Neural basis of stress induced sleep disturbances

OTKA KH research project 126879

Final report

Summary: The major aim of the present two years project was to establish *the experimental conditions and design* to investigate the neuronal basis of stress-induced behavioral disturbances in a preclinical mouse model. To this end, we set up a research pipeline that included the analysis of motor activity, behavior, sleep, neuronal and hormonal changes before and after a brief exposure to a natural stressor. As detailed below, we discovered a surprisingly long term beneficial effect of modulating the neuronal activity at a key center of the stress circuits. This led the us to an in depth investigation of the phenomenon.

Behavioral apparatus: The core of the set-up is a behavioral box that allows continuous monitoring of movements, behavior, and neuronal activity and, in addition, provides an opportunity for cell type-specific experimental manipulation using an optogenetic approach. In these chronic experiments, the animals were recorded for several hours every day for at least 2-3 weeks. They were connected by multiple wires AND optic fibers to a recording/stimulating system. These allowed recording the activity of the brain and, at the same time, to use a targeted optogenetic approach to influence neuronal activity. However, while the animals are moving around in their cage these cables got entangled which blocks the animals' movement and prevent assessing their movement, sleep, and arousal state. This is especially true following exposure to a stressor when locomotion increases. To prevent this technical problem a special device (so called "commutator") is needed. Commutators for the electrical wires and the optic fibers *separately* are readily available in the market, however, combined commutators are not trivial to find. According to the plans of this project, the majority of the budget was dedicated to purchasing a novel, special type of commutator. As indicated in our previous reports we had problems obtaining the optimal solutions but finally working together with a company (Immetronics) we acquired the appropriate system which allows parallel recording and stimulation. (Figure 1). Behavioral tests now demonstrated that the commutator system as used in this project works properly. We also had to design appropriate sound-insulated behavioral boxes which allow recording the animals' movement and brain activity in a noise-free environment.

Experimental design: We aimed to establish an experimental paradigm, where the stressor evokes natural innate fear in mice. Based on a literature research, we selected predator (fox urine) odor as an innate stressor that robustly induces avoidance and freezing behavior, respectively. To reveal how acute stress affects sleep and sleep-related behavior, we used an auto-controlled

experimental design. Application of the aforementioned behavioral apparatus made it possible to record

Figure 1. Identical behavioral boxes to study the mechanisms of stress induced sleep disturbances in parallel experiments. *Commutators are visible on the top of the boxes, laser apparatus for optogenetic manipulations are placed below the boxes. The box contains sound insulation, adjustable led illumination and digital cameras.*

stress related behavioral changes and EEG/EMG activity. Initially, mice were habituated to the behavioral apparatus and cables for three days. Subsequently, during the next 5 days, we recorded baseline pre-stress behavior (via video recording) and EEG/EMG activity for 3 hours. On the 6th day, mice were exposed to fox odor in a novel environment for 10 min then their activity pattern was recorded for another five days.

In our previous study (Mátyás, Komlósi, et al. Nat Nsci 2018) we established that calretinin-containing neurons in the dorsal medial thalamus (DMT/CR+ cells) are poised to initate arousal and recruited during stress exposure. Thus, we decided to utilize precisely timed optogenetic inhibition (SwiChR) to interfere with the activity of DMT/CR+ cells. The critical step in our approach was that we used optogenetic inhibition not during but after stress exposure since we do not intend to interfere with the perception of the stressor, instead we aimed at understanding what brain activity leads to long-term changes. We utilized 2s ON 15 OFF laser pulses in both control group (receiving EYFP containing viruses in the DMT) and in the experimental group (receiving SwiChR) for one hour in CR-Cre animals after exposure to the stressor (**Figure 2**).

