NEUROSCIENCE

Zona incerta subpopulations differentially encode and modulate anxiety

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Despite recent clinical observations linking the zona incerta (ZI) to anxiety, little is known about whether and how the ZI processes anxiety. Here, we subject mice to anxious experiences and observe an increase in ZI c-fos–labeled neurons and single-cell calcium activity as well as an efficient effect of ZI infusion of diazepam, a classical anxiolytic drug. We further identify that somatostatin (SOM)–, calretinin (CR)–, and vesicular glutamate transporter-2 (Vglut2)–expressing cells display unique electrophysiological profiles; however, they similarly respond to anxiety-provoking stimuli and to diazepam. Optogenetic manipulations reveal that each of these ZI neuronal populations triggers specific anxiety-related behavioral phenotypes. Activation of SOM-expressing neurons induced anxiety, while photoactivation of CR-positive cells and photoinhibition of Vglut2-expressing neurons produce anxiolysis. Furthermore, activation of CR- and Vglut2-positive cells provokes rearing and jumps, respectively. Our findings provide the first experimental evidence that ZI subpopulations encode and modulate different components of anxiety.

INTRODUCTION

Anxiety is a negative emotional state triggered by potential threats and generates autonomic responses, avoidance, and stress-like behaviors (1, 2). Several brain regions—including the amygdala (3–5), the bed nucleus of stria terminalis (5, 6), the medial prefrontal cortex (7, 8), the hippocampus (4, 8, 9), and the hypothalamus (9, 10)—have been reported to play an important role in anxiety. Very recently, the understudied zona incerta (ZI) has also been associated with it. Specifically, patients with Parkinson's disease (PD) treated with deep brain stimulation (DBS) in the ZI self-reported lower levels of anxiety (11, 12). However, there is little to no experimental evidence supporting these interesting clinical observations.

Located between the thalamus and the hypothalamus, the ZI is an elongated string-like structure that has been implicated in a wide range of functions, varying from neuronal development (13), hormonal regulation (14, 15), ingestion (16–18), sleep (19, 20), sensory processing (21, 22), and pain (23). More recently, a stream of research showcased the implication of the ZI in emotionally related experiences and adaptive defensive behaviors. Exposure to stressful events such as sleep deprivation was shown to potentiate the expression of the immediate early gene *c-fos* in the ZI (20). Furthermore, electrical stimulation in the ZI induced pupil dilation (24) and increased the blood flow to the extremities (24) and the heart rate (25); all of these are physiological reactions similar to that of the acute stress response (2). In addition, ZI loss of function impaired mice in their ability to respond to cues predictive of incoming threat. Specifically, mice with ZI electrolytic lesions displayed reduced avoidance in a conditioned avoidance response test (26). Similarly, ZI tetanus toxin infusion to block synaptic transmission decreased the ability to associate a predictive cue with the negative outcome in a fear conditioning task for mice (27). In contrast, optogenetic inactivation of ZI vesicular γ -aminobutyric acid (GABA) transporter (Vgat)–expressing cells during the presentations of loud tones exacerbated the triggered flight response (28). These previous studies

imply that the ZI could be influenced by negative cues and modulate adaptative responses. However, how the ZI senses stressful stimuli when mice experience anxiety-triggering states is not known.

Most of the current knowledge on the ZI was gained from non-specific manipulations, yet the ZI is a highly heterogeneous structure. Histological investigations revealed diverse cell types having the capacity to release chemical signaling molecules such as glutamate, GABA, neuropeptides, and, potentially, dopamine (tyrosine hydroxylase–expressing cells) (29, 30). While the GABAergic neurons are abundant in number and thoroughly expressed across the ZI, the existence of somatostatin (SOM), calretinin (CR), calbindin, parvalbumin (PV), and vasopressin indicate diversity within the GABAergic cells (29–31). Such biochemical heterogeneity may explain why the ZI has been implicated in quite a wide range of functions (30).

