Light affects the prefrontal cortex via intrinsically photosensitive retinal ganglion cells

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Abstract

The ventromedial prefrontal cortex (vmPFC) is a part of the limbic system engaged in the regulation of social, emotional and cognitive states, which are characteristically impaired in disorders of the brain such as schizophrenia and depression. Here, we show that light, through intrinsically photosensitive retinal ganglion cells (ipRGCs), modulate the integrity, activity and function of the vmPFC. This regulatory role, which is independent of circadian and mood alterations, is mediated by an ipRGC-thalamic-corticolimbic pathway. Lack of ipRGC signaling in mice causes dendritic degeneration and depressed neuronal activity in the vmPFC. These alterations primarily undermine the ability of the vmPFC to regulate emotions. Our discovery provides a potential light-dependent mechanism for certain PFC-centric disorders in humans.

Introduction

In mammals, non-image-forming visual functions such as circadian photoentrainment, pupillary light response and mood regulation by light exposure are dependent on a subpopulation of retinal ganglion cells (RGCs) made intrinsically photosensitive (thus ipRGCs) by their expression of the photopigment melanopsin (Opn4) (Provencio & Warthen, 2012; Hattar et al, 2002; Berson et al, 2002; Provencio et al, 2000; Güler et al, 2008). These ipRGCs are diverse, comprising at least six subtypes (M1-6) (Ecker et al., 2010; Quattrochi et al., 2019; Schmidt et al., 2014) distinguished by size, dendritic stratification, and electrophysiological properties. Their central projections also differ, reflecting the diverse biological functions that ipRGCs regulate (Lazzerini Ospri et al., 2017). Among the brain regions ipRGCs innervate is the perihabenula (PHb) (Fernandez, Fogerson, Lazzerini Ospri et al., 2018), a nucleus of the dorsal thalamus that mediates the direct effects of light on mood (Fernandez et al., 2018, LeGates et al., 2012, Fernandez, Fogerson, Lazzerini Ospri et al., 2018). The PHb densely innervates the ventromedial subdivision of the prefrontal cortex (PFC) (Fernandez, Fogerson, Lazzerini Ospri et al., 2018), and thus relays retinal input directly to this region.

The prefrontal cortex organizes goal-directed behaviors, its main domains being planning, decision making, executive attention and emotional regulation (Fuster, 2001, Franklin and Chudasama, 2012). A distinction is drawn between the dorsolateral PFC, implicated in purely cognitive functions, and a paralimbic PFC, comprising the orbital and ventromedial areas, involved in evaluating the emotional significance of events and regulating emotions for the purposes of goal-directed behavior (Fuster, 2001, Morgan et al., 1993, Franklin and Chudasama, 2012). In mice, the ventromedial prefrontal cortex (vmPFC) encompasses the prelimbic and infralimbic cortex. It is part of a broader network interconnecting limbic and associative areas such as the basomedial amygdala and the ventral

striatum, the hypothalamus and the brainstem (Adhikari et al., 2015; Vertes, 2004), and thereby contributes to a wide range of functions. Lesion studies in mice implicate the vmPFC in social cognition (Bicks et al., 2015), the regulation of fear and anxiety (Sotres-Bayon and Quirk, 2010), and the cognitive control of actions (Latif-hernandez et al., 2016). These behavioral deficits are a core feature of many clinical populations including those suffering from depression and schizophrenia, who are known to have a compromised vmPFC (Chudasama and Robbins 2006, Bicks et al., 2015). Despite its clinical importance, and the fact that it is interconnected with many areas directly or indirectly receiving photic input, to date, there is no indication that this region may be affected by light exposure, an important environmental variable. Here we show that light exposure regulates the integrity, activity, and function of the vmPFC through an ipRGC-thalamic-prefrontal pathway that is independent of the visual cortex.

Results

Dark-reared mice or mice lacking ipRGC exhibit reduced apical dendritic arborization in the vmPFC

Within the vmPFC, PHb innervation encompasses layers I and III/IV of the infralimbic and prelimbic cortex. Previous studies showed that chronic exposure of animals to constant darkness (known as a dark-dark or DD) delays the normal development of the primary visual cortex (Gianfranceschi, et al., 2003, Izquierdo et al., 2006, Perez-Cruz et al., 2009). To address whether light exposure affects the vmPFC, we prepared and analyzed Golgi-stained coronal sections along the anterior-posterior extent of the infralimbic and prelimbic cortex. One group of control mice were maintained on a standard 12-hour light – 12-hour dark cycle (12:12 LD), and another group of experimental mice were kept in DD prior to birth and up to 4 months after birth. We then reconstructed the apical dendritic arbors of pyramidal neurons in the vmPFC **well-known for their plasticity in response to environmental stimuli (Izquierdo et al., 2006)** (Fig. S1A-B, Fig. 1A). We observed significant differences between light regimes, with mice in the DD group displaying severe dendritic atrophy, with a reduction in total apical dendritic length of ~36% relative to LD controls, and a similar (~40%) significant reduction in the number of branch points (Fig. 1B-C). This shows that light is required for the normal development of the vmPFC.

Dark rearing causes depression-like mood alterations (Zhou et al., 2018) and lengthens the period of circadian rhythms in rodents (Chew et al., 2017). Depression is associated with metabolic changes in the vmPFC (Drevets et al., 1992). This raised the possibility that our DD results might have been secondary to these alterations. To address this, we used two mouse lines that detect light but lack a subset of ipRGCs (Güler et al., 2008; Chen et al., 2011). We analyzed the vmPFC pyramidal neurons of

a transgenic mouse line that expresses an attenuated form of diphtheria toxin A ("aDTA") under the control of the melanopsin promoter (*Opn4*$^{aDTA/aDTA}$). In aDTA mice, M1 ipRGCs develop and function normally until about 8 weeks of age, when they start degenerating; the loss progresses linearly with complete die-off by 6 months (Güler et al., 2008). Basal mood, as assessed by forced swim and sucrose preference tests, is normal in these animals, their visual system develops normally, and they have intact circadian rhythms (Güler et al., 2008; LeGates et al., 2012). In addition, ipRGC degeneration in aDTA mice occur without traumatic or stressful procedures known to affect PFC neuronal morphology (Izquierdo et al., 2006). In mice older than 6 months, we observed major differences between genotypes, with reconstructed neurons from aDTAs displaying severe dendritic atrophy (Fig. 1D-F), similar to what was observed in wildtype mice reared in DD. The reduced number of intersections in Scholl analysis after ipRGC loss (Fig.1D) confirmed a reduction in total apical dendritic length by ~30% in aDTA mice relative to controls (Fig.1I), with a ~36% significant reduction in the number of branch points (Fig. 1M).

The time course of ipRGC loss in aDTAs afforded an internal control. We repeated the morphometric analysis in aDTA mice at 7 weeks old, before the onset of cell degeneration, and at 3-4 months old, when ~50% of ipRGCs had degenerated. At 3-4 months of age, aDTA mice displayed an early form of the atrophic phenotype showing ~10% reduction in total apical dendritic length and ~33% reduction in number of branch points compared age matched control mice (Fig. 1H, L). In contrast, the atrophic phenotype was not observed in 7-week-old aDTA mice, when degeneration of ipRGCs had not started (Fig. 1E, G, K, J, N). Thus, the severity of apical dendritic atrophy seen in this neuronal population was proportional to the extent of ipRGC loss and confirms an essential role for light in the dendritic development of the vmPFC.

To investigate if the atrophic phenotype we observed is specific to regions of the cortex that receive ipRGC input, we reconstructed neurons in the motor cortex as a control region. No differences between the wildtype and aDTA mice in the motor cortex were observed (Fig. S1C), indicating that the atrophic phenotype was specific to the vmPFC.

Chronic dark rearing and the aDTA mice lack the ability to adjust their day/night activity to the 24-hour day in what is known as circadian photoentrainment (Chew et al., 2017; Güler et al., 2008). The lack of circadian photoentrainment could underlie the atrophic phenotype we observed in the vmPFC. Therefore, we repeated our analysis in a transgenic line, *Opn4*$^{Cre/+}$; *Brn3b*$^{zDTA/+}$ (Brn3bDTAs) mice that have normal circadian photoentrainment but lack ipRGC input to the PHb-vmPFC (Chen et al., 2011). These mice express a strong variant of diphtheria toxin A under the control of the Brn3b promoter in a Cre-dependent manner, and Cre itself under the control of the melanopsin promoter.