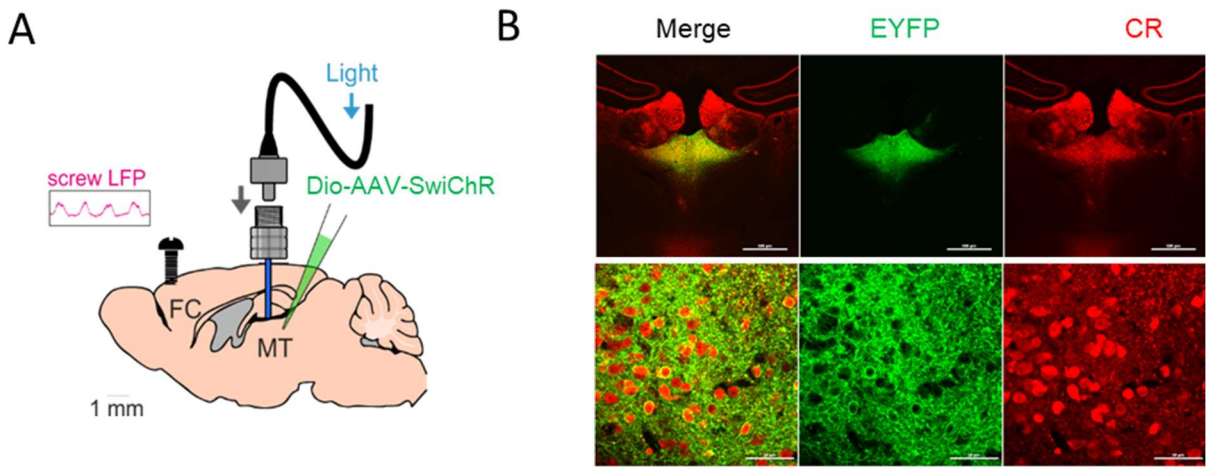

Figure 2. A, Experimental design*. A virus vector containing an inhibitory version of channelrhodopsin (floxed SwiChR) was injected into the medial thalamus of calretinin-Cre mice. This resulted in stable expression of the inhibitory opsin for up to a year. Two 100 micron diameter optic fibers were inserted in two anteroposterior portions of the midline nuclei. The animals were equipped with three screw electrodes to measure cortical EEG activity. B,* ***Low and high magnification confocal images*** *showing calretinin-immunopositive cells (CR+, red) expressing SwiChR opsin (green) in the DMT of a CR-Cre mice*

Results

Short term behavioral, hormonal and molecular changes following acute stress

In our first experiment, we tested whether fox odor stress exposure causes behavioral, hormonal and neuronal changes (**Figure 3A**). After a 5 days long baseline period, mice were transferred into a new room and placed into a new box (n=13 mice). After 2 min mice were presented with **fox odor**. In this novel environment, mice showed novelty-induced exploratory activity (rearing behavior). Upon fox odor presentation the stressed control mice (i.e. mice expressing EYFP fluorophore) displayed robust defensive behaviors (~80% time), particularly freezing and escape jumps conforming to the acute effects of the stressor at the behavioral level (**Figure 3B**). After the stress exposure, mice were returned to the recording environment where we applied the optic fibers to illuminate the DMT/CR+ with blue laser light (**Figure 3A**). During this period, mice showed increased locomotion in their home-cage which was associated with reduced nest-building activity (**Figure 3C**), a core feature of awake pre-sleep behavioral repertoire. Furthermore, we also observed a reduction in time spent with comfort movements (**Figure 3D**). Noteworthy, on the stress day, stress exposure did not affect sleep onset latency (**Figure 3E**). Non-stressed controls animals served as comparison on these measures (n=5). On the stress day, following blue light termination, trunk blood and brains were collected for the measurement of stress reactivity (circulating stress hormone level - corticosterone) and c-Fos upregulation in stress-responsive and stress response regulating brain regions.

