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Cholinergic neurons of the pedunculopontine nucleus as targets of orexinergic neuromodulation

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INTRODUCTION

Anatomy, cell types and connectivity of the pedunculopontine nucleus

The pedunculopontine nucleus (PPN) is a structure in the midbrain extending in a rostrocaudal direction bordered medially by the superior cerebellar peduncle, laterally by the lateral lemniscus, and rostrally the substantia nigra. The parabrachial nucleus provides the caudal borders of the nucleus while the PPN is surrounded by the cuneiform and precuneiform nuclei in the dorsal direction. The pars dissipata corresponds to the rostral while the pars compacta corresponds to the caudal part of the PPN. In addition to numerous cholinergic cells it contains GABAergic and glutamatergic neurons, which appear in different proportions and densities along the PPN rostrocaudal axis. The number of cholinergic and glutamatergic neurons increases in the caudal region of the nucleus whereas that of the GABAergic neurons increases in the rostral direction.

The PPN receives inputs from several regions such as the spinal cord, superior colliculus, nucleus propositus hypoglossi, entopeduncular nucleus, subthalamic nucleus, substantia nigra pars reticularis and the cortex. The axon collaterals of cholinergic neurons follow ascending dorsal and ventral pathways. The dorsal pathway innervates the superior

and inferior colliculi and the thalamus. Synapses of the ventral pathway are detectable in some regions of the basal ganglia, and the substantia nigra as well as in the regions of ventral tegmental area, subthalamic nucleus, lateral hypothalamus, ventral pallidum, amygdala, mediolateral septum and the striatum. The descending projections of the cholinergic neurons provide innervation of the nucleus pontis oralis, the gigantocellular nucleus and the spinal cord. The descending projections of cholinergic neurons provide innervation of the oral pontine reticular nucleus, the gigantocellular nucleus, and the spinal cord. A great deal less information is available on the synapses of the GABAergic and glutamatergic neurons. Their axons possess less collaterals and their pathways are restricted to the midbrain structures and the caudal region of the brainstem.

Physiological roles of the PPN

The PPN is part of the reticular activation system (RAS), which plays a role in, among other things, regulating the sleep-wakefulness cycle and movement as well as sensory gating. During wakefulness and REM sleep PPN cholinergic neurons fire at higher frequencies than during slow-wave sleep, thereby showing that RAS plays a role in the regulation of the cortical active state. However, it has been demonstrated that damage to the nucleus causes no changes in the full sleep-wakefulness cycle. The question then arises what role cholinergic neurons of the nucleus may play in determining sleep-wakefulness cycles. Examination of the activity of the cholinergic neurons of the nucleus in *in vivo* experimental conditions revealed that their activity is episodic, contradicting the assumption that tonic activity is necessary to maintain wakefulness. It is the M-current and the astrocyte dependent as well as endocannabinoid-mediated processes that are responsible for the transient nature and control of the cholinergic neurons. In addition to the cholinergic neurons the GABAergic and glutamatergic neurons of the PPN also play a role in wakefulness state and cortical activation.

The role of the PPN in sensory gating can be observed on the startle reflex and its prepulse inhibiton. The startle reflex is a sudden, rapid motor response to potentially harmful effects. Reflex amplitude

decreases in progressive supranuclear palsy, while in schizophrenia a decrease in prepulse inhibiton is observed. Using selective optogenetic activation of cholinergic neurons it has been demonstrated that stimulation of PPN cholinergic neurons increases startle reflex amplitude without influencing prepulse inhibition. Activation of non-cholinergic neurons is likely to be responsible for the latter.

It is a well-known fact that the PPN is part of the mesencephalic locomotors region. Stimulation of this region inn different locations creates diverse movement patterns. It has been demonstrated in electrophysiological experiments that this may be attributed to the stimulation of the different neuron subpopulations. Stimulation of the PPN inhibits movement by decreasing muscle tone, while the cuneiform nucleus situtated dorsally to the PPN increases muscle tone and stimulates movement.

Functional classification of PPN neurons

Examination of the different subgroups of the PPN has been conducted by several workgroups. These examinations used primarily NADPH diaphorase, neuronal nitrogen-monoxide synthetase (bNOS) and choline acetyltransferase ChAT) immunohistochemical

identification. These methods made cholinergic neurons visible; however, no examinations have previuosly been carried out on transgenic mice.

