trace. The tonic current elicited by different manipulations was defined as the difference of the current values belonging to the histogram peaks from the traces corresponding to after and before conditions. To assess the spontaneous changes of the holding current 10 minutes long recordings were performed under control conditions, which was analyzed with the same protocols described above.

In some experiments, the presynaptic fibers innervating the investigated neuron were stimulated by a monopolar electrode, inserted 50-100 μm away from the soma. The stimulation electrode was connected to a BioStim STC-7a stimulator device (Supertech, Pécs, Hungary). After finding the ideal position of the stimulatory electrode, the amplitude of the stimuli was set to a value (approximately 1.5 times above minimal stimulation) where failures were almost fully absent. The paired stimuli were delivered with a frequency of 50

Hz; with 10 s delay between the consecutive trials. Postsynaptic depolarization of the neurons was achieved by setting a holding potential of 0 mV for 10 s.

Optogenetic stimulation of samples taken from GFAP-ChR2 mice was achieved by epifluorescent stimulation (480 ± 5 nm) of the whole visual field using a xenon bulb-based Polychrome V light source (Till Photonics GmbH, Gräfeling, Germany) and the Till Vision software (version 4.0.1.3). The stimulation protocol was either a 1 minute long continuous illumination or pulsatile stimulation (10 Hz) with 10 ms-long illumination periods.

Pharmacology

The CB1 receptor agonist, WIN55,212-2, was administered at a concentration of 1 µM, whereas arachidonyl–2'–chloroethylamide (ACEA) was used in 5 µM. In certain experiments, slices were continuously perfused with 10 µM 2,3–dihydroxy–6–nitro–7–sulfamoyl-benzo[f]quinoxaline–2,3–dione (NBQX), 50 µM D–2–amino–5–phosphonopentanoate (D-AP5), 1 µM strychnine, and 10 µM bicuculline (Tocris Cookson Ltd., Bristol, UK) in order to block ionotropic glutamatergic, glycinergic and GABAergic neurotransmissions. Spontaneous action potential firing was blocked by 1 µM tetrodotoxin (TTX). In some experiments, slices were incubated with 1 µM thapsigargin for 45 minutes to suppress astrocytic activity (Kőszeghy et al, 2015). 100 µM 7– (Hydroxyimino)cyclopropa[*b*]chrome–n–1a–carboxylate ethyl ester (CPCCOEt) and 10 µM 2–Methyl–6– (phenylethynyl)pyridine hydrochloride (MPEP) were used to block type 1 and 5 metabotropic glutamate receptors (group I mGluRs), respectively; while 10 µM LY341495 was administered to block group II mGluRs (Tocris Cookson Ltd., Bristol, UK).

Calcium imaging

In order to confirm astrocyte activation by optogenetic stimulation in GFAP-ChR2 mice, calcium imaging experiments were performed. Slices were preincubated for 45 minutes with either 33 µM Oregon

Green BAPTA I AM (OGB) or 20 μM Rhod-2 AM (Invitrogen-Molecular Probes, Carlsbad, CA, USA; courtesy of Dr. Péter Szentesi; University of Debrecen, Hungary). A Zeiss Axioskop microscope (Carl Zeiss AG). was equipped with a fluorescent imaging system (Till Photonics GmbH, Gräfeling, Germany) containing a xenon bulb-based Polychrome V light source, a CCD camera (SensiCam, PCO AG, Kelheim, Germany), an imaging control unit (ICU), and the Till Vision software (version 4.0.1.3). The excitation wavelength for the OGB experiments was set to 480 nm. The fluorescent filter set was composed of a dichroic mirror (Omega XF2031 505DRLPXR; Omega Drive, Brattleboro, VT, USA) and an emission filter (LP 515, Till Photonics). Frames with 344 x 260 pixel resolution were employed with a frame rate of 10 Hz. As the excitation wavelength of OGB also stimulates ChR2 opening, data obtained from GFAP-ChR2 mice were compared to data recorded from wild type animals.

Rhod-2 imaging was performed as described above, except that excitation wavelength was set to 550 nm and a different filter set was used (dichroic mirror: Omega XF2019 590DRLP, emission filter: Omega 3RD590LP; Omega Drive, Brattleboro, VT, USA). In these experiments, optogenetic stimulation of ChR2 was achieved by an external LED light source (470 nm; Thorlabs, Newton, NJ, USA).

Calcium signals were recorded from the somata of the cells loaded with calcium indicators. Areas of calcium waves were calculated by the numerical integration of data points from recordings of the same duration.

Visualization of the labeled neurons

Recorded neurons were filled with biocytin and slices containing the filled neurons were fixed overnight (4% paraformaldehyde in 0.1M phosphate buffer; *pH* 7.4; 4 °C) for post hoc identification of the cells. Permeabilization was achieved in Tris buffered saline (in mM, Tris base, 8; Trisma HCl, 42; NaCl, 150; *pH* 7.4) supplemented with 0.1% Triton X-100 and 10% bovine serum (60 min). Incubation was performed in

phosphate buffer containing streptavidin-conjugated Alexa488 (1:300; Molecular Probes Inc., Eugene, OR, USA) for 90 min. The cells were visualized using a Zeiss LSM 510 confocal microscope (Carl Zeiss AG).

Immunohistochemistry

Experiments were carried out on 3 wild type Bl6 mice. Animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with Tyrode's solution (oxygenated with a mixture of 95% O_2, 5% CO_2), followed by a fixative containing 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB, pH 7.4). After the transcardial fixation, the mesencephalon was removed, postfixed in the original fixative for 4 hours, 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.

Free-floating sections were first incubated with a mixture of antibodies that contained rabbit anti-CB1-R (1:2000, Cayman Chemical), goat anti-ChAT (1:500, Millipore) and one of the following antibodies: guinea pig anti-VGLUT2 (1:2000, Millipore), guinea pig anti-VGAT (1:500, Synaptic Systems) or mouse anti-GFAP (1:1000, Millipore). The sections were first incubated in the primary antibody solutions for 2 days at 4 °C, followed by a treatment with an appropriate mixtures of secondary antibodies that were selected from the following: donkey anti-rabbit IgG conjugated with Alexa Fluor 555 (1:1000, Invitrogen), donkey anti-goat IgG conjugated with Alexa Fluor 488 (1:1000, Invitrogen), donkey anti-guinea pig IgG conjugated with Alexa Fluor 647 (1:1000, Invitrogen) and donkey anti-mouse IgG conjugated with Alexa Fluor 647 (1:1000, Invitrogen). The co-localization of CB1 receptor immunoreactive puncta with glutamatergic (VGLUT2-IR) or GABAergic (VGAT-IR) axon terminals, as well as with astrocytic (GFAP-IR) profiles was quantitatively analyzed in the multiple stained sections. Antibodies were diluted in PBS (pH 7.4) containing 1% normal goat serum (Vector Labs., Burlingame, CA). Sections were mounted on glass slides and covered with Vectashield (Vector Labs, Burlingame, CA).

The specificity of the primary antibody against CB1 receptor (Cayman Chemical, Ann Arbor, MI) has extensively been characterized previously (Hegyi et al, 2009). 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. No immunostaining was observed in these sections.

