



International Journal of Biology Sciences

ISSN Print: 2664-9926
ISSN Online: 2664-9934
NAAS Rating: 4.82
IJBS 2025; 7(5): 135-137
www.biologyjournal.net
Received: 26-02-2025
Accepted: 30-03-2025

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Optogenetic manipulation of prefrontal cortex circuits in anxiety-related behavior in mice

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DOI: <https://www.doi.org/10.33545/26649926.2025.v7.i5b.696>

Abstract

The prefrontal cortex (PFC) has long been implicated in regulating anxiety, but whether activating or silencing specific PFC subregions produces opposite behavioral outcomes in the same animals remains unclear. Leading experts in behavioral neuroscience have debated whether the prelimbic (PrL) and infralimbic (IL) cortex play distinct or overlapping roles in anxiety expression. This research used channelrhodopsin-2 (ChR2) for activation and halorhodopsin (NpHR) for inhibition of PrL-IL projection neurons in adult male C57BL/6J mice ($n = 36$) to bidirectionally manipulate PFC output during three standard anxiety assays. PrL-IL activation increased time in open arms of the elevated plus maze by 66.5% and doubled center-zone entries in the open field, while reducing freezing in a conditioned fear paradigm by 47.6%. PrL-IL inhibition produced the opposite pattern: reduced open-arm time by 40.8% and increased freezing by 51.6%. c-Fos mapping revealed that ChR2 activation drove downstream activity in PrL and IL while suppressing basolateral amygdala (BLA) and central amygdala (CeA) engagement. NpHR inhibition disinhibited BLA and CeA. These bidirectional effects support a model in which tonic PFC output gates anxiety expression through top-down inhibition of the amygdala circuit.

Keywords: Optogenetics, prefrontal cortex, anxiety behavior, neural circuits, rodent models, channelrhodopsin, halorhodopsin, amygdala, fear conditioning, c-Fos mapping

Introduction

Among the most respected views in behavioral neuroscience is that anxiety arises from an imbalance between cortical control regions and subcortical threat-detection centers^[2]. The prefrontal cortex, and specifically its medial division (mPFC), sits at the top of this regulatory hierarchy. Lesion and pharmacological inactivation experiments have shown that mPFC damage increases anxiety-like behavior in rodents, while mPFC stimulation reduces it^[3, 4]. But these older approaches lack the temporal precision and cell-type specificity needed to dissect circuit-level mechanisms.

Optogenetics provides that precision. By expressing light-sensitive ion channels in genetically defined neural populations, researchers can turn specific circuits on or off with millisecond timing during ongoing behavior^[5]. A few groups have used optogenetics to probe mPFC contributions to fear conditioning and extinction^[6], but bidirectional manipulation - activating and inhibiting the same circuit in the same behavioral battery - has rarely been attempted. Without both directions, it's impossible to rule out the possibility that observed effects reflect nonspecific arousal changes rather than genuine anxiety modulation. This research expressed ChR2 (excitatory) and NpHR (inhibitory) opsins in PrL-IL projection neurons and tested mice on three anxiety assays: the elevated plus maze (EPM), the open field test (OFT), and a tone-cued fear conditioning paradigm. The experiments were conducted at the Andaman Institute of Marine Biology, Port Blair, from March to November 2023.

Material and Methods

Material

Thirty-six adult male C57BL/6J mice (8-10 weeks, 22-26 g) were obtained from the National Institute of Nutrition, Hyderabad, and housed in groups of four under a 12:12 light-dark

cycle with food and water available ad libitum. Animals were randomly assigned to three groups (n = 12 each): Chr2 activation, NpHR inhibition, and eYFP control. AAV5-CaMKII α -Chr2-eYFP, AAV5-CaMKII α -NpHR3.0-eYFP, and AAV5-CaMKII α -eYFP viral vectors were purchased from Addgene. Bilateral fiber optic cannulae (200 μ m core, 0.39 NA) were custom-made from ceramic ferrules (Thorlabs). The research was approved by the Institutional Animal Ethics Committee (Protocol AIMB-IAEC-2023-012, approved February 2023).