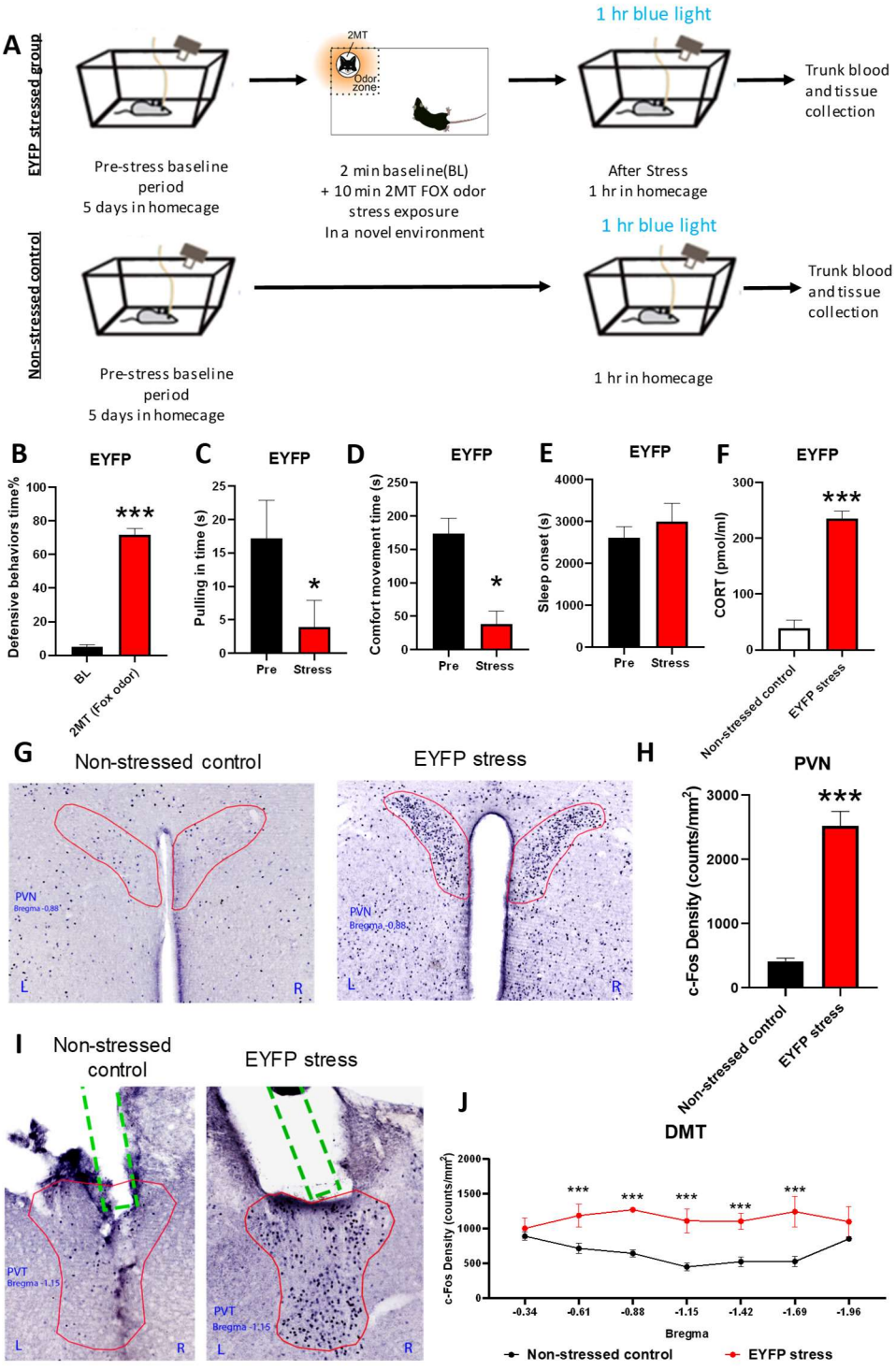

Figure 3. Fox odor stress exposure induces acute behavioral, hormonal, and neuronal changes. A*, schematic representation of the experimental design. Mice expressing the EYFP control virus were recorded during a 5-day long pre-stress period. On the 6th day mice were exposed to 2MT for 10 min. After stress exposure PVT was illuminated for 1 hr long followed by trunk blood and brain tissue collection. The non-stressed control group underwent the same behavioral protocol except stress exposure.* ***B****, during 2MT fox odor exposure mice, exhibited increased defensive behaviors (freezing behavior and escape jumps) compared to the 2 min pre-odor baseline period.* ***C****, After stress exposure EYFP mice, were placed back to the home cage where they spent significantly less time with nesting material collection movements compared to the pre-stress baseline period.* ***Dm*** *stress exposure reduced the time spend with comfort movements compared to the pre-stress baseline period.* ***E****, acute*

*stress exposure did not affect sleep onset latency. **F**, stress exposure elevated blood CORT level. **G**, bright-field images showing c-Fox expression in the PVN of stressed mice (right) and non-stressed mice (left). **H**, bar graph showing c-Fos density in the PVN of stressed and non-stressed mice. **I**, bright-field images showing c-Fox expression in the DMT of stressed mice (right) and non-stressed mice (left).* **J**, *Line graph showing c-Fos density in the DMT across different rostrocaudal levels in stressed and non-stressed mice. CORT, corticosterone; PVN, paraventricular hypothalamic nucleus, DMT, dorsal midline thalamic nucleus; 2MT, 2methyl-2-thiazoline.*

At the hormonal level, stress exposure significantly increased corticosterone levels compared to non-stressed animals (**Figure 3F**). In line with this, at the neuronal level, stress exposure induced robust c-Fos expression both in the DMT (**Figure 3I and 3J**) and in the paraventricular hypothalamic nucleus (PVN), a key brain region mediating hormonal stress response (**Figure 3G and 3H**).

From the point of view of major aims of the project it is important to emphasize that in non-stressed controls we observed low corticosterone levels and lack of marked c-Fos upregulation in the PVN which proved that the assembled behavioral apparatus did not hinder the animal's normal home-cage behavior (locomotion, sleep) and did not induce a stress response. Collectively, we were able to demonstrate that acute fox odor exposure on the stress day heavily disturbed normal home-cage behavior and sleep onset pattern, increased stress reactivity, and neuronal activity of DMT and PVN neurons.

