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Neuromodulatory actions on ion channels of neuronal populations of the reticular activating system

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INTRODUCTION

The reticular activating system

The reticular activating system (RAS) is a part of the reticular formation in the brainstem. It sends ascending projections to the hypothalamus and the thalamus, as well as directly to the cortex for activation of awake, desynchronized cortical EEG patterns and to affect behavioral modulation. The RAS is composed of three main cell groups, which consists the cholinergic pedunculopontine nucleus (PPN) and its medial partner, the laterodorsal tegmental nucleus (LDT); the noradrenergic locus coeruleus (LC), and the serotonergic raphe nuclei (RN). The RAS receives inputs from all afferent sensory systems in parallel to primary afferent sensory projections.

Cholinergic structures of the basal forebrain and midbrain

Immunohistochemical labeling studies have demonstrated four major groups of ChAT-positive neurons: (1) in the striatum; (2) in the magnocellular basal nucleus; (3) in the pontine tegmentum; and (4) in the cranial nerve motor nuclei. The well known brainstem and forebrain cholinergic structures are also grouped into six main central pathways (Ch1–Ch6) associated to the origin of the nuclei where the cholinergic fibres arise Ch1-Ch6.

The most abundant cholinergic neuronal populations are found in the basal forebrain (BF) and centered on the output of the nucleus basalis. The human nucleus basalis comprises approx. 200,000 neurons. As a complex structure, the cholinergic basal forebrain plays an

important role in attention, learning, memory, perception, and consciousness.

One of the main cholinergic areas of mesopontine is the pedunculopontine nucleus (PPN) which is not only the source of cholinergic fibers but receives cholinergic fibers from the neighboring laterodorsal tegmental nucleus (LDT) and the contralateral PPN. Apart from cholinergic fibers from neighboring structures, local cholinergic collaterals exist in the nucleus. The PPN is composed of cholinergic and non-cholinergic neurons (GABAergic, glutamatergic neurons), which present different activity pattern during modulation of global brain states. The PPN shows different neuronal activity pattern in slow wave sleep (SWS), paradoxical sleep (PS) and wakefulness (W).

Cell types and markers of PPN

Morphological markers. The PPN consists of at least three main neuronal populations such as cholinergic, GABAergic, and glutamatergic neurons and a variety of other neuronal markers, including calcium binding proteins and neuropeptides. The density of GABAergic and glutamatergic neurons has a strong gradient on rostrocaudal location. Out of these three main cell types, other neurochemical markers and different subpopulations can also be defined within these neuronal groups.

Membrane properties and firing pattern. Based on the electrophysiological properties of membrane, the PPN neurons are grouped to three types (type I, II and III). The three neuronal types above can be distinguished based on following membrane properties:

type I with A-current (transient outward current, which delays repolarization to the initial membrane potential after a hyperpolarizing pulse), type II with low-threshold calcium spikes and type III, which possess both. Based on certain studies, one can also distinguish type IV or type IIIK, whose membranes do not possess these features, thus does not fit previous groups.

In vivo properties. Cholinergic and non-cholinergic neurons of PPN can also be grouped according their *in vivo* firing properties. In anesthetized rats, which condition resembles physiological slow wave activity, synchronized cortical activity is seen together with characteristic activity patterns of PPN cholinergic cells with two different firing patterns. The larger population consisted of slow firing cholinergic neurons, which operate synchronously with the active, "up state" phase of the cortical slow oscillations. There was also a smaller population of fast firing cholinergic neurons, which were active at the same time as slow cortical oscillations occurred. Besides the cholinergic neurons, some non-cholinergic (glutamatergic and GABAergic) neurons are also found in the PPN. These are highly heterogeneous and composed of at least three different groups: (1) "quiescent" neurons, which are much silent during slow-wave activity (SWA) but respond mightily to neocortical activation; (2) "tonic firing" neurons, which have a stationary firing rate that is independent of neocortical activity across different brain areas; and (3) "irregular firing" neurons, show a variable level of correlation with neocortical activity.

PPN neurons are synchronized during slow wave sleep (SWS) but the level of synchronization is decreased during REM and wakefulness (W). This synchronization mechanism has not been

presented yet in detailed, indicating that the local neuronal networks of the PPN have not been mapped well.