Series of 1 µm thick optical sections with 500 nm separation in the z-axis were scanned with an Olympus FV1000 confocal microscope. Scanning was carried out using a 60x PlanApo N oil-immersion objective (NA: 1.42) and FV10-ASW software (Olympus). The scanned images were processed with Adobe Photoshop CS6 software. Confocal settings including confocal aperture, laser power, gain, offset and pixel size were identical for all scans. By filtering the background staining out with a high-pass intensity filter, threshold values were set for both CB1-R and the other markers.

A 10x10 standard square grid in which the edge-length of the unit square was 5 µm (the whole grid was 50 µm x 50 µm in size) was placed onto the regions of confocal images corresponding to the PPN. 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. The quantitative measurement was carried out in three sections that were randomly selected from three animals. Thus, the calculation of quantitative data, mean values and standard error of means (SEM), was based on the investigation of nine sections.

All data represent mean ± SEM. Statistical significance was determined using Student's *t*-test. The level of significance was $p < 0.05$.

Results

Synaptic and extrasynaptic actions of CB1 receptor stimulation on PPN neurons

In our previous work we focused on cannabinoid actions on extrasynaptic neuronal currents. It was shown that distinct neuronal populations within the PPN were depolarized, hyperpolarized or unaffected upon application of CB1 receptor agonists. These tonic actions did not seem to depend on neuronal network activity, as either blockade of fast synaptic neurotransmission or application of tetrodotoxin did not affect the changes of the membrane potential (Kőszeghy et al, 2015). This finding is in seeming contradiction with the well established fact that CB1 receptors predominantly exert presynaptic actions (see e.g. Lovinger, 2008; Kano, 2014; Katona and Freund, 2012). To resolve this issue, in the present work, we attempted to observe the tonic currents together with the changes of postsynaptic currents elicited by cannabinoids for separating the different endocannabinoid actions. Spontaneous currents were recorded from 17 PPN neurons in voltage-clamp mode at a holding potential of -60 mV. After application of the CB1 receptor agonist WIN55,212-2 (1 μM), tonic currents, ranging between +22 and -22 pA, developed (Fig.1A-F). In 7 cases, an inward current exceeding -3.5 pA occurred, whereas an outward current greater than +3.5 pA was recorded on 3 neurons. In the remaining 7 cases, changes of the holding current did not exceed 3.5 pA (Fig.1I). These changes of the holding current showed only a weak correlation with the location of the neuronal soma along the rostrocaudal axis of the nucleus (Suppl. Fig. 1). In order to exclude the possibility that WIN55,212-2 activates targets other than the CB1 receptor, the same experiments were performed on 9 PPN neurons from CB1 receptor knockout mice (Fig.1G-I). We found that the change of the holding current during application of WIN55,212-2 did not exceed 3.5 pA in 8 cases, and -4 pA inward current developed on a single neuron. The mean absolute value of the holding current changes was significantly greater in the wild type than in the CB1 knockout samples (7.54 ± 1.8 pA vs. 1.49 ± 0.37 pA, $p = 0.01$; Fig.1I). These data were also compared with the spontaneous fluctuations of the holding current. The absolute value of the fluctuations was 1.41 ± 0.25 pA, which did not differ

significantly from the changes observed on CB1 knockout mice (n = 18; p = 0.41), whereas WIN55,212-2 elicited significantly greater changes in wild type animals. Maximal values of the fluctuation were -2.8 and +3.2 pA (n = 18; p = 0.002; Fig.1I).

Next, we tried to distinguish direct neuronal actions of CB1 receptor stimulation from the indirect, astrocyte-dependent mechanisms. Slices were pre-incubated with 1 μM thapsigargin for 45 minutes prior to the recordings to deplete internal calcium stores. This treatment mostly affects astrocytes and has less influence on neurons (e.g. Navarrete and Araque, 2008, 2010; Perea et al, 2014; Kőszeghy et al, 2015). Spontaneous fluctuation of the holding current after thapsigargin significantly increased (n=8), compared to the untreated samples (5.24 ± 1.32 pA with thapsigargin and 1.41 ± 0.25 pA in control; p = 0.0001; red symbols and columns from Fig.1I, M). When the CB1 receptor agonist WIN55,212-2 was applied on these thapsigargin-treated samples, the holding current of the neurons displayed the same level of fluctuation as observed in control (5.81 ± 1.02 pA; p = 0.25; Fig.1K-M).

In 3 cases out of the 17 recorded PPN neurons, application of the CB1 receptor agonist elicited slow inward currents (SICs; Fig.1E, J). These currents were not present under control conditions, but appeared with a frequency of 0.23 ± 0.17/min when WIN55,212-2 was applied. Their average amplitude (35.6 ± 11.4 pA), rise time (69.8 ± 18.3 ms) and decay time (201 ± 39 ms; fit with single exponential function) clearly distinguished these events from sEPSCs (amplitude: 13.04 ± 0.54 pA; rise time: 5.35 ± 0.72 ms; decay time: $\tau_1 = 6.93 \pm 1.06$ ms; $\tau_2 = 15.5 \pm 3.19$ ms; fit with double exponential function; $p < 0.0001$ for all parameters).

The results above confirmed our previous findings with changes of the membrane potential and action potential firing (Kőszeghy et al, 2015). However, parallel changes of the synaptic events were out of focus of the previous publication; thus, direct presynaptic and astrocyte-dependent changes on these currents are shown by the present paper. During recording of the holding current at -60 mV holding potential, spontaneous excitatory (sEPSCs) and inhibitory postsynaptic currents (sIPSCs) were seen. Under control conditions, the average frequency of the sEPSCs was 0.18 ± 0.04 Hz, with average amplitude of 13.04 ± 0.54 pA. When

WIN55,212-2 was applied, the frequency changed to 0.25 ± 0.06 Hz with amplitude of 12.22 ± 0.47 pA. None of these changes proved to be significant due to the variability of the values under control conditions. However, when data obtained with CB1 receptor agonist were normalized to the control data, 59 ± 23 % increase of the frequency and 5.6 ± 2.1 % decrease of the amplitude was seen (n = 17; Fig1N).

From the 17 recorded neurons, spontaneous IPSCs were only detected in 5 cases. IPSCs were less frequent (0.05 ± 0.02 Hz), with an amplitude of 10.5 ± 1.67 pA. In the presence of the CB1 receptor agonist, the frequency decreased to 0.02 ± 0.01 Hz (37.6 ± 8% of the control; $p < 0.001$), with average amplitude of 8.1 ± 0.15 pA (19 ± 7% decrease, p= 0.07; Fig.1N).

Thapsigargin preincubation also affected cannabinoid-induced changes of sEPSC frequency. Instead of observing frequency increase of the sEPSC, the frequency significantly decreased (0.17 ± 0.04 Hz prior to WIN55,212-2 application, 0.12 ± 0.04 Hz in the presence of the CB1 receptor agonist; p = 0.045, 62.6 ± 9.7 % of control; Fig.1O). The amplitude of sEPSCs did not show significant changes to control (15.22 ± 0.86 pA in control and 15.05 ± 1.13 pA with WIN55,212-2; 99 ± 0.04%; Fig.1O).

