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Cholinergic and endocannabinoid neuromodulatory effects overlap on neurons of the pedunculopontine nucleus of mice

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Abstract

The pedunculopontine nucleus (PPN) is a part of the reticular activating system (RAS) and one of the main sources of the cholinergic fibers in the midbrain, while it is also subject to cholinergic modulation. This nucleus is thought to have an important role in REM sleep and wakefulness. Several neuromodulatory mechanisms were described in the PPN, but overlaps of the endocannabinoid and cholinergic effects have not been demonstrated yet.

We showed that PPN neurons respond to carbachol in a heterogeneous way: they were depolarized and increase firing rate, hyperpolarized and decrease firing frequency, or lack response. The effect of carbachol was similar to our previous observations with type 1 cannabinoid (CB1) receptor agonists; therefore, we investigated whether different neuromodulatory effects elicit the same action on a certain neuron. A marked but not full overlap was revealed: all neurons depolarized by carbachol were depolarized by the CB1 receptor agonist ACEA, and all neurons lacking response to carbachol lacked response to ACEA, as well. However, neurons hyperpolarized by carbachol were depolarized, hyperpolarized or not affected by the ACEA .

Summarizing our data, we found that certain neurons of the PPN respond to muscarinic and cannabinoid stimulations in a similar but not identical way: the same cells were depolarized or not affected by both drugs, whereas neurons hyperpolarized by carbachol responded to ACEA in a heterogeneous way.

Keywords

Pedunculopontine nucleus, cannabinoids, acetylcholine, neuromodulation

Introduction

The pedunculopontine nucleus (PPN) is part of the reticular activating system and is known as one of the main sources of the cholinergic fibers in the midbrain. Besides the cholinergic neurons, the nucleus is also composed of non-cholinergic cells, which are mostly GABAergic and glutamatergic [1,2].

The PPN does not only provide cholinergic neuromodulation for several brain areas, but it is also target of neuromodulatory mechanisms. Muscarinic and nicotinic cholinergic [3,4,5] serotonergic [6,7], GABAergic [8] effects have been already described, as well as modulatory effects by orexin, ghrelin [9] or endocannabinoids [10,11]. These effects overlap in certain cases: neurons hyperpolarized by serotonin are hyperpolarized by stimulation of muscarinic acetylcholine receptors (mAChR) as well [6], and orexin and ghrelin depolarizes the same neuronal population [9].

The PPN receives cholinergic fibers from the laterodorsal tegmental nucleus and the contralateral PPN [4,12]. Acetylcholine causes neuronal hyperpolarization by activating inward rectifier potassium current via M2 and M4 muscarinic receptors [6,4]. Activation of M1 muscarinic or nicotinic acetylcholine receptor (nAChR) leads to activation of an inward current, whereas a smaller proportion of neurons showed biphasic response or did not respond to cholinergic stimulation [4].

According to our recent observations on midbrain slices, activation of CB1 receptor can depolarize or hyperpolarize neurons eliciting inward and outward currents, whereas a smaller population of neurons lack response. These effects are largely due to activation and glutamate release of astrocytes; which, in turn activates different

subgroups of metabotropic glutamate receptors [11]. The effects of CB1 receptor stimulation resembled the ones elicited by carbachol, where neurons responded with inward or outward currents, or lacked response [4].

Although cholinergic and endocannabinoid neuromodulatory mechanisms elicit virtually identical effects of the PPN neurons, it has not been investigated yet whether these effects activate the same or different populations of neurons. According to the observations of this project, carbachol and ACEA depolarizes the same neuronal population, but the ones hyperpolarized by cholinergic effects can respond to cannabinoid effects in an independent way. Neurons lacking response to carbachol do not respond to ACEA as well.

Materials and methods

Solutions, chemicals

Experiments were performed in an artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl, 125; 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. For the slice preparation, 100 mM NaCl was replaced by sucrose (130 mM) and glycerol (60 mM; low Na aCSF). All chemicals were purchased from Sigma, unless stated otherwise.

Animals, preparation, recordings

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. 9-13 days old C3H mice were used (n = 16).

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). Brain slices were visualized with a Zeiss Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany). Patch pipettes with 5 MΩ pipette resistance were fabricated, 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; Na_3-GTP, 0.3; Na_2- phosphocreatinine, 10; biocytin, 8. Whole-cell patch-clamp recordings were

performed using an Axopatch 200A amplifier (Molecular Devices, Union City, CA, USA). Carbachol was used at 50 μM concentration, whereas arachidonyl – 2'-chloroethylamide (ACEA), a CB1 receptor agonist was administered at 5 μM concentration. Data acquisition was achieved using the Clampex 10.0 software (Molecular Devices, Union City, CA, USA), while data analysis was performed using the Clampfit 10.0 (Molecular Devices) and MiniAnalysis (Synaptosoft, Decatur, GA, USA) programs. For comparing the membrane potentials, a 120-second-long trace segment were selected from the control period and another starting at 3 minutes after the beginning of drug administration or after 10 minutes of washout with control solution. Histograms of the membrane potential values were constructed from these periods and the value corresponding to the largest bin was considered as resting membrane potential [11].