Brn3b is a transcription factor expressed in all ipRGCs except a subset of M1 cells that project to the SCN and are sufficient for circadian photoentrainment (Chen et al., 2011). So, Brn3bDTA mice can synchronize their circadian rhythms to the environmental light cycle but lose in developmental stages non-SCN-projecting ipRGCs. In this mouse model, we found vmPFC abnormalities identical to those present in dark reared and aDTA mice showing an ~33% average reduction in total apical dendritic length and ~37% fewer branch points vs. littermate controls (Fig. 1D, I, M). We conclude that lack of light-driven ipRGC input leads to apical dendritic degeneration in the vmPFC, and that this effect is not secondary to any SCN-based circadian disruptions, mood alterations, or developmental effects.

vmPFC response to a stressful stimulus is reduced in ipRGC-ablated mice

We then measured the activity of the vmPFC in ipRGC-ablated mice by photometry recording in freely moving mice. We transduced vmPFC neurons of Brn3bDTA mice and wildtype littermates with the Ca^{2+} sensor, GCaMP6f, under the control of the synapsin promoter. An optical fiber was implanted above the vmPFC region (Fig. 2A) and patched to a photometry apparatus capable of exciting GCaMP and measuring emitted fluorescence without restricting the movement of animals. We recorded the activity of the vmPFC at different circadian times, since the vmPFC is known to display strong circadian rhythms (Perez-Cruz et al., 2009). Contrary to the presence of strong molecular circadian clock in the vmPFC, the activity of the vmPFC did not show robust daily variations. However, the vmPFC of ipRGC-ablated animals, indeed exhibits lower activity, specifically by showing fewer endogenous activity peaks compared to controls (Fig. 2C, D). These data suggest that ipRGC input to the PHb maintain vmPFC integrity and function.

The changes in the basal activity and morphology of the vmPFC prompted us to investigate the intra-vmPFC Ca^{2+} dynamics in response to a stressful stimulus. A sudden loud sound was chosen (Fig. 2B) because it is independent of the visual system ensuring that any difference between ipRGC-ablated mice and controls could not be due directly to loss of the visual sensory input. Neuronal activity in controls and Brn3bDTAs surges after the auditory stimulation (Fig. 2E), reaching a peak ~500 ms on average after the sound is emitted, with no significant difference between genotypes on time to peak. The amplitude of the peak response was also not significantly different between genotypes. However, the time course of the decay differed significantly (Fig. 2F) in that fluorescence decayed to baseline (T_{decay}) in 4.13 s in Brn3bDTAs vs. 22.08 s in controls. This longer decay means that the cumulative activity, quantified by area under the curve (AUC), is significantly higher in controls (Fig. 2G). Thus, the absence of ipRGC input causes deficits in the vmPFC that are manifested at the morphological and neuronal activity levels and its response to stressful stimulations.

ipRGC-ablated mice show impaired regulation of aversive emotions

ipRGC-ablated mice have normal mood, as assessed by forced swim and sucrose preference tests, and their hippocampal-dependent memory is unimpaired (Fernandez et al., 2018). Prefrontal injuries in humans show changes in general cognition and social demeanor, and in both primates and rodents, prefrontal cortex lesions result in anomalous social behavior (Brody & Roxvold, 1952, Sato, 1971, Franzen & Myers, 1973). We opted, therefore, to examine the behavior of ipRGC-ablated mice in a social context. The ipRGC-ablated and littermate control animals were housed in standard colony conditions with their same-sex littermates for at least 6 months and no exposure to females after weaning. Male mice were tested in a free social interaction test (Fig. 3A) in which the mouse was exposed for 5 minutes in an unfamiliar setting to a strange conspecific of the same sex. The interactions between the two mice were tracked and scored for many different parameters of social behavior, including total time interacting together. Moreover, since we have noticed in our lab that socially isolated aDTA mice become hyper-reactive relative to wildtypes, and social isolation is a known stressor to rodents capable of altering PFC myelination patterns (Liu et al, 2012), we tested mice before social isolation (Fig. S3B), after a short 4-day period of social isolation, and again after long-term 23-day isolation (Fig. 3A).

After long-term isolation, ipRGC-ablated mice substantially reduced social interactions with their conspecific (Fig. 3B) by avoiding the conspecific when approached and were averse to interacting closely by spending less time engaging in social behaviors such as nose-touching and sniffing (Fig. 3B, C, Supplementary Video 1). As these mice were never exposed to females, there was little immobility and severe fighting was uncommon (Fig. S3A). Thus, the decreased social interactions (Fig. 3C) were not due to aggressive behavior.

To assess social preference further, we used the 3-chamber test, where a mouse is given a choice between a conspecific and an object (Fig. S2A). ipRGC-ablated mice were significantly less likely to spend time in the conspecific-mouse compartment during the first 2 minutes of the trial—consistent with heightened fear towards unfamiliar conspecifics displayed in the free interaction assay (Fig. S2B and C, Supplementary Videos 2, 3). The reduced social contact exhibited by the ipRGC-ablated mice suggested to us an increased state of generalized anxiety. We first used the marble burying test and measured defensive burying of 20 marbles perceived as a potential threat. The number of marbles buried by ipRGC-ablated mice was not different to littermate controls (Fig. S3C) confirming that ipRGC ablation does not heighten fear towards potentially threatening stimuli.

We then subjected the ipRGC-ablated mice to the looming test, where fear is induced by stimulating a predator attack. In this test, mice typically react to an expanding dark disc used to mimic a predator approaching or looming from above by escaping to the shelter, freezing or rattling their tails (Fig. 3D, Supplementary Video 4). Tail rattling during the looming test is directly mediated by the medial PFC (Salay et al., 2018), and has been used as a proxy for social fear associated with prefrontal cortex dysregulation, whereas the shelter escape and the freezing behaviors are dependent on image-forming vision. Consistent with previous data, the ipRGC-ablated mice showed normal image functions as they escaped to the shelter and froze in a similar proportion to their littermate controls (Fig. 3E). Remarkably, the ipRGC-ablated mice were significantly less prone to rattling their tails (only 20.0% of ipRGC-ablated mice did vs 57.1% of controls, Fig. 3E), indicating a dysfunction in the medial PFC.

We then used the defensive burying assay using a noxious odor stimulus, a behavior that is independent of image-forming functions, but still depends on the vmPFC (De Boer & Koolhaas, 2003). In this test (Fig. 3F), a Q-tip dipped in concentrated fox-urine odorant (TMT) was placed in the cage (Fig. S3D). As expected, the mice buried the Q-tip as a defensive strategy except that only 40% of the ipRGC-ablated mice buried within the 20-minute limit of the test relative to 80% of the wildtypes (Fig. 3G). The ipRGC-ablated mice also took significantly longer to bury the Q-tip relative to controls (650 vs. 270 seconds) (Fig. 3H). Moreover, these mice typically distanced themselves from the Q-tip, often confining themselves to the opposite end of the arena (Supplementary Video 5). Thus, an olfactory stimulus elicited behavioral abnormalities just like visual stimuli did. Together, these data indicate that the behavioral deficits observed in ipRGC-ablated mice are not due to sensory dysfunction, but instead are a manifestation of emotional dysregulation due to an impaired vmPFC.

We also tested mice for high-order cognitive processing of focused attention and flexibility using a classic vmPFC test, the IntraDimensional/ExtraDimensional (ID/ED) set shifting task (Heisler et al., 2015). Briefly, mice were trained to dig into ramekins for a buried pellet of food. The ramekins were filled with different digging media, and during the test (Fig. 3I), the mouse performed a series of discriminations that differed along two dimensions (i.e., odor and texture). Two measures of performance were recorded: a) the number of trials to reach a criterion level of performance as a measure of accuracy, and b) the latency to correct their choice following an error (counter choice). In the Simple Discrimination (SD) stage, the mouse learnt that a specific digging medium was associated with reward. In the subsequent Compound Discrimination (CD) phase, an additional dimension was introduced; now the ramekins differed by both digging medium and scent, but the mouse must focus on the digging medium as the relevant cue. In the Reversal (Rev) stage, the reward contingency was reversed such that the previously unrewarding scent was now rewarded. In all these stages of learning,

(SD, CD and Rev) the ipRGC-ablated mice and the controls did not differ in their performance (Fig. 3J, and Fig. S2D) indicating normal learning capabilities in ipRGC-ablated mice.

We then subjected the mice to the IntraDimensional Shift (IDS) during which the mice were required to discriminate three new exemplars of digging media/odors (IDS1, IDS2, and IDS3). In all cases, the reward was associated with the digging media and not the odor. For the most part, the animals performed normally on the IDS discriminations with no major changes between the ipRGC-ablated mice and the controls in terms of accuracy and the latency to counter-choice (Fig. 3J, and Fig. S2D). We observed an unexpected deficit in rule acquisition in IDS2, potentially indicative of executive memory deficits because the animals were trained in this stage on the following day (Fig. S2D). The main impairment, however, was evident during the ExtraDimensional Shift (EDS) test when for the first time in the course of testing, odor became the rewarding stimulus and the digging medium had to be ignored. Now the ipRGC-ablated mice were markedly impaired, evidenced by long latencies to make their response to the new rewarding dimension (Fig.4J). Performance in this complex cognitive task is linked to specific prefrontal areas: The Rev stage recruits an orbitofrontal network, while the EDS relies on the medial portions of the PFC. Therefore, our data indicate that cognitive networks in the orbitofrontal and dorsomedial PFC are normal, and importantly, the deficits in the vmPFC we observed are not due to a generalized prefrontal dysfunction.