The role of PPN and the cuneiform nucleus in the regulation of locomotion

The mesencephalic locomotor region (MLR) is a functionally-defined midbrain area consisted of the PPN and the cuneiform nucleus (CnF). The latter structure has been typically identified as an output station of forebrain systems reaching lower motor structures. The motor function of the MLR is related to the excitatory transmission of glutamatergic neurons, which are the most prominent type of cells in the MLR. In the last two decades, its role has been proposed in the functional circuits and posture. Furthermore, in Parkinson's disease, some of the motor abnormalities may underlie by degeneration of neurons in the MLR.

The deep brain stimulation of the PPN has been revealed some improvements in abnormal gait, which may have caused by the stimulation of the excitatory output from the MLR. However, it is not fully clear how excitatory MLR neurons contribute to motor activity and how motor functions are associated with different neuronal types in the MLR. The PPN is the largest component of the MLR and it is highly heterogeneous. As mentioned in previous chapter, the nucleus is composed of three neurotransmitter-defined cell types such as cholinergic, GABAergic and glutamatergic neurons. Among PPN glutamatergic neurons, a high degree of difference has been shown in their neurochemical composition, connectivity and firing properties. It is comparatively less known about the CnF. Recent studies showed that

activation of CnF glutamatergic neurons generates a robust motor activity that is functionally distinct from the activation of PPN neurons, suggesting a functional specialization of MLR neurons.

Raphe nuclei

Serotonin is an important modulator of neural circuits that mediate anxiety-related behavior. However, the mechanisms through these circuits and the associated physiological and behavioral responses are not fully clear. The serotonergic system is well organized; and the serotonergic neurons in topographically different regions of the brainstem raphe nuclei give rise to projection to distinct functional systems.

Caudal serotonergic structures (as the nucleus raphe magnus, obscurus and pallidus) send projection to the spinal cord and the brainstem, whereas the rostral nuclei as median raphe (MR) and the dorsal raphe (DR) nuclei project to forebrain targets. The MR innervates hippocampus, nucleus accumbens, medial septum and hypothalamic nuclei. The DR sends projections to the frontoparietal cortex, amygdala, lateral septum, nucleus accumbens shell, ventral hippocampus and several hypothalamic nuclei.

The DR and MR are members of the reticular activating system and play important role in the regulation of sleep-wakefulness cycles, movement and affective states. During wakefulness, the activity of DR is increased and it is less active in paradoxical or REM-sleep. It was shown that DR and MR serotonergic neurons have several subgroups

based on expressed markers, morphological characteristics, and in vitro and in vivo electrophysiological properties.

The M-current

The M-current is a non-inactivating voltage-gated potassium current, which is under the regulation of several receptors and neuromodulatory actions. Presumably the best known neuromodulatory action on these channels is the inhibition by muscarinic acetylcholine receptors. The neuronal M-current contributes to setting resting membrane potential and regulates action potential firing. The M-current controls synaptic vesicle release in presynaptic location.

Homo- or heterotetrameric ion channels formed by KCNQ (Kv7) subunits are responsible for M-current. KCNQ2-5 (Kv7.2-5) subunits are found in the central nervous system (CNS), from which KCNQ2, 3 and 5 are more abundant in several brain areas, but KCNQ4 is restricted to the brainstem. These composed of auditory brainstem nuclei as the cochlear nuclei, nuclei of the lateral lemniscus and the inferior colliculus; the principal and spinal trigeminal nuclei; and members of the reticular activating system (RAS) as raphe nuclei and the ventral tegmental area.

KCNQ4 gene mutations lead to an autosomal progressive nonsyndromic hearing loss (DFNA2) due to the degeneration of outer, and in a lesser extent, inner hair cells of the cochlea, known as DFNA2. This disease was reproduced in mouse models that expressed a human KCNQ4 mutation or lacked KCNQ4 channel expression. The mutation changes somatosensory functions as well due to the lack of expression

on skin somatosensory receptors and dorsal root ganglia also in human and mouse (DRG).

It has been revealed that the M-current formed by KCNQ4 subunits contribute to neuromodulatory autoregulation. Therefore, we sought evidence based on the hypothesis that M-current formed by KCNQ4 subunits presents on mesopontine cholinergic neurons.

M-current on the PPN. In a study from our laboratory, it was revealed that the vast majority of pedunculopontine nucleus cholinergic neurons have M-current, whereas glutamatergic and GABAergic neurons completely lack it. There are certain electrophysiological differences between cholinergic and GABAergic neurons, as amplitude of medium afterhyperpolarization, spike frequency adaptation and firing frequency caused by presence or absence of M-current. The inhibition of the M-current completely terminated the oscillations of cholinergic neurons and reduced it at other frequencies.

