Cellular and network mechanisms of physiological and pathological brain states

Ph.D. thesis

Szabina Horváth-Furdan

Supervisor:

Magor László Lőrincz Ph.D.

associate professor

Ph.D. School in Biology

University of Szeged

Faculty of Science and Informatics Department of

Physiology, Anatomy and Neuroscience

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List of abbreviations

5-HT: 5-hydroxy-tryptamine; serotonin

AE: absence epilepsy

ChR2: Channelrhodopsin2

DPP: depolarizing postsynaptic potential

DRN: dorsal raphé nucleus

EEG: electroencephalogram

HCN: hyperpolarization-activated cyclic nucleotide gated

HPP: hyperpolarizing postsynaptic potential

IPSP: inhibitory postsynaptic potential

LH: lateral hypothalamus

NREM: non rapid eye movement sleep

REM: rapid eye movement sleep

STG: Stargazer

SWD: spike-and wave discharges

TC: talamocortical

VB: ventrobasal

Vgat: vesicular GABA transporter

WT: wild-type

Introduction

In my doctoral dissertation, I present two research topics: exploring the cellular and network mechanisms of physiological (sleep-wake cycle) and pathological (epilepsy) brain states.

In the absence of sensory input the mammalian brain exhibits a wide array of structured brain state dependent spontaneous activity patterns as happens during relaxed wakefulness, sleep and epilepsy. In cortical areas brain state dependent neuronal activity is determined by both intrinsic and thalamo-cortico-thalamic network interactions fine tuned by neuromodulation.

Physiological brain state: sleep-wake cycle

Brain states are generated within the thalamo-cortico-thalamic system and modulated by various other structures. The main difficulty to a mechanistic understanding of brain states is the heterogenity of the cellular elements, the multitude of interacting brain areas and their neuromodulatory systems.

The serotonergic system is an evolutionarily conserved, complex neuromodulator system that projects to almost all brain regions. Serotonin (5-hydroxy-tryptamine; 5-HT) is released from the axons terminals of serotonergic neurons located in the raphé nucleus of the brainstem and affects numerous brain function. 5-HT regulates the sleep-wake cycle, moreover is involved in fine-tuning certain sensory, motor, and cognitive functions, while it's dysfunction can be associated with pathological conditions such as schizofrenia, depression, autism, and anxiety. Contradictory experimental results have been reported on the role of serotonergic dorsal raphé nucleus (DRN) neurons in the regulation of the sleep-wake cycle. According to the results of early studies, 5-HT has hypnogenic effect, however, electrophysiologycal studies have reported that the activity of putative 5-HT neurons is highest during wakefulness, decreases during non rapid eye movement sleep (NREM) and almost completely ceases during rapid eye movement sleep (REM). Interestingly, contradictiory results can also be found in more recent studies. Optogenetic stimaultion of 5-HT neurons was shown to promote wakefulness and reduce sleep, while in other studies it was claimed to have a hypnogenic effect. Possible reasons for these contradictiory results are the heterogeneity of DRN cell types and differences between the stimulation protocols used.

The anatomical connection between lateral hypothalamus (LH) and DRN is well known, yet it's functional relevance is not fully understood. LH is a neurochemically heterogenous brain region, that contains several well-distinguished nuclei regulating numerous brain functions. It

plays an important role in the control of the sleep-wake cycle, participates in the regulation of emotions, and contributes to the regulation of goal-directed behaviors such as eating, drinking, agression, and certain cognitive functions. Within the LH, there are also two neuropeptide synthesizing cell populations involved in the control of the sleep-wake cycle. Orexin-producing neuron activation promotes wakefulness in mice, but their silencing results in sleep disturbance and narcolepsy. In contrast to orexinergic neurons, optogenetic activation of melanin-concentrating homone-producing neurons leads to the development of NREM and REM sleep. In additon to orexin and melanin-concentrating homone synthesizing neurons, the vesicular GABA transporter (Vgat) expressing LH neurons (LH_{Vgat}) have also been shown to play an important regulatory function during sleep-wake cycle. Specific opotogenetic stimaulation of LH_{Vgat} neurons increased the duration of wakefulness and resulted in awakening from NREM sleep. Furthermore, selective silencing of LH_{Vgat} neurons had a hypnogenic effect. Otpogenetic activation of LH_{Vgat} axons in the *locus coeruleus* caused awakening from both NREM and REM sleep. The functional relationship of LH_{Vgat} neurons other brainstem neuromodulatory systems has remained elusive.