Discussion

We report, using two genetic lines and constant dark rearing, that light via ipRGCs affects the activity and function of vmPFC in mice, independent of circadian, developmental, and mood alterations. Using a genetic line (Brn3bDTA mice), which has normal circadian photoentrainment since only non-SCN projecting ipRGCs are ablated at early stages, we uncover that the effects of ipRGCs on vmPFC are not a corollary of circadian disruptions. In addition, using a genetic line (aDTA mice) where ipRGCs are ablated only at adult stages starting at 2 months and completed by ~6 months (Güler et al., 2008), we demonstrate similar deficits to those of Brn3DTA mice, indicating that there is no developmental critical period for the effects of ipRGCs on vmPFC. Finally, both genetic lines show normal basal mood as assessed by classical depression tests (Fernandez et al., 2018; LeGates et al., 2012), highlighting that the effects of ipRGCs on vmPFC are not a consequence of mood alterations. Importantly, even in the presence of ipRGCs, the absence of light (dark rearing) leads to similar deficits in the vmPFC dendritic structures as those observed in the two ipRGC-ablated genetic lines.

We decided to investigate the vmPFC because it receives input from the PHb that is innervated directly by ipRGCs (Fernandez et al., 2018). In this study, we refer to the PHb-recipient area of the prefrontal cortex, broadly encompassing the infralimbic and prelimbic cortex, as vmPFC (Hiser & Koenigs, 2018). Previously, it was shown that the PFC dendritic structure of pyramidal neurons are sensitive to circadian disruptions and stress (Perez-Cruz et al., 2009), with stress inducing the largest deficits. Remarkably, the deficits in the dendritic structures we observe in this study, using the two genetic lines and dark rearing, are greater than those observed with stress. Simply ablating ipRGCs or just depriving a nocturnal animal from light led to changes in the vmPFC that are more substantial than stress or circadian disruptions. We also investigated the time course of the changes in the dendritic structures and correlated them to ipRGC loss in aDTA mice. We observed that deficits in the dendritic structures of pyramidal neurons directly correspond with the extent of ipRGC loss, showing normal morphology at ages below 2 months and then showing progressive disruptions starting at 3-4 months. The morphological changes we observed are correlated with physiological deficits in the response of the vmPFC to acute stressors such a loud sound. The use of a noxious auditory stimulus avoids the complications of using a retina-dependent stressor. We used photometry to show that vmPFC of ipRGC-ablated animals respond initially to the sound but show faster decay and smaller area under the curve in response to the acute stressor. Our studies indicate that ipRGCs of the retina, and their light reception has a profound influence on the morphology and the physiology of the vmPFC.

In rodents, the prefrontal cortex is important for social behaviors. We have conducted several behavioral tests (free interaction and 3- chamber) to examine the social behavior of the animals lacking input to the PHb, and a selected set of behaviors (Looming, Defensive Burying and Attention Set Shift tests) that are known to be associated with the medial PFC. Animals lacking input to the PHb showed severe social dysregulation. This emotional dysregulation was not due to an increased state of generalized anxiety (Marble Burying Test), in agreement with previous studies (LeGates et al., 2012). The Looming, Defensive Burying, and Attention Set Shift tests measure mPFC function directly. The looming test is particularly informative because it probes two aspects: vision, by the freezing and sheltering of the mouse, and mPFC function by the tail rattling. Remarkably, animals lacking input to the PHb showed deficits only in tail rattling, indicating cortical vision to be intact. The defensive burying test, a non-visual behavior for the mPFC function, further confirmed that the deficits in the mPFC are not due to visual cortical effects. Finally, the most comprehensive test was the Attention Shift Set, which measure 7 aspects that involves memory and discrimination, and the animals lacking input to the PHb only showed deficits in the EDS stage, which is highly dependent on the medial portions of the PFC, but not the Rev stage, which is dependent on the orbitofrontal region that does not

receive input from ipRGCs. The light-modulated circuit appears to be mainly involved in the top-down regulation of negative emotions, possibly via an attentional effect, as revealed by the performance of ipRGC-ablated mice in the EDS stage of the attention set shift test. Based on the impaired rule-acquisition of ipRGC-ablated animals in the IDS2 stage of the same test, a deficit in the retention of procedural memory also cannot be ruled out; it has been suggested that the PFC may mediate “episodic control”, i.e. the storage of a rule in memory and its use to adapt behavior (Koechlin et al., 2003). These results show that the morphological and physiological deficits in the vmPFC lead to PFC-specific behavioral deficits.

Our results allow for a simple model. Light through ipRGCs that innervate the PHb activates the vmPFC, and this activation is important for the integrity of the morphological, physiological and molecular components of the vmPFC. Our discovery of modulation by light of the vmPFC raises an intriguing possibility. The PFC is the most evolutionarily recent area of the neocortex, and its most primordial core is the vmPFC. Its modulation by ipRGCs, simple photodetectors found in all vertebrates, invites the speculation that its evolutionary origin and ancestral function may lie in the regulation of light-driven daily activity patterns.

A striking aspect of this study is the fact that dark rearing led to major deficits in the vmPFC. Dark rearing has been shown to affect the primary visual cortex. This is not surprising considering the massive input from the retina to the primary visual cortex. But are the deficits we observed in dark rearing occurring secondarily through the primary visual cortex? Remarkably, animals that lack only ipRGCs and have normal input to the primary visual cortex still exhibit deficits in the vmPFC, even though the PFC receives input broadly from sensory areas, including visual ones (Noudoost et al., 2021). In other words, the absence of an ancient photoreceptive system that is subconscious in nature is sufficient to cause deficits in the vmPFC, even in the context of functional cortical vision.

In humans, the PFC expands during development eventually adding up to 29% of the overall volume of the cortex (Brodmann, 1912), a large proportion to total brain size, which sets a record for the entire animal kingdom (Uylings & Van Eden, 1990). It is well established in the literature that the PFC is one of the first brain region to undergo age-dependent degeneration, a phenomenon characterized by morphological changes (dendritic atrophy) strikingly similar to those observed in this study after light deprivation. Aging causes a progressive yellowing of the eye lens, an effect that reduces the intensity of blue-shifted light reaching the retina. It was shown that the transmission of 480 nm light decreased by 72% at age 80 relative to age 10 (Kessel et al., 2010). It is precisely to these short-wavelength photons that ipRGCs are most responsive. The outcome is an age-dependent, progressive loss of ipRGC signaling. This was thought only to result in the characteristic circadian and

sleep disturbances seen in the elderly. We may now have reason to suspect a role of ipRGC signaling in PFC age-dependent degeneration.

A marked trend in recent decades has been the proliferation of artificial blue-light sources, and human nighttime exposure to them, an unprecedented behavior in the natural history of our species known to disrupt physiological ipRGC signaling. The emotion-regulatory function of the vmPFC is specifically affected in many disorders, from schizophrenia to autism to syndromes characterized by fear generalization (Hiser & Koenigs, 2018). In this study we show that light, via a retinal cell population, profoundly affects the prefrontal cortex across multiple domains—neuronal morphology, network activity, and behavior—, with effect sizes so considerable as to suggest an ecologically important regulation. This unexpected feature, if confirmed in humans, may prove to be of considerable interest.

Materials & Methods

Animals

The *Opn4*$^{aDTA/aDTA}$ and *Opn4*$^{Cre/+}$*;Brn3b*$^{zDTA/+}$ mouse lines used in this study were provided by Dr. Samer Hattar's laboratory at the National Institutes of Health. They were housed under a 12 h light: 12 h dark (T24) cycle at a temperature of 22--25°C with food and water ad libitum unless otherwise noted. In Brn3bDTAs, ~200 ipRGCs survive per retina, which are the only ipRGCs that do not express the transcription factor Brn3b (POU4F2); these cells exclusively innervate the SCN. Brn3bDTA mice were obtained by mating mice that express the Cre-dependent diphtheria toxin under the control of the Brn3b promoter (Brn3b$^{zDTA/+}$), with Opn4$^{Cre/Cre}$ mice. Opn4$^{Cre/+}$ littermates were used as controls. All animals were handled in accordance with guidelines of the Animal Care and Use Committees of Johns Hopkins University and the National Institutes of Health.