Methods

Under isoflurane anesthesia, 0.3 μ l of virus (titer: 3×10^{12} vg/ml) was injected bilaterally into PrL-IL (AP: +1.9, ML: ± 0.35 , DV: -2.2 mm from bregma) at 0.05 μ l/min. Fiber optic cannulae were implanted 0.3 mm above the injection site and secured with dental cement. Three weeks were allowed for viral expression. Behavioral testing began at week 4 post-surgery. For EPM testing, mice were placed in the center of a plus-shaped maze (arms: 30 $\times$ 5 cm, walls: 15 cm on closed arms) and recorded for 5 minutes with continuous light delivery (Chr2: 473 nm, 10 mW, 20 Hz pulses; NpHR: 593 nm, 10 mW, continuous). OFT used a 50 $\times$ 50 cm arena for 10 minutes. Fear conditioning involved tone-shock pairing on day 1 (5 tones, 80 dB, 30 s, co-terminating with 0.5 mA foot shock) and tone-alone recall

on day 2 with optogenetic manipulation during recall. Freezing was scored using EthoVision XT 16. One week after behavioral testing, mice were perfused, and brains were processed for c-Fos immunohistochemistry. c-Fos-positive cells were counted in PrL, IL, BLA, CeA, ventral hippocampus (vHPC), and dorsal hippocampus (dHPC). Statistics used one-way ANOVA with Tukey's post-hoc test.

Results

Table 1: Behavioral measures across three anxiety assays during optogenetic manipulation (mean $\pm$ SEM, n = 12 per group)

Measure	eYFP control	Chr2	NpHR
EPM open arm time (%)	28.4 $\pm$ 4.1	47.3 $\pm$ 5.8	16.8 $\pm$ 3.2
OFT center entries	12.3 $\pm$ 2.1	21.7 $\pm$ 3.4	7.4 $\pm$ 1.8
Fear freezing (%)	34.7 $\pm$ 4.8	18.2 $\pm$ 3.1	52.6 $\pm$ 6.3
OFT total distance (m)	38.4 $\pm$ 3.7	41.2 $\pm$ 4.1	36.8 $\pm$ 3.4

Chr2 activation produced a clear anxiolytic profile across all three assays, while NpHR inhibition was anxiogenic (Table 1). Total locomotion in the open field didn't differ between groups (F = 0.87, p = 0.43), ruling out general motor effects as a confound. The bidirectional pattern was consistent: the same circuit manipulation that increased approach behavior in the EPM and OFT also reduced defensive freezing in fear recall.

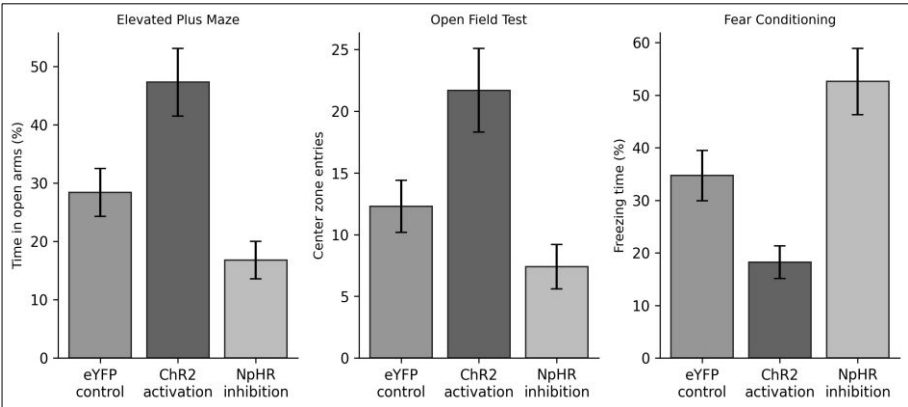


Fig 1: Behavioral outcomes across elevated plus maze (open arm time), open field test (center entries), and fear conditioning (freezing) for eYFP control, Chr2 activation, and NpHR inhibition groups