Here, we aimed at investigating how the ZI neuronal population responds to stressful and anxious events. On the basis of a recent report that the rostral ZI modulates cue-triggered defense behaviors (28), we speculated that the rostral ZI could also sense stressful anxiety-provoking cues. Thus, in our study, we targeted the rostral ZI (referred throughout as ZI). We first show that the ZI indeed encodes anxiety. We applied a three-step strategy with histological labeling against *c-fos* and *in vivo* calcium recordings as mice experience anxious conditions, as well as the assessment of the efficacy of a pharmacological anxiety-relieving drug directly infused in the ZI. We then focused on two GABAergic subpopulations, the SOM- and CR-expressing neurons, along with the glutamatergic ones. They exhibit distinct electrophysiological properties. Although they are all activated by exposure to anxious conditions and are similarly modulated by diazepam, their bidirectional optogenetic modulation revealed distinct behavioral phenotypes. Specifically, activation of SOM-expressing cells induced anxiogenesis. Conversely, activation of CR-expressing neurons triggered anxiolysis and exploratory rearing. Last, the activation of vesicular glutamate transporter-2 (Vglut2)–expressing cells induced flight-like jumps. Our data provide unprecedented information about how the ZI and some of its neuronal subpopulations encode anxiety-related behaviors. Our study hence represents an important first step into understanding the neuronal

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and circuit basis of the clinical observation for ZI DBS to relieve anxiety in patients with PD (11, 12).

RESULTS

ZI neurons encode anxiety

On the basis of the recently reported clinical observations (11, 12), we first verified and assessed that the ZI encodes anxiety. We performed three experiments among different levels of investigation. First, if indeed the ZI encodes anxiety, then an anxious stimulus is expected to increase the expression of the immediate early gene *c-fos* in the ZI. To test this hypothesis, we forced mice to experience a stay on an elevated rod, which is also exposed, meeting the two common criteria to trigger anxiety in rodents (1, 32). Mice were hence placed on an elevated rod (100 cm above the ground and 3 cm in diameter; Fig. 1A) for 30 min and brought back to their home cage for an additional 30 min before intracardiac perfusion. Control mice stayed in their home cage for 60 min (Fig. 1B). Post hoc immunostaining against *c-fos* (Fig. 1, C and D) followed by quantification revealed a significantly higher number of *c-fos*-labeled neurons in the ZI of mice that experienced the elevated rod as compared to control mice (elevated rod, 3.38 ± 0.20 cells versus home cage, 1.00 ± 0.14 cells; 951 and 243 cells, respectively, in 12 slices from three mice; Fig. 1E). These data confirm that an anxiety- or stress-triggering event increased the activity of ZI cells.

We next further investigated this with a higher temporal precision through in vivo calcium imaging. An adeno-associated virus (AAV) construct carrying the calcium indicator GCaMP6m gene was expressed in ZI neurons and a gradient refractive index (GRIN) lens implanted slightly above (Fig. 2, A and B). To investigate changes in ZI populational activity due to shifts in anxiety levels, we needed an apparatus where mice can freely explore between areas with relatively high and low anxiety-triggering values. While the elevated rod is a strong and unambiguous anxiogenic stimulus, it does not provide such a contrast. We therefore recorded the calcium dynamics of ZI neurons while mice explored an elevated platform

