Ultrafast Functional Ultrasound (fUS) Imaging for Highly-Resolved Targeted Mapping of Functional Connectivity in the Awake Mouse Brain

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Final report

Summary

The Hungarian partner of this FLAG ERA international consortium had two major aims. The methodological aim was to establish an easily reproducible experimental paradigm which allows the combination of optogenetics, electrophysiology and the novel imaging method (fast ultrasound imaging) in a behaviorally relevant condition. In order to do so we needed to identify the role of the midline thalamic nucleus (also called paraventricular nucleus) in mediating the sleep-to-awake transition (i.e. arousal), which consisted of the second aim of the project. In our experiments we utilized the fact that a neuronal marker (calretinin, CR) is selectively present in midline thalamic neurons. Using the appropriate CR-Cre transgenic mouse line, viral mediated gene transfer, optogenetics and electrophysiology we were able the selectively record and activate this specific cell population and determine its input-output characteristics. We also had a chance to compare the organization of rodent and human midline thalamic nuclei. During the experiments we discovered that:

i; neuronal activity CR+ midline cells predict behavioral arousal

ii; graded optogenetic activation of CR+ midline cells are able to evoke behaviorally relevant arousal patterns

iii; arousal mediated by CR+ midline cells are distinctively different than arousal evoked by sensory thalamic systems

iv; the input-output characteristics of CR+ midline cells are consistent with a role as a major forebrain arousal center

v; the organization of human and rodent midline nucleus is similar, indicating that it is an evolutionary ancient, conserved system.

According to these data using the CR+ cells of the midline thalamic nuclei and arousal may form an easily reproducible, biologically relevant experimental paradigm for future fUS investigations.

Results

In our previous experiments we have established that calretinin is a selective marker of the midline thalamic neurons (MT) which have widespread forebrain connections. To test the ability of calretinin-containing MT neurons to initiate state transitions in freely sleeping animals we delivered 10 Hz optogenetic stimulation for variable durations and intensities via optic fibers implanted to the midline thalami of calretinin-Cre mice. The animals received either AAV-DIO-ChR2-eYFP or control AAV-DIO-eYFP virus injections to their MT. During the experiments we recorded EEG and EMG activities and the movements of the animals. The animals were in their home cage, sleeping in their nest. Sleep state was determined by a combination of EEG and EMG signals as slow wave sleep (SWS) or rapid eye movement (REM) sleep.

Long duration (10 sec) optogenetic stimulations

Without exception ten second long, 10 Hz stimulation delivered during SWS in 8 animals receiving the ChR2-containing virus construct resulted in arousal, characterized by an immediate drop in delta power, a sudden increase in EMG activity and locomotion. **(Figure 1.)** Behavioral arousal far outlasted the stimulation and resulted in 3-34 minutes of uninterrupted active awake states. The animals performed normal awakening behavior, (e.g. stretching, grooming) did not display startle or freezing responses, left their nest and started active locomotion. In animals injected with control virus no significant change in EEG, EMG or locomotor activity could be observed following the stimulation.

Short duration (1 sec) stimulations

For quantitative evaluations, which may be useful for fast ultrasound imaging, we aimed to use a stimulation parameter which could be utilized many times. Thus, we switched to 1 sec long laser stimulation instead of 10 sec. In these cases, following the stimulation the animals never left their nest and displayed only short neck and head movements **(Figure 2).** The stimulation induced changes in EMG activity were short and returned to baseline after 3-9 seconds. The EEG activity was also only transiently interrupted during these evoked events. After a large initial drop in delta power this value returned to prestimulation baseline within 15-45 sec. These transient interruptions of sleep, known as microarousals, are considered to be part of the normal sleep behavior both in humans and rodents. In order to compare the stimulation evoked microarousals to spontaneous microarousals we systematically varied stimulation duration from 0.5 sec to 2 sec and compared the duration of evoked microarousal to the duration of natural, spontaneous microarousals of the same animals. In case of 0.5 and 1 sec long stimulations these values were not statistically different from the duration of spontaneous microarousals, demonstrating that we evoked a behaviorally relevant arousal behavior by brief CR+ neuron stimulations.