M-current on dorsal and median raphe nuclei. Previous electrophysiological studies showed that there are differences in membrane properties of DR and MR serotonergic neurons. It was shown that the M-current is present in the DR but less is revealed about its functional significance and distribution. It was recently published that approximately 60% of DR serotonergic neurons express KCNQ4, the main ion channel subunit for M-current. Whereas, this proportion of the neurons possessing M-current in the MR, the topographical distribution of the M-current possessing neurons in both nuclei, as well as their significance in distinct electrophysiological parameters of the serotonergic neurons have not been reported yet.

OBJECTIVES

1. Is the M-current an electrophysiological hallmark of PPN cholinergic neurons?
2. How M-current contributes to synchronization of PPN cholinergic neuronal populations?
3. Do cholinergic neurons possess the KCNQ4 subunits?
4. Can M-current by channels with KCNQ4 subunit modulate circadian rhythm?
5. What proportion of serotonergic neurons in the DR and MR possess M-current and whether is there a topographical organization of neurons possessing M-current?
6. Are glutamatergic neurons in the PPN and CnF functionally distinct or do they form overlapping populations?

MATERIALS AND METHODS

Solutions

The artificial cerebrospinal fluid (aCSF) was used with the composition such as (in mM): NaCl, 120; KCl, 2.5; $NaHCO_3$, 26; glucose, 10; myo-inositol, 3; NaH_2PO_4, 1.25; sodium-pyruvate, 2; CaCl2, 2; $MgCl_2$, 1; ascorbic acid, 0.5; pH 7.4 for experimental

solutions. Low Na^+ aCSF was administered for procedure of slice preparation. In the solution, 95 mM NaCl was replaced by glycerol (60 mM) and sucrose (130 mM). The chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise. Internal solution was used for micropipette with following composition: 120 mM potassium gluconate; 5 mM NaCl; 10 mM 4- (2-hydroxyethyl) -1-piperenethanesulfonic acid (HEPES); 2 mM EGTA; 0.1 mM $CaCl_2$; 5 mM Mg-ATP; 0.3 mM Na_3 -GTP; 10 mM Na_2 - phosphocreatinine; 8 mM biocytin and the osmolarity 280-290 mOsm / l, pH 7.3.

Animals and preparation

Animal experiments were performed according to the appropriate institutional, national and international laws (EU Directive 2010/63/EU for animal experiments) and guidelines on the care of research animals. The protocols used for experiments below were approved by the Committee of Animal Research of the University of Debrecen (6/2011/DEMÁB; 5/2015/DEMÁB; 19/2019/DEMÁB). For slice electrophysiology, 10-22 days old mice were employed and 52-69 days old mice were used for behavioral tests. Mice expressing tdTomato fluorescent protein or channelrhodopsin2 (ChR2) optogenetic

actuator in a choline acetyltransferase- (ChAT) dependent way (n = 59 and 5, respectively), as well as mice expressing tdTomato in a type 2 vesicular glutamate transporter (Vglut2) dependent way (n = 13), and in a Serotonin transporter- (Sert) dependent way (n = 12) from both sexes were employed. The KCNQ4 knockout (KO) strain was kindly provided by Prof. Thomas Jentsch. Coronal midbrain slices with 200 µm thick were prepared in ice-cold (cca. 0 - -2 ºC) low Na+ aCSF by a vibratome Microm HM 650V (Microm International GmbH, Walldorf, Germany) while continuously oxygenated with 95% O_2 and 5% CO_2. The slices were incubated for 1 hour before starting recording in a chamber while bubbling continuously with carbogen in normal aCSF on 37°C.

Electrophysiology

Our experimental setup included a Zeiss Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany) which was used to visualize the slices. The microscope was connected to a fluorescent imaging system (Till Photonics GmbH, Grafeling, Germany), which was including a xenon-based Polychrome V light source (Till Photonics GmbH, Grafeling, Germany), a CCD camera (SensiCam, PCO AG, Kelheim, Germany), the imaging control unit (ICU) and the Till Vision software

(4.0.1.3. version). Axopatch 200A amplifiers (Molecular Devices, Union City, CA, USA) for whole cell patch-clamp measurements with both voltage clamp and current in clamp mode were used. Data processing and recording was performed with Clampex 10.0 software (Molecular Devices, Union City, CA, USA), while data analysis by Clampfit 10.0 (Molecular Devices).