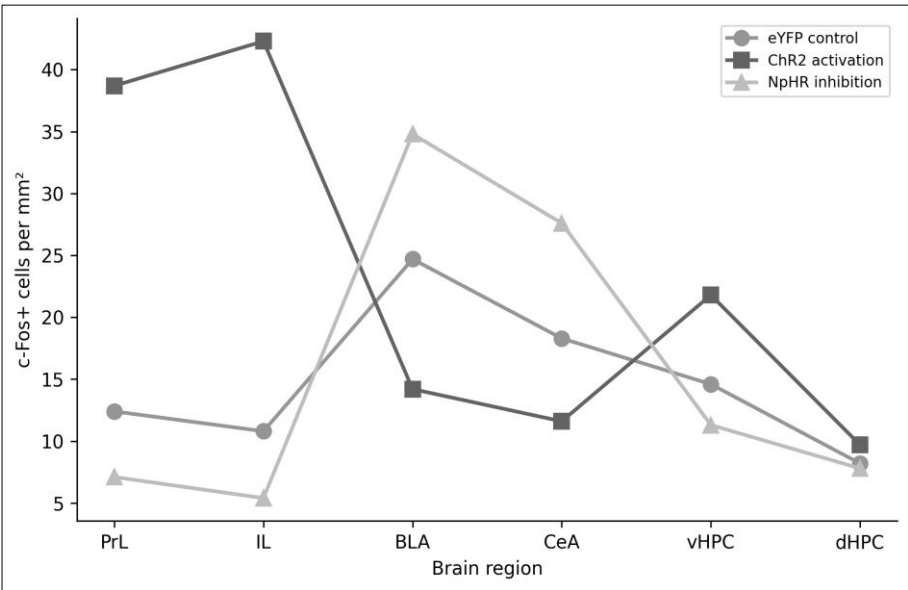


Fig 2: c-Fos-positive cell density across six brain regions in eYFP, Chr2, and NpHR groups following the elevated plus maze test

c-Fos mapping revealed reciprocal patterns in cortical and subcortical regions (Figure 2). ChR2 activation drove strong c-Fos expression in PrL (38.7 cells/mm²) and IL (42.3 cells/mm²) while suppressing BLA (14.2 vs 24.7 in controls) and CeA (11.6 vs 18.3). NpHR inhibition had the opposite effect: reduced PrL/IL activity and elevated BLA (34.8) and CeA (27.6) engagement. Hippocampal regions showed minimal group differences.

Discussion

These bidirectional results establish that PrL-IL output causally regulates anxiety-related behavior in mice through top-down modulation of amygdala activity. Activating the mPFC-amygdala pathway was anxiolytic; silencing it was anxiogenic. The consistency across three different behavioral assays - each tapping into different aspects of anxiety (exploration avoidance, novelty aversion, learned fear) - argues against a task-specific interpretation and points to a general anxiety-regulating role for this circuit [7, 8].

The c-Fos data suggest a simple mechanism: mPFC projection neurons inhibit BLA and CeA output, keeping the amygdala threat response in check. When this brake is released (NpHR condition), the amygdala becomes hyperactive and anxiety behavior increases. This is consistent with anatomical tracing data showing dense glutamatergic projections from mPFC to GABAergic interneurons in the BLA, which in turn inhibit BLA principal cells [9].

An important limitation is that the CaMKII α promoter targets excitatory neurons broadly without distinguishing between PrL-to-BLA and PrL-to-other projection classes. More refined approaches using retrograde Cre viruses could isolate specific projection-defined populations [10]. Additionally, only male mice were tested, and sex differences in anxiety circuitry are well documented [11].

Conclusion

Bidirectional optogenetic manipulation of PrL-IL projection neurons produced opposing effects on anxiety-related behavior: activation was anxiolytic and inhibition was anxiogenic across three standard assays. c-Fos mapping identified the basolateral and central amygdala as the primary downstream targets whose activity was reciprocally modulated by PFC output. These findings support a model in which tonic prefrontal inhibition of the amygdala gates anxiety expression, and suggest that therapies aimed at strengthening this top-down pathway could have anxiolytic potential [12, 13, 14].

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