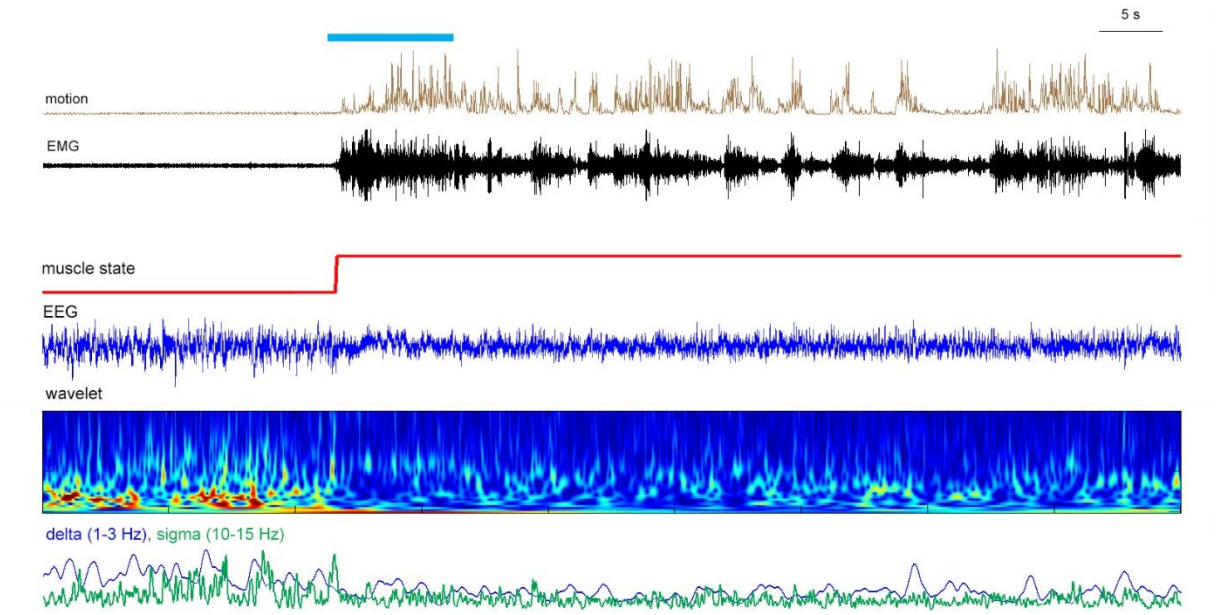

Figure 1. Stimulation of CR+ midline neurons induces persistent arousal.

10 sec long, 10 Hz stimulation (blue bar) was delivered, while the animal was naturally sleeping in its home cage. Videotracking of head movement (brown) along with EMG, (black) and EEG signals (blue) was monitored to determine sleep/wake states. The muscle state, calculated from EMG signal was used to quantify sleep (0) and wake (1) durations and latencies. The figure also shows the wavelet spectrum and the delta and sigma power of the EEG signal. Note the sudden increase in EMG activity and movement at the onset of the stimulation (blue bar on top) together with an immediate drop in sleep oscillations

One sec long stimulation not always evoked detectable EMG activity i.e microarousal (**Figure 2)**. The probability of these "sleepthroughs" depended on the stimulation power. We wondered whether stimulation induced changes in EEG and EMG are dissociable i.e. the lack of EMG activity during these subthreshold stimulation is coupled with a lack of detectable effect on cortical oscillations. We found that in the absence of EMG changes EEG power was still affected by midline stimulations during sleepthroughs in a frequency dependent manner. Compared to microarousals delta power displayed a similar drop in power in case of sleepthroughs which, however, returned to prestimulation baseline much faster. Microarousals could be evoked not only during SWS but also during REM sleep as well albeit with lower probability. Interestingly, however, following REM microarousals the animals never returned to REM sleep but entered SWS by gradually increasing delta power. This sequence of events (i.e. REM-microarousal-SWS) is a well-known phenomenon in sleep literature and suggests that midline thalamic stimulation is able to induce normal state transition from REM to SWS sleep via inducing a REM microarousal. **(Figure 3.)**

