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RESEARCH HIGHLIGHT

Stay Active to Cope with Fear: A Cortico-Intrathalamic Pathway for Conditioned Flight Behavior

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When facing fear-provoking situations, animals and humans alternate between active and passive coping responses [1]. Active coping such as flight or escape is thought to occur when animals perceive the situation to be controllable. In contrast, passive coping such as immobility or freezing is evoked if the situation is perceived to be inescapable. Previous studies have focused predominantly on the neural mechanisms underlying passive fear behaviors (or freezing) with very few published reports on the neural control of active fear responses (or flight). So, what are the neural mechanisms that regulate active fear responses and that permit flexible switching between passive and active coping strategies? A recent study published in *Nature Neuroscience* entitled "A novel cortico-intrathalamic circuit for flight behavior", from Dr. Xiao-Ming Li's lab at Zhejiang University, sheds new light on these important questions [2].

In this study, the authors adopted a new fear conditioning protocol that was first developed by Dr. Andreas Luthi's team [3] (Fig. 1A). Similar to conventional fear conditioning protocols, this protocol uses an auditory stimulus as the conditioned stimulus (CS) and pairs it with an aversive unconditioned stimulus (US), an electrical foot shock, to elicit fear responses. However, rather than just using a pure tone as the CS, which would lead to freezing as the dominant conditioned fear response, the authors used a serial compound stimulus (SCS) consisting of a pure tone followed by white noise as the CS. Over two days of training (5 paired shocks each day), gradually mice freeze in response to the pure tone but exhibit flight, as characterized by increases in locomotor speed and escape jumps, during the white noise (Fig. 1A). In this way, the authors were able to measure both passive and active fear responses and rapid switches between these coping strategies in a single behavioral trial. Importantly, SCS prior to training does not elicit either freezing or flight behavior. Moreover, on the day after training, SCS delivered in a novel context (retrieval test) elicits freezing to both the pure tone and the white noise, while SCS delivered in the pairing context (extinction test) elicits flight to the white noise that quickly extinguishes in the absence of a foot shock. Together, these results demonstrate the highly flexible and context-dependent display of the conditioned flight response.

To dissect the neural mechanism that underlies the conditioned flight response and the switch between freezing and flight, the authors hypothesized that the thalamic reticular nucleus (TRN), a shell-shaped relay structure located between the cortex and thalamus, may play a role. The TRN receives excitatory inputs from both cortex and thalamus and sends inhibitory projections throughout the thalamus [4]. It is thus well-situated as a "gate" to control cortico-thalamic communication in order to regulate attention and behavioral switching [5–9]. Anatomically, the TRN is divided into a limbic sector anterior-dorsally, and a sensory sector posteriorly that connects topographically with sensory thalamic nuclei. Notably, parvalbumin (PV) labels both the limbic and the sensory sectors of the TRN.

To explore whether the TRN is involved in conditioned flight behavior, the authors injected a Cre-dependent