(Fig. 2C), adapted from the elevated plus maze (EPM) by blocking off the closed arms. This configuration allows mice to freely explore between the relatively safer (center) and anxiety-triggering (open arms, especially the edges) areas and, at the same time, improves maneuverability of the miniscope mounted mice and maximizes the amount of time mice explored the anxiety-evoking edges. First, we observed that the mice spent more time in the center as compared to the edges (center, $48.0 \pm 3.3\%$ versus edges, $14.3 \pm 3.3\%$ time; Fig. 2D). This is in line with the reported values of the standard EPM (33) and hence validates that our elevated platform task evokes anxiety-like phenotypes. We observed that a substantial proportion of cells exhibited higher levels of activity when mice explored the safe center while other cells showed elevated calcium activity in the open edges (Fig. 2, E and F, and movie S1). To confirm and strengthen this observation, we further analyzed and evaluated the correlation between neuronal activity and the absolute distance of the recorded animal away from the safe center. Twenty-three percent of the imaged ZI cells displayed a positive correlation and were more active when mice explored the anxious edges as compared to the safer center (+ve Corr, 0.96 ± 0.07 versus not Corr, 0.06 ± 0.04 z scores; 128 cells from five mice; Fig. 2, G to I). Conversely, 9% of the imaged cells exhibited a negative correlation and were less active when the animals were in the edges compared to the center (–ve Corr, -0.75 ± 0.06 versus not Corr, 0.06 ± 0.04 z scores; Fig. 2, G to I). We also analyzed the calcium dynamics as mice transitioned into a safer or more anxious location (Fig. 2, J to M). To do so, we extracted from the cells exhibiting a positive, negative, and no correlation [from Fig. 2 (G to I)]; the calcium activity immediately before and after mice transitioned into and out of the edges (edge entry and exit, respectively; Fig. 2, J and K); and the center (center exit and entry; Fig. 2, L and M). On average, the positively correlated neurons increased their activity when entering the edge or leaving the center [Fig. 2J, edge entry (center/transition, 0.00 ± 0.07 versus edge, 0.32 ± 0.06 z scores); Fig. 2L, center exit (center, 0.00 ± 0.03 versus edge/transition, 0.15 ± 0.04 z scores)] and significantly decreased their activity when leaving the edge or entering the center [Fig. 2K, edge exit (edge, 0.00 ± 0.10 versus center/transition, -0.36 ± 0.03 z scores); Fig. 2M, center entry (edge/transition, 0.00 ± 0.03 versus center, -0.20 ± 0.05 z scores)]. Furthermore, the magnitude of these changes was significantly larger than the not correlated cells during edge entry (+ve Corr, 0.32 ± 0.11 versus not Corr, -0.07 ± 0.05 z scores; Fig. 2J) and edge exit (+ve Corr, -0.36 ± 0.13 versus not Corr, -0.08 ± 0.05 z scores; Fig. 2K) but not during center entry (+ve Corr, -0.20 ± 0.04 versus not Corr, -0.10 ± 0.04 z scores; Fig. 2M) or center exit (+ve Corr, 0.15 ± 0.03 versus not Corr, 0.10 ± 0.04 z scores; Fig. 2L). On the other hand, the negatively correlated cells showed the opposite trend on average; reaching significant decreases in activity when the animals exited the edges [Fig. 2J, edge entry, (center/transition, 0.00 ± 0.06 versus edge, -0.259 ± 0.03 z scores); Fig. 2K, edge exit (edge, 0.00 ± 0.03 versus center/transition, 0.18 ± 0.07 z scores); Fig. 2L, center exit (center, 0.00 ± 0.03 versus edge/transition, -0.07 ± 0.04 z scores); Fig. 2M, center entry (edge/transition, 0.00 ± 0.02 versus center, -0.06 ± 0.02 z scores)]. However, the magnitude of their responses did not differ significantly from the not correlated cells [Fig. 2J, edge entry (–ve Corr, -0.26 ± 0.10 versus not Corr, -0.07 ± 0.05 z scores); Fig. 2K, edge exit (–ve Corr, 0.18 ± 0.11 versus not Corr, -0.08 ± 0.05 z scores); Fig. 2L, center exit (–ve Corr, -0.07 ± 0.08 versus not Corr, 0.10 ± 0.04 z scores); Fig. 2M, center entry (–ve Corr, -0.06 ± 0.11 versus not

Fig. 1. Anxious experience enhances ZI neuronal activation. (A) Experimental setup where mice were placed on a narrow elevated rod. (B) Schematic of the control home cage condition. (C) Example confocal image of the immunohistochemical (IHC) staining against *c-fos* (green) in the ZI-containing coronal brain slices from mice that were exposed to the anxious elevated rod experience. Scale bar, 100 μ m. (D) Example confocal image of the IHC labeling of *c-fos* in the ZI-containing coronal brain slices of mice that were left in the control home cage condition. Scale bar, 100 μ m. (E) Bar graphs reporting the relative number of *c-fos*-labeled cells (normalized to the control mice) in the ZI for both experimental groups. Data are presented with means $\pm$ SEM. **** $P < 0.0001$.