Membrane potential changes within 2 mV were considered as spontaneous fluctuation, and only changes exceeding this cutoff value were considered as depolarization or hyperpolarization [11].

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

Visualization of the labeled neurons

The neurons were filled with biocytin during the electrophysiological recordings. The slices accommodating the filled neurons were fixed overnight (4% paraformaldehyde in 0.1M phosphate buffer; *pH* 7.4; 4 °C). 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). The slices were incubated 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).

Results

Whole-cell patch-clamp recordings were performed in order to characterize the effects of cholinergic stimulation on the membrane potential and the firing pattern of the PPN neurons. To achieve this, 22 PPN neurons were patched. All cells were located dorsally from the superior cerebellar peduncle in its vicinity (within 100 μm), and medially from the lateral lemniscus, both from the pars compacta and pars dissipata. The majority of the neurons (19 out of 22) fired action potentials spontaneously, with a rate of 3.4 ± 0.6 Hz. The average resting membrane potential was -53.7 ± 0.9 mV. Cells responded to 50 μM carbachol in different ways: 10 of them were depolarized with increase of action potential firing frequency and 7 of them were hyperpolarized and decreased firing frequency or completely stopped activity. The rest of the cells (5) did not respond to carbachol (Fig. 1A-F). The magnitude of these changes significantly exceeded the changes of these parameters by spontaneous fluctuations ($p = 0.004$; Fig. 1G). Neuronal location and morphology was determined by post-hoc reconstruction [11]. The absolute value and the heterogeneity of the response to carbachol did not differ significantly from our previous observations with ACEA ($p = 0.054$; Fig1H; [11]). Because of these overlaps, we next investigated whether the responses to the cholinergic and cannabinoid stimulations affect the same or different neurons in the same way. In order to judge this, whole-cell patch-clamp experiments were performed again on 16 PPN neurons, where carbachol was applied first, and, after achieving a good recovery from the effects of carbachol (or washing out carbachol for a similar duration in case when effects were not seen), 5 μM ACEA, a CB1 receptor agonist was applied.

In 7 cases carbachol caused depolarization. On these neurons, application of ACEA resulted depolarization, as well (5 ± 0.6 mV for carbachol and 3.8 ± 0.38 mV to ACEA). No significant difference was found in the magnitude of depolarizations caused by the two drugs ($p = 0.053$). In 4 cases carbachol did not result any change; on these cells ACEA application was ineffective in the same way. 5 neurons were hyperpolarized by carbachol. On these cells, application of ACEA did not show correlation with this effect, showing significant difference from the effect of carbachol: 1 from these cells were depolarized, 1 of them were hyperpolarized and 3 of them did not respond to ACEA (in average, -7.7 ± 1.9 mV for carbachol and 0.48 ± 2.1 mV for ACEA). The difference between the effects of the drugs were proved to be significant in this population ($p = 0.009$). Changes of the firing frequency showed similar tendencies. Those cells which displayed an increase of the firing frequency with carbachol, responded to ACEA in a similar way (1.24 ± 0.26 Hz change to carbachol and 0.91 ± 0.4 Hz to ACEA). The cells having a decreased firing frequency with carbachol, had various responses to ACEA. (-2.17 ± 1.3 Hz change to carbachol and -0.066 ± 0.6 Hz with ACEA; Fig. 2A-D, Table 1.).

Discussion

We revealed that neurons of the PPN respond to carbachol (muscarinic agonist) and ACEA (CB1 receptor agonist) in an overlapping but not fully identical way. With both agonists, neurons were depolarized, hyperpolarized or lacked response. The neurons depolarized or did not respond to carbachol displayed the same response to ACEA, whereas the ones hyperpolarized by carbachol responded to ACEA by depolarization, hyperpolarization or lacked response.

The PPN is formed by heterogeneously reacting cholinergic and non-cholinergic cells. Depending on the behavioral state, certain cells are active only during REM sleep („REM-on") or wakefulness („Wake-on"), other ones increase their firing activity during both wakefulness and REM sleep ("Wake-REM-on"), whereas a small population does not show correlation with sleep or wakefulness [13, 14]. The heterogeneous behavior of the PPN neurons was demonstrated in correlation with the stages of slow wave sleep and gamma-activity, as well [12, 15].

Several agonists can elicit heterogeneous response on the PPN neurons. With application of serotonin, „REM-on" neurons were suppressed, while „Wake/REM-on" cells were not affected [7, 16]. Carbachol activated an inward or outward current on the majority of the thalamic projecting cholinergic PPN neurons, and no effect was observed in a small population [4]. According to our observations, carbachol had the same multiple effect on a mixed population of PPN neurons. Comparing to our previous observations, the baseline membrane potential of the neurons was identical in the populations investigated, but the proportion of the spontaneously firing neurons was

higher in the present study. This indicates that neuronal populations might differ in the two studies [11].