Cell-level classification

Based on the membrane properties of neurons, in 1990, Kang and Kitai identified low threshold spiking (LTS) cells and named them Type I cells, while the neurons with A-current (transient outward current) and LTS were identified as Type II. A third group was also established for cells that did not possess either of the above properties. In 1988 Leonard and Llinas also established three categories. In their classification, Type I contained neurons that had LTS, Type II contained neurons with A-current and Type 3 contained neurons that possessed both properties that the other two types had. Classition by Takakusai et al., and Ye et al. was in accordance with the 1988 grouping. Takakusaki et al. classified cell groups that simultaneously possessed both A-current and LTS as II/2, while they belonged to Type III in Ye et al.'s classification. The latter workgroup named neurons that did not have either of the above properties Group IV. In addition to the above properties (A-current and LTS) M-current (slowly activating, non-inctivating voltage-gated potassium current) and HTO (High

threshold membrane potential oscillation) are also important markers of cholinergic neurons. The presence of these properties in cholinergic neurons alone was demonstrated by Bordás et al. in 2015.

In vivo electrophysiological classification

In vivo classification is based on the firing properties of neurons and their cortical activity, which served to distinguish two main groups of cholinergic neurons. The firing frequency of cholinergic neurons during the active periods of cortical slow wave activity and gamma activity was low (0.9 Hz). On the other hand, high action potential firing frequency was found (31 Hz) in neurons firing primarily during the second half of the passive periods of slow wave activity and were independent of gamma oscillations. Non-cholinergic neurons fell into one of three populations: „silent neurons", „tonically firing neurons", and „irregularly firing" neurons. A further classification of PPN neurons can be carried out in terms of their relationship to REM and non-REM sleep. The main groups are as follows: neurons active during wakefulness and REM sleep, neurons active during REM sleep and neurons active during wakefulness.

Astrocyte-neuron communication

Astrocytes are cells that belong to macroglial cells and play a role in the functioning and regulation of neurons. One of their recently recognised tasks is the regulation of synapses, playing a role as a third component in a so-called „tripartite synapse". They also participate in the setting of synaptic neurotransmission. They also release gliotransmitters. They contribute to neuronal homeostasis, regulate potassium ion concentration and mediate uptake and elimination of several important neurotransmitters, including glutamate. They contribute to certain neuromodulatory effects and synchronisation of neuronal networks. In addition to their physiological role, their pathological significance is also known; they contribute to the development of several diseases (including neuropsychiatric and neurodegenerative disorders). Astrocytes are not electrically excitable, are incapable of action potential firing, however, by changing the intracellular calcium concentraton, they generate calcium waves, resulting in the release of various gliotransmitters. One of the most important gliotransmitters is glutamate. By regulating extrasynaptic neurotransmitter concentration astrocytes are able to influence neuron excitability.

Astrocyte-dependent currents stimulating neuronal excitability

Slow inward currents (SIC) are phasic extrasynaptic excitatory events that are consequences of astrocyte activation, distinguished from excitatory postsynaptic currents (EPSC). SICs have larger amplitudes as well as slower rise and decay times than EPSCs. SICs are thought to be consequences of extrasynaptic, GluN2B-containing NMDA receptors that are freely detectable in the central nervous system. Astrocyte stimulation elicits generation of SICs on neighbouring neurons while astrocyte inhibition prevents their generation. These events are related to changes in the intracellular calcium concentration of astrocytes. Our workgroup has demonstrated that, in the presence of TTX, the frequency of SICs does not change but its amplitude decreases in the PPN, which suggests that SICs are probably consequences of astrocyte-neuron interactions. Kinetics of the event are greatly affected by gliotransmitter concentration and the number of transmitter receptors.

Tonic inward currents are slow, non-adapting currents, and their relationship with astrocytes is less straightforward. They are thought to be consequences of mainly, although not exclusively, extrasynaptic glutamate- and glycine-mediated events and to occur simultaneously with SICs.

Features of PPN SICs and neuromodular effects exerted on them

Like in other parts of the brain, SICs generated on PPN cells are characterised by slower kinetics and larger amplitudes than excitatory postsynaptic currents. The features of PPN slow inward currents are as follows: (1.) They are consequences of astrocyte activation, consequential glutamate release and activation of extrasynaptic, GluN2B-containing NMDA receptors. (2.) PPN SICs occur in both cholinergic and non-cholinergic neurons in the same proportion, thus they are not related to the neurochemical cell types of PPN neurons. (3.) SICs do not synchronise neighbouring neurons. (4.) Their occurrence greatly depends on the neuron-astrocyte distance. (5.) – Neuromodulatory actions on SICs depends on the original SIC activity. (6.) The neuromodulatory effects observed on SICs are likely consequences of NMDA receptor desensitisation.