Long term behavioral effect of acute stress

After validating our stress paradigm, we next investigated the **long-term effects of stress exposure** on sleep-related behaviors, sleep-wake profiles (EEG/EMG activity), and the activity of DMT/CR+ cells (**Figure 4A**). At the behavioral level, compared to pre-stress days, **during the post-stress period (4 days)**, we observed a progressively increased locomotion (total distance traveled) and EMG activity, respectively (**Figure 4F**). Furthermore, during the post-stress period mice spent significantly less time with nest building (**Figure 4D**) in conjunction with increased awake nest time (high EMG activity without an increase in delta activity) (**Figure 4C**)**.** We also observed a persistent and progressive increase in time spent with shiver (freezing-like behavior characterized by tiny movements that are not aimed at nest building) by the stressed EYFP mice (**Figure 4B**)**.**

Regarding the sleep-wake profiles, we did not observe sleep fragmentation after stress exposure in the first four days. Intriguingly, our analyses focusing on the assessment of sleep depth using spectral slopes calculated based on the power-law scaling of the Fourier spectra, revealed a decrease in the spectral slope during NREM sleep (**Figure 4E**), indicating disturbed sleep.

Long term effect of acute stress on neuronal activity

To study how stress exposure affects DMT/CR+ neurons activity at the long-term we used movable tetrodes and opto-tagging technique after expressing ChR2 in DMT/CR+ neurons in CR-Cre mice. We observed that DMT/CR+ cells displayed strongly state-dependent activity during pre-stress days (**Figure 4J**), which displayed a significantly altered pattern during the post-stress period (**Figure 4K**). Wake activity in the nest was lower than outside the nest and firing further decreased at the onset of sleep. On the day of stress exposure, both firing rate and synchrony during wakefulness (out of nest) among DMT/CR+ cells increased. Firing-rate remained elevated during the post-stress period (**Figure 4G**), with the strongest change in the time spend in the nest (**Figure 4H**). Importantly, during sleep behavior opto-tagged units showed a significantly increased firing rate change in the post-stress period compared to the pre-stress period (**Figure 4I**). The synchrony and high frequency action potential clusters of DMT/CR+ cells increased during sleep after the stress. Collectively, these data strongly suggest that post-stress activity of PVT/CR+ cells might contribute to the emergence of stress-induced sleep behavior.

Photoinhibition of DMT/CR+ neurons prevent stress-induced alteration in behavior

The next experiments aimed to determine whether inhibition of DMT/CR+ neurons immediately after the stress exposure can alleviate stress-induced behavioral, hormonal, and neuronal changes. Using AAV vectors we expressed inhibitory step-function opsin SwiChR in the DMT of CR-cre and mice were submitted to the aforementioned habituation protocol (**Figure 5A**).

As expected, similarly to EYFP control animals SwiChR mice showed a significant increase in defensive behaviors compared during fox odor stress exposure (**Figure 5B**). However, in contrast to the EYFP control animals SwiChR mice did not display the stress induced behavioral alterations during the post-stress photoinhibition. Relative to pre-stress baseline days SwiChR mice showed reduced locomotor activity indicated by a reduction in EMG activity and a decrease in total distance traveled. In accordance with the wake promoting role of DMT/CR+ neurons, immediate post-stress photoinhibition of DMT/CR+ neurons readily reduced sleep onset latency (**Figure 5D)**. As a consequence of photoinhibtion, SwiChR mice spent very little time with nest building (**Figure 5C**) and exhibited a reduction in time spent with comfort movements (**Figure 5E**).