Pathological brain state: epilepsy

Absence epilepsy (AE) is the most common type of epilepsy during childhood, that can be classified as generalized epilepsy. The main sympotoms of AE are sudden, relatively brief lapses of consciousness asoociated with the lack of voluntary movements and disinctive electographic spike-and wave discharges (SWD) at 2,5-4 Hz. SWDs appear in the oscillations of thalamo-cortico-talamic network during relaxed wakefulness and drawsiness. Numerous recent studies investigated the involvement of various ion channels in the rhythmic oscillations. Hyperpolarization-activated cyclic nucleotide gated (HCN) channels regulate resting membrane potential and membrane resistance. By depolarizing the membrane, they have a positive effect on the initiation of the action potential. Furhermore, by influencing the duration of inhibitory postsynaptic potentials (IPSP), they affect the neuronal responses and play an imprortant role in the generation of rhythmic oscillations. By influencing neural network excitability, synaptic responses, and network activity, HCN channels play an important role in many physiological processes such as learning and memory. Their altered expression and function associated with pathophysiological conditions such as epileptic seizures. Their role in pathological function is supported by the observation of HCN channel mutations and altered HCN1 or HCN2 expression in temporal lobe or AE patients. Experimental results suggest that

upregulation or downregulation of HCN channels is associated with the onset of epileptic seizures, however the exact role of thalamic HCN2 channels in ictogenesis is still unclear.

Aims

During constant adaptation to a complex and dynamic enviroment, the behavior of living beings is constantly changing. The most conspicuous change is observed during the sleep-wake transition. Precise neuronal control of this transition is essencial and is one of major function of neuromodulatory systems. However, the precise regulation of transitions between different brain states is very complex and accordingly actively researched. In our experiments, we examined the synaptic connections of LH and DRN neurons and their effect on the sleep-wake cycle.

My aims were:

- To explore the synaptic effects of LH fiber stimulation on different DRN target cells using a combination of *in vitro* whole-cell patch clamp and optogenetic techniques.
- To reveal the neurochemical identity of the recorded DRN neurons using immunohistochemical methods.
- To investigate the effect of selective stimulation of LH GABAergic fibers on identified DRN neurons in intact animals.

Given the multifaceted cellular and synaptic effect of HCN channels, it is difficult to determine the relationship between channel function and inctogenesis. We investigated the role of thalamic HCN2 channels in absence seizures using both pharmagological and genetic tools to selectively supress HCN2 channel function in talamocortical (TC) neurons of the ventrobasal thalamus (VB) in rodent models of AE.

My aims were:

- To observe the cellular effect of genetic silencing of HCN2 channels in TC neurons of VB thalamus using *in vitro* electrophyisiology in Stargater (STG) mice.
- To investigate the effect of genetic silencing on the expression of HCN2 channels using immunohistochemistry.

Materials and methods

Surgery: LH (AP: -0,94 mm; ML: ±1.00 mm; DV: -5,2 mm and -5,4 mm) of wild type (WT), GAD67-GFP and Vgat-ires-Cre mice, and VB thalamus (AP: -1,8mm; ML: ±1,5;mm DV:-3.0 mm) of STG and WT mice were infected with different viruses (Channelrhodopsin2 (ChR2)-expressing (LH); HCN2-targeting shRNA (VB thalamus), control (LH; VB thalamus).

***In vitro* electrophysiology:** Following virus infection, whole-cell patch clamp recordings were performed from DRN neurons and VB thalamocortical relay cells. During the recording of DRN neurons, the local axons of LH were photostimulated (475 nm; 0.5–0.8 mW; 5 light pulses (10 ms, 20 Hz)).

***In vivo* electrophysiology and juxtacellular labeling:** In order to examine the activity of DRN neurons in intact animals, single unit recordings were performed from anesthetized and awake head-fixed mice. During anesthetized measurements, recorded cells were labeled using 0.5–4 nA 500 ms current pulses for 2–10 min. Photostimulation contained 5, 10 ms light pulses at 20 Hz.