Orexinergic neuromodulation

Orexins also affect PPN cells. Two types of this peptide, released from orexinergic neurons in the lateral hypothalamus are known: orexin-A and orexin-B. It exerts its effect through Gq-protein coupled receptors, OXR1 and OXR2, which are present in different

parts of the brain. It was originally identified as a hormone related to food uptake. Today we are familiar with its role in the regulation of sleep and wakefulness. Furthermore, it is also recognised as a participant in several pathophysiologocal processes, including narcolepsy. In mice, loss of prepro-orexin leads to a condition similar to human narcolepsy with daytime sleepiness and cataplexy. Loss of OXR2 resulted in milder symptoms but lack of OXR1 did not affect wakefulness stability. In people with narcolepsy there was a marked lack of orexinergic neurons and decrease in the quantity of orexin. Proopiomelanocortin (POMC) and the NPY-expressing neurons of the arcuate nucleus provide neuronal inputs to orexinergic neurons. This indicates a relationship between the orexinergic system and food uptake. The fact that calory intake decreases in patients with narcolepsy while body mass index increases also suggests some further relationship. In addition to the above, several other roles have been attributed to orexin, of which the one related to reward should be highlighted. The orexinergic neurons project into several areas of the reward system, including locus coeruleus, the ventral tegmental area or nucleus accumbens.

OBJECTIVES

Our aim was to examine whether the earlier *in vitro* electrophysiological classification of PPN neurons, which was primiarily based on the immunohistochemical properties of cholinergic neurons, are indeed valid in cholinergic neurons identified using transgenic techniques. We sought to answer the following questions:

1. Is tdTomato really expressed in cholinergic neurons?
2. Can functional subgroups according to former categories be identified in transgenic mice?
3. Does a new functional classification of cholinergic neurons, different from earlier groupings exist?
4. Do the neurons that have different functional properties possess a rostrocaudal gradient?
5. Are the different functional and morphological properties of cholinergic neurons related to one another?

Further, we wished to examine the effect of orexin as a neuromodulator affecting PPN neuron, including:

1. We wished to examine the effect of orexin on SICs.
2. We looked for the existence of a common astrocyte-dependent neuromodulatory action.

MATERIALS AND METHODS

Solutions

In our experiments artificial cerebrospinal fluid (aCSF) with a pH of 7.4 was used while the midbrain slices were prepared in ice-cold low Na+ artificial cerebrospinal fluid („low Na aCSF"), where 95 mM NaCl was replaced by glycerol (60 mM) and sucrose (130 mM). For the recordings, tetrodotoxin (TTX)-citrate, $CdCl_2$, orexin-A, 2,3dihidroxi-6-nitro-7-sulfonamil-benzol[f]quinoxalin (NBQX) D-(-)-2-amino-5-phosphono pentanoate (D-AP5), bicucullin and strychnine were used. As the internal solution of the pipette used for recordings a potassium-gluconate based solution with a pH of 7.3 was used and biocytin was added immediately before the recordings.

Animals, tissue preparation

In our study 9-22 day-old mice were used (n= 92). A larger group of transgenic mice expressed tdTomato fluorescent protein in a choline acetyltransferase-dependent way (ChAT-tdTomato), another group expressed tdTomato in Type 2 vesicular glutamate transporter-dependent way (Vglut2-), and a smaller group in a glial fibrillary acidic protein-dependent way (GFAP-tdTomato). For mouse crossing the cre-lox system was used. In this system, one parent is involved in the

promoter-specific expression of cre-recombinase and the other parent's genome has the protein to be expressed whose transcription was inhibited by a stop codon surrounded by lox-P sites. For the crossing, as a result of the cleavage of the stop codon, the protein occurs in a promoter-specific way. The mice were decapitated, the brains were carefully removed and, using a vibratome, 200 μm coronal slices were prepared. During the entire process the slices were kept in ice-cold low Na^+ aCSF. Following the fast preparation, the slices intended for use were kept in normal aCSF for 60 minutes at 37 ºC and oxygenated.