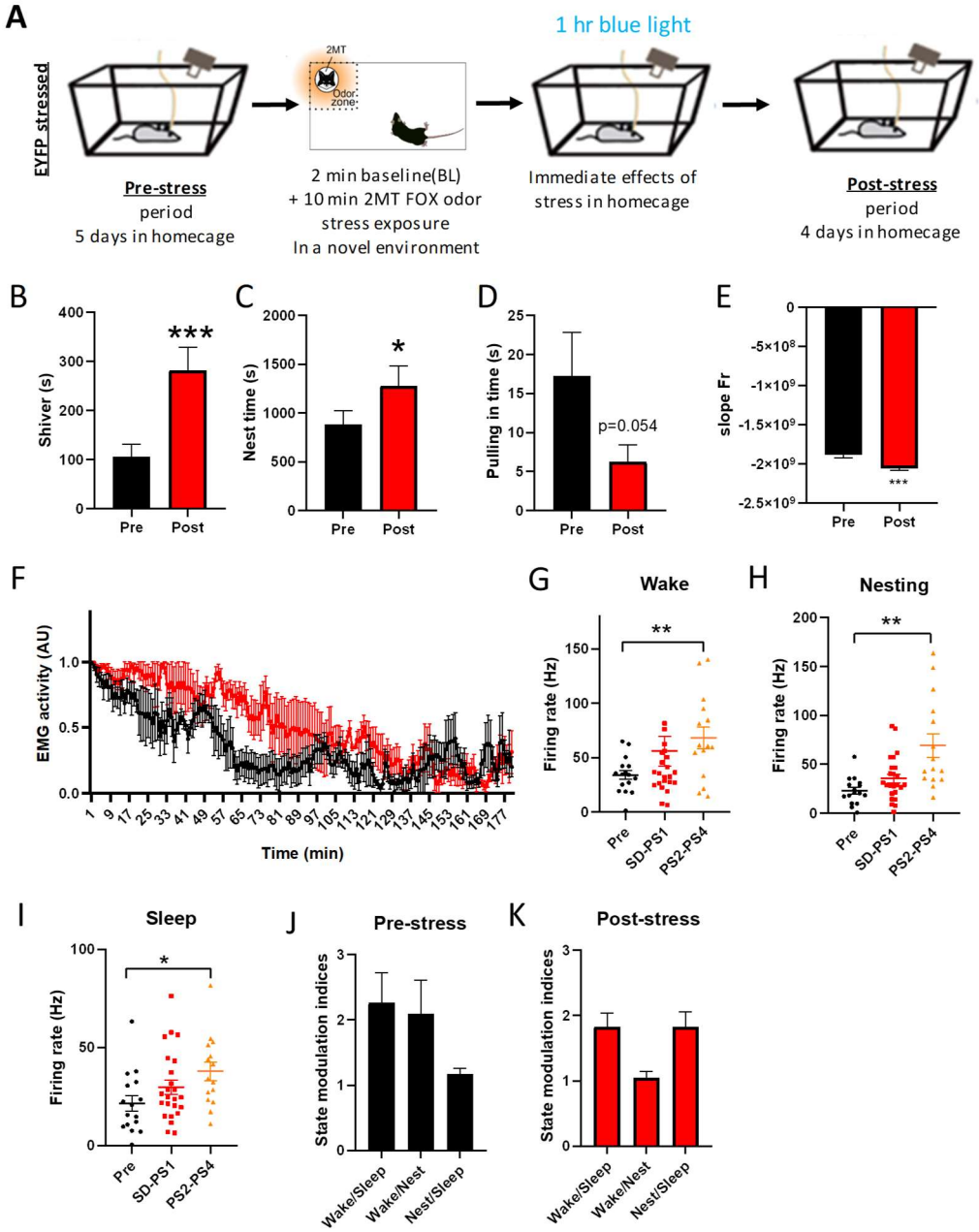

Figure 4. ***Predator odor stress exposure induces long-term sleep-related behavioral changes and sleep disturbance and affects the activity of DMT/CR+ neurons in a state-dependent manner.*** ***A,*** *schematic representation of the experimental design. Mice expressing the EYFP control virus were recorded during a 5-day long pre-stress period followed by stress exposure with a subsequent post-stress 1 hr long blue light illumination. After stress day, mice were recorded during the next 4 days which served as a post-stress period.* ***B****, during the post-stress period EYFP mice showed increased shivering behavior compared to the pre-stress period.* ***C****, during the post-stress period EYFP mice showed increased awake nest time compared to the pre-stress period.* ***D****, during the post-stress period EYFP mice spent significantly less time with nesting material collection movements compared to the pre-stress baseline period.* **E***, during the post-stress period EYFP mice showed a decrease in the spectral slope during NREM sleep compared to the pre-stress period.* ***F****, During the post-stress period EYFP mice showed increased EMG activity compared to the pre-stress period.* ***G****, During the post-stress period (PS2-PS4), opto-tagged photo-responsive units showed an increased firing rate during the awake*

state compared to the pre-stress period. ***H****, During the post-stress period (PS2-PS4), opto-tagged photo-responsive units showed an increased firing rate during nesting* behavior *compared to the pre-stress period.* **I***, During the post-stress period (PS2-PS4), opto-tagged photo-responsive units showed an increased firing rate during sleeping compared to* the pre-stress period. ***J, K,*** *Bar graph showing behavior state-dependent modulation indices of opto-tagged units during the pre-stress period and post-stress period.*

After post-stress photoinhibition, we found a significant reduction in c-Fos expression selectively in the vicinity of the optic fibers confirming that photoinhibition was effective at the level of DMT in SwiChR mice (**Figure 5I and 5J**). However, post-stress photoinhibition of DMT/CR+ cells was not able to prevent the stress induced increase in blood corticosterone levels (**Figure 5F)** and the marked stress-induced c-Fos expression in the PVN (**Figure 5G and 5H**). This suggests that while the treatment is effective at the behavioral level photoinhibiton of DMT/CR+ neurons did not affect hypothalamic-pituitary-adrenal stress response.