Patch pipettes were made of borosilicate glass capillaries using Narishige PC-10 pipette puller (Narishige Group, Tokyo, Japan) with 5-8 MΩ resistance. Whole-cell patch-clamp experiments were performed on neuronal somata using the Axopatch amplifiers. Simultaneous recordings were performed on synaptically non-coupled, neighboring cholinergic neurons and both neurons were simultaneously depolarized by using 1-s-long depolarizing square current injections with amplitude of 100 pA in current clamp configuration. For the optogenetic experiments, 500 μm thick coronal midbrain slices including the laterodorsal tegmental nucleus (LDT) and the pedunculopontine nucleus (PPN) were prepared. An optical fiber was attached to the LDT and 50 ms long illuminations with 470 nm wavelength and 1 Hz frequency were used. Before and during optogenetic illuminations, whole cell patch clamp recordings of the M-current (see above) were performed on PPN cholinergic neurons.

Activity wheel test

Activity cycle duration and distances moved per day were tested by activity wheel test (Campden Instruments Ltd., Loughborough, UK). KCNQ4 knockout young adult (52-69 days old) mice (n = 16) and wild type littermates (n =16) were employed. Mice were settled individually in cages with activity wheel. Animals were in a room for 7 days to accommodate to alternating 6 h illumination and 6 h darkness periods and had voluntary and unlimited access to the activity wheel. After accommodation, recording was started with the same conditions of illumination (LD conditions) and lasted for 5 days. After that, recording process was continued during 5 days of complete darkness (DD conditions). The recording data of last 3 days were taken into account.

Statistics

Student's t-test, one way ANOVA and post hoc Tukey Multiple Comparison tests were used for assessing statistical significance for pairwise comparison in case if the datasets had normal distribution, whereas Bonferroni's multiple comparison test was employed for multiple comparisons. Statistical analysis was performed using Microsoft Excel or IBM SPSS Statistics 25, SigmaPlot 12.0 and

GraphPad Prism 5.01 (GraphPad software, San Diego, USA). In analysis, an alpha of 0.05 was used as the cutoff for significance. If the p-value is less than 0.05, we rejected the null hypothesis.

RESULTS

Electrophysiological characterization of M-current and localization in neurons from PPN

We previously hypothesized that the M-current is only present on cholinergic neurons in the PPN, according to the data obtained from genetically labelled cholinergic (ChAT+) and GABAergic (GAD65+), but not glutamatergic neurons. The first aim was to confirm it by analyzing M-current in genetically labelled PPN glutamatergic neurons (Vglut2+). In line with the previously published data, the M-current was present in almost all ChAT+ PPN-neurons and absent in the GAD65+. 6.8% of glutamatergic neurons (n=32) possessed the M-current. One of them proved to be ChAT-positive, possibly belonging to the population of glutamatergic-cholinergic neurons. In conclusion, the vast majority of non-cholinergic neurons lack the M-current, but very few exceptions may exist for the glutamatergic neuron population.

The next aim of our experiments was to test whether a near-physiological activation of a cholinergic input of the PPN neurons (as the laterodorsal tegmental nucleus (LDT)) can effectively inhibit the M-current on PPN cholinergic neurons. In this experiment, the M-current of PPN cholinergic neurons was recorded from 500-µm-thick coronal brainstem block, which was prepared from ChAT-ChR2 mice and the LDT was optogenetically stimulated with 1 Hz frequency. This stimulation lowered the holding current to 60.45 ± 6.49% of control ($p = 0.008$). The relaxation current decreased at all voltage steps tested as well. The magnitude of the current amplitude during stimulation was in between 51-73% of control. 5 minutes after the optogenetic stimulation, the M-current was partially regenerated. We concluded that the optogenetic stimulation for a single cholinergic input of the PPN can effectively inhibit the M-current.

During slow wave sleep, neighboring PPN neurons fire synchronously. To describe the contribution of the M-current to this synchronization, we used the KCNQ channel inhibitor XE991 and recorded the response of two synaptically non-coupled neurons, depolarized simultaneously with 100 pA. Inhibition of the M-current by XE991 reduced the adaptation index of the recorded neurons, to a similar magnitude as in KCNQ4 KO PPN cholinergic neurons, showing the action of the drug. We recorded action potential trains and assessed

time differences between the closest action potentials from two synaptically non-coupled neurons. For the neuron pair in control conditions, a high number of short delay values were observed after administration of XE991, delay values were exhibited longer and more variable values. In control, greater than 50.0% of the delay values were shorter than 40 ms. In the presence of XE991, delay values became more uniformly distributed for the first 100 ms. Furthermore, decay tau of the distribution histograms was significantly reduced.