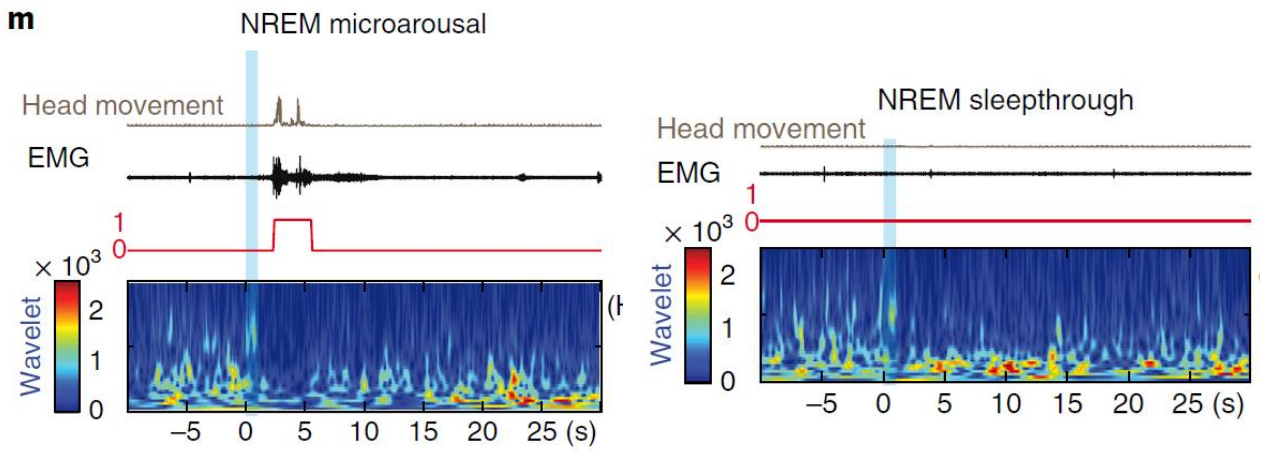

Figure 2. Brief stimulation may induce sleepthrough or microarousals.

Same arrangement as in Fig 1. Sleep-through (right) and microarousal (left) followed by 1 sec long 10 Hz stimulation (blue vertical bar). Microarousals consisted of a brief head movement (brown), increased EMG activity (black) and a transient decrease in low frequency EEG bands. Sleep-through cases were defined as stimulation failed to induce any increased muscle activity and detectable motion.

The data together show that variable stimulus durations is able to evoke distinct, behaviorally relevant arousal patterns ranging from full-blown persistent arousal, through microarousals to transient, subthreshold disruption of specific sleep rhythms as well as state transitions from REM to SWS.

Comparison to sensory arousal

In order to systematically study these MT vs sensory arousals we injected syn-AAV-ChR2 to the somatosensory nuclei of the mouse (VB including the ventral postero-medial, and ventro posterolateral nuclei, n=4 and compared the properties of arousals following laser stimulation in MT and VPM. The two systems displayed pronounced difference in the latencies and duration of microarousals. The latencies of VB stimulations were significantly shorter (VB, 0.8 sec +/-0.11 scec; MT, 3,34 sec +/-0,59 scec $p<0.05$) but, more importantly, it showed a significant negative correlation with laser intensity. In contrast, laser intensity and latency of microarousals did not correlate in case of MT stimulations. Similar to latency the duration of microarousals showed positive correlation in case of VB but no correlation in case of MT stimulation. This shows that whereas the properties of microarousals depend on the laser power (i.e. the size of the neuronal population involved) in case of sensory arousal in case of MT arousal once a threshold is achieved microarousals follow a stereotyped pattern.

The data together showed that midline and sensory arousal can be reliably induced and display significant differences which are worth to study using the fUS system.