Thapsigargin treated neurons displayed sIPSCs (3 out of 8), which showed similar changes in frequency to WIN55,212-2 as observed without preincubation with thapsigargin (0.1 ± 0.001 Hz in control; 0.038 ± 0.002 Hz with WIN55,212-2, p = 0.013; 38.2 ± 1.8% of control). Similar to the samples without preincubation with thapsigargin, amplitudes of sIPSCs were not affected (11 ± 0.2 pA in control and 11 ± 1.01 pA with WIN55,212-2; 100 ± 7% of control; Fig.1O).

These findings raised the possibility that cannabinoid actions on synaptic events might be the mixture of the canonical presynaptic inhibitory action of CB1 receptor activation and an indirect stimulatory effect of astrocytes on excitatory neurotransmission.

Effects of optogenetic activation of astrocytes on PPN neurons

To better distinguish astrocyte-dependent actions from the direct neuronal CB1 receptor stimulation, we chose an optogenetic approach to selectively activate astrocytes in the PPN. For these experiments, homozygous mice expressing cre recombinase in glial fibrillary acidic protein (GFAP) dependent way were crossed with flox-stop channelrhodopsin-2 (ChR2) mice, resulting in ChR2 expression in a GFAP-dependent way.

To test whether photostimulation (470-480 nm) can activate astrocytes, two different set of calcium imaging experiments were performed. First, slices prepared from wild type and GFAP-ChR2 mice were incubated with Oregon Green BAPTA I AM (OGB), and changes of the intracellular calcium concentration were recorded from cell bodies presenting only slow calcium waves as glial cells were earlier shown to lack fast calcium transients (Nimmerjahn et al., 2004; Kőszeghy et al, 2012; 2015). Cells lacking spontaneous calcium waves were also excluded from evaluation. As the wavelength necessary for excitation of OGB also facilitates the opening of channelrhodopsin 2 (ChR2), comparison of calcium wave areas proved that astrocytes of GFAP-ChR2 mice were stimulated by the pulsatile light used for calcium imaging (10 ms illumination with 10 Hz frequency), whereas astrocytes of wild type mice were unaffected (14.2 ± 2.2 %*s in wild type and 44.93 ± 8.2 %*s in GFAP-ChR2 ; p = 0.0028; n = 17 and 20 from 4 and 3 mice; Fig.2 A-C).

To avoid unwanted stimulation of astrocytes during calcium imaging, in the next experiments, we used a calcium indicator, the Rhod2 fluorophore, with an excitation wavelength far away from that of ChR2. In these experiments, after recording calcium waves under control conditions, the whole visual field was illuminated with continuous blue light (470 nm) for 1 minute, by using an external LED light source (Fig.2D). In this case, the areas of the calcium events showed a significant increase during the illumination (15.5 ± 3.6 %*s before and 93.5 ± 30.3 %*s during photostimulation; p = 0.012; n = 16 from 3 mice; Fig.2E). To demonstrate the photocurrent evoked in the astrocytes by the stimulation of ChR2, cells producing slow calcium waves were patched and holding currents were recorded at -80 mV holding potential. As expected, an inward current with amplitude of 65.7 ± 2.6 pA developed, and declined rapidly after finishing illumination (n = 4; Fig.2F).

In order to test the neuronal responses for the photoactivation of astrocytes neurons were recorded in whole-cell patch clamp experiments while astrocytes in the same visual field were activated by epifluorescent illumination via the 40X objective (480 ± 5 nm; 1 min). In the majority of the cases (8 out of 9) neurons were depolarized together with a consequential increase of the firing frequency (Fig.2G). The average depolarization was 5.07 ± 1.4 mV and the firing rate increased with 1.42 ± 0.54 Hz (Fig.2G-I). Hyperpolarization was only seen in a single case.

In voltage-clamp experiments continuous illumination (1 minute) elicited a tonic current on almost all neurons. In the majority of the cases, this tonic current was inward (n = 9 from 12). Outward current was observed in one case, while in the remaining two cases no tonic current was detected. The absolute change of the tonic currents was 11.45 ± 3.39 pA (Fig.2J-L).

Optogenetic stimulation of astrocytes with pulsatile light (10 ms illumination at 10 Hz) induced similar changes on the neuronal holding current (inward current in 5 cases, outward in one and no change in the remaining 2). The absolute change of the holding current was 7.86 ± 2.36 pA. Changes of the holding current showed no significant difference between pulsatile and continuous light stimulation (p = 0.19; Fig.2K-L).

In our previous work (Kőszeghy et al, 2015), we showed neuronal tonic currents elicited by astrocytic activation depend on activation of metabotropic glutamate receptors (mGluRs). To confirm this finding with the specific optogenetic activation of astrocytes, slices were continuously washed with 100 µM CPCCOEt and 10 µM MPEP (blockers of group I mGluRs; n = 6) or 10 µM LY341495 (blocker of group II mGluRs; n = 7). Blockers of mGluR I did not affect the absolute amplitude of tonic current elicited by photostimulation (12.8 ± 4.67 pA; p = 0.35; Fig.2K-L). In the presence of group I mGluR blockers no outward current was observed. Inhibition of group II mGluRs, on the other hand, largely reduced the magnitude of the inward currents. The absolute change of the holding current was 3.66 ± 1.9 pA, which significantly differed from data obtained under control conditions (p = 0.04).

Optogenetic stimulation of astrocytes also influenced spontaneous synaptic events. Continuous illumination increased the amplitude of sEPSCs recorded on neurons from 14.6 ± 1.2 pA, to 15.18 ± 0.79 pA (117 ± 10%; p = 0.049) and boosted sEPSC frequency from 0.13 ± 0.02 Hz to 0.25 ± 0.64 Hz (320 ± 116 %; n = 8). Pulsatile illumination again similarly increased the sEPSC frequency (from 0.13 ± 0.02 Hz to 0.27 ± 0.07 Hz; 224 ± 28% of control). Application of mGluR I blockers CPCCOEt and MPEP was less powerful in prevention of frequency changes (from 0.18 ± 0.02 Hz to 0.29 ± 0.08 Hz during illumination; 189 ± 30 % of control; n = 6), whereas the mGluR II blocker LY341495 almost fully prevented changes caused by astrocytic activation (from 0.22 ± 0.04 Hz to 0.26 ± 0.04 Hz during illumination; 104 ± 6%; n = 7; Fig.2M).

Spontaneous IPSCs were less frequent in all the recordings and did not show any significant change during photoactivation (amplitude from 11.25 ± 1.24 pA to 13.5 ± 3.1 pA during illumination; 118 ± 14% of control; p = 0.16; frequency from 0.175 ± 0.075 to 0.11 ± 0.01 Hz; 74 ± 26% of control; p = 0.21; n = 3).

Furthermore, when astrocytes were activated by illumination, 4 neurons from 7 displayed SICs (Fig.2J, asterisk). No spontaneous SIC appeared under control conditions, while the frequency of these events was 0.93 ± 0.28/min during illumination.