M-current properties mediated by KCNQ4 subunits

In our further experiments, we studied the electrical properties of PPN neurons from KCNQ4 KO animals using electrophysiological experiments. We performed tests for the presence of the M-current in these neurons. For determination of neuron identity, we labeled all recorded neurons with biocytin and checked their cholinergic nature with post hoc ChAT immunohistochemistry. The holding current at -20 mV was much lower in KO than WT animals. Also, we identified that the M-current was absent in 62.5% of the KO cases while WT animals only exhibited its absence in 7.7% of the cases. On ChAT-negative neurons, M-current was recorded neither in WT nor in KO.

Next, we tested and analyzed whether the absence of KCNQ4-mediated M-current can affect SFA. By depolarizing square current injections, we obtained trains of action potentials and then we calculated the adaptation index (AI) of the trains. In cholinergic neurons from KCNQ4 KO animals, the AI was significantly lower than WT. 66.67% of the KO neurons presented lower AI, in agreement with the experiments shown above. In this experiment, the SFA for non-cholinergic neurons from KCNQ4 KO mice was also studied. As expected on the basis of our previous studies, the AI of the cholinergic neurons was significantly lower than in non-cholinergic. Interestingly, a marked decrease of AI was observed in KO compared to WT.

Presence of functional KCNQ4 subunits in PPN

In order to differentiate the function of KCNQ4 in PPN cholinergic neurons under WT conditions, we dissected its contribution by using different subunit-specific KCNQ channel openers. First, we conducted holding current analysis using 20 μM retigabine, a non-specific KCNQ channel opener. An outward shift of the holding current at -20 mV on 100% of the neurons was elicited by retigabine (n = 11). The holding current at -20 mV had 43.3 ± 8.4% increase. Next, we administered ML213 (20 μM), a KCNQ2- and KCNQ4-specific M-

current opener. This generated an outward shift in the holding current in 69.2% of the cholinergic neurons tested and the rest were non-sensitive to it. Then, we tested a KCNQ2- and KCNQ3-specific opener ICA27243 (20 μM). In the majority of the cases, the opener did not show holding current changes. Signs of KCNQ channels activation were showed on 42.9% of the neurons.

In a next series of experiments, we identified the effects of the openers on the SFA and similar percentages of rise in AI were seen.

Functional roles of KCNQ4 in the RAS

The PPN is a member of the RAS which participates in modulation of the circadian rhythm. We identified the presence of KCNQ4 in this nucleus and its functional role in neuronal properties. At that point, we investigated if this subunit contributes to the regulation of the circadian rhythm by the PPN by voluntary wheel running test in young adult KCNQ4 KO and WT mice. For discarding any potential impairment in the visual pathway, we made circadian bifurcation for mice by altering the LD cycle to a 6-h LDLD periods. Actogram showed two activity bouts during both scotophases for each genotype in this condition, demonstrating a proper function of the visual pathway. No difference of the period time between genotypes under LDLD

condition was shown. Next, we measured the distance ran by both mice genotypes under LDLD conditions. There was no significant difference in distance traveled between WT and KO in LDLD conditions.

For identifying the intrinsic circadian rhythm in both genotypes, we made analysis of mice in the free-running or constant darkness conditions (DD). In DD condition, the period time was significantly reduced in WT compared to LDLD, but exhibited only an increasing tendency in KO. Regarding distance traveled in DD conditions, we observed an increasing tendency for WT animals, though it was not statistically significant compared to LDLD. Nevertheless, a ~2-fold increase was revealed in the distance traveled of KO mice in DD compared to LDLD condition. All parameters exhibited a higher variability of individual measurements in DD conditions. The period time was significantly longer in KO mice than in WT under DD condition and for the distance traveled, it was not statistically significant. Also the period time under DD condition was normalized to LDLD condition in each cases, this parameter was 0.71 ± 0.56 in WT and 1.15 ± 0.76 in KO. However, when distances under DD conditions were normalized to LDLD conditions, this parameter proved to be significantly higher in KO with a much higher standard deviation.