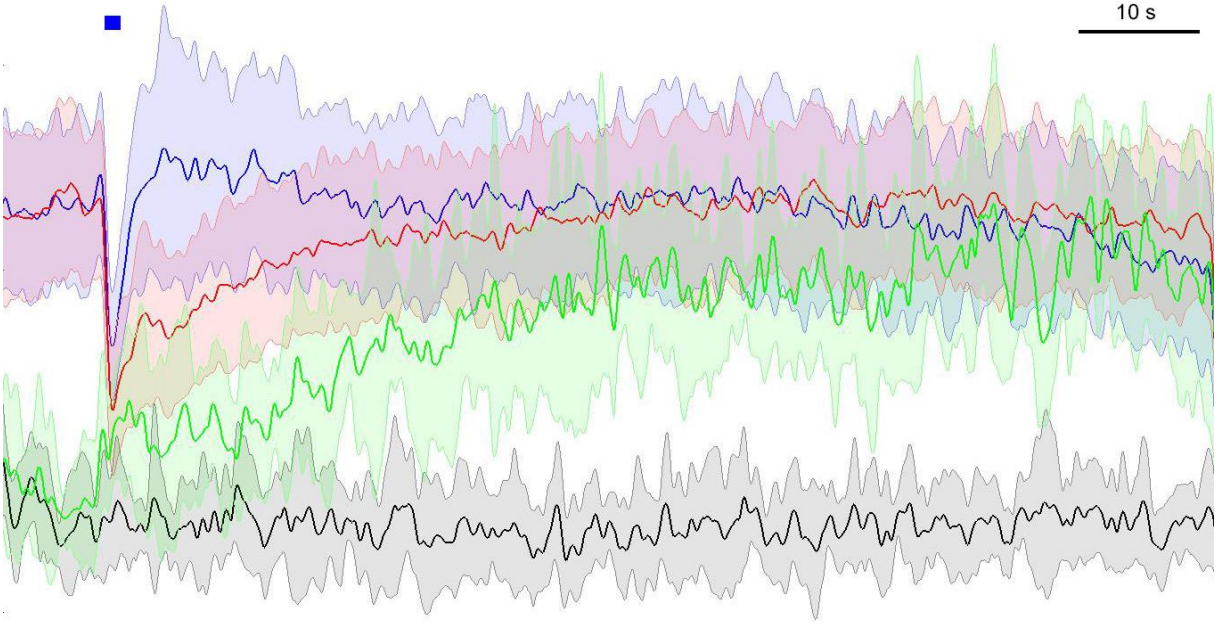

Figure 3. *Changes of delta power as a signiture of state transitions*

During NREM sleep delta power is initially high (red, blue), and decrease transiently in response to 1 sec 10 Hz stimulation (blue bar). Recovery of high delta is longer in microarousal (red), than in sleep-through cases (blue). In REM sleep, however, delta power is significantly lower. When stimulation induces microarousal in REM, mice fall back to NREM sleep, as indicated by high delta power (green). In comparison when stimulation in REM sleep failed to induce microarousals, mice stayed in REM, with low delta power (black). Traces are averages of 6 mice, shaded area are half S.D

Output of CR+ midline thalamic neurons

Next we characterized the connectivity of midline CR+ cells as well as the postsynaptic physiological consequences of activating these neurons . These data are necessary if we want to understand the nature if the fUS signal. By mapping the axons of AAV-DIO-ChR2-eYFP infected DMT/CR+ cells, we found that these cells provided widespread projection to extensive cortical as well as subcortical forebrain targets **(Figure 4).** We observed profuse axon arborizations in several layers of the prelimbic (PrL), insular, perirhinal and entorhinal cortices as well as in the subiculum. In addition layer 6 of almost every cortical regions were innervated at a lower density. Rich innervation reached the core and shell of nucleus accumbens (NAc), the olfactory tubercle, the basolateral and central amygdala (AMY) and the lateral septum. In addition, the hypothalamus, the dorsal striatum and the bed nucleus of stria terminalis also received significant amount of DMT/CR+ fibers.