In control experiments where GFAP-cre animals (n = 7) were used under the same conditions as GFAP-ChR2 mice, no changes were observed. The absolute change of the holding current was 1.36 ± 0.45 pA, which did not differ significantly from the spontaneous fluctuations of the holding current (1.41 ± 0.25 pA; Fig.2J-L). Similarly, no changes in the frequency and amplitude of postsynaptic currents and SICs appeared. Prior to the illumination, sEPSCs had a frequency of 0.53 ± 0.12 Hz and amplitude of 14.8 ± 0.65 pA. During light stimulation, the frequency was 0.56 ± 0.12 Hz, whereas the amplitude proved to be 14.6 ± 0.87 pA. Spontaneous IPSCs occurred only on 3 neurons from GFAP-cre samples. The frequency of these events was 0.07 ± 0.04 Hz with amplitude of 13.5 ± 3.5 pA. When illuminated, the frequency changed to 0.04 ± 0.002 Hz and the amplitude was 14.56 ± 1.8 pA. During these recordings, no SICs appeared.

Direct synaptic actions of CB1 receptor stimulation

In the next set of experiments, actions of presynaptic CB1 receptor activation on excitatory postsynaptic currents (EPSCs), elicited by 50 Hz paired pulse stimulation of the presynaptic fibers within the nucleus (stimulation electrode placed approximately 50-100 μm away from the patched soma), were revealed (Fig.3A-B). The average amplitude of the first EPSC was 46.28 ± 1.54 pA and the paired pulse ratio (PPR, ratio of the second and first EPSC) showed mild facilitation (1.17 ± 0.04). After achieving stable amplitude, the postsynaptic cell was depolarized to 0 mV for 10 s with continuous detection of the EPSCs, in order to test whether depolarization-induced suppression of excitation (DSE) exists in the PPN (see Ohno-Shosaku et al., 2002). During the consecutive 60 seconds, the amplitude of the first EPSC was significantly decreased to 30.83 ± 1.69 pA ($p = 0.0001$), together with the increase of the PPR to 1.49 ± 0.08 ($p < 0.001$). After 4 minutes, values close to the original amplitude (44.63 ± 1.36 pA) and PPR (1.17 ± 0.03) were recovered ($n = 11$). Following the recovery, WIN55,212-2 (1 μM) was applied, which resulted in a similar change (amplitude = 31 ± 1.56 pA; PPR = 1.42 ± 0.05; $n = 11$; $p < 0.001$ for both parameters) to the one provoked by the depolarization (Fig.3A-B.). Another CB1 receptor agonist, ACEA (5 μM) exerted a similar action: the amplitude was reduced to 33.6 ± 1.85 pA with a PPR of 1.48 ± 0.17 ($n = 6$; $p = 0.002$ for the amplitude and 0.03 for the PPR).

The above cannabinoid actions were absent in CB1 knockout animals ($n = 6$). Under control conditions, the amplitude of the first EPSC was 56 ± 1.17 pA with a PPR of 1.23 ± 0.04. After postsynaptic depolarization, the amplitude of the first event changed to 55.6 ± 3.5 pA, whereas the PPR was 1.4 ± 0.12. During application of WIN55,212-2, the first amplitude was 58.6 ± 1.2 pA with a PPR of 1.29 ± 0.05. None of these changes proved to be significant (Fig.3C-D).

In accordance with the literature, decrease of the amplitude together with increase of the PPR suggests the existence of presynaptic inhibition (see e.g. Zucker and Regehr, 2002). This finding is in accordance with our data with sEPSCs in the presence of thapsigargin (Fig.1O), but it is in contrast with experiments on sEPSCs under control conditions when these events had an increased frequency (Fig.1N). This observation raised the

possibility that astrocytic activation has a facilitatory effect on excitatory synaptic transmission. Thus, in the next series of experiments we tested whether an astrocyte-dependent indirect presynaptic action of cannabinoids exists in the PPN. Slices prepared from GFAP-ChR2 mice were loaded with OGB, and neurons in the slices were recorded in whole-cell mode (n=7) while their excitatory input was stimulated with paired pulses delivered at 50 Hz frequency (Fig.4A). Evoked EPSCs were recorded under control conditions, during pulsatile illumination (when calcium waves were also recorded from neighboring cells), and after achieving recovery from the effects of photostimulation (Fig.4B-C). No changes of the first EPSC or the paired pulse ratio was observed during these recordings: the amplitude was 37.3 ± 9.4 pA before and 36.4 ± 9.8 pA during illumination; whereas the PPR was 1.48 ± 0.3 before and 1.59 ± 0.33 during astrocytic photostimulation (p = 0.47 and 0.41, respectively). The only change observed during photostimulation of astrocytes was the appearance of the tonic inward current (-11.14 ± 4.8 pA) in 6 cases, whereas no tonic current developed in a single case (Fig.4C-D).

After testing evoked EPSCs, we aimed to observe possible direct and indirect endocannabinoid actions on IPSCs, as well. From 17 experiments when evoked postsynaptic potentials were tested, only a single case resulted appearance of IPSCs. Increasing the efficacy of the experiments, miniature IPSCs (mIPSCs) were investigated in patch clamp experiments with KCl-based internal solution and in the presence of tetrodotoxin (TTX) and the ionotropic glutamate receptor antagonists D-AP5 and NBQX (Fig.5A-F, M). Under these conditions, previous findings with sIPSCs were confirmed: both depolarization of the postsynaptic neuron to 0 mV for 10 s and application of WIN55,212-2 caused reduction of the frequency of mIPSCs with unchanged amplitude (Fig.1N-O). The mIPSC frequency was 0.249 ± 0.075 Hz under control conditions, whereas it was decreased to 0.188 ± 0.089 Hz (64.5 ± 2.2 % of control; p = 0.02) after postsynaptic depolarization and to 0.128 ± 0.059 Hz (46.7 ± 7.8 % of control; p = 0.004) during application of the CB1 receptor agonist (n = 7). When using CB1 knockout animals (n=5), these effects were absent. Under control conditions, the frequency of mIPSCs was 0.27 ± 0.09 Hz, which became 0.26 ± 0.06 Hz (107 ± 36% of control; p = 0.43) after postsynaptic

depolarization and 0.27 ± 0.15 Hz (89 ± 20%; $p = 0.49$) when WIN55,212-2 was used. The amplitude of the mIPSCs did not change significantly (20.35 ± 1.8 pA in wild type animals under control conditions, 18.3 ± 2.9 pA (91 ± 5%; $p = 0.09$) after postsynaptic depolarization and 19.99 ± 2.2 pA (99.1 ± 9.5%; $p = 0.13$) in the presence of the CB1 receptor agonist (Fig.5F). In CB1 receptor knockout mice, the amplitude did not change as well (21.5 ± 1.8 pA under control conditions, 17.9 ± 0.3 pA after postsynaptic depolarization and 18.5 ± 3 pA with WIN55,212-2; $n = 5$; Fig.5L).