Localization of M-current in DR and MR neurons

We examined whether the raphe serotonergic neurons possessing the M-current have topographical organization and whether this current influences other electrophysiological properties. It was described by patching 56 genetically identified serotonergic neurons (n =31 from the DR and n =25 from the MR).

It was shown that neurons possessing M-current are more likely located rostrally in the DR (-4.74 ± 0.05 mm from the bregma for the ones having and -4.9 ± 0.05 mm for the ones lacking M-current, $p = 0.02$) and rather located dorsally in the MR (3.99 ± 0.07 mm from the top for the ones having and 4.36 ± 0.09 mm for the ones lacking M-current; $p = 0.007$).

PPN glutamatergic neurons are physiologically different from CnF neurons

We defined functional subgroups based on changes of spike frequency adaptation with increasing depolarization. We observed that certain neurons have a more-or-less constant adaptation index, other ones have a gradual increase of this parameter with increasing depolarizing steps, and further neurons cease firing at the end of the

greater depolarizing step, but have a high frequency burst at the beginning of the trace. We grouped the neurons according to the following criteria: the neurons with constant adaptation index (called „non-adapting") had less than 50% increase in the adaptation index of the action potential trains obtained with 50 and 120 pA current injections; the ones with increasing adaptation index („slowly adapting") had more than 50% change of the adaptation index between the same traces, but fired during the whole 1-s-long depolarizing step, whereas the rest of the neurons paused firing after application of greater depolarizing steps („rapidly adapting"). 30.2% of all neurons (13/43 neurons) in the PPN were non-adapting and were located mostly in the lateral regions, whereas 21% (9/43 neurons) were slowly adapting and 48.8% (21/43 neurons) were rapidly adapting. Contrarily, in the CnF, the large majority of neurons (85.7%, 24/28 neurons) were rapidly adapting, and non-adapting and slowly adapting composed equally small proportions (7.15%, 2/28 neurons for each category). Therefore, the responses of MLR glutamatergic neurons to spike adaptation show important biophysical group differences in the composition of the PPN and the CnF ranging from firing frequency to adaptation index. Our results thus support differences in the input/output connectivity of PPN and CnF glutamatergic neurons with separate motor circuits.

DISCUSSION

The M-current is a hallmark of cholinergic neurons of the PPN

By adding data on glutamatergic PPN neurons, our study supports the functional results that the cholinergic neurons possess M-current and absent on GABAergic and glutamatergic subpopulations. The single exception is the existence of glutamatergic-cholinergic neurons having both neurotransmitters as co-transmitter. In terms of M-current, this population belongs to cholinergic neurons.

The M-current synchronizes neighboring PPN cholinergic neurons

In accordance with the modelling and data from other brain areas, we reported that SFA helps synchronization of neuronal populations. SFA was decreased by inhibition of the M-current and the synchronization between two neighboring neurons also decreased. Moreover, we showed that the M-current is effectively blocked by the cholinergic inputs of the PPN.

It seems to be likely that cholinergic activation desynchronizes a neuronal population in cholinergic nucleus. As the PPN provides local

axon collaterals for itself, sends projections to the contralateral PPN and receives cholinergic input fibers from the LDT, cholinergic activation might spread to all mesopontine cholinergic areas and contributes to the desynchronization of cholinergic neuronal populations. Desynchronization of PPN neuronal population takes place in parallel with cortical desynchronization. We can conclude that cholinergic blockade of the M-current contributes to autoregulatory desynchronization of cholinergic areas, and in turn, to regulation of global brain states.

The KCNQ4 subunit is present on a subset of PPN cholinergic neurons

In our study, morphological and pharmacological methods and transgenic animals were used to demonstrate the presence of the KCNQ4 subunit in the PPN. All of these methods uniformly showed that only a group of them expressed KCNQ4 in a proportion in between 9 to 62.5%. This study is similar to that reported by Heidenreich et al. (2011) in the DRG, where also only a fraction of neurons expressed KCNQ4. Similar findings were presented in the dorsal raphe. However, further studies are needed to demonstrate whether KCNQ4-positive cholinergic neurons have different roles than KCNQ4-negative ones. In

KCNQ4 KO, the KCNQ4 subunit disappears both on mRNA- and protein levels. In parallel with these alterations, KCNQ2- and 5 levels remain unchanged but KCNQ3 is upregulated. Interestingly, KCNQ3 subunit alters its expression pattern from cholinergic to non-cholinergic neurons. We did not find any evidence for the presence of M-current on the non-cholinergic ones, indicating that KCNQ3 does not form functional channels or they may form homomeric channels with very low membrane expression on non-cholinergic neurons. However, we demonstrated further changes in the electrical properties of these neurons.