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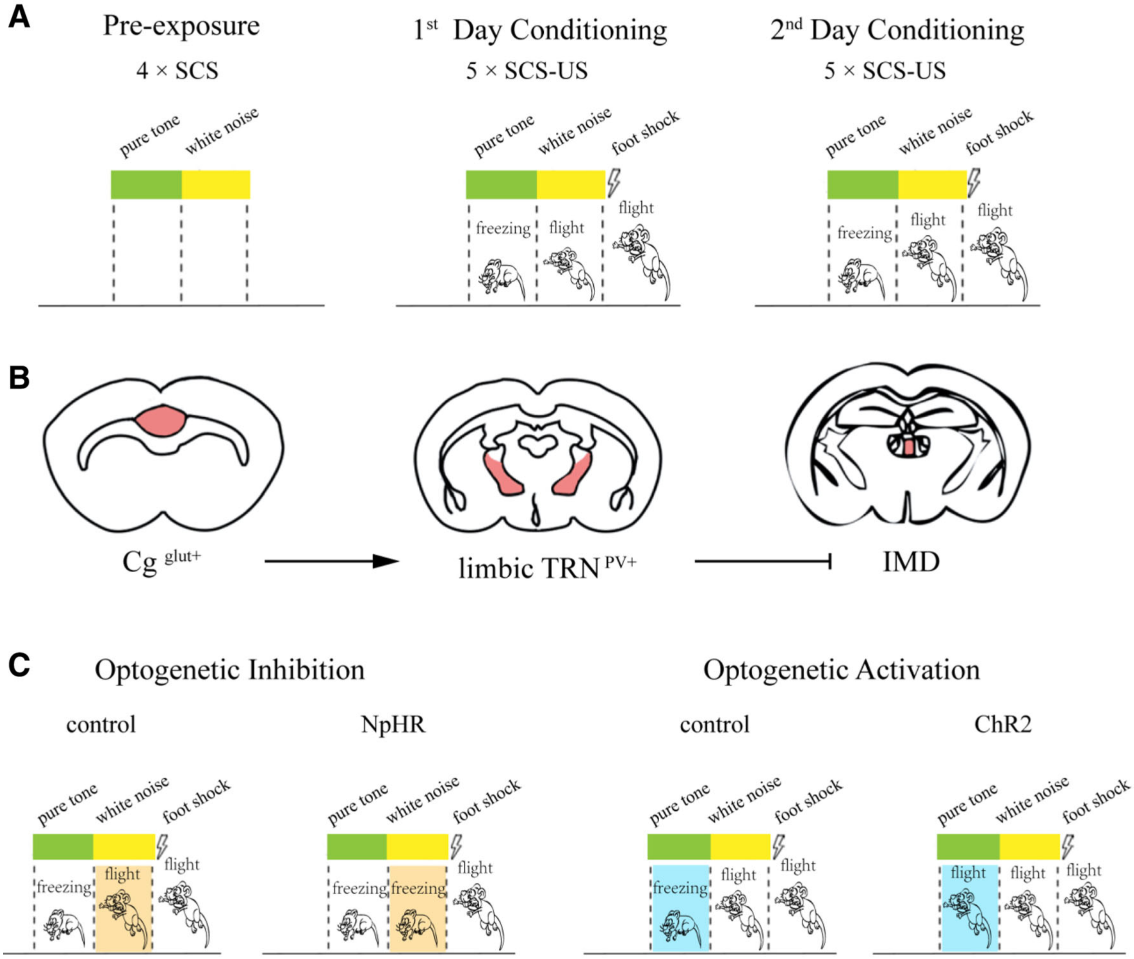

Fig. 1 The $\text{Cg}^{\text{glut+}}$ → limbic $\text{TRN}^{\text{PV+}}$ → IMD circuit bi-directionally regulates conditioned flight. **A** Modified fear conditioning training with the gradual appearance of a conditioned flight response to the white noise over two days of training. **B** Diagrams of the anatomical structures associated with the $\text{Cg}^{\text{glut+}}$ → limbic $\text{TRN}^{\text{PV+}}$ → IMD circuit. **C** Left, optogenetic inhibition of limbic TRN PV+ neurons or $\text{Cg}^{\text{glut+}}$ → limbic TRN projections during the white noise segment of SCS on day 2 of training blocks the conditioned flight response in the NpHR-expressing group but not in controls. Right, optogenetic activation of limbic TRN PV+ neurons, or $\text{Cg}^{\text{glut+}}$ → limbic TRN projections, or limbic $\text{TRN}^{\text{PV+}}$ → IMD projections during the pure tone segment of the SCS on day 2 of training elicits the conditioned flight response in the ChR2-expressing group but not in controls. Cg, cingulate cortex; glut, glutamate; TRN, thalamic reticular nucleus; PV, parvalbumin; IMD, intermediodorsal nucleus; NpHR, halorhodopsin from *Natronomonas*; ChR2, channelrhodopsin-2