Golgi staining and cell morphometry

Animals were socially housed with littermates in standard colony conditions till the indicated age; rapidly anesthetized with 5% isoflurane, their brain was extracted without any preliminary fixation, rinsed with PBS, and processed with FD Neurotechnologies' Rapid Golgi staining kit, in accordance with the kit protocol. The resulting 200-mm coronal sections were imaged at 40x to 100x magnification. The dendritic morphology of neurons in the Golgi-stained sections was reconstructed using the Neurolucida tracing system (MicroBrightField). The neurons to be traced were selected according to the following criteria: 1. Position in the prefrontal cortex area shown by previous tracing to receive PHb afferents (PL and IL cortices). 2. Pyramidal type, with a roughly triangular soma, a clear single

apical dendrite extending from the soma towards the pial surface, and two or more dendritic trees extending from the base of the soma. 3. Distance from the soma to the pial surface <390 µm and >280 µm (~layer 3). 4. Shape of the apical dendritic tree not suggestive of a layer 5 or short-shaft layer 2/3 neuron. 5. Apical dendritic tree mainly unfolding within the middle third of the section, well isolated and not obscured by neighbors. 6-10 cells typically qualified to be analyzed per animal (with little discretion for the experimenter). The cells were traced and reconstructed by two independent investigators, one of whom was blind to genotype. Analysis of the variables of interest was performed automatically by Neurolucida Explorer.

Fiber Photometry

Mice were maintained under 1-1.5% isoflurane anesthesia, and stereotactically injected with 120 nL of AAVDJ-syn-GCaMP6f, titer 10^{12} gc/mL, over approximately 10 minutes. The empirically-optimized injection coordinates were ML 0.15, AP 1.72, DV 2.84-2.64, relative to bregma; the DV coordinate was adjusted for each animal (the goal being to inject 2±0.1mm from the dorsal surface of the brain). An optical fiber ⌀400mm with conical tip (Doric Lenses) was implanted over the injection site, in the PL cortex (DV ~2.5) (See Fig. S4A). After 5 weeks, the animals were transferred into a special cage and connected to a photometry apparatus (Doric Lenses), where all experimental manipulations were conducted. Ca^{2+}-dependent and isosbestic fluorescence wavelengths were recorded, the latter as an internal control. Every trace was rectified and corrected by fitting first for exponential decay and secondly linearly using the isosbestic fluorescence as the regressor. A classic problem with photometry is inter-individual comparisons, as many hard-to-control-for variables affect even normalized fluorescence amplitude (e.g. transfection efficiency, distance of transfected cells from fiber tip, differences sometimes in LED optical power output). To get around this hurdle, we took advantage of the “background noise” produced, in each recording, by uncorrelated single-cell activity at rest. We calculated the median absolute deviation (MAD) of stretches of such minimal activity for every trace and equalized them across animals. The possibility of differential GCaMP expression between groups, which would result in different amplitude of this activity, was thus accounted for**.** MAD normalization affects only measures of fluorescence amplitude, not time- or frequency- based variables.
Recordings from mice in which the transfection site was subsequently found to be outside the ILC were excluded (these recordings typically did not show sound- and/or light- evoked responses). All the mice used in this experiment had completed the behavioral tests described below and were 11 to 13 months old (age-matched between genotypes).

Behavioral Tests

Tests were performed during the mice's active phase under dim red light. In every case, the animals were habituated by keeping them at least 1 h in the test room before the test was performed. All test sessions, except for IDED, were videotaped and every single video is available for the reader's viewing pleasure at the link provided in the next section. Particularly salient examples are enclosed here as Supplementary Material. IDED sessions require 5 to 10 hours' total time per animal, and were therefore not recorded, except for the representative snippets in Supplementary Material. All tests were performed with the experimenter blind to genotype.

Free Interaction Test

The test mouse was placed in an unfamiliar cage with new bedding, acclimatized for 20 minutes, and exposed for 5 minutes to a strange wildtype conspecific of the same sex of approximately 7-12 weeks of age. Neither participant was a breeder mouse (to reduce aggression). Interactions between mice were analyzed by TopScan (CleverSys, Inc).

3-Chamber Test

The test mouse was placed in the middle chamber and acclimatized for 10 minutes, then partitions were removed allowing the animal to explore the other 2 chambers, for 10 minutes. Again, movements were analyzed with TopScan.

Marble Burying Test

The mouse was dropped into a square-footprint arena with 10-cm-thick corncob bedding and a regular array of 20 1-cm black marbles. After 10 minutes, he was extracted from the cage and the number of completely or partially buried marbles counted.

Looming Test

As previously described, the test mouse was placed in a custom-built arena and allowed to acclimatize for 10 minutes. A maximum of 10 iterations of the expanding disc animations were played (the series was cut short if the mouse displayed all the three behaviors of interest).

Defensive Burying Test

Mice were acclimatized to the unfamiliar test cage, with a 5-cm-thick layer of new bedding, for 10 minutes. A Q-tip soaked in 4 mL of TMT was dropped into the cage in the second quadrant, and the mouse's reactions videotaped for 20 minutes.

IDED Test

Every mouse was preliminarily handled by the experiment for 7 days, and weighed to determine a body weight baseline. Food restriction followed, for 4 days, with 1 to 2 g of food dispensed every day in the ramekins subsequently used for the test. Then the mice were acclimated to the test arena for 2 days; and trained to dig into bedding-filled ramekins for food for 1 or 2 days depending on performance. Body weight typically dropped to 80% of baseline by the beginning of the test sessions.

Statistical Analysis

Statistical analysis of results was made by a Student's t test or a two-way analysis of variance (ANOVA), unless otherwise stated.

Data Availability

All videos of recorded behavioral tests, as well as all original databases, are available on request.

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Authors' Contributions

LLO designed the study, and performed and analyzed the fiber photometry experiments and the behavioral tests. JZ and LLO performed the morphometric analysis, and wrote the manuscript.

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Figure Legends

Figure 1. Loss of ipRGCs leads to apical dendritic degeneration in the vmPFC. We reconstructed the apical dendritic trees of pyramidal neurons in layer III-, throughout the anterio-posterior extension of the IL and PL areas (+1.9 to +1.3 mm relative to bregma), from Golgi-stained coronal sections. **A.** Sholl analysis revealed a significant degeneration of both proximal branches and distal tufts in animals (littermate controls) conceived and reared in constant darkness (DD), and ipRGC-ablated animals (specifically, Brn3bDTA) ($p<0.001$, repeated-measures ANOVA). **B.** The total apical dendritic length is significantly ($p<0.001$) reduced in DD mice (849.37±43.13) relative to LD controls (1327.89±36.22). **C.** The number of branch nodes was also significantly reduced ($p<0.001$) in DD mice (9.70±0.68) relative to LD controls (17.39±0.46). **D.** Sholl analysis revealed a significant degeneration of both proximal branches and distal tufts in LD littermate controls (Cntrl) relative to aDTA and Brn3bDTA animals ($p<0.001$, repeated-measures ANOVA). **E.** In aDTA mice, at 7 weeks of age (light red), apical dendrites had a wildtype morphology, significantly different than at 6 months (dark red, re-plotted from C)($p<0.001$, repeated-measures ANOVA). **F.** Dramatic examples of apical dendritic shrinkage; compare neurons from wild-type (leftmost) and young (7 week old) aDTA mice (center-left) with affected neurons in old (>6 month old) aDTA (center-right) and brn3bDTA mice (rightmost). **G.** The total apical dendritic length was not significantly different between controls (1360.06±85.18) and aDTAs (1374.83±94.25) at 7 weeks of age; **H.** it is still not significantly different at 3 to 4 months of age (controls 1327±36.22 vs. aDTAs 1196.33±104.34); **I.** it becomes significantly different ($p<0.0001$) at older than 6 months (controls 1358.62±31.28 vs. aDTAs 950.07±48.60); the same significant difference is observed in age-matched Brn3bDTAs (894.12±81.77). **J.** This panel replots data from

aDTAs pre- and post- ipRGC degeneration for between-ages comparison within this genotype ($p<0.001$)(t-test). **K.** The number of branch points was not significantly different between controls and aDTAs at 7 weeks of age (14.19±1.04 vs. 14.58±0.97 respectively); **L.** an incipient significant difference was observed at 3 to 4 months (17.39±0.46 vs. 11.68±0.94, $p<0.001$); **M.** which increased at older ages than 6 months (16.14±0.63 vs. 10.39±0.59, $p<0.001$); **N.** There is a significant difference within the aDTA genotype between 7 weeks and over 6 months of age ($p<0.001$)(t-test). All values are mean±SEM. In the plots, each dot represents one animal.