In conclusion, these observations rise the probability that KCNQ4 is not only crucial as one of the subunits forming channels for M-current but might be a potential modulator of the expression of other M-current subunits, setting their physiological function. In agreement with this, RAS-related behavioral alterations were found in KCNQ4 KO mice.

Ion channels formed by KCNQ4 subunits critically affect adaptations to changes in light-darkness conditions

We demonstrated that deletion of KCNQ4 subunit, partially due to its expression in the PPN, caused disturbances in adaptation to

changes in the circadian rhythm. In complete darkness, the activity time is increased in KCNQ4 KO mice, accompanied with longer distances ran under this condition with a higher standard deviation of the dataset compared to WT littermates. Consequently, KCNQ4 KO mice exhibited a lower capacity to modulate sleep-wakefulness cycles upon environmental light condition changes, suggesting a contribution to regulation of RAS functions. In 2-month-old animals, the hearing loss is already present but less prominent than in older ones, therefore, actions seen on activity cycles are potentially at least partially caused the lack of the ion channel subunit in brainstem structures. There is no significant change in activity cycles and movement in alternating LD conditions. Alteration in outer light conditions perhaps does not affect hearing and tactile sensation, rather the structures regulating activity cycles. It is also known that raphe nuclei and the VTA have functional KCNQ4 subunits, which contribute to neuronal functions in these regions. One can predict that these structures, which are members of the RAS, contribute to the changes in activity cycles found in KCNQ4 KO animals. The PPN, raphe nuclei and VTA are all involved in regulation of both sleep-wakefulness and movement. As it was predicted, distances ran under different environmental light conditions were significantly different in WT and KO animals. Based on our study and literature data, we can conclude that potassium channels formed by

KCNQ4 subunits in the RAS (including the PPN) are regulating activity cycles and movement.

We can conclude that the KCNQ4-related M-current of the RAS might have a regulatory role on adaptation of activity cycles to environmental changes and on the dependent changes in the motor activity. The PPN modulates sleep-wakefulness cycles and motor activity through its M-current, which may have a crucial role on these functions. Different roles were demonstrated for the three main neuronal populations of the PPN to transitions between brain states.

The M-current of raphe serotonergic neurons is topographically distributed

It was an important finding of this project that M-current positive neurons seem to be topographically organized in both DR and MR. In the DR, they form a rostral population, whereas they are dorsal in the MR. In addition, we found that certain other electrophysiological parameters correlate with each other, as high threshold oscillations are related to the input resistance and the delay of the first action potential is inversely proportional to the maximal firing rate.

Several studies demonstrated that DR and MR serotonergic neurons are not homogenous but form different subgroups in terms of development, gene expression, projection, neurochemical markers, as well as in vitro and in vivo electrophysiological properties. Developmentally, raphe serotonergic neurons arise from six rhombomeres and neurons with different origin have distinct gene expression patterns. Raphe serotonergic neurons form 11 transcriptomic clusters partially based on differences in expression of genes encoding ion channels. Differential ion channel subunit expression seems to contribute to defining genetically identified subgroups of serotonergic neurons.

According to their function, the MR and the DR, as well as subregions of the DR have distinct functions. If DR activity overwhelms MR activity, it might explain development of phobic and anxietic syndromes, whereas the opposite rather supports development of psychotic syndromes. Within the DR, amygdala-projecting serotonergic neurons are active during anxiety, whereas frontal cortex projecting ones facilitate active coping with challenges.

We also demonstrated that the M-current contributes to autoregulation and synchronization of mesopontine cholinergic structures. The M-current is widely known to be inhibited by muscarinic acetylcholine receptor activation, thus serving as an

important neuromodulatory mechanism. One can assume that serotonergic neurons with M-current are subjects of this pathway of muscarinic cholinergic neuromodulation. Alternatively, since it is known that –similar to acetylcholine- serotonin is also capable of influencing M-current (via 5HT2C receptor), we hypothesize that M-current possessing neurons are subject of serotonergic autoregulation within the raphe nuclei. This cholinergic regulation or serotonergic autoregulation might be specific to subregions in the rostral DR and the dorsal MR. As the rostral DR has a role in movement-related stress tolerance and the MR is important for adaptation to chronic psychosocial stress, neuromodulatory regulation via M-current might play a role in adaptation to stress. These literature results and our findings might provide further support to literature findings that the M-current might be a good candidate as a target for anxiolytic therapy.