Presynaptic and astrocytic locations of the CB1 receptor

According to our functional experiments, CB1 receptor agonists had indirect astrocyte-dependent and direct presynaptic actions as well. To confirm these findings the presence of the CB1 receptor in presynaptic and astrocytic locations was proven with immunohistochemistry (Fig.6.). The CB1 receptor and the choline acetyltransferase (a marker for the dominant cholinergic neuronal population within the PPN) were labeled together with GFAP, VGLUT2 or VGAT, the markers of astrocytes, glutamatergic and GABAergic terminals, respectively. CB1 receptor-immunopositive puncta were colocalized with GFAP, VGLUT2 and VGAT, but almost no colocalization was found with ChAT. In total, 21.28 ± 1.91% of 624 CB1 receptor-positive puncta was colocalized with GFAP-labelling, whereas 33.18 ± 1.88% of 684 CB1 receptor positive puncta overlapped with VGLUT2. 13.49 ± 0.82% CB1 receptor positive patches were colocalized with VGAT-positive puncta ($n = 614$).

Discussion

In the present paper we showed that cannabinoid signaling has a direct presynaptic and an indirect, astrocytic modulatory pathway in the pedunculopontine nucleus. Specific optogenetic activation of astrocytes confirmed our previous results that astrocytic actions elicit tonic inward and outward currents, with the involvement of mGluRs. In parallel with tonic changes of neuronal excitability, CB1 receptor activation exerted stimulatory effect on excitatory neurotransmission, together with the inhibition of inhibitory neurotransmission. The latter effect was likely the consequence of activation of presynaptic CB1 receptors, whereas the cannabinoid actions on excitatory neurotransmission are combination of a presynaptic inhibition through CB1 receptors located on excitatory axon terminals, and an increase of neuronal activity via stimulation of astrocytic glutamate release.

Neuronal tonic currents elicited by astrocytic activity

In our present and previous studies we observed tonic inward and outward currents elicited by the activation of astrocytes (Kőszeghy et al, 2015; Kovács et al, 2015). According to our previous findings (Kőszeghy et al, 2015), the depolarization by astrocytic activation via CB1 receptor was prevented by blockers of group II mGluRs, whereas the hyperpolarization is blocked by the group I mGluR inhibitors. Astrocytes can release glutamate by vesicular exocytosis and via different channels and transporters, and modulate ambient glutamate levels (Parpura et al, 1994; Szatkowski et al, 1990; Warr et al, 1999; Cotrina et al, 1998; Ye et al, 2003; Duan et al, 2003; Rosenberg et al, 1994; Pasantes Morales and Schousboe, 1988). This released glutamate might exert its actions on different sets of neuronal mGluRs. Several studies described extrasynaptic mGluRs associated with changes of the neuronal membrane potential and tonic currents. Group I mGluR activation is associated with neuronal depolarization via activation of L-type calcium channel or a non-selective cationic conductance (Libri et al, 1997; Partridge et al, 2014; Kato et al, 2012; Smith et al, 2009), whereas

group II mGluR stimulation inhibits L-type calcium channel or activates potassium current (e.g. Chavis et al, 1994, 1995; Irie et al, 2006; Hermes and Renaud, 2011). More similar to our observations, the opposite effects of group I and II mGluR activation were also seen: mGluR5 (group I) stimulation led to activation of potassium currents (e.g. Jian et al, 2010; Rainnie et al, 1994; Kohlmeier et al, 2013) and group II mGluR activation inhibited a potassium conductance and stimulated a cationic conductance (Ster et al, 2011). Besides this, tonic currents can be elicited by activation of other neuronal targets or release of other gliotransmitters. The release of glutamate can be the consequence of inhibition of the glutamate uptake by excitatory amino acid transporters (EAAT1-3), as TBOA, the specific blocker of these transporters initiates inward currents (Angulo et al, 2004; Jabaudon et al, 1999; Le Meur et al., 2007). Such increase of the ambient glutamate activates extrasynaptic NMDA receptors composed of NR2B subunits (Kozlov et al, 2006; Angulo et al, 2004; D'Ascenso et al., 2007; Fellin et al., 2004).

Tonic outward currents can also be elicited or modulated by astrocytic activation: taurine and GABA-mediated tonic chloride currents have been already described (Belluzzi et al, 2004; Lee et al, 2010). A similar effect was found in the laterodorsal tegmental nucleus where WIN55,212-2 elicited an outward current in a magnitude comparable with our findings (Soni et al, 2014).

In the present paper we confirmed our previous results on tonic neuronal depolarization and hyperpolarization by showing cannabinoid actions on tonic inward and outward currents.

Optogenetic activation of astrocytes

Experiments with optogenetic activation of astrocytes further supported our theory that tonic neuronal changes of excitability have glial origin in the PPN. In the last decade, the revolutionary technique of optogenetics opened the way for stimulation or inhibition of cell populations in a selective way (e.g. Fenno et al, 2011; Zhang et al, 2006; Deisseroth et al, 2006). Recent attempt were done for selective activation of astrocytes, where ChR2 was expressed in a GFAP-dependent way (Gourine et al, 2010; Figueiredo et al, 2011;

Chen et al, 2012; Perea et al, 2014). ChR2 is a non-selective cationic channel, allowing influx of Na^+, H^+-, and Ca^{2+}-ions (Ji and Wang, 2015; Gourine et al, 2010; Figueiredo et al, 2011; Chen et al, 2013). This way of stimulation potentiates calcium wave generation not only via entry of extracellular calcium. Indeed, Ca^{2+} entering via ChR2 mostly triggers generation of astrocytic calcium waves via autocrine action of ATP (Figueiredo et al, 2014). Similarly, facilitation of gliotransmitter release is also not driven by a single release mechanism, but activation of multiple pathways ways can lead to this process after optogenetic activation as membrane potential depolarization, intracellular acidification or elevation of the intracellular calcium concentration (Ji and Wang, 2015; Gourine et al, 2010; Figueiredo et al, 2011; Perea et al, 2014; Beppu et al, 2014).

Optogenetic activation of astrocytes led to stimulation of neuronal structures via different indirect ways. Neurons of the retrotrapezoid nucleus were depolarized and increased firing rate via astrocytic ATP release (Gourine et al, 2010, Figueiredo et al, 2011), and optogenetic stimulation of Bergmann glia led to release of glutamate as gliotransmitter, which activated AMPA receptors on the Purkinje-cells and metabotropic glutamate receptors on parallel fiber-Purkinje-cell synapses (Sasaki et al, 2012). In the visual cortex, optogenetic stimulation of astrocytes increased the frequency of sEPSCs, sIPSCs and SICs by glutamate release and activation of mGluR1a receptors. Furthermore, firing frequency of parvalbumin-positive inhibitory interneurons was increased, whereas somatostatin-positive interneurons either displayed an increase or decrease of their firing rate (Perea et al, 2014). Results obtained with optogenetic methods on PPN neurons are in accordance with the observations above, with our previous data (Kőszeghy et al, 2015) and with the present results with CB1 receptor agonists. The observed changes during optogenetic activation of astrocytes have similarities with the actions of endocannabinoids, further supporting the hypothesis that endocannabinoid actions in the PPN have an astrocytic, indirect component. Neuronal tonic inward currents and depolarization elicited by either CB1 receptor agonists or optogenetic activation of astrocytes have a similar magnitude and can be prevented by blockade of mGluR II.