Single post-stress photoinhibition of DMT/CR+ neurons prevent long term alteration in behavior

During the 4 days of post-stress period (Figure 6A), SwiChR mice exhibited no stress-induced behavioral changes indicated by indistinguishable levels of locomotor activity (**Figure 6F**), nest building (**Figure 6D**), and awake nest time (**Figure 6C**) relative to the pre-stress baseline period. Remarkably, shivering was also abolished by single post-stress photoinhibition for the entire period (**Figure 6B**). Regarding sleep-wake profiles, relative to pre-stress baseline period and similarly to EYFP group we did not observe sleep fragmentation. Furthermore, photoinhibition prevented stress-induced alteration in the steepness of spectral slope during NREM sleep suggesting that post-stress activity of DMT/CR+ neurons contribute to stress evoked sleep disturbances (**Figure 6E**).

Since we previously showed that the *firing rate of DMT/CR+ neurons* was increased **during the post-stress period, next,** we asked whether photoinhibiton of DMT/CR+ neurons after stress exposure could interfere with their activity changes during the post-stress period. To achieve this, we again used tetrode recordings and opto-tagging on SwiChR-expressing DMT/CR+ neurons in CR-Cre mice.

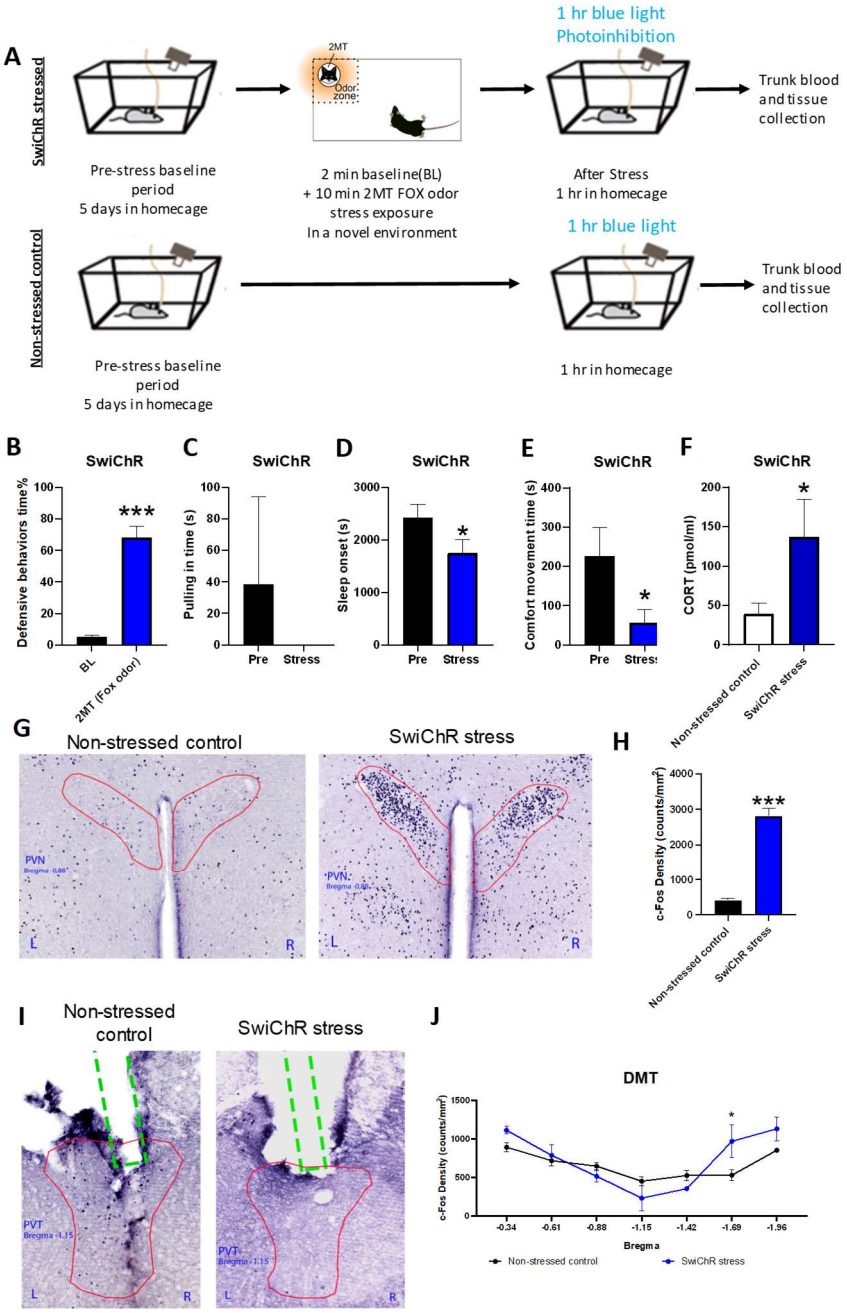

Figure 5. ***Effects of post-stress photoinhibition of DMT/CR+ neurons on fox odor stress-induced acute behavioral, hormonal, and neuronal changes. A,*** *schematic representation of the experimental design. Mice expressing SwiChR inhibitory opsin were recorded during a 5-day long pre-stress period. On the 6th day, mice were exposed to 2MT for 10 min. After stress exposure PVT photoinhibition was applied for 1 hr long followed by trunk blood and tissue collection. The non-stressed control group underwent the same behavioral protocol except stress exposure.* ***B,*** *during 2MT fox odor exposure mice, exhibited increased defensive behaviors compared to the 2 min pre-odor baseline period.* ***C,*** *after stress exposure SwiChR mice spent significantly less time with nesting material collection compared to the pre-stress baseline period.