Electrophysiology

Whole-cell patch-clamp recordings and data acquisition were performed in magnesium-free naCSF at room temperature containg the following systems: Axopatch 200A amplifier, Zeiss Axioskop microscope with fluorescent imaging system with further components: Polychrome V5 light source and a CCD camera with an imaging unit. Recording and data acquisiton were carried out using Clampex 10 software while for analysis Clampfit 10 programme was used. The slices were continually washed in oxygenated aCSF during the recordings, too, uisng a peristaltic pump. Patch pipettes with 6–8 MΩ pipette resistance were prepared using Narishige P-10 puller.

The following recordings were performed in current-clamp configuration, using 1 s current steps between -30 pA and +120 pA, in 10 pA increments. Resting membrane potential was kept at -60 or -80 mV. Input resistance, maximum firing frequency, average firing frequency, action potential delay, adaptation index, existence and amplitude of low threshold spikes and rebound spikes were recorded. For high threshold membrane potential oscillations 2 s, ramp depolarising current injection rising from 0 pA to 800 pA was used. In some cases simultaneous recordings were made on the somata and proximal dendrites of the same cholinergic cells. A-current and all orexin-dependent events were recorded in voltage-clamp configuration.

Neuron reconstruction

To visualise cholinergic cells biocytin was used. It was placed in the internal pipette solution immediately before the measurement and then reached the inside of the cell during the recordings. After the recordings the slices were fixed overnight . Antigen retreval was performed in Tris Buffered saline solution completed with 0.1% Triton-X-100 and 10% bovine serum for 60 minutes. The slices were incubated for 90 minutes using Alexa 488 conjugated streptavidin, and then photomicrographs were taken using a Zeiss LSM 510 confocal microscope. Cells were reconstructed using Neurolucida software while

to record and visualise topography we relied on the contours and parameteres of the Paxinos mouse brain atlas.

In three cases choline acetyltransferase immunohistochemistry was used on slices prepared from transcardially perfused ChAT-tdTomato mice with a view to assessing the overlap between tdTomato expression and ChAT immunopositivity.

RESULTS

There is considerable overlap between ChAT immunohistochemistry and tdTomato expression

We carried out our invstigation in 3 ChAT- tdTomato mice by manually analysing 379 cell bodies. 98± 1% of tdTomato positive cell bodies were found to be ChAT positive. When all cells that were either tdTomato expressed or ChAT-immunopositive were considered, 86,8% ± 3,5% of the examined cells overlapped. 11,9 ± 3,3% of the examined cells were only ChAT immunopositive while 1,76 ± 0.9% showed only tdTomato expression. Thus, tdTomato expression proved to be a suitable marker that can be efficiently used to identify cholinergic neurons.

There is a correlation between the orientation of neuron dendrites and the existence of LTSs

We examined the orientation of dendrites originating from the somata in 17 neurons, 4 of which had a bipolar dendritic tree, in other words, the two longest processes were arranged in opposite directions. The rest of the neurons were multipolar with the dendrites pointing in

more than two directions. As a relationship between the orientation of dendrites and the functional properties of neurons we found that, cells with LTS are bipolar while those without LTS tend to be multipolar.

We classified a total of 91 genetically identified cholinergic neurons along the following lines:

- Type I neurons: had low threshold depolarising spikes (LTS) and rebound spikes.
- Type II neurons: possessed A-current and were characterised by hyperpolarisation-induced delay.
- Type III neurons: were characterised by LTS and A-current as well as hyperpolarisation-induced delay.
- Type IIIK neurons: had none of the above properties.

We were able to clearly determine rostrocaudal localisation of the somata in 33 cases. 12 of these were Type I neurons, 45.45% belonged to Type II, 21.21% to type III, and another 21.21% to Type IIIK. We identified 13 neurons in the pars compacta of the PPN, in which the dominant type of neurons were Type II with 69.2%, Types I and III neurons were represented by 7.69% each and Type IIIK by 15.38%. In the rostral part of the PPN we identified 20 neurons, of which 15% were

Type I neurons, 30% Type II. and Type III, respectively, and 25% were Type IIIK.

There were relatively few neurons (15.4%) in the pars compacta of the PPN that had LTS or rebound firing (Types I and III). The corresponding ratio in the pars disspata was 47.3%.