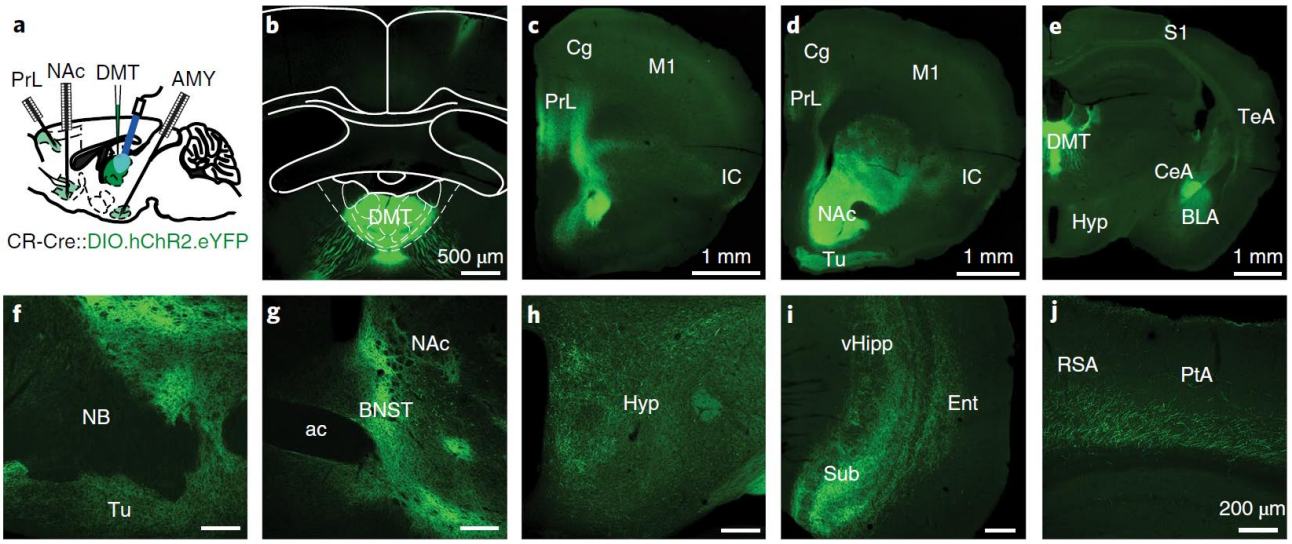

Figure 4. Forebrain targets of CR+ midline thalamic neurons

Schematic of the experiment. b) Thalamic injection site in the CR-Cre mouse. c-j) Postsynaptic targets in various forebrain regions. Abbrev anterior commissure; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; Cg, cingulate cortex; Ent, entorhinal cortex; Hyp, hypothalamus; IC, insular cortex; M1, primary motor cortex; NB, nucleus basalis; PtA; parietal association cortex; RSA, retrosplenial agranular cortex; S1, primary somatosensory cortex; Sub, subiculum; TeA, temporal association cortex; Tu, olfactory tubercle; vHipp, ventral hippocampus

To assess the impact of DMT/CR+ cells on their targets we simultaneously recorded in vivo multiunit activity in the PrL, the AMY and the NAc, while optical stimulation was delivered to DMT under urethane anesthesia, following AAV-DIO-ChR2-eYFP injection into the DMT of CR-Cre mice. One Hz stimulation reliably activated neurons in all three postsynaptic targets with fast onset (<10ms), consistent with a monosynaptic glutamatergic pathway. Ten Hz stimulation was still effective in driving the targets and did not cause a delay in the timing of response . The magnitude of the response depended on stimulus intensity. The multiunit and cortical LFP signal displayed depression at 10 Hz. These data show that DMT/CR+ have widespread projections and can effectively drive their main cortical and subcortical targets.

These data clearly demonstrate that fast and significant fUS signal can be expected in the AMY, NAc and PrL following the stimulation of DMT/CR+ cells. Using three different anatomical methods as well as a physiological approach we consistently demonstrated that a single CR+ midline cell can project to distinct forebrain targets, consistent with its proposed role in broadcasting brainstem arousal signals simultaneously to widespread cortical regions.

Selective inputs of CR+ midline cells, comparison of rodent and human conditions

In mice thalami, orexin-immunopositive fibers provided a highly selective innervation of DMT/CR+ cells irrespective of the exact nuclear position (**Figure 5)**. CR+ cells located both in the paraventricular nucleus, as well as those scattered in the rostral intralaminar nuclei, received dense orexinergic inputs, whereas nearby DMT regions were devoid of orexin+ fibers. Similar observations were made for subcortical glutamatergic terminals labeled by vesicular glutamate transporter 2 (vGLUT2). To study the DMT/CR+ system in humans and its selective subcortical innervation, we performed parallel experiments in postmortem human tissue. In

humans (*n* = 4 brains), CR+ cells were distributed along the ventricular wall of the thalamus. As in mice, a substantial number of CR+ cells were also distributed in the intralaminar nuclei. Irrespective of the shape or size of the DMT/CR+ region in humans, orexinergic axon terminals selectively innervated the CR+ cell groups in a pattern similar to that observed in mice **(Figure 5)**. Also as in mice, heat maps of vGLUT2 fiber density displayed high values in midline and intralaminar regions in correspondence with the distribution of DMT/CR+ cells, whereas the adjacent regions of the mediodorsal nucleus were practically free of any vGLUT2+ axons, demonstrating highly selective innervation of the DMT/CR+ cells