* ***D,*** *immediate post-stress photoinhibition of DMT/CR+ neurons reduced sleep onset latency compared to the pre-stress baseline period.* ***E,*** *stress exposure reduced the time spend with comfort movements compared to the pre-stress baseline period.* ***F,*** *SwiChR mice showed elevated blood CORT levels compared to non-stressed mice.* ***G,*** *bright-field images showing c-Fox expression in the PVN of stressed SwiChR mice (right) and non-stressed EYFP mice (left).* ***H,*** *bar graph showing c-Fos density in the PVN of stressed SwiChR and non-stressed EYFP mice.* ***I,*** *bright-field images*

depicting DMT c-Fos expression in stressed SwiChR mice (right) and non-stressed EYFP mice (left). ***J****, line graph showing comparable c-Fos density in the DMT across different rostrocaudal levels in stressed and non-stressed mice. CORT, corticosterone; PVN, paraventricular hypothalamic nucleus, DMT, dorsal midline thalamic nucleus; 2MT, 2methyl-2-thiazoline.*

Immediately after stress exposure during the 1 hr long photoinhibition treatment, we observed that upon blue light illumination the photo-responsive units (putative DMT/CR+ neurons) showed a sharp activity decrease. Based on this and our c-Fos immunohistochemical results, we confirmed that SwiChR-dependent photoinhibiton effectively diminish CR+ neuron activity *in vivo*. Importantly, immediate post-stress photoinhibion of DMT/CR+ neurons prevented long-term post-stress unit activity increase during waking (**Figure 6G)**, nesting (**Figure 6H),** and sleeping (**Figure 6I)** thus mechanistically supporting the notion that DMT/CR+ neurons contribute to the organization of stress-induced sleep-related behavioral disturbances. In addition, the behavioral state-dependent indices remained unaltered during post-stress period (**Figure 6K)** compared to pre-stress period (**Figure 6J)**. Intriguingly, we observed that photoinhibition lastingly and persistently reduced the synchronicity of opto-tagged units in all the studied behavioral states and the return rate of synchronicity showed a gradual trend staring days after the photoinhibtion.