adeno-associated virus into PV-Cre mice to selectively express GCaMP6s, a genetically-encoded Ca^{2+} indicator, in PV+ neurons of both the limbic and sensory TRN ($\text{PV}^{\text{limbic-TRN}}$ and $\text{PV}^{\text{sensory-TRN}}$) and monitored the activity in each of them during fear conditioning. Intriguingly, as the animals begin to display flight behavior during the white noise phase of the SCS, the authors found a tightly-coupled increase in GCaMP6s fluorescence signals in $\text{PV}^{\text{limbic-TRN}}$ but not $\text{PV}^{\text{sensory-TRN}}$ neurons. In comparison, both $\text{PV}^{\text{limbic-TRN}}$ and $\text{PV}^{\text{sensory-TRN}}$ neurons are strongly activated during the electric shock. Intriguingly, during the extinction test, the GCaMP6s signals in $\text{PV}^{\text{limbic-TRN}}$ also increase during white noise along with the onset of flight behavior. Importantly, no changes in GCaMP6s signals in $\text{PV}^{\text{limbic-TRN}}$ or $\text{PV}^{\text{sensory-TRN}}$ neurons occur during any other behavioral stages. Furthermore, no changes in the fluorescent signals occur in control animals that express enhanced green fluorescent protein (EGFP) during flight behavior, suggesting that the fluorescent signals in the limbic TRN during flight are not due to a motion artifact. Together, these recordings establish that the activity of $\text{PV}^{\text{limbic-TRN}}$ neurons is tightly correlated with the onset of flight behaviors.

To test whether the activity of $\text{PV}^{\text{limbic-TRN}}$ neurons is required for the conditioned flight response, the authors selectively expressed the inhibitory opsin halorhodopsin (NpHR) in these neurons. They confirmed with *in vitro* electrophysiological recording that yellow light effectively silences NpHR-expressing $\text{PV}^{\text{limbic-TRN}}$ neurons. Yellow light delivered on day 2 of training during the white noise phase of SCS nearly abolishes the conditioned flight behaviors and at the same time promotes freezing (Fig. 1C). In comparison, light inhibition of PV$^{\text{limbic-TRN}}$ neurons during other behavioral stages has no behavioral effects, consistent with little activation of PV$^{\text{limbic-TRN}}$ neurons during these stages. As a control, inhibition of PV$^{\text{sensory-TRN}}$ or light delivery in control animals that express EYFP (enhanced yellow fluorescent protein) also does not affect flight or freezing. Together, these results show that the activity of PV$^{\text{limbic-TRN}}$ neurons is indeed necessary for the conditioned flight response and for the switch from freezing to flight during the white noise.

Next, *via* the selective expression of channelrhodopsin-2 and delivery of pulses of blue light, the authors showed that optogenetic activation of PV$^{\text{limbic-TRN}}$ neurons during the pure tone phase of SCS on day 2 of training decreases freezing and promotes flight (Fig. 1C), in other words switching the fear response from passive to active. A telling observation is that optogenetic stimulation of PV$^{\text{limbic-TRN}}$ in the pairing context alone increases the heart rate of the animal. Such a somatic effect perhaps predisposes an animal for active behavioral responses during fear. In comparison, optogenetic activation of PV$^{\text{limbic-TRN}}$ neurons during other behavioral stages or optogenetic activation of PV$^{\text{sensory-TRN}}$ neurons or light delivery in EYFP-expressing control animals has no effects on freezing or flight behavior. Thus, the activity of PV$^{\text{limbic-TRN}}$ neurons is sufficient to elicit a conditioned flight response during the pure tone.

Interestingly, the acute behavioral effects caused by inhibition or activation of PV$^{\text{limbic-TRN}}$ neurons are carried over to the next day in the extinction test, such that inhibition of PV$^{\text{limbic-TRN}}$ neurons during white noise on day 2 of training results in decreased flight and increased freezing to white noise during the extinction test. Similarly, activation of PV$^{\text{limbic-TRN}}$ neurons during the pure tone on day 2 of training increases flight and decreases freezing to the pure tone during the extinction test. Thus, manipulation of PV$^{\text{limbic-TRN}}$ neuronal activity during training also influences fear memory formation.