However, neuronal actions of optogenetic activation of astrocytes and astrocyte-dependent actions of cannabinoid agonists on neurons might have differences, as tonic outward currents were elicited less frequently by optogenetic methods. This finding raises the possibility that generation of tonic outward currents is achieved by complex mechanisms where neuronal activity is also affected. Alternatively, gliotransmitter release by astrocytes might be affected by the different ways of activation; suggesting that -although currently being one of the best possibilities for selective astrocytic activation- optogenetic stimulation of astrocytes triggers non-physiological gliotransmitter release.

Endocannabinoid- and astrocyte-dependent modulatory actions on synaptic currents

Besides confirming our previous data on astrocyte-dependent tonic activation and inhibition of PPN neurons with different methods, we showed that synaptic neurotransmission is also affected by direct and indirect cannabinoid actions in the PPN.

In accordance with the literature, we showed that both presynaptic and astrocytic CB1 receptors exist in the PPN. It is well established that CB1 receptors are predominantly found in a presynaptic location; both on excitatory and inhibitory synapses (Katona et al, 1999, 2006; Kreitzer and Regehr, 2001; Katona and Freund, 2012). There is a growing body of evidence that, besides the presynaptic location, astrocytes also possess CB1 receptor in several brain regions (nucleus accumbens, cingulate cortex, medial forebrain bundle, amygdala, spinal cord and hippocampus; Rodríguez et al, 2001; Moldrich and Wenger, 2000; Salio et al, 2002; Navarrete and Araque, 2008; Hegyi et al, 2009)

The presynaptic inhibition of inhibitory (Llano et al, 1991; Katona et al, 1999) and excitatory (Ohno-Shosaku et al, 2002) neurotransmission by CB1 receptor was described in the cerebellum and hippocampus, and later, in other structures e.g. the cerebral cortex (Trettel and Levine, 2003), and the substantia nigra (Yanovsky et al, 2003). In this paper, we provided the first evidence that the mechanisms of DSE and DSI exist in the PPN. CB1 receptor agonists always decreased frequency but not amplitude of sIPSCs and mIPSCs. In contrast

with it, sEPSCs was not reduced by cannabinoids. Instead, a tendency of increase in sEPSC frequency was seen when the CB1 receptor agonist WIN55,212-2 was applied. In the related cholinergic nucleus, the laterodorsal tegmental nucleus, similar observations were done on spontaneous and miniature IPSCs, where the frequency of these events was decreased but the amplitude did not change. In contrast, the frequency of EPSCs only changed in a minority of cases (Soni et al, 2014; Soni and Kohlmeier, 2015).

When EPSCs were evoked by stimulation of the presynaptic fibers, both postsynaptic depolarization and CB1 receptor agonists exerted presynaptic inhibition in the majority of the cases. However, it seems to be in contrast with the finding that sEPSCs had a mild increase of frequency. The presynaptic facilitation of EPSCs was demonstrated on hippocampal CA1 pyramidal neurons, where astrocytes contributed to this phenomenon (Navarrete and Araque, 2008, 2010; Han et al., 2012; Coiret et al., 2012; Castillo et al., 2012). It was shown that increased neuronal activity which leads to endocannabinoid release activates astrocytic glutamate release, which, in turn, can potentiate glutamatergic synapses and initiate long term depression via NMDA receptors. Not only glutamate as neurotransmitter, but ATP and D-serine release can also lead to long term potentiation in the neocortex (Rasooli-Nejad et al, 2014).

When slices were incubated with thapsigargin, the CB1 receptor agonist WIN55,212-2 caused a frequency decrease of sEPSC. It was demonstrated in our previous work that preincubation with thapsigargin prevented astrocytic activation but did not inhibit development of neuronal depolarization elicited by glutamate (Kőszeghy et al, 2015). It is therefore likely that increase of sEPSC frequency by astrocytic mechanisms and decrease of sEPSC frequency by direct presynaptic inhibition participate together in endocannabinoid actions on excitatory neurotransmission in the PPN.

As a further support for the existence of astrocyte-dependent stimulatory actions on excitatory neurotransmission, it was also shown that optogenetic activation of astrocytes increased sEPSC frequency via group II mGluR activation, but did not affect sIPSCs.

According to recent publications using either optogenetic or pharmacological approaches for astrocytic stimulation, glutamate released from astrocytes acts on presynaptic group I mGluRs and potentiate hippocampal CA3-CA1 synapses (Navarrete and Araque, 2010), induces LTP (Navarrete et al, 2012), enhances excitatory and inhibitory neurotransmission in the visual cortex (Perea et al, 2014) or induces LTP in cerebellar parallel fiber-Purkinje-cell synapses (Sasaki et al, 2012). We observed that an astrocyte-dependent stimulatory action on sEPSC frequency exists in the PPN, and it is able to mask inhibition by presynaptic CB1 receptors. Furthermore, optogenetic activation of astrocytes increased sEPSC frequency in a group II mGluR dependent way. These observations raised the possibility that presynaptic mGluR-dependent mechanisms regulate synaptic strength in the PPN as well. Surprisingly, when EPSCs were elicited by stimulation of presynaptic excitatory fibers and parallel optogenetic activation of astrocytes was achieved, only an inward shift of the holding current and no change of the postsynaptic currents was seen. Although one can not clearly exclude the possibility that presynaptic mGluRs regulate neurotransmitter release in the PPN on certain synapses being out of focus of our experimental arrangement, our findings might lead to the conclusion that the stimulation of excitatory neurotransmission is rather due to the overall increase of excitability of excitatory neurons in the PPN, instead of an effect on synaptic vesicle release. The hypothesis that the increase of sEPSC frequency is due to the activation of glutamatergic neurons in the slice preparation suggests that the targets of this astrocyte-dependent stimulatory mechanism are the glutamatergic non-cholinergic neurons of the PPN. Although the neurochemical cell type (i.e. whether a neuron is cholinergic, GABAergic or glutamatergic) was not investigated in the present study, our previous study supports the hypothesis above. We previously found that the majority of non-cholinergic (i.e. GABAergic and glutamatergic) neurons are depolarized by CB1 receptor agonists, whereas depolarization, hyperpolarization and the lack of response was seen on cholinergic neurons in the same proportion (Kőszeghy et al, 2015). The reason that inhibitory neurotransmission was not stimulated by indirect cannabinoid mechanisms, raises the possibility that local GABAergic collaterals are less abundant than local glutamatergic inputs in a coronal slice preparation. This theory is supported by morphological

observations: putative glutamatergic neurons gave rise to local axon collaterals, whereas putative GABAergic neurons lacked local varicosities and thought to function as projection neurons (Ros et al, 2010).

Functional implications

Our previous and present results demonstrated that application of cannabinoids elicits a special activity pattern of PPN neurons. The hyperpolarization, lack of response or depolarization (or the tonic outward or inward currents in their background) has a dependence on neurochemical character of the neurons (Kőszeghy et al, 2015) but shows only a weak correlation with the rostrocaudal location of the somata.

It was demonstrated that exogenous cannabinoids increase the duration of slow wave- and REM-sleep (Murillo-Rodriguez et al, 2008; Herrera-Solís et al, 2010), and endocannabinoid release has a significant contribution to the homeostatic drive of sleep (see Murillo-Rodriguez, 2008). Therefore, the condition observed during our *ex vivo* experiments might resemble to the activity of the PPN neurons either during cannabinoid-induced REM- or slow wave sleep.