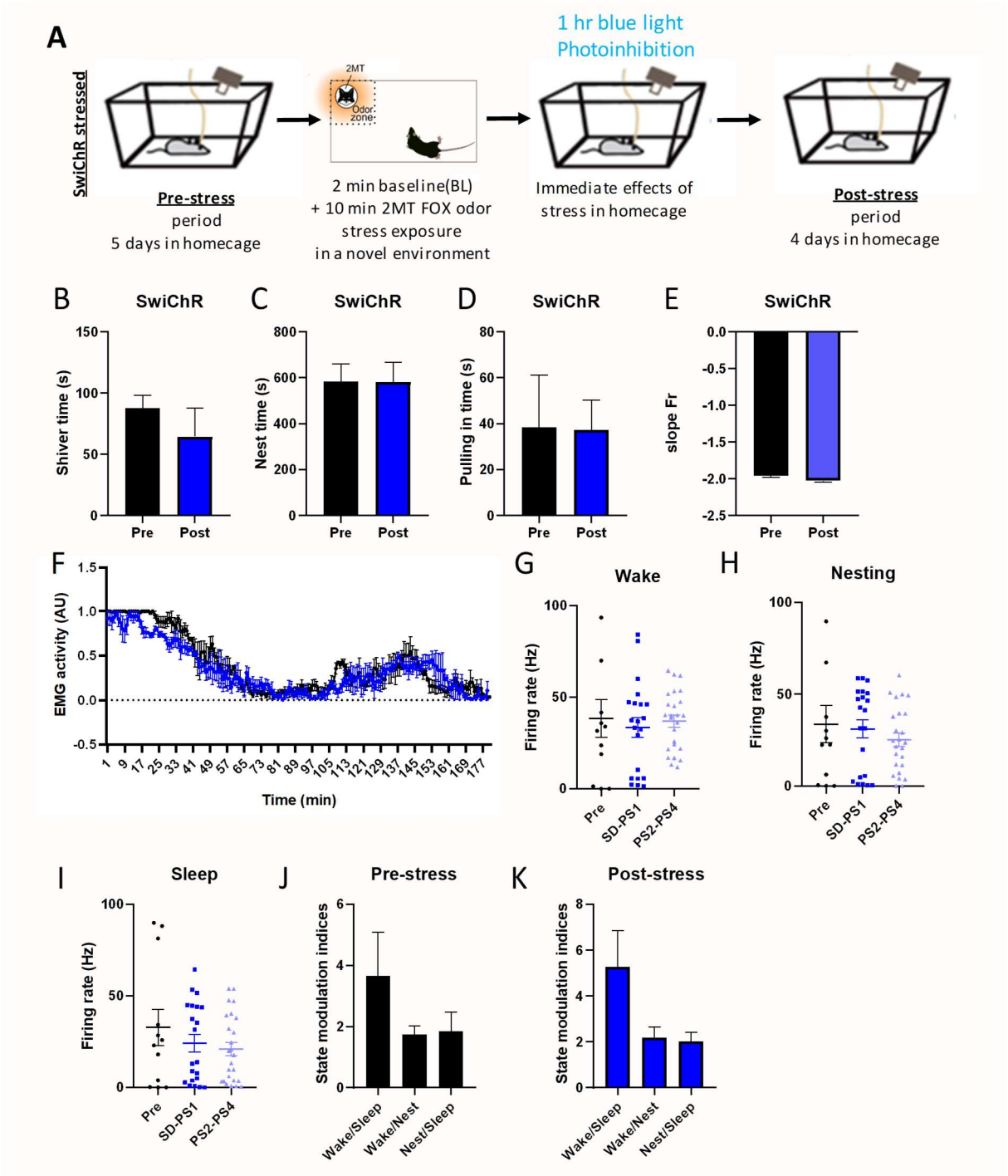

Figure 6. **Immediate post-stress photoinhibition of *DMT/CR+ neurons prevents stress-induced long-term sleep-related behavioral changes, sleep disturbance, and neuronal firing changes. A,*** *schematic representation of the experimental design. SwiChR inhibitory opsin expressing mice were recorded during a 5-day long pre-stress period followed by stress exposure with an immediate post-stress 1 hr long photoinhibition in their home cage. Following stress day, mice were recorded during the next 4 days which served as a post-stress period.* ***B****, SwiChR mice spend similar time with shivering behavior between pre-stress and post-stress periods.* ***C****, SwiChR mice exhibited indistinguishable levels of awake nest time during pre-stress and post-stress periods.* ***D****, SwiChR mice exhibited indistinguishable levels of awake nest time between pre-stress and post-stress periods.* **E***, SwiChR mice showed no changes in NREM spectral slope steepness between pre-stress and post-stress periods.* ***F****, During the post-stress period SwiChR mice showed similar EMG activity as during the pre-stress period.* ***G, H, I****, Immediate*

post-stress photoinhibition prevented long-term effects of stress exposure on DMT CR unit activity during the awake state, nesting, and sleeping in opto-tagged units. ***J, K,*** *Bar graphs showing behavior state-dependent modulation indices of opto-tagged units during pre-stress and post-stress periods.*

Summary

The assemblage and utilization of the behavioral apparatus, as outlined in the project proposal, permitted the monitoring and manipulation of behavior and neuronal activity. This enabled us to establish an animal model to study the neuronal underpinnings of stress-induced long-term changes in behavior. Below we summarize our main achievements:

1. The behavioral apparatus enabled long-term behavioral and neuronal recordings and diminished unintended stress during recordings as indicated by the mice' lack of stress reactivity during pre-stress baseline recording conditions.
2. The newly established predator odor stress paradigm recapitulated stress readouts at the behavioral, hormonal, and neuronal levels and uncovered that the activity of DMT/CR+ neurons was heavily affected by the stressor.
3. The established stress paradigm caused long-term disturbances of normal sleep-related behaviors (sleep preparatory behaviors), sleep quality, and a shift in the activity profile of DMT/CR+ neurons.
4. Photoinhibition of DMT/CR+ neurons after the stress exposure prevented stress-induced behavior and sleep disturbances as well as stress-induced DMT/CR+ activity changes.

Conclusion

Here we revealed that post-stress activity of DMT/CR+ neurons necessary to the development to the stress-induced long-term sleep-related behavioral and sleep disturbances.

Final notes:

Discovery of the long term effect of a ***single*** post-stress modulation of neuronal activity has obvious direct and important therapeutical consequences. This is why the originally short, pilot, project grew into a detailed multidisciplinary study. To enhance its impact details of the study have not been published independently so far. The project, as presented here, is about to submitted to a major multidisciplinary journal in the first half of 2022.