Having established a critical role for PV$^{\text{limbic-TRN}}$ but not PV$^{\text{sensory-TRN}}$ neurons in the conditioned flight response, the authors next explored the neural circuit mechanism through which PV$^{\text{limbic-TRN}}$ neurons function. By retrograde tracing and electrophysiological recording, they first demonstrated a monosynaptic excitatory input from the cingulate cortex (Cg) that specifically targets PV$^{\text{limbic-TRN}}$ but not PV$^{\text{sensory-TRN}}$ neurons. Optogenetic activation or inhibition of Cg terminals in the limbic TRN largely recapitulates the behavioral effects on the conditioned flight response found with the functional manipulation of PV$^{\text{limbic-TRN}}$ neurons (Fig. 1C), suggesting that the Cg$^{\text{glut+}}$ → limbic TRN pathway mediates the behavioral effect. Next, by tracing the axonal terminals of PV+ neurons, the authors demonstrated that PV$^{\text{limbic-TRN}}$ neurons project to two major targets, the intermediodorsal thalamic nucleus (IMD) and the lateral part of the mediodorsal thalamus (MDL), whereas PV$^{\text{sensory-TRN}}$ neurons preferentially target the medial geniculate, again pointing to two segregated pathways. As expected, *in vitro* electrophysiological recording revealed that the PV$^{\text{limbic-TRN}}$ projection to the IMD is inhibitory. Furthermore, optogenetic activation or inhibition of PV$^{\text{limbic-TRN}}$ terminals in the IMD but not the MDL repeats the behavioral effects on the conditioned flight response found with the manipulation of PV$^{\text{sensory-TRN}}$ neurons (Fig. 1C), demonstrating that the limbic TRN$^{\text{PV+}}$ → IMD pathway, in other words inhibition of the IMD, underlies the conditioned flight response.

Excitingly, using the cTRIO technique (a cell-type-specific tracing method used to determine the relationship between inputs and outputs) and electrophysiological recordings in brain slices, the authors further showed that the Cg preferentially innervates IMD-projecting PV$^{\text{limbic-TRN}}$ neurons, thus connecting the Cg$^{\text{glut+}}$ → limbic TRN pathway with the limbic TRN$^{\text{PV+}}$ → IMD pathway (Fig. 1B). More importantly, by combining region-specific pharmacology with optogenetic manipulation, they showed that, while optogenetic activation of the Cg$^{\text{glut+}}$ → limbic TRN terminals promotes flight behavior and suppresses freezing during the pure tone, such effects depend on inhibition of the IMD, as infusion of picrotoxin (PTX, a selective GABA$_A$ receptor antagonist) into the IMD blocks these effects. Furthermore, infusion of PTX without activation of the Cg$^{\text{glut+}}$ → limbic TRN pathway also significantly decreases flight and promotes freezing in control animals, suggesting that GABA$_A$ receptor-mediated inhibition in the IMD is essential for conditioned flight behavior. In summary, these results suggest that the bi-synaptic Cg$^{\text{glut+}}$ → limbic TRN$^{\text{PV+}}$ → IMD circuit bi-directionally regulates the conditioned flight response and the behavioral switch from freezing to flight through inhibition of the IMD (Fig. 1).

What then might be the downstream targets of IMD neurons? Given that these neurons are predominantly excitatory and that inhibition of the IMD promotes the conditioned flight response, the logic would be that neurons downstream of IMD neurons likely promote freezing. Indeed, by anterograde trans-synaptic tracing from the IMD, the authors identified somatostatin-expressing (SOM+) neurons in the central amygdala (CEA), a population with a well-documented role in promoting conditioned freezing. This suggests the exciting possibility that the CEA SOM+ neurons might be the major downstream target of the Cg$^{\text{glut+}}$ → limbic

$\mathrm{TRN}^{\mathrm{PV}+}$ → IMD circuit to mediate conditioned flight behavior; this remains to be tested.

In conclusion, in this exhaustive and comprehensive study, the authors show that the limbic TRN is selectively activated during the conditioned flight response and that the $\mathrm{Cg}^{\mathrm{glut}+}$ → limbic $\mathrm{TRN}^{\mathrm{PV}+}$ → IMD circuit bi-directionally regulates this behavior through inhibition of the IMD (Fig. 1). These findings provide novel and piercing insights into the function of the TRN, an elusive and seemingly impenetrable brain structure that has so far only been investigated in the context of sleep and selective attention [8, 9]. Discoveries from this article suggest perhaps a broader view of the function of the limbic TRN in regulating behavioral selection, through interaction with a subcortical circuit. This study also reaffirms the complex and layered control of what appear to be very simple motor functions [10]. Previous studies using a similar behavioral paradigm have shown that CEA CRF+ and SOM+ neurons reciprocally inhibit each other to tilt the behavioral response between freezing and flight [3]. It will be interesting to trace CEA CRF+ neurons to see whether they too have a similar upstream cortical-thalamic regulatory circuit. At a more fundamental level, what might be the neural mechanisms that activate the cortical regions during SCS training? Undoubtedly, this study from Dr. Xiao-Ming Li's team paves the way for future research to answer these exciting questions.

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