It is proposed by previous and recent publications that neurons of the PPN, regardless of their neurochemical character, fire action potentials in the gamma band range and maintain active states (as wakefulness and REM sleep) by this activity (see e.g. Urbano et al, 2012; Garcia-Rill et al, 2013). Our *ex vivo* findings are partially in contrast with these results, as a pronounced, cell type-dependent heterogeneity of a neuromodulatory action was seen, and uniform changes of neurons were absent. Furthermore, gamma-band activity of the PPN neurons sustained by excitatory inputs might facililtate depolarization-induced production of endocannabinoids, and endocannabinoid blockade of stimulatory inputs to the depolarized neurons with gamma-band firing rates might antagonize long-range high frequency firing.

However, our results represent the local changes of the PPN dissected from its distant connections, which limits the possibility of the comparison between the literature data and our findings . Furthermore, the

dual nature of the cannabinoid regulation was also demonstrated here, thus presynaptic inhibition of excitatory inputs can be counterbalanced by astrocyte-dependent increase of sEPSC frequency.

Heterogeneity in neuronal responses to neuromodulatory stimuli or in correlation with transitions between global states seems to be a general feature of the PPN. Application of carbachol induces heterogenous responses (including development of inward, outward or biphasic currents and lack of response) on PPN neurons (Ye et al, 2010). Furthermore, the activity of most PPN neurons coupled to cortical slow oscillations, but this coupling is reduced during global active states. Transitions between cortical slow oscillations and active states are associated with phasic excitation, tonic excitation or tonic inhibition of cholinergic and non-cholinergic neurons, respectively (Petzold et al, 2015). Although it might be speculative to compare data obtained with different experimental approaches, our findings represent a similarly characteristic change of the activity pattern of PPN neurons, which might also correspond to a similar change in the PPN activity coupled with cannabinoid-induced transitions of global brain states.

Summary

Considering our previous (Kőszeghy et al, 2015) and present results, we propose the following model of the cannabinoid neuromodulatory actions in the PPN (Fig.7). Depolarized neurons with trains of action potentials release endocannabinoids (Lovinger, 2008; Katona and Freund, 2012; Kano, 2014). These molecules diffuse to the neighboring neuronal and astrocytic targets and activate structures being in close contact with the neuron is question via presynaptic and astrocytic CB1 receptors (Kőszeghy et al, 2015). Activated astrocytes release glutamate (and maybe other gliotransmitters), which act on extrasynaptic and postsynaptic neuronal glutamate receptors. These receptors can be the mGluRs, as blockade of group I mGluRs prevented hyperpolarization, and inhibition of group II mGluRs prevented neuronal depolarization. The exact location of these mGluRs is not investigated; therefore, either neuronal extrasynaptic or astrocytic mGluRs can be hypothesized. Besides astrocyte-dependent actions, excitatory and inhibitory inputs are inhibited by the

endocannabinoid release from the depolarized neuron. Local glutamatergic neurotransmission, however, might "escape" from this inhibitory cannabinoid action, via the increase of excitability of local glutamatergic neurons giving collaterals within the nucleus. Taken together, endocannabinoid actions seem to disconnect PPN from its synaptic inputs and to support an intrinsic activity pattern including depolarization and hyperpolarization of different neuronal populations.

Furthermore, although the present study focused exclusively on the endocannabinoid signaling, it might be a support for the hypothesis that several known neuromodulatory actions have a direct neuronal and indirect astrocytic component, providing parallel channels for fine tuning of neuromodulatory actions. Distinguishing astrocytic and neural components might be a significant help for the better knowledge of complex neuromodulatory mechanisms.

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Figure legends

Fig.1. Compound actions of CB1 receptor stimulation on PPN neurons. A-F. The CB1 receptor agonist WIN55,212-2 (1 μM) elicits tonic inward or outward currents on PPN neurons. **A.** Representative trace for development of tonic inward current. Dashed lines indicate the holding current level under control conditions and during drug application. **B.** Histograms of the control trace (black) and the holding current recorded under WIN application (blue). WIN55,212-2 was applied in the same time on traces shown on panels A, C and E. **C-D.** Current trace and histogram of a neuron where the CB1 receptor agonist did not elicit changes in the tonic current. **E-F.** Current trace and histogram recorded from a neuron where WIN elicited tonic outward current. Note that slow inward currents (SICs, asterisk) were also developed during drug application. **G-H.** Representative current trace and histogram recorded from a CB1 receptor knockout sample. **I.** Statistical comparison of individual changes of the holding current (hollow dots) induced by WIN and averages ± SEM of the absolute values of current changes (columns) from wild type samples (W, black), spontaneous fluctuations of the holding current (S, red) and changes of the holding current recorded from CB1 knockout samples in the presence of WIN (-/-, green). **J.** Current traces of an EPSC (a) and a SIC (b), magnified from panel E. **K-L.** Current trace and histogram from a sample preincubated with thapsigargin. Note that although spontaneous fluctuations increased but application of WIN did not elicit tonic currents. **M.** Statistical comparison of individual changes of the holding current (hollow dots) induced by WIN and averages ± SEM of the absolute values of current changes (columns) on wild type samples preincubated with thapsigargin (W, black) and spontaneous fluctuations of the holding current of the same samples (S, red).

N. Statistical summary of changes of spontaneous EPSCs and IPSCs under control conditions (C, black), in the presence of WIN (W, blue), and data normalized on control (hollow columns). Note that the frequency of sEPSCs displayed a mild increase, whereas the sIPSC frequency was reduced. **O.** Statistical summary of

changes of sEPSC and sIPSC parameters after application of WIN on samples preincubated with thapsigargin. Note that the frequency of both sEPSCs and sIPSCs decreased.

Fig.2. Optogenetic activation of astrocytes induces changes of the neuronal holding currents and sEPSC frequency in an mGluR II-dependent way. A. Spontaneous slow calcium waves recorded from a wild type sample with Oregon Green 488 BAPTA-I (OGB). **B.** Slow calcium waves recorded from a sample expressing channelrhodopsin-2 in a GFAP-dependent way (GFAP-ChR2) with the same indicator. **C.** Statistical summary of observations with areas of calcium waves using Oregon Green 488 BAPTA-I (green column, wt: OGB, wild type sample; blue column, GFAP-ChR2: OGB, sample expressing channelrhodopsin-2 in a GFAP-dependent way) **D.** Calcium waves recorded from GFAP-ChR2 samples with Rhod-2 calcium indicator before, during and after 1 minute continuous illumination with 470 nm light (blue square). **E.** Statistical summary of observations with areas of calcium waves using Rhod-2 (black, control: GFAP-ChR2 sample, prior to illumination; red, 470 nm: same sample, during illumination). **F.** Photocurrent recorded at -80 mV holding potential, from the cell producing the medium calcium trace from panel D. **G.** Representative current clamp recording from a neuron in a GFAP-ChR2 sample. The blue square indicates the duration of illumination with 480 nm light, dashed lines represent the membrane potential value prior to and during illumination. **H.** Histograms of the current clamp trace from panel G (black: control, blue: photostimulation). **I.** Changes of the resting membrane potential (ΔRMP) and firing rate (ΔFreq.) after photostimulation. **J.** Changes of the neuronal holding current on GFAP-ChR2-samples and on GFAP-cre sample which does not express ChR2. Note that both inward and outward tonic currents and SICs (asterisk) occur during photostimulation. Histograms of the representative current traces can be seen at the right side of the panel (black: control, blue: photostimulation). **K.** Individual changes of the neuronal holding current during photostimulation of GFAP-ChR2 samples (cont: continuous photostimulation for 1 minute; puls: pulsatile photostimulation, 10 ms illumination with 10 Hz; spont: spontaneous membrane potential fluctuations; Cre: GFAP-cre sample used as negative control; C+M: 100 μM