Figure 2. vmPFC activity in response to a stressful stimulus is depressed in ipRGC-ablated mice. **A.** Neurons were transduced with GCaMP6f; an optical fiber was implanted above the area. **B.** Mice were exposed to a loud sound—a non-visual fear-evoking stimulus. **C.** We recorded in vivo neuronal activity from the vmPFC. Recordings of endogenous activity showed a significantly higher number of peaks in controls relative to Brn3bDTAs at ZT12 ($p=0.037$), ZT18 ($p=0.028$), and ZT6 ($p=0.002$). A trend in the same direction is present at ZT0 ($p=0.116$). All values are mean±SEM. **D.** Examples to highlight high intrinsic activity recording from a control mouse (top) vs. a low intrinsic activity trace from a Brn3bDTA (bottom). **Traces are individual recordings; the thicker line is a rolling time average. E.** Normalized Ca^{2+}-dependent fluorescence in response to the noise (delivered at time=0) in controls (left) and Brn3bDTA mice (right). Traces are mean (±SEM, hued area) of recordings from 3 animals, each averaged over multiple trials. **F.** Fluorescence decayed to baseline more slowly in controls than in ipRGC-ablated mutants (22.08±5.85 s vs. 4.13±0.92 s)($p=0.039$). **G.** The Area Under the Curve of vmPFC activity in response to this stimulus was significantly higher in controls (0.204±0.0230) vs. ipRGC-ablated mice (0.030±0.0048)($p=0.002$).

Figure 3. ipRGC-ablated mice show impaired regulation of emotion and attention. **A.** In a free-interaction assay, a test mouse is exposed to a strange conspecific in an unfamiliar arena. **B.** ipRGC-ablated mice were less likely to interact socially, spending 30.85±5.07% of their time in this activity vs. 51.4±6.44% for controls ($p=0.025$). **C.** The reason for decreased social interaction time was increased distancing. **D.** In the looming test, a mouse can react to a simulated predator by running under a shelter, freezing and/or rattling his tail. The last is a known mPFC-dependent behavior. **E.** A similar proportion of ipRGC-ablated mice ran under the shelter and froze relative to littermate controls (sheltering: 73.33% vs. 85.71%; freezing: 40% vs. 50%), but they were less likely to rattle their tails (20% vs. 57.14%, $p=0.027$) (Normal Approximation proportion test). The plot shows what proportion of specified behavior was displayed by control vs. ipRGC-ablated mice (normalized). **F.** In the defensive burying

test, mice bury a fox-urine-soaked Q-tip if they can overcome their fear of the scent. **G.** ipRGC-ablated mice were less likely to bury the noxious object: 40% did (dark red), vs. 80% of littermate controls (dark blue), p=0.025) (Normal Approximation proportion test). **H.** Restricting the analysis to those that dared to bury it, a difference was also evident in the latency to enact this behavior (647±147.4 s for ipRGC-ablated mice vs. 271.1±66.2 s for controls, p=0.015). **I.** The Attention Set Shift Test is a task in which cognitive flexibility and attention interact with emotional drives. **J.** We recorded the latency necessary for a mouse to enact a correction for an initial incorrect choice after a reward-association rule was learned. For each stage of the test, the p-value of the performance difference between genotypes is reported over the bars. ipRGC-ablated mice took longer to enact corrective behavior in the EDS (10.61±1.32 s vs. 6.9±0.44 s, p=0.023). In the plots, each dot represents one animal.

Supplementary Figure 1. A. The motor cortex, a control region, is unaffected. **B.** Sholl analysis consists in counting the number of intersections between a dendritic tree and concentric circles of increasing radius centered on the soma. **C.** Neurons in the motor cortex of control and ipRGC-ablated mice do not display morphological difference.

Supplementary Figure 2. A. In a 3-chamber test a mouse has to choose whether to spend time in a chamber with a caged conspecific or in another chamber with an object. **B.** During the first two minutes of the test, ipRGC-ablated mice displayed decreased social-chamber time (p=0.014). **C.** Individual paths and averaged heatmaps of mice in the 3-chamber test. **D.** In the attention set shift test, ipRGC-ablated mice surprisingly demonstrated an impaired rule-learning ability in the IDS2, which is the first stage tested during the second day of the test. They required significantly more trials to learn the reward-association rule (19.9±1.49 vs. 12.25±2.07, p=0.007).

Supplementary Figure 3. A. The number of fights initiated by test mice in the Reciprocal Interaction Test was not different between genotypes, and neither was the time spent immobile. **B.** Without prior social isolation there was no significant difference between genotypes in social contact (although a trend exists). **C.** The marble burying test assesses anxiety elicited by a novel environment. ipRGC-ablated mice did not differ in this behavior from littermate controls (6.4±1.7 marbles buried vs. 6.2±1.1, p=0.93). **D.** A mouse approaches an object soaked with a chemical (TMT) giving fox urine its characteristic odor (from the defensive burying test described in Fig. 4F). **E.** In the Attention Set Shift Test, ipRGC-ablated mice were significantly impaired in learning the association rule in IDS2. This

may suggest dysregulation in additional prefrontal areas, possibly as a secondary effect from the vmPFC alterations.

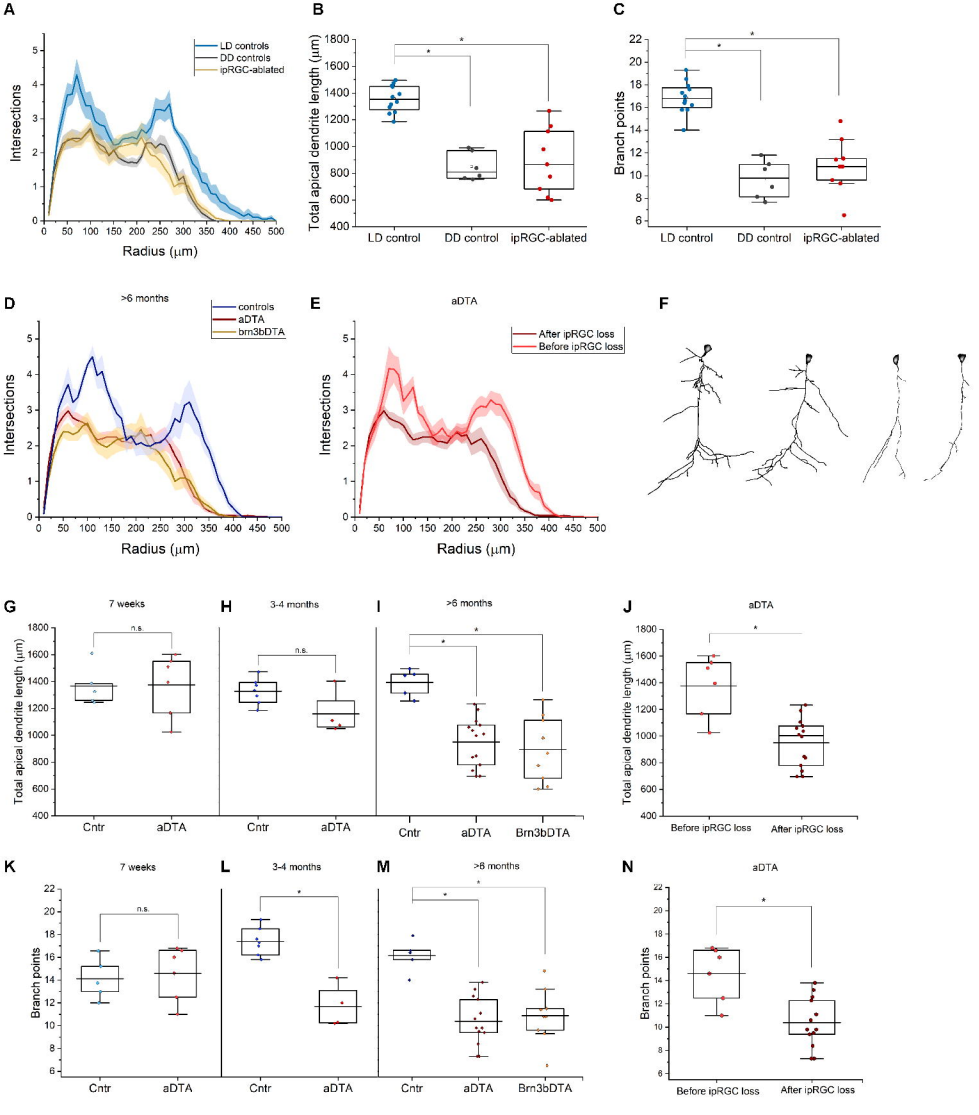
Supplementary Video 1. *Free Interaction test of Brn3DTA mouse*. Mouse is in cage for 1 minute. At ~1 minute a wildtype mouse is introduced. Total length is ~9mins.