Electroencephalogram (EEG) registration: EEG registration was performed after virus infection to detect absence seizures in STG mice and to monitor the sleep-wake cycle of Vgat-ires-Cre animals. In case of Vgat-ires-Cre mice, during EEG registration LH_{Vgat} axons were fotostimulated (5 ms at 5 or 20 Hz).

Immunohistochemistry: Following our *in vitro* and *in vivo* recordings, Biocytin-filled DRN neurons were visualized using Cy3- or Alexa 488-Streptavidin antibody. The neurochemical identity of *in vitro* recorded DRN neurons was determined using serotonin or GABA immunostaining. HCN2 immunostaining was used to examine the expression of HCN2 channels.

Data analysis: In vitro results were analyzed with FitMaster, OriginPro 8.5 and IgorPro 8. Data from in vivo experiments were analyzed using the Spike2 program.

Statistics: Data are reported as means ± S.E.M. and statistical comparisons were performed by Wilcoxon rank-sum test, Wilcoxon signed-rank test, linear regression, and three-way ANOVA. P values less than 0.05 were considered significant.

Results and Discussion

Investigation of the functional connection of LH-DRN projection

- Following LH infection of WT and GAD67-GFP mice, whole-cell patch clamp recordings were performed from DRN, while ChR2-expressing LH axons werelocally photostimulated. We found that photostimulation of ChR2 expressing LH terminals in the DRN evoked hyperpolarizing postsynaptic potentials (HPPs) in 7 (14%) DRN neurons and depolarizing postsynaptic potentials (DPPs) were recorded in 10 (20%) DRN cells. No detectable postsinaptic potenctials could be recorded in the remaining population (n=34, 66%).

- The AMPA/kainate receptor blocker, NBQX, blocked stimulus-evoked DPPs in all cases. In our experiments, the NBQX did not eliminate HPPs, however, by washing $GABA_A$ blocker (gabazine) into slices, we were able to block stimulus-evoked HPPs each time. $GABA_A$ receptor-mediated IPSPs were also well detectable after TTX and 4-AP application.

- All DRN neurons exhibiting IPSPs following LH axonal photostimulation were confirmed to be GABAergic, by post-hoc immunohistochemistry (n=5) or by recording DRN_{GABA} neurons (n=2) in GAD-GFP mice. The five cells we examined by immunophistochemistry that responded to LH photostimulation with excitatory postsinaptic potentials were 5-HT immunoreactive.

- Following LH infection, extracellular single unit recordings were performed from DRN neurons from anesthetized mice, while locally ChR2-expressing LH_{Vgat} axons were photostimulated. During our recordings, local photostimulation of LH_{Vgat} axons resulted in a rapid decrease in the spontaneous activity of the recorded DRN neurons. We identified the morphology of 8 neurons recorded and filled with the juxtacellular labeling technique. These neurons were classified as putative DRN_{GABA} neurons based on their high baseline firing rate (≥6 Hz), morphology and location within the DRN.

- In awake, head-fixed animals, the fotostimulation confirmed the suppressive effect in a subset of DRN neurons (2/12, 17%), while the activity of the remaining neurons was

increased (10/12, 83%). The overall activity of DRN neurons was slowly (~200 ms), but persistently (~1 sec) increased.

- Stages of the sleep-wake cycle were separated by simultaneous recording of EEG and electromyogram. Optogenetic activation of LH_{Vgat} projections to the DRN during NREM sleep led to a rapid arousal at both 5 and 20 Hz within <7s, whereas optical activation of these fibers failed to significantly change REM sleep duration.

Based on our *in vitro* electrophysiology results, local photostimulation of ChR2-expressing axons from LH evoked AMPA-/kainate receptor-mediated excitatory in some DRN neurons, while $GABA_A$ receptor-mediated inhibitory postsynaptic potentials in other DRN cells, and the polarity of postsynaptic potentials correlates well with the neurochemical identity of DRN neurons. The results of the pharmacological experiments demonstrated the existance of monosynaptic excitatory and inhibitory connections. Based on our *in vivo* electrophysiological results, we can conclude that the effect of LH → DRN projections is relevant, as photostimulation of LH can greatly influence the spontaneous electrical activity of DRN neurons in both anesthetized and awake animals. Our results demonstrate, that all the neurons exhibiting IPSPs following LH axonal photostimulation are GABAergic and that DR_{GABA} neurons are preferentially inhibited by LH_{GABA} fibers. In addition, in our *in vivo* recordings from anesthetized animals, local photostimulation of GABAergic LH axons resulted in a rapid decrease in activity in putative DRN GABAergic neurons. Furtehermore, our in *vivo* results show that LH GABAergic projections promote arousal from NREM but not REM sleep by selectively inhibiting DRN GABAergic neurons via $GABA_A$ receptors, resulting in a prominent disinhibition of DRN output neurons. Our results support and complement the observations of previous studies that LH_{Vgat} neurons regulate the transition from sleep to wakefulness.