Type III neurons have smaller maximum frequencies and smaller average firing frequencies

Type III. neurons had significantly smaller maximum firing frequencies than did Types I and IIIK. In Type III neurons maximum firing frequency was 16.49 ± 1.99 Hz, in Type I it was 30.76 ± 5.04 Hz, in Type I. neurons it was 23.99 ± 1.69 Hz with 32.94 ± 7.44 Hz in Type IIIK. We obtained similar results with regards to average firing frequency. Average firing frequency of Type III neurons was 7.06 ± 1.54 Hz during 100 pA current injection, compared with 14.16 ± 3.24, 11.3 ± 0.94, and 12 ± 2.3 Hz in Type I, II, and IIIK groups, respectively.

Neurons with A-current can be either early or late firing

We further classified neurons that were characterised by firing delays larger than 50 ms at resting membrane potential. Neurons with A-current can be classified into two subgroups:

1. The group of early firing neurons, where the occurrence of the first action potential from the beginning of the stimulus was less than 250 ms.
2. The group of late firing neurons, where latency was longer than 250 ms. Thus the second group represents a smaller subgroup of cholinergic neurons with A-current that are located caudally.

Average latency in the pars compacta was 205.3 ± 59 ms compared with 71.4 ± 15.5 ms in the pars dissipata. This can lead us to conclude that average latency in the caudal neurons was significantly longer than the examined rostral neurons.

High threshold membrane potential oscillations

Our workgroup has previously demonstrated that HTOs are a characteristic feature of cholinergic neurons and this study has confirmed that finding.

Oscillation frequency of the neurons located in the caudal part of the PPN was higher (23.07 ± 4.9 Hz) and their amplitudes were low (6.59± 3.8 mV^2/Hz power), while in the rostral part of the PPN frequency of oscillation was low (12.08 ± 2.01 Hz), with greater amplitudes (18.85 ± 5.08 mV^2/Hz power). In order to confirm the relationship between firing frequency and oscillation frequency we used $CdCl_2$ in our later recordings to block oscillations. Parallel to the inhibition of oscillation activity, the firing frequency of the action potentials increased significantly from 6.71 ± 104 to 15.38 ± 1.42 Hz.

We looked for the place of origin of the oscillations while making simultaneous dendritic and somatic patch clamp recordings. Oscillation on the dendrites was characterised by lower (9.77 ± 6.57 mV^2/Hz) power compared to the data of the somata (20.13 ± 8.55 mV^2/Hz power) and frequency was almost identical on the two (1,41 Hz). It is clear that the value of oscillation power recorded on the dendrites was 39.7 ± 12% of those recorded on the somata with frequency being practically the same (102 ± 11%). This ratio

corresponds to that found during the passive spread of the electric impulses from the somata to the dendrites.

The effect of orexin on tonic currents, EPSCs and low threshold oscillation activity

The neuromodulatory actions (muscarinic cholinergic, serotoninergic, and cannabinoid) that have been examined so far have, at least partly in an astrocyte-dependent way, elicited tonic inward currents on some neurons and tonic outward currents on some other neurons. In contrast with the above neuromodulators, the only effect orexin exerts is depolarisation of PPN neurons. During our experiments we examined whether orexin, which affects tonic currents in a way different from that of other examined neuromodulators, also affects SICs differently from other neuromodulators.

We conducted all our experiments involving orexin in a nominally magnesium-free solution in order to make the spontaneous occurrence of SICs more likely. Using 200 nmol/l orexin-A we recorded a -17.32 ± 3.11 pA amplitude tonic inward current, which was significantly different from the -0.98 ± 3.11 pA amplitude fluctuation of the holding membrane potential recorded in control conditions. Parallel to the development of the tonic current sEPSC frequency increased

significantly. There was no significant increase in sEPSC amplitude but it did show an increasing tendency.

sEPSC frequency increased to 279 ± 41% of the control during orexin application, and its amplitude rose to 119 ± 12% of the control. Orexin also activated noise-like low threshold oscillations. Fittings the power spectra of currents under control conditions and with orexin with a single exponential function were significantly different.

In the case of 8 neurons no SICs occurred either in controls or during orexin use. In a further 13 cases orexin changed the appearance of SICs. In the 9 cases when SIC activity was low in controls, orexin changed the frequency from 0.057 ± 0.031/minute to 0.34 ± 0.08/minute, and increased charge transfer by SICs from 0.49 ± 0.26 pC/minute to 5.6 ± 2.1 pC/minute. In the four other cases where SIC activity was already high in the control (charge movement was above 2 pC/minute) orexin caused a significant decrease in SIC frequency and charge movement. Frequency decreased to 52 ± 12% of the control (from 0.52 ± 0.05/minute to 0.28 ± 0.12/minute), with the corresponding decrease in charge movement being -32 ± 10% of the control (from 60.95 ± 30.6 pC/minute to 22.7 ±12.4 pC/minute).