Similar to the effect of carbachol, PPN neurons responded to ACEA in three different ways: several cells were depolarized and increased their firing frequency [11]. On those cells which were depolarized by carbachol, ACEA exerted the same depolarizing effect. It was shown that the postsynaptic excitatory effect of carbachol on the PPN thalamic projecting neurons can be inhibited by the blockade of M1 and nicotinic acetylcholine receptors [4]. Nicotinic acetylcholine receptor is thought to be inhibited directly by anandamide and 2-arachydonoylglycerol independently from CB1 or CB2 receptor activation [17], therefore the involvement of nicotinic receptors in the overlapping depolarizing effect of carbachol and ACEA seems to be unlikely. M1 receptor activation might activate similar mechanisms as CB1 receptor activation, and this might explain the similarity of the effect. A similar overlap between M3 and CB1 receptors via activation of rho-kinase and protein kinase C was described on ciliary muscles [18].

It was revealed in our previous study that CB1 receptor activation can depolarize PPN neurons via stimulating group II metabotropic glutamate receptors, which is activated by the glutamate released from the astrocytes [11]. As an alternative of the activation of signaling pathways shared by M1 and CB1 receptors, group II mGluRs and M1 receptors can activate or inhibit similar conductances, and this might lead to depolarization of the same neuron.

Activation of muscarinic receptors can stimulate endocannabinoid synthesis, as it was shown on cerebellar Purkinje-cells [19] and in the hippocampus [9]. If this is the

case in the PPN, activation of M1 receptor increase endocannabinoid synthesis on neurons possessing it, and, in turn, this endocannabinoid acts on astrocytes [11]. Neurons lacking response to carbachol did not respond to ACEA, as well. It was shown by us that non-responding cells stay silent due to the increased endocannabinoid tone around them [11]. This previous finding either supports the theory of M1-stimulated endocannabinoid release or raises the possibility that there is an increased acetylcholine tone around these neurons in the same time.

The hyperpolarization by carbachol does not overlap with the hyperpolarization caused by ACEA, as the cells hyperpolarized by carbachol respond in a heterogeneous way to ACEA. In accordance with the hypothesis of the M1-mediated endocannabinoid release, one can assume that the neurons hyperpolarized by carbachol possess M2 or M4 receptors, and activation of these receptors might overwrite the heterogeneous effect of endocannabinoids synthesized in an M1-dependent way. As a further possibility, neurons expressing different muscarinic receptors can express different mGluRs, which can also explain our findings.

Conclusions

To summarize our findings, CB1 receptor stimulation has a profound effect on cells of the PPN. Cholinergic and cannabinoid stimulations can depolarize the same cell population, whereas the cells hyperpolarized by carbachol respond to ACEA in an independent way. Overlaps of different neuromodulatory effects might indicate that a common neuromodulatory pathway exists is the PPN.

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Figure legends

Fig. 1. Effects of carbachol (50 μm) on the membrane potential and spontaneous action potential firing of PPN neurons. A. A group of neurons was depolarized and increased spontaneous firing rate. B. Histogram of the membrane potential data points of traces under control conditions (black) and during application of carbachol (gray). The highest peak of the histogram was defined as the "resting" membrane potential. C-D. Certain neurons lacked response to carbachol, showed by a voltage trace (C) and a membrane potential histogram (D). E-F. Other neurons were hyperpolarized by carbachol and ceased spontaneous firing.

G. Statistical summary of the effects of carbachol on the membrane potential (X axis) and firing rate (Y axis; black dots), compared to the spontaneous fluctuations of the membrane potential and firing rate. H. Comparison of the changes of resting membrane potential and firing rate by carbachol (black dots) and ACEA (gray; from Kőszeghy, Kovács et al, 2014; Fig. 1K)

Fig. 2. Effects of sequentially applied carbachol and ACEA on the same neurons. A. An example of a neuron with spontaneous action potential firing (control, first panel), depolarized and increased firing rate with carbachol (CCh, second panel). After washout of carbachol (third panel) ACEA elicited depolarization as well (fourth panel). B. Another neuron, hyperpolarized by carbachol and depolarized by ACEA. C. A third neuron, hyperpolarized by both agonists. D. Statistical summary of the resting

membrane potential changes with carbachol and the sequentially applied ACEA (following washout of the first drug).

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Parameter	**Depolarized cell (n = 7)**	**Hyperpolarized cell (n = 5)**	**Non-responding cell (n = 4)**
Change of the RMP to carbachol (mV)	5 ± 0.6	-7.7 ± 1.9	0.4 ±0.2
Change of the RMP to ACEA (mV)	3.8 ± 0.4	0.5 ± 2.1	0.75 ± 0.6
Change of the firing frequency to carbachol (Hz)	1.2 ± 0.3	-2.2 ± 1.3	0.15 ± 0.1
Change of the firing frequency to ACEA (Hz)	0.9 ± 0.4	-0.1 ± 0.6	-0.15 ± 0.5

Table 1. Data of cell groups defined according to their response to carbachol. All changes were compared to control.