Fig. 2. ZI neurons are sensitive to anxiety-related cues. (A) Surgical setup to express and visualize GCaMP6m in the ZI of wild-type (WT) C57BL/6 mice. (B) Example confocal image of a partial coronal section containing the GRIN lens tract and the GCaMP6m-expressing (green) cells in the ZI. Scale bars, 500 and 50 μ m. (C) Schematics of the elevated-platform design with the compartments (edge, transition, and center) annotated. (D) Bar graph reporting the percentage of time mice explored the center (C) and edges (E). (E) Raw frame extracted from a ZI calcium recording (left) and maximum intensity projection visualization of identified cells (right) with example cells color coded. (F) Track plot of a single mouse (top) along the elevated platform (different compartments highlighted). Time-locked heatmap of calcium activity (represented in z scores) of all recorded cells (middle) and traces from example cells [(bottom; corresponding to E)]. (G) Scatterplots of example recorded cells with calcium activity positively (red), negatively (blue), and not (black) correlated to the distance from the center in the recorded mouse. (H) Pie chart depicting the proportion of cells whose calcium activity correlate to the center distance. (I) Bar graphs reporting the differences in calcium activity while mice were in the edges versus the center. (J) Transient changes in activity as mice entered the edge. Schematic of the positional change (top). Line plot (left) depicting the average activity 2.5 s before and after the positional change. Heatmap (right) of the activity of all cells with a vertical bar depicting the type of correlation. Bar graphs (right) comparing the change in calcium activity between following the positional change. (K) Transient changes in activity as mice exited the edge. (L) Transient changes in activity as mice exited the center. (M) Transient changes in activity as mice entered the center. Data are presented with means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Corr, -0.10 ± 0.04 z scores)]. Corresponding increases and decreases in activity when mice entered more anxiety-provoking and safer areas, respectively, provide further support that this subset of ZI neurons encode anxiety-related information. To exclude a possible motor component, we correlated the calcium activity of individual ZI cells with the movement speed of the recorded mice. Of the cells that displayed positive and negative correlation with the mouse position on the platform, a majority of 69 and 91% were not correlated with the movement speed, respectively (20 of 29 and 10 of 11 cells; fig. S1, A and B). On average, the speed mice that traveled in the center and the edges were similar (center, 0.038 ± 0.003 m/s versus edges, 0.024 ± 0.007 m/s; fig. S1C) and thus further suggest that movement speed does not explain the differences in neuronal activity while mice were in these two areas. Together, these data reveal that a notable subset of ZI neurons is sensitive to anxiety-related cues irrespective of locomotion.

So far, we have shown that the ZI can detect changes in environments provoking relatively lower and higher levels of anxiety. We then wanted to assess the ability of the ZI to influence anxiety. We took a pharmacological approach where the anxiolytic properties of

diazepam applied directly into the ZI were tested. The much smaller cannula implants posed little physical constraint on maneuverability, and thus, mice were tested in a standard paradigm for studying anxiety-like behaviors in mice (34). Of the two popular elevated mazes [the EPM and the elevated O-maze (EOM)], the EOM was selected because it allows straightforward interpretations given the simple design with equally sized safe closed and anxiogenic open arms (35). Furthermore, to ensure that a potential anxiolysis effect is not overlooked, we used BALB/c mice for their relatively heightened baseline levels of anxiety (36, 37). Infusion cannulas were bilaterally implanted above the ZI (Fig. 3, A and B). Diazepam or vehicle infusions were made 30 min before placing mice in the EOM (Fig. 3C). It was previously reported that diazepam (10 mg/kg, provided intraperitoneally) was anxiolytic without affecting general locomotion (38), and thus, we provided a dose 10 times smaller for infusion. The order of the treatments was counterbalanced and reversed 2 weeks later. When mice received diazepam, they spent significantly more time in the open arms as compared to when they were treated with the vehicle (vehicle, 130.8 ± 27.85 s versus diazepam, 219.9 ± 30.03 s; 13 mice; Fig. 3, D and E, and movie S2). They also made more entries into