When we plotted changes in the charge transfer of SICs against control charge transfer, fitting of the points revealed a linear

relationship. When we plotted changes in the charge transfer of SICs as a function of changes in sEPSCs or the emerging tonic currents, we found no or only weak linear correlations.

Comparison of the effect of orexin on SICs and the formerly published effects of other neuromodulators (muscarinic cholinergic, serotoninergic, and cannabinoid) on SICs showed that the effect of orexin on SICs did not differ from that of other neuromodulators. In sharp contrast, however, we found that while other neuromodulators can even evoke inward and outward tonic currents, orexin was characterised by a strong dominance of inward tonic currents.

DISCUSSION

In this dissertation we sought to answer the question whether the „canonical” *in vitro* electrophysiological classification of PPN cholinergic neurons is valid for cholinergic neurons identified using transgenic techniques. We also investigated whether it was possible to establish a new classification or possibly a rostrocaudal gradient of functional properties. Another important question was whether orexinergic neuromodulation had an astrocyte-dependent component. If so, we wanted to find out whether this component was identical to the ones experienced during other neuromodulatory effects.

In short, we concluded that the „canonical” electrophysiological classification of PPN cholinergic neurons is, indeed, valid for cholinergic neurons identified using transgenic techniques, too. We demonstrated the existence of two further groups of cholinergic neurons with A-current: the categories of early and late firing neurons. We demonstrated that early and late firing neurons as well as neurons showing low and high frequency membrane potential oscillation were present in different proportions along the rostrocaudal axis of the nucleus.

Further, we demonstrated that, in terms of its effect on SICs, the part of orexinergic neuromodulation through astrocytes is identical to that of other neuromodulators. The neuromodulatory effects on SICs probably form an aspecific, common component of the effects that change the activity of the neurons of the nucleus.

Functional classification of PPN cholinergic neurons

Based on our results we concluded that the ChAT-tdTomato mouse model is suitable for the examination of the functional properties of cholinergic neurons and its use enabled us to confirm the existence of previously described functional groups. Furthermore, we confirmed that cholinergic neurons were characterised by high threshold membrane potential oscillation. We also demonstrated that it determines action potential firing frequency.

We demonstrated that the diverse functional groups of cholinergic neurons occur in different proportions in the caudal and rostral parts of the PPN. LTSs rarely elicit action potential in the cholinergic neurons of the PPN and this phenomenon was mostly found on rostrally located PPN neurons. We further classified neurons with A-current into two subgroups. These are early and late firing neurons, of which the latter tended to be detected in the caudal part of the PPN.

HTOs that had higher frequency and lower power were detected on the caudal part of the PPN whereas HTOs with higher power and lower frequency tended to occur in the rostral part. In addition, we concluded that HTOs showed higher amplitudes on somata than on proximal dendrites.

Previous studies have used NADPH diaphorase, bNOS and ChAT immunohistochemical methods to identify cholinergic neurons. However, examination of membrane properties in genetically identified PPN cholinergic neurons has not taken place yet. In our experiments a negligible number of tdTomato-positive neurons were found to be ChAT-negative during immunohistochemical examinations. We can claim, in accordance with the literature, that the ChAT–tdTomato mouse model can be effectively used in the examination of the functional parameters of neurons.

Neurons in the PPN have traditionally been classified into 3 large groups based on their membrane properties. We integrated and unified the diverse markings in the literature, creating a useful classification. Type I neurons possess LTS caused by calcium conductances. Type II neurons have A-current. Type III neurons possess both properties, whereas Type IIIK comoprises neurons that possess neither.

Roughly in accordance with literature data, our experiment carried out on transgenic mice revealed that only a minority (12%) of the examined neurons belonged to Type I, and most (48%) belonged to Type II. In our data analysis we were the first to establish that a greater proportion of Type II neurons were present in the caudal part of the PPN with one third of rostrally located neurons belonging to this subgroup. In addition to the parameters used for grouping the only functional difference between the functional goups was that Type III neurons showed lower maximum firing frequency as a result of polarisation.