Taken together, our results identify a novel long range inhibitory projection implicated in the control of arousal.

Relationship between HCN2 channels and absence seizures

- A statistically significant reduction in the total time spent in seizures and the average lengt of individual seizures was observed in HCN2-targeting compared with non-targeting shRNA-injected mice at 28 and 32 day after injection.

- We investigated the functional effect of genetic silencing by monitoring the electrophysiological properties of VB thalamus TC neurons in slices taken from mice previously given injections of either HCN2-targeting or non-targeting shRNA (control) in VB thalamus. The resting membrane potential of HCN2-targeting shRNA infected TC neurons was more hyperpolarized than in control neurons. Moreover, the depolarizing sag in response to hyperpolarizing voltage steps was almost abolished in TC neurons infected with HCN2-targeting shRNA compared to control. In addition, the steady-state and peak input resistance ratio was significantly larger in neurons from HCN2-targeting than control, indicating that the sag difference is not a consequence of a difference in input resistance. In contrast, action potential properties were not affected.

- Following the immunostaining the fluorophore intensities of HCN2 and GFP were correlated. HCN2-targeting shRNA infected TC neurons that were immunopositive for GFP had a low HCN2 immunoreactivity compared with non targeting shRNA infected slices. Indeed, a negative correlation was observed between HCN2 and GFP immunostaining in slices that had received the HCN2 targeting shRNA, whereas no correlation was observed in slices infected with the non-targeting RNA. Neocortical HCN2 immunofluorescence was unaltered.

Our present results show that block of HCN2 channels in TC neurons reduces the lenght and ferquency of absence seizures in freely moving Stargazer mice. Our *in vitro* data demonstrate that HCN2-targeting shRNA selectively affects I_h-dependent membrane properties of VB TC neurons without altering other neuronal properties. Furthermore, the results of our immunostainig show that HCN2-targeting shRNA silencing reduces the expression of HCN2 channels exclusively in VB TC neurons, thus allowing a region-specific examination of the channel defect.

Publications

Publication related to the thesis

Gazea M*, **Furdan S***, Sere P, Oetsch L, Molnár B, Di Giovanni G, Fenno L, Ramakrishnan C, Mattis J, Deisseroth K, Dymecki S, Adamantidis A, Lőrincz ML (2021) Reciprocal lateral hypothalamic and raphé GABAergic projections promote wakefulness. *J Neurosci;* DOI: https://doi.org/10.1523/JNEUROSCI.2850-20.2021

* The authors contributed equally to this work.

IF: 5,673

David F*, Carcak N*, **Furdan S**, Onat F, Gould T, Meszaros A, Di Giovanni G, Hernandez VM, Chan CS, Lőrincz ML, Crunelli V (2018) Suppression of Hyperpolarization-Activated Cyclic Nucleotide-Gated Channel Function in Thalamocortical Neurons Prevents Genetically Determined and Pharmacologically Induced Absence Seizures. *J Neurosci;* 38:6615-6627.

IF: 6,074

Publication not related to the thesis

Csajbok EA, Kocsis AK, Farago N, **Furdan S**, Kovacs B, Lovas S, Molnar G, Liko I, Zvara A, Puskas LG, Patocs A, Tamas G (2019) Expression of GLP-1 receptors in insulin-containing interneurons of rat cerebral cortex. *Diabetologia;* 62:717-725.

IF: 7,518

Borbely E, Horvath J, **Furdan S**, Bozso Z, Penke B, Fulop L (2014) Simultaneous changes of spatial memory and spine density after intrahippocampal administratiooff fibrillar abeta1-42 to the rat brain. *Biomed Res Int*; 2014:345305.

IF: 1,579

Cumulative IF: 20,844