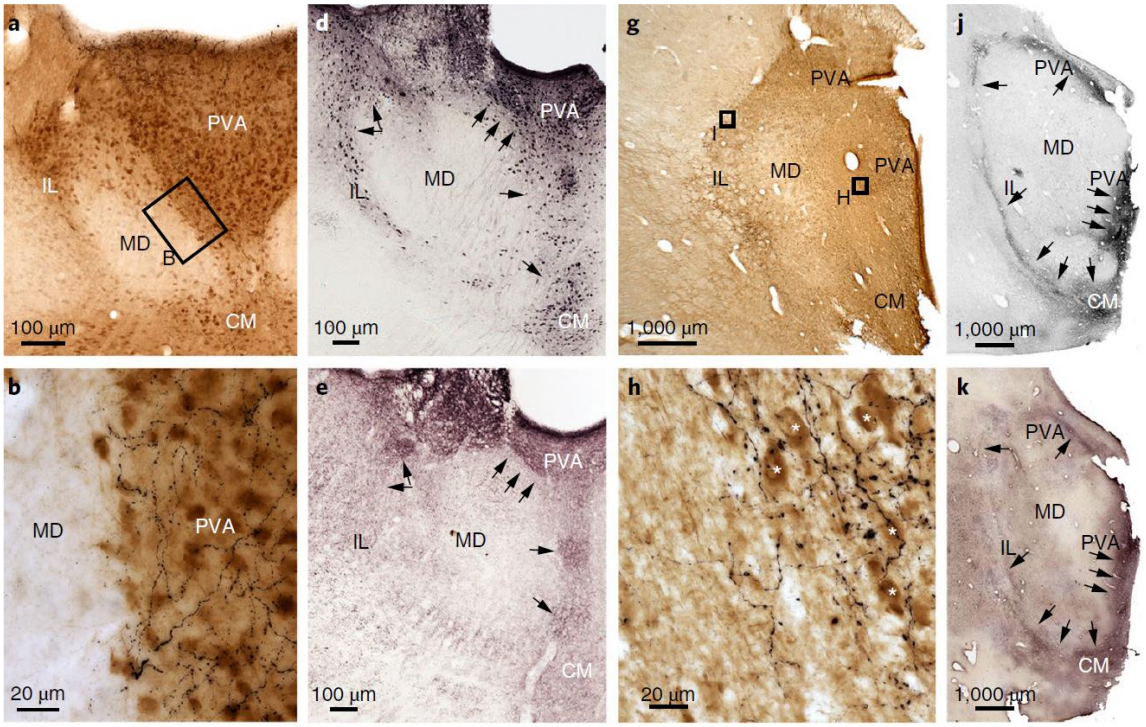

Figure 5 | *Selective subcortical innervation of DMT/CR+ cells in mice and human.* ***a****, Low-power double-immunostaining of mouse DMT for CR (brown) and orexin (Orx, black; n = 4 mice). Small box represents the area enlarged in* ***b****.* ***b,c****, High-power images from the midline (****b****) and intralaminar (****c****) regions. Note that orexin+ fibers are restricted to regions populated by CR+ cells.* ***d,e****, Low-power immunostaining for CR (****d****) and vGluT2 (****e****) of the mouse DMT.* ***g-k,*** *same as a-e but in human thalamic sections.*

Conclusions

The project fulfilled both of its aims.

- **A)** Using morphological and physiological criteria we established that the CR+ midline thalamic neurons form a pivotal arousal system in the forebrain with evolutionary conserved features between rodents and humans.
- **B)** Brief (1 sec) optogenetic stimulation of CR+ midline cells, which can be repeated many times, evokes microarousal pattern in a probabilistic manner. This paradigm allows to test large scale network changes using the unprecedented spatial and temporal resolution of fUS in a biologically relevant behavior. Comparison of microarousal and sleep-through cases with fUS will be especially useful to determine network activity which leads to arousal with motor output. In 2022 the Institute of Experimental Medicine purchased an fUS which will be used as a core facility.