CPCCOEt and 10 μM MPEP –mGluR I antagonists; Ly: 10 μM LY341495 – mGluR II antagonist). **L.** Statistical comparison of the absolute values of holding current changes under different conditions (see panel J). **M.** Changes of sEPSC frequency during photostimulation (C, black: control; I, blue: illumination with 480 nm light; hollow column: data normalized to control). Left columns: without prior manipulations; middle: in the presence of mGluR I blockers CPCCOEt and MPEP; right columns: in the presence of the mGluR II antagonist LY341495.

Fig.3. Both postsynaptic depolarization and the CB1 receptor agonist WIN55,212-2 cause presynaptic inhibition of evoked EPSCs in a CB1 receptor-dependent way. A. Representative current traces of EPSCs evoked by 50 Hz paired stimulation of the presynaptic fibers under control conditions, after depolarization of the recorded neuron to 0 mV for 10 s (postsynaptic depolarization; PD), after recovery from the effects of postsynaptic depolarization, in the presence of WIN55,212-2, and in the presence of the ionotropic glutamate receptor antagonists NBQX and D-AP5. The holding currents were aligned to zero and each trace is the average of 10 concomitant individual sweeps. The stimulatory artefact was removed for the better visibility of the traces. **B.** Changes of the amplitude of the first EPSC (S1) and the paired pulse ratio (PPR) during postsynaptic depolarization and the application of WIN55,212-2. All symbols represent average ± SEM. **C-D.** Recordings from CB1 knockout mice, with the same arrangement as in panel A and B.

Fig.4. Optogenetic stimulation of astrocytes does not affect the amplitude and paired pulse ratio of the evoked EPSCs. A. Image representing the experimental arrangement. Slices from GFAP-ChR2 mice were loaded with Oregon Green BAPTA I AM (1.-5.; bright dots bordered by red lines), a neuron was patched (rec.) and a stimulatory electrode (stim.) was placed approximately 100 μm away from soma. **B.** Calcium waves from areas numbered on panel A during photostimulation. **C.** Representative current traces of EPSCs evoked by 50 Hz paired stimulation under control conditions, during photostimulation (blue square), after recovery from the

effects of photostimulation and in the presence of the ionotropic glutamate receptor antagonists NBQX and D-AP5. Each trace is the average of 10 concomitant individual sweeps, and the holding current is presented at its original value. **D.** Changes of the holding current, the amplitude of the first evoked EPSC and the PPR prior to, during and after photostimulation. Note that an inward current developed without changes in parameters of EPSCs during photostimulation. All symbols represent average ± SEM.

Fig.5. The frequency of the miniature IPSCs decreased both after postsynaptic depolarization and during application of WIN55,212-2. A-D. Current traces recorded from a wild type sample under control conditions (A), after depolarization of the recorded neuron to 0 mV for 10 s (B), after recovery from the effects of postsynaptic depolarization (C) and in the presence of WIN55,212-2 (D). **E.** Cumulative histogram of inter-event intervals of events on the representative current traces from panels A-D. under control conditions (black), after postsynaptic depolarization (orange), after recovery (blue) and in the presence of WIN55,212-2 (red). **F.** Cumulative histogram of amplitudes, using the same color code as in panel E. **G-J.** Representative current traces from a CB1 receptor knockout sample; with the same arrangement as on panels A-D. **K-L.** Cumulative histograms of the inter-event intervals and amplitude of events from records of panel G-J, using the same color code as above. **M.** Statistical summary of the results with mIPSC frequency under control conditions (C, black), after postsynaptic depolarization (PD, orange) and in the presence of WIN55,212-2 (W, red). Data shown on the right panel were normalized to control. **N.** Statistical summary of results with miniature EPSC frequency recorded from CB1 knockout samples, with the same arrangement as on panel M.

Fig.6. CB1 receptor immunopositivity colocalizes with GFAP-, VGLUT2- and VGAT-immunolabelings. A-D. Colocalization of GFAP- and CB1 receptor immunolabelings. **A.** Overview of GFAP- (green), CB1 receptor- (red) and ChAT immunolabelings (blue). Numbered areas indicate magnified and labeled columns of panels B-D. Scale bar: 10 µm. **B.** GFAP-positive areas of panel A. **C.** CB1 receptor immunopositivity. **D.**

Merged image of B and C. Scale bar: 1 μm. **E-H.** Colocalization of VGLUT2- (green) and CB1 receptor-immunopositivity (red) with ChAT-immunolabeling (blue). **E.** Overview of VGLUT2- (green), CB1 receptor- (red) and ChAT-(blue) immunopositivity. Numbered areas indicate magnified and labeled columns of panels F-G. Scale bar: 10 μm. **F.** VGLUT2-positivity of the numbered areas on panel E. **G.** CB1 receptor positivity of the same areas. **H.** Merged images of F and G. Scale bar: 1 μm. **I-L.** Colocalization of VGAT- (green), CB1 receptor- (red) and ChAT-(blue) immunolabelings. **I.** Overview of VGAT- (green), CB1 receptor- (red) and ChAT-(blue) immunopositivity. Numbered areas indicate magnified and labelled columns of panels J-L. Scale bar: 10 μm. **J.** VGAT-positivity of the numbered areas on panel I. **K.** CB1 receptor positivity of the same areas. **L.** Merged images of J and K. Scale bar: 1 μm.

Fig.7. Schematic drawing of endocannabinoid effects on the neurons and astrocytes of the PPN (based on the results of this study and Kőszeghy et al, 2014.) **1.** A depolarized neuron (green, middle) releases endocannabinoids (eCB; yellow arrows). **2.** These endocannabinoids act on astrocytic CB1 receptors (astrocyte: orange, CB1 receptor: red), which activates astrocytes. **3.** Activated astrocytes release glutamate (or increase ambient glutamate concentration with different mechanisms). **4.** This glutamate might act on neuronal extrasynaptic mGluRs, and hyperpolarizes certain neurons by eliciting a tonic outward current via activation of mGluR I (4a) or depolarize others via mGluRII and NMDA receptors (4b). **5.** Besides these mechanisms, the "canonical" presynaptic inhibition of the excitatory and inhibitory inputs of the neurons is also present.

Summarizing the actions above, endocannabinoids might decrease the influence of synaptic inputs of the PPN and support a local spontaneous activity pattern of the PPN neurons.

Besides these suggested pathways, other gliotransmitters and network effects might also contribute to endocannabinoid effects. Furthermore, astrocytic mGluRs might also contribute to the observed actions (indicated with red arrows and question marks).