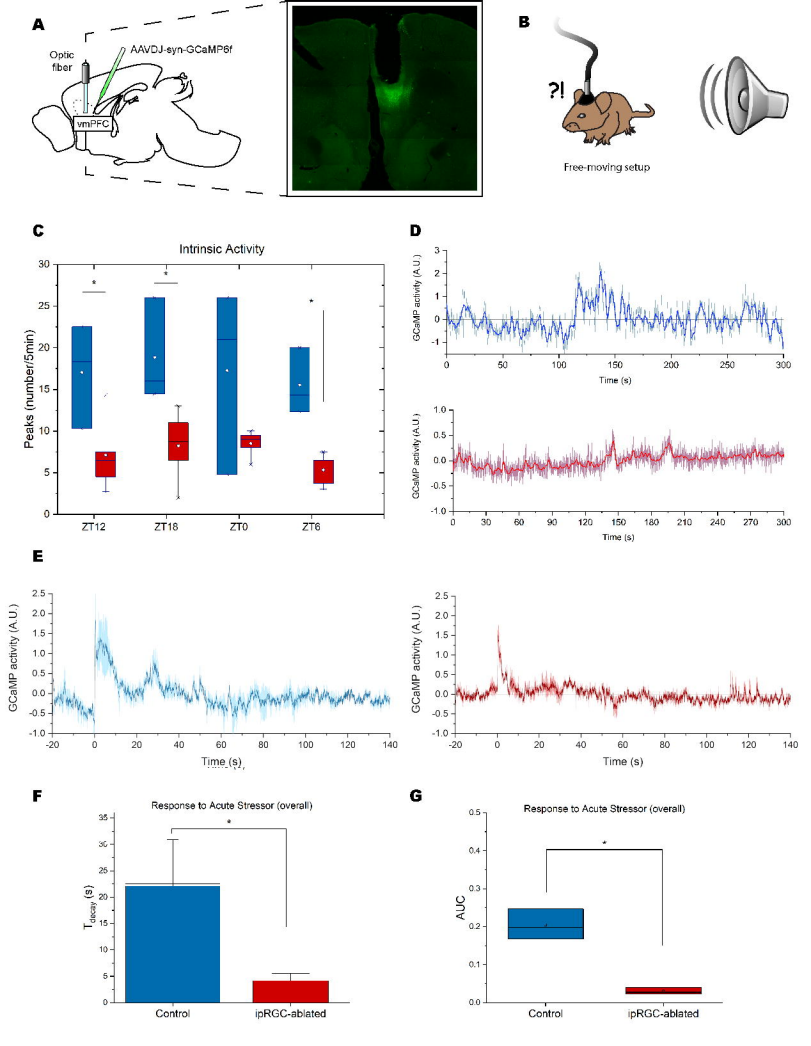
Supplementary Video 2. *Three Chamber test*. A control mouse explores the left side of the three chambers, where a mouse is located. In the right side of the three chambers, an object is provided. Total length is ~2mins.

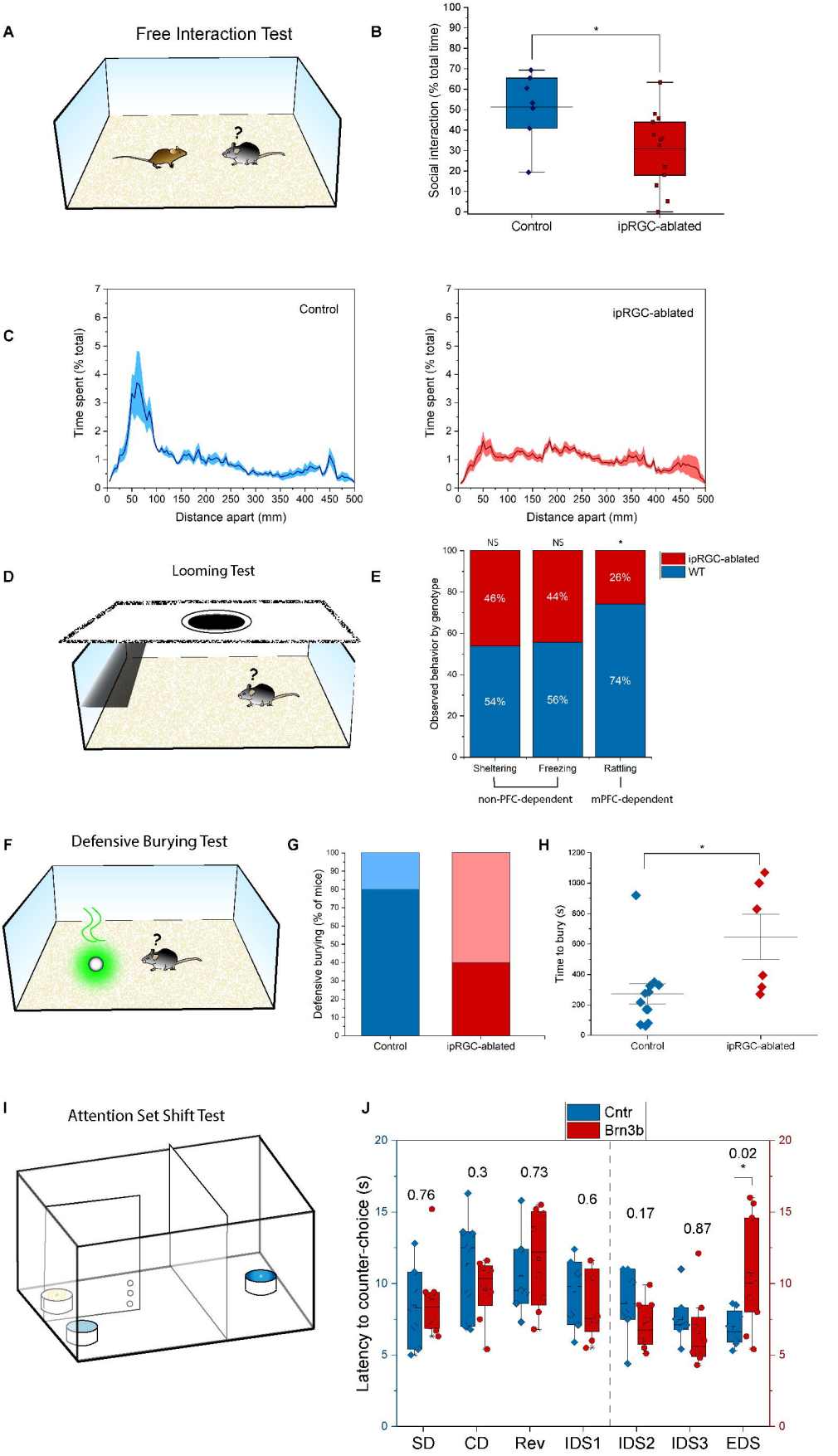
Supplementary Video 3. *Three Chamber test*. A Brn3DTA mouse avoids the left side of the three chambers, where a mouse is located. In the right side of the three chambers, an object is provided. Total length is ~2mins.

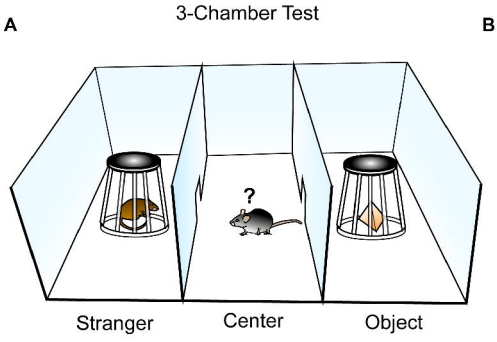
Supplementary Video 4. *Tail rattling in the looming test*. A Brn3DTA mouse rattles his tail in response to a looming stimulus. Total length is less than half a minute.

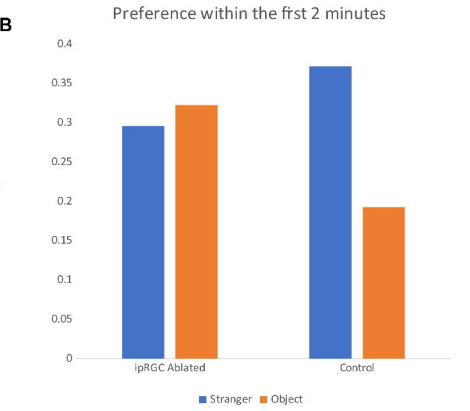
Supplementary Video 5. *Defensive burying test*. At ~10 seconds, the fox urine is introduced to the cage. Note how the Brn3bDTA mouse avoids the scent and only tries to bury it ~3mins into the video. Total length is ~6mins