In our study the presence of LTSs also showed a rostrocaudal gradient, and was a feature of rostrally located neurons. We found a further important relationship in relation to LTSs: neurons with LTSs possessed a bipolar dendritic tree, while those without LTSs tended to be multipolar.

Our results have led us to conclude that the „canonical" classification used in the literature can still be used to describe the functional membrane properties of PPN neurons. The combination of the rostrocaudal differences of the presence of LTS and the low firing frequency of Type III neurons may suggest that the function of cholinergic neurons along the rostrocaudal axis may be different.

A feature of Type II and III neurons is that they possess A-current, a transient, voltage gated potassium current which is known to

cause action potential delay compared to the beginning of the depoliarising stimulus. According to literary data, A-current latency varies between 50 and 500 ms. In the course of our examinations we classified PPN cholinergic neurons with A-current into early and late firing types. In addition, we could detect a correlation in the PPN between A-current kinetics and delay of the action potential delay. As a further rostrocaudal difference we established that the late firing neurons tended to be located in the caudal part of the PPN. In addition to the role of the A-current in physiological action potential firing it is assumed to contribute to various patho-physiologocal processes, too. It has been found that the amplitude of the A- current of cardyomyocytes decreased in artificially induced inflammatory conditions. Accordingly, we can assume that neuroinflammatory processes can also affect the firing pattern of PPN cholinergic neurons by reducing A-current amplitude and action potantial delay contributing to changes in sleep-wakefulness cycles.

High threshold oscillations can be detected in PPN cholinergic neurons. Literature data suggest that oscillation frequency varies between 4-16 Hz and 4-80 Hz across theta and beta domains. A characteristic feature of PPN cholinergic neurons is high-frequency oscillation regardless of „canonical” functional subgroups or morphological properties. Confirming literary data, in genetically

identified cholinergic neurons we detected low-frequency, high-amplitude oscillations in the rostral part of the PPN. Cholinergic neurons with a caudal location were characterised by low-amplitude, high frequency oscillations. Our simultaneous recordings revealed that oscillation activity recorded on somata appears on the dendrites with lower amplitude but identical frequency and time. This led us to assume that the location of the emergence of the HTO is the soma, and the oscillation spreads passively towards the dendrite.

We have also demonstrated that the frequency of HTOs is directly proportinal to the average firing frequency of the same neuron and inhibition of HTOs significantly increases firing potential. We find it probable that The relationship between membrane potential oscillations and action potential firing frequency is likely to be that the same ion channel set is responsible for both processes.

As a summary we can conclude that we have confirmed existence of HTOs and demonstrated their rostracaudal differerences. It is likely that HTOs are generated in the somata rather than in the dendrites and are able to determine neuron action potential frequency.

The diverse projections of the PPN are known to be topographically organised. Caudal PPNs provide input for the ventral tegmental area and the dorsomedial striatum while the rostral PPN innervates the area of substantia nigra pars compacta as well as the

dorsomedial striatum. These facts are in accordance with the already known differences between the caudal and rostral areas of the PPN. In our study we have demonstrated that several membrane properties possess rostrocaudal distribution. The presence of LTS is typical of cholinergic neurons in the rostral location whereas in the caudal area we found late-firing cholinergic neurons with slow A-current. In addition, the presence of HTOs (and the consequential firing frequency) also showed a rostrocaudal distribution. These *in vitro* rostrocaudal differences may account for the differences encountered in *in vivo* conditions. We assume that the *in vitro* results that we have demonstrated promote the design and assessment of further *in vivo* experiments that use a transgenic mouse model. We also hope that the above data contribute to the understanding of the different outcomes of deep brain stimulation used therapeutically in different areas of the PPN.

Investigtion of the effects of orexinergic neuromodulation on the PPN

As a continuation of our laboratory studies, we examined the effect of orexin on the tonic neronal currents, EPSCs, and SICs of the PPN, and looked for correlations between these parameters and the

results of our earlier research exploring other neuromodular effects exerted on SICS.

We found that, elicited by orexin, SIC activity increased when control activity was low, while in the case of high SIC activity in the control, it decreased. The effect of orexin on SICs was independent from its effects on EPSCs and tonic inward currents. We found one important difference between orexin and other neuromodulators: orexin almost never elicited tonic outward current.