Fig. 3. Local ZI diazepam infusion reduces anxiety without affecting movement. (A) Surgical setup to place cannulas above the ZI. (B) Example confocal image of a partial coronal section containing the cannula tract with the red RetroBeads in the ZI to show the extent of spread. Scale bars, 500 and 250 μ m. (C) Schematic of the setup to directly infuse diazepam (1 mg/kg) into the ZI or vehicle 30 min before testing the mouse on the EOM. (D) Track plot of a single example mouse on the EOM (dashed lines denote the open arms, while the filled lines indicate the closed arms) after vehicle (left) and diazepam (right) infusion with the speed color coded. (E) Bar graph of time the mice spent in the open arms after vehicle and diazepam infusions. (F) Bar graph depicting the number of times mice entered the open arm after vehicle and diazepam infusions. (G) Track plot of an example mouse in the OFT after vehicle (left) and diazepam (right) infusion with the speed color coded. (H) Bar graph depicting the distance mice traveled after vehicle or diazepam infusions. (I) Bar graph showing the time mice spent immobile after vehicle or diazepam infusions. Data are presented with means $\pm$ SEM. * $P < 0.05$.

the open arms (Fig. 3F, vehicle 24.38 ± 4.90 entries versus diazepam 37.92 ± 4.77 entries). In addition, we observed no significant differences across distance and immobility measures between diazepam- and vehicle-treated mice in the EOM (fig. S2). To make sure that the diazepam-mediated anxiolytic effects in the EOM were not confounded by changes in locomotion, we investigated general locomotion with the open field test (OFT). The distance traveled and the time mice spent immobile in the OFT were similar with both treatment conditions (distance traveled: vehicle, 43.76 ± 3.82 versus diazepam, 46.33 ± 3.22 ; time immobile: vehicle, 50.05 ± 13.17 s versus diazepam, 64.39 ± 17.09 s; Fig. 3, G to I). These findings suggest that local diazepam treatment in the ZI reduces anxiety without affecting locomotion, further consolidating our biochemical and in vivo calcium imaging data. To briefly summarize here, our multilevel experimental observations converge onto the ZI as a brain structure processing anxiety-related stimulus and modulating exploration into anxiety-evoking environments.

ZI SOM-, CR-, and Vglut2-expressing cells exhibit unique electrophysiological profiles and are all sensitive to anxious stimuli and diazepam.

So far, we have shown with complementary approaches that the ZI encodes anxiety. However, note that we observed subsets of ZI neurons displaying anxiety-induced *cfos* expression and anxiety-induced changes in calcium transients. Previous literature reported the cellular diversity within the ZI (29–31). We therefore wondered about the specific role that an individual ZI subpopulation plays in the encoding of anxiety. To address this question, we first examined the ZI biochemical diversity with fluorescent in situ hybridization (FISH), focusing on the GABAergic and glutamatergic markers with Vgat and Vglut2, respectively. We report that Vgat-expressing cells are more abundant than Vglut2-expressing ones (Vgat-only, 78.8% and Vglut2-only, 19.0%; 3390 cells from three mice; Fig. 4, A and B). Probing against specific inhibitory markers, namely, SOM, CR, and PV, revealed that the SOM- and CR-expressing subpopulations are larger than the PV-expressing one (SOM-only, 36.6%; CR-only, 53.2%; and PV-only, 2.0%; 1684 cells from three mice; Fig. 4, C and D). As expected, SOM- and CR-expressing cells coexpress Vgat (fig. S3).

We also investigated the coexpression of calcium/calmodulin-dependent protein kinase II α (CaMKII α) with SOM $^{-}$ and CR $^{-}$ (fig. S4, A to F) or with Vglut2 $^{-}$ (fig. S4, G to K) in the ZI in separate preparations. We observed that subsets of CaMKII α -expressing cells also coexpress SOM $^{-}$ (16.3%), CR $^{-}$ (26.4%), or Vglut2 $^{-}$ (19.7%) (fig. S4, A to K). With these data, we confirmed that the imaged neurons (GCaMP6m under the expression of CaMKII α promoter) recorded in Fig. 2 indeed included SOM-, CR-, and Vglut2-expressing cell types.