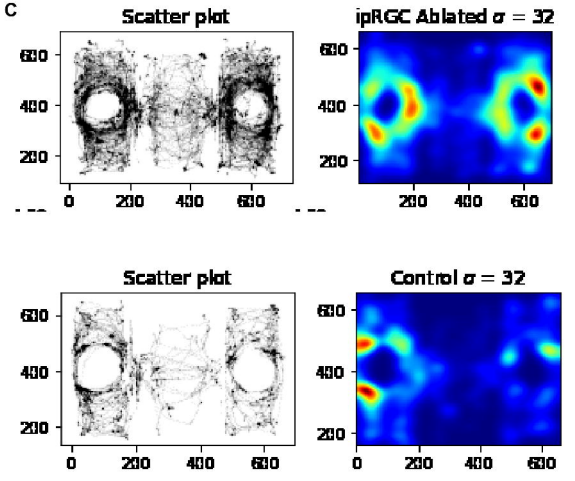

A
LD controls
DD controls
ipRGC-ablated
Intersections
Radius (μm)
B
Total apical dendrite length (μm)
LD control
DD control
ipRGC-ablated
C
Branch points
LD control
DD control
ipRGC-ablated
D
>6 months
controls
aDTA
brn3bDTA
Intersections
Radius (μm)
E
aDTA
After ipRGC loss
Before ipRGC loss
Intersections
Radius (μm)
F
G
7 weeks
n.s.
Total apical dendrite length (μm)
Cntr
aDTA
H
3-4 months
n.s.
Cntr
aDTA
I
>6 months
Cntr
aDTA
Brn3bDTA
J
aDTA
Total apical dendrite length (μm)
Before ipRGC loss
After ipRGC loss
K
7 weeks
n.s.
Branch points
Cntr
aDTA
L
3-4 months
Cntr
aDTA
M
>6 months
Cntr
aDTA
Brn3bDTA
N
aDTA
Branch points
Before ipRGC loss
After ipRGC loss

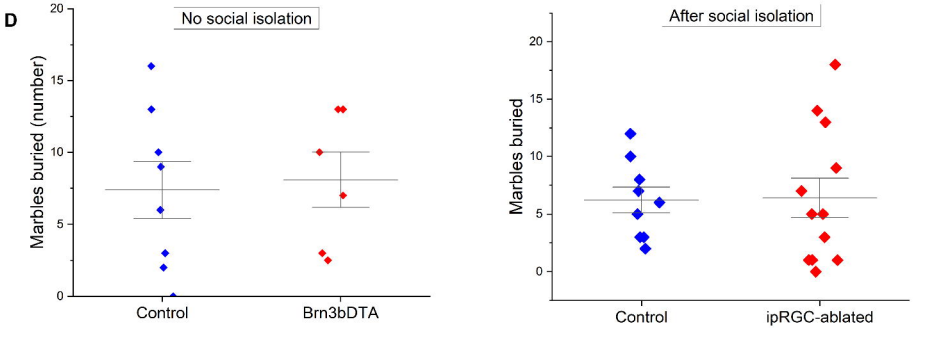

A
Optic fiber
AAVDJ-syn-GCaMP6f
vmPFC
B
?!
Free-moving setup
C
Intrinsic Activity
Peaks (number/5min)
ZT12
ZT18
ZT0
ZT6
D
GCaMP activity (A.U.)
Time (s)
E
GCaMP activity (A.U.)
Time (s)
F
Response to Acute Stressor (overall)
T_{decay} (s)
Control
ipRGC-ablated
G
Response to Acute Stressor (overall)
AUC
Control
ipRGC-ablated

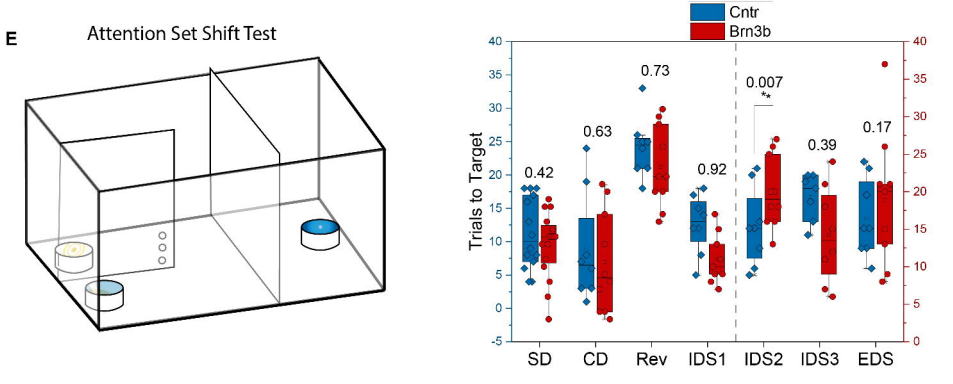

A
Free Interaction Test
B
Social interaction (% total time)
Control
ipRGC-ablated
C
Control
ipRGC-ablated
Time spent (% total)
Distance apart (mm)
D
Looming Test
E
NS
NS
*
ipRGC-ablated
WT
46%
44%
26%
54%
56%
74%
Observed behavior by genotype
Sheltering
Freezing
Rattling
non-PFC-dependent
mPFC-dependent
F
Defensive Burying Test
G
Defensive burying (% of mice)
Control
ipRGC-ablated
H
Time to bury (s)
Control
ipRGC-ablated
I
Attention Set Shift Test
J
Cntr
Brn3b
Latency to counter-choice (s)
0.76
0.3
0.73
0.6
0.17
0.87
0.02
SD
CD
Rev
IDS1
IDS2
IDS3
EDS

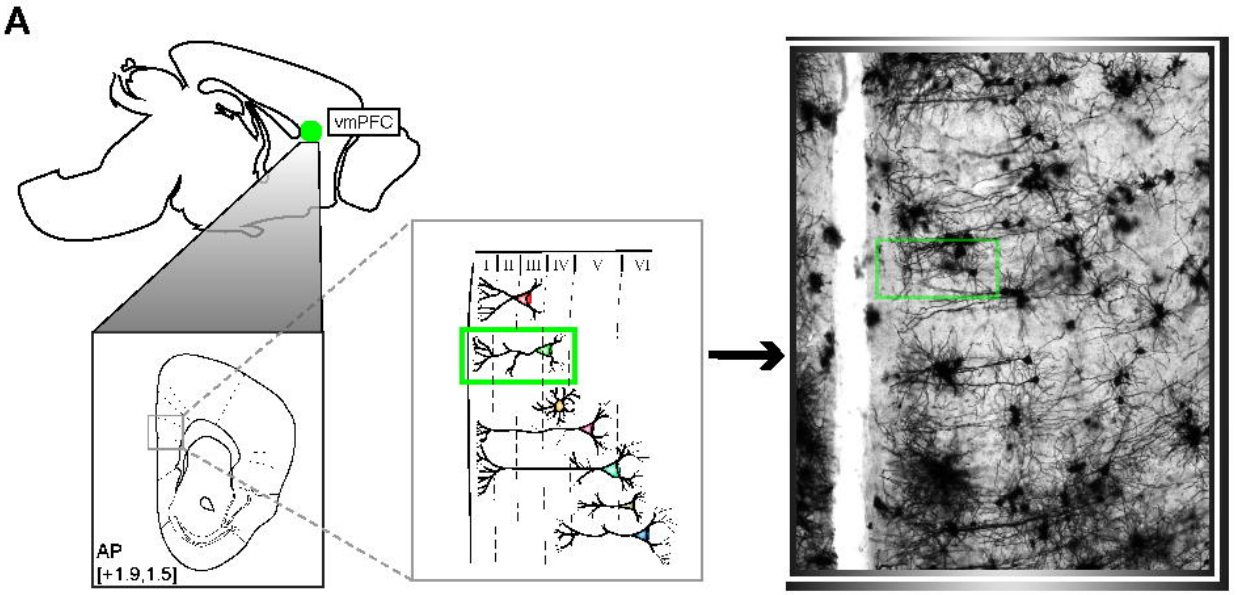

A
3-Chamber Test
?
Stranger
Center
Object

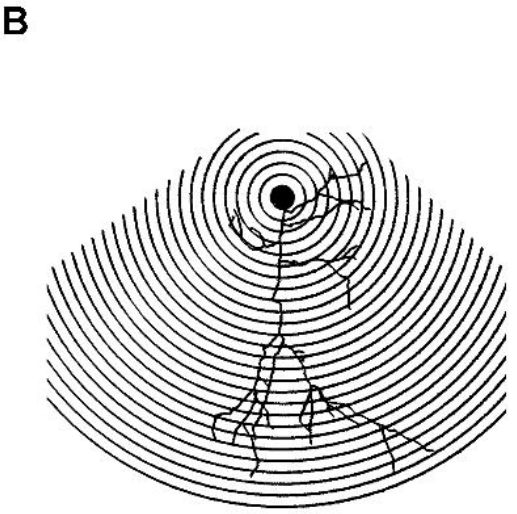

B
Preference within the first 2 minutes
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ipRGC Ablated
Control
Stranger
Object