CnF and PPN glutamatergic neurons are functionally distinct

The MLR is a functionally-defined midbrain area consisting of the PPN and the cuneiform nucleus (CnF), which has been typically identified as an output station of forebrain systems reaching lower motor circuits and their activation has been proposed to evoke different types of movement, but how the differences in connectivity and

physiological properties explain their contributions to motor activity is not clear yet.

Based on our findings, CnF glutamatergic neurons are electrophysiologically more homogeneous than PPN neurons. For the physiological characteristics, we showed that PPN neurons composed a heterogeneous group displaying a range of adapting responses, whereas the majority of CnF neurons are fast-adapting. In the PPN, 48.8% of recorded neurons were fast-adapting neurons, 30.2% non-adapting neurons, and 21% slow adapting, suggesting a greater diversity of neuronal profiles than the CnF.

Furthermore, in accordance with the data from our collaborating partner (Dr. Juan Mena-Segovia), CnF glutamatergic neurons have mostly short-range connectivity, but PPN glutamatergic neurons are heterogeneous and maintain long-range connections, particularly with the basal ganglia. CnF neurons produced short-lasting muscle activation driving involuntary motor activity by optogenetic activation. On the other hand, PPN neurons activation produced long-lasting increases in muscle tone that reduced motor activity and disrupted gait. These results highlight a key biophysical and functional feature among MLR neurons that support their differential contribution to motor behavior in normal and pathological conditions.

SUMMARY

The pedunculopontine nucleus (PPN) is a cholinergic area of the reticular activating system (RAS). It contributes to regulation of sleep-wakefulness cycles and movement, as it is a part of the mesencephalic locomotor region (MLR). Rostral serotonergic members of the RAS are the dorsal and median raphe nuclei (DR and MR, respectively) which also targeted by cholinergic neuromodulation and play important role in regulation of sleep-wakefulness cycles, movement and affective states.

We showed that deletion of KCNQ4 leads to alterations in adaptation of activity to light-darkness cycles. Although the M-current is an electrophysiological hallmark of the cholinergic neurons, only a subpopulation of these neurons possessed KCNQ4-dependent M-current. It synchronizes neighboring PPN neurons and inhibition of the M-current decreases neuronal synchronization.

Serotonergic neurons with M-current are located rostrally in the DR and dorsally in the MR. The M-current seems to have a strong impact on firing properties of certain serotonergic neuronal subpopulations and it might serve as an effective contributor to cholinergic and local serotonergic neuromodulatory actions.

The MLR is a functionally-defined midbrain area consisted of the PPN and the cuneiform nucleus (CnF). We showed that CnF glutamatergic neurons are electrophysiologically more homogeneous than PPN neurons. PPN neurons composed a heterogeneous group displaying a range of adapting responses, whereas the majority of CnF neurons are fast-adapting; suggesting a greater diversity of neuronal profiles than the CnF.

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1. **Bayasgalan, T.**, Stupniki, S., Kovács, A., Csemer, A., Szentesi, P., Pocsai, K., Dionisio, L., Spitzmaul, G., Pál, B.: Alteration of mesopontine cholinergic function by the lack of KCNQ4 subunit.
   Front. Cell. Neurosci. 15, 1-16, 2021.
   IF: 5.505 (2020)

2. Dautan, D., Kovács, A., **Bayasgalan, T.**, Diaz-Acevedo, M. A., Pál, B., Mena-Segovia, J.: Modulation of motor behavior by the mesencephalic locomotor region.
   Cell Reports. 36 (8), 1-53, 2021.
   IF: 9.423 (2020)

3. **Bayasgalan, T.**, Csemer, A., Kovács, A., Pocsai, K., Pál, B.: Topographical Organization of M-Current on Dorsal and Median Raphe Serotonergic Neurons.
   Front. Cell. Neurosci. 15, 1-8, 2021.
   DOI: http://dx.doi.org/10.3389/fncel.2021.614947
   IF: 5.505 (2020)

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További közlemények

4. 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.
Cell. Mol. Life Sci. 76 (14), 2799-2815, 2019.
DOI: http://dx.doi.org/10.1007/s00018-019-03025-4
IF: 6.496

5. 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: 1.394

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