It can be a matter of debate whether the astrocyte activation eliciting SICs is a direct or indirect effect of orexin. The orexinergic effect on SICs might also be a consequence of direct astrocytic action. It is well known that OX1R and OX2R receptors can also be detected in astrocyte cell cultures but there were no OX1R receptors in *in situ* PPN astrocytes, only in neurons. Since OX2R can be found on both neurons and astrocytes in the PPN, it is plausible that their direct activation leads to the appearance of SICs. However, we can also assume that astrocytes react to the increased average neuronal activity.

Another important issue is whether the effect of orexin on SICs can be regarded as a model of physiological phenomena or it respresents pathophysiological mechanisms. The number of orexin receptors and glutamate transporters increases in certain neurodegenerative diseases and cerebral ischemia and can play a protective role in these processes.

We assume that SICs and orexinergic effects on SICs are present in physiological conditions, too, but probably intensify in pathological conditions.
The effects of various neuromodulators and agonists on SICs depend on the SIC activity of the control. A possible explanation for this is that astrocytes, responding to increased neuronal effects under the effect of neuromodulators, increase extrasynaptic glutamate concentration. If extrasynaptic glutamate concentration was originally low, the increase in glutamate level activates extrasynaptic NMDA receptors, leading to an increase in SIC activity. If original extrasynaptic glutamate concentration was high, a further increase in glutamate level results in NMDA receptor desensitisations. .

Our results support the theory that SICs are uniformly modulated by various neuromodulatory mechanisms in the PPN, contributing to the emergence of a common, astrocyte dependent component of neuromodulatory mechanisms. This astrocyte dependent component is presumably not or only partly is a consequency of the direct activation of astrocytes, instead, it is a response to the neuronal activity altered by neuromodulators. SICs in the PPN might be part of an astrocyte dependent homeostatic regulation.

SUMMARY

The pedunculopontine nucleus is a cholinergic nucleus of the reticular activation system. Its physiological significance lies in the fact that it plays an important role in the regulation of sleep-wakefulness cycles, sensory gating, and movement regulation.

Although the nucleus is known as a cholinergic structure, large numbers of GABAergic and glutamergic neurons are also present there. Several attempts have been made to classify PPN neurons. The classification based on *in vitro* experiments distinguished 3 or 4 functional groups based on the presence of A-current and low threshold membrane potential dendrites.

In the first part of this work we revised an earlier grouping based on *post hoc* neurochemical cell identification using cell identification based on transgenic technique. We concluded that cholinergic neurons with A-current can further be divided into two subgroups: that of early-firing and late-firing neurons. We also demonstrated that A-current, low-threshould spikes and high-threshold membrane potential oscillations all showed varying distribution along the rostrocaudal axis. The differences along the rostrocaudal axis can be a cell-level elecrophysiological explanation for the locally different outcomes of deep brain stimulation in Parkinson's disease.

The PPN is a target for several neuromodulatory mechanisms affecting sleep-wakefulness cycles. In our earlier work we demonstrated that cholinergic, serotoninergic and cannabinoid neuromodulatory effects had overlapping, astrocyte-dependent components, too. Some of these were tonic inward and outward currents evoked by astrocyte activity and the composite effect on phasic slow inward currents (SICs).

Orexinergic neuromodulation somewhat differs from the ones studied so far since it known to cause the homogenous depolarisation of neurons rather than the different tonic effects. Our aim was to examine the effect of orexin on SICs. We concluded that if starting SIC activity was low, it was increased by orexin, whereas if SIC activity was high in control conditions, it was decreased by it. This phenomenon probably caused by NMDA receptor activation or desensitisation was the same as all neuromodulatoty effects that we examined. The observed phenomenon is a probably aspecific, common, astrocyte-dependent component of the neuromodulary actions which might be capable of regulating neuronal desychronisation.

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List of publications related to the dissertation

1. **Baksa, B.**, Kovács, A., Bayasgalan, T., Szentesi, P., Kőszeghy, Á., Szűcs, P., Pál, B.: Characterization of functional subgroups among genetically identified cholinergic neurons in the pedunculopontine nucleus.
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2. Kovács, A., **Baksa, B.**, Bayasgalan, T., Szentesi, P., Csemer, A., Pál, B.: Orexinergic actions modify occurrence of slow inward currents on neurons in the pedunculopontine nucleus.
Neuroreport. 30 (14), 933-938, 2019.
DOI: http://dx.doi.org/10.1097/WNR.0000000000001298
IF: 1.146 (2018)

List of other publications

3. Gebri, E. Z., Kiss, A., Hegedűs, C., **Baksa, B.**: Symptoms of acute leukemias in the oral cavity.
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