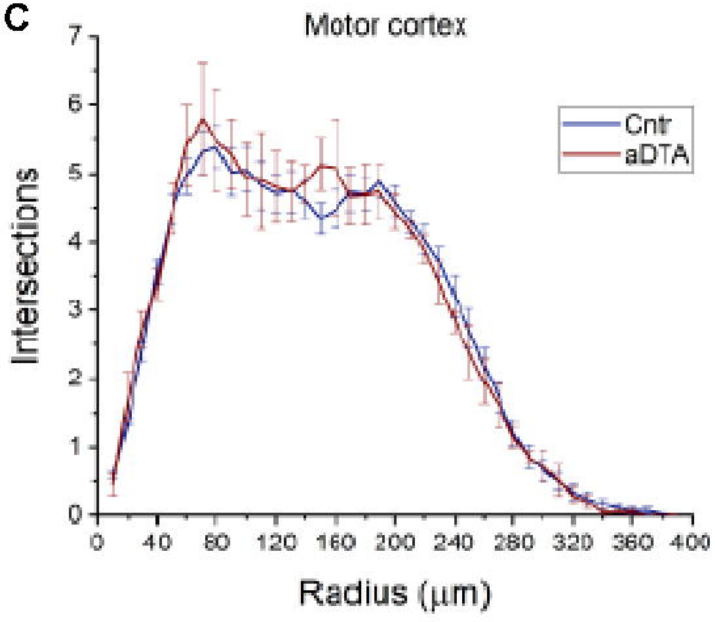

C
Scatter plot
ipRGC Ablated σ = 32
Scatter plot
Control σ = 32
600
400
200
0
200
400
600

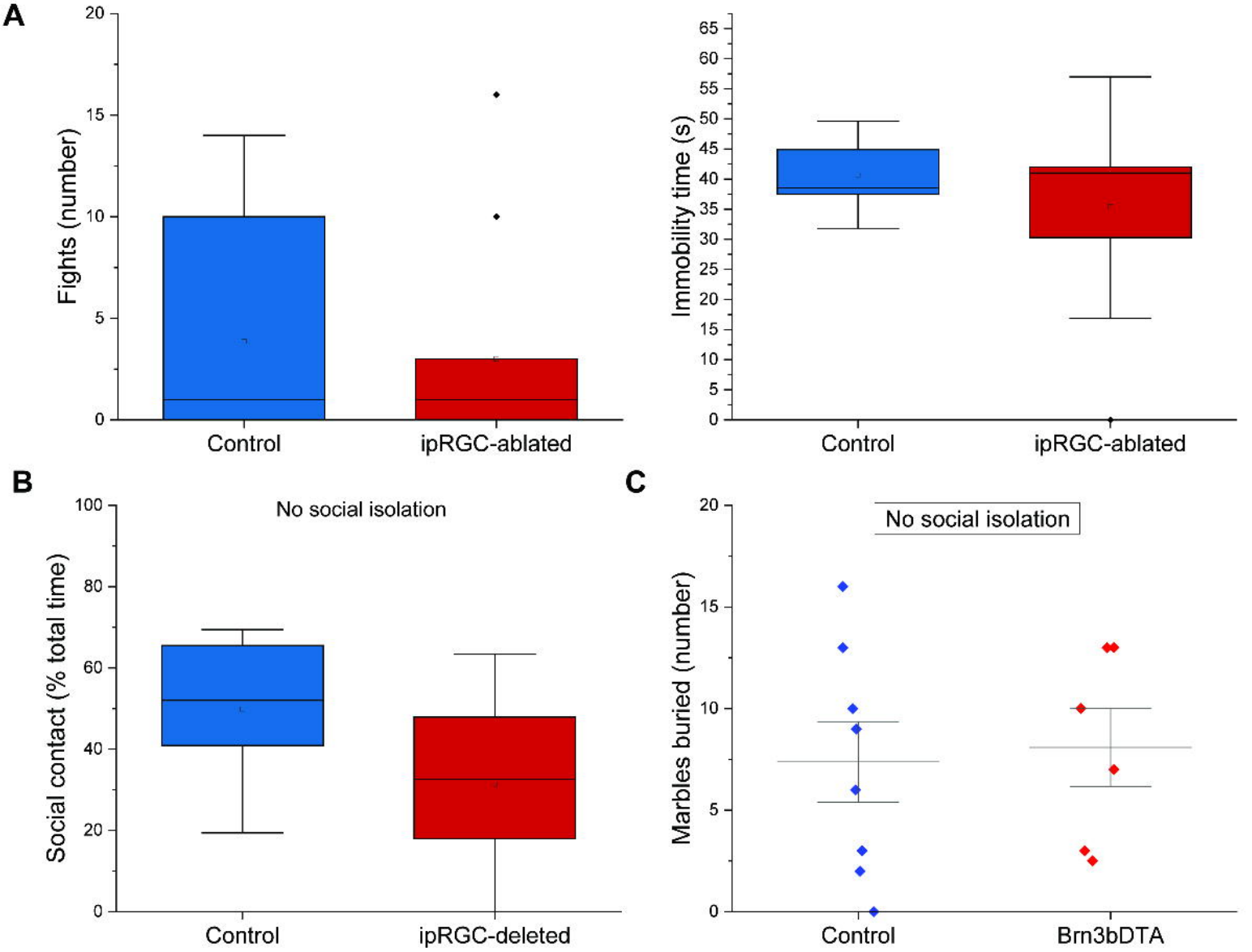

D
No social isolation
Marbles buried (number)
20
15
10
5
0
Control
Brn3bDTA
After social isolation
Marbles buried
20
15
10
5
0
Control
ipRGC-ablated


E
Attention Set Shift Test
Cntr
Brn3b
Trials to Target
40
35
30
25
20
15
10
5
0
-5
0.42
0.63
0.73
0.92
0.007
**
0.39
0.17
SD
CD
Rev
IDS1
IDS2
IDS3
EDS

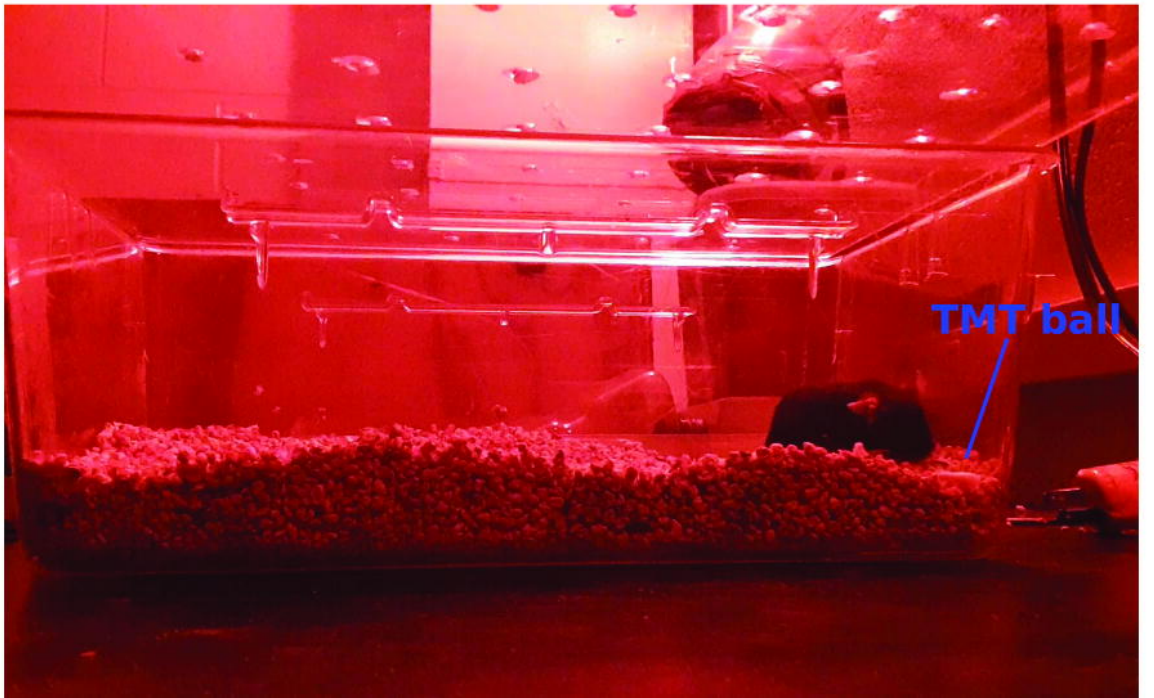

A
vmPFC
AP
[+1.9,1.5]
I
II
III
IV
V
VI


B


C
Motor cortex
Cntr
aDTA
Intersections
Radius (μm)
0
1
2
3
4
5
6
7
0
40
80
120
160
200
240
280
320
360
400


A
Fights (number)
Control
ipRGC-ablated
Immobility time (s)
Control
ipRGC-ablated
B
No social isolation
Social contact (% total time)
Control
ipRGC-deleted
C
No social isolation
Marbles buried (number)
Control
Brn3bDTA

D


TMT ball