Considering that SOM- and CR-expressing cells represent a sizable portion of the Vgat-positive neurons (as opposed to the PV-expressing cells; Fig. 4 and fig. S3), we sought to focus the study onto these two inhibitory subpopulations and the rare excitatory Vglut2-expressing one.

Next, we functionally characterized these ZI subpopulations performing in vitro whole-cell patch-clamp recordings (Fig. 4, E to M). To unequivocally study SOM-, CR-, and Vglut2-expressing cells, reporter lines (SOM-TOM and CR-TOM) or CRE-dependent EYFP (enhanced yellow fluorescent protein; AAV5-DIO-EYFP) that injected Vglut2-CRE-mouse line was used (Fig. 4, E and F). Despite exhibiting similar capacitance (SOM, 14.27 ± 0.80 pF; CR, 16.94 ± 2.16 pF; and Vglut2, 15.15 ± 1.09 pF; 13 to 16 cells; four to five mice;

Fig. 4G), resting membrane potential (SOM, -72.20 ± 2.12 mV; CR, -74.31 ± 3.37 mV; and Vglut2, -68.15 ± 2.88 mV; Fig. 4H), and action potential width (SOM, 8.46 ± 0.16 ms; CR, 8.46 ± 0.13 ms; and Vglut2, 9.16 ± 0.47 ms; Fig. 4I), SOM-expressing neurons are less excitable and display a higher rheobase (SOM, 41.33 ± 5.68 pA; CR, 23.13 ± 2.54 pA; and Vglut2 29.17 ± 3.362 pA; Fig. 4J) while Vglut2-expressing cells sustain higher maximum firing frequencies (SOM, 45.43 ± 5.34 Hz; CR, 42.00 ± 6.38 Hz; and Vglut2, 105.20 ± 11.42 Hz; Fig. 4, K to M).

To characterize further these three neuronal populations in a circuit mapping context, we first assessed their local connectivity probability and, in a second step, their long-range anatomical mapping. We injected an AAV vector containing CRE-dependent ChR2 (Channelrhodopsin-2) (AAV5-DIO-ChR2-EYFP) in the ZI of CR-, SOM-, and Vglut2-CRE mice (fig. S5A). We performed in vitro electrophysiology experiments in brain slices containing the ZI and recorded from ChR2-negative cells identified by the lack of a 400-ms blue (473 nm) light-induced desensitizing current. Local postsynaptically connected cells exhibited a brief 5-ms light-induced current response. We observed no (0 of 20 patched cells) locally connected cells to the SOM-expressing subpopulation (fig. S5B). In contrast, we observed 21.5% connected cells to the CR-expressing subpopulation (fig. S5D) and 59% connected cells to the Vglut2 $^{-}$ subpopulation (fig. S5F). These connections are all monosynaptic, as challenged by the successive bath application of tetrodotoxin (TTX) and 4-aminopyridine (4-AP), and are inhibitory and excitatory, as abolished by subsequent bath application of picrotoxin (PTX) and kynurenic acid (KA), respectively (fig. S5, D to G).

We next identified SOM-, CR-, and Vglut2-expressing cells' long-range projections to other brain structures. To do so, we injected an AAV construct containing CRE-dependent tdTOM and synaptophysin-tagged enhanced green fluorescent protein (SynEGFP) into the ZI of SOM-, CR-, and Vglut2-CRE mice and comprehensively explored the diversity of the efferent projections across the brain (fig. S6). Across all three ZI subpopulations, the most noticeable tdTOM and SynEGFP signals were observed in the lateral habenula (LHb) and the periaqueductal gray (PAG) (fig. S6). While CR-expressing ZI cells sparsely project to the dorsal medial PAG (dmPAG), the Vglut2-expressing cells densely project to the lateral and ventrolateral PAG (l/vlPAG), and the SOM-expressing cells project to all three (fig. S6).

We next assessed whether SOM-, CR-, and Vglut2-expressing ZI neurons are activated by anxious conditions. The elevated rod experiment followed by *cfos* labeling was performed (fig. S7). Our quantification reports that coexpression of *cfos* with either SOM $^{-}$, CR $^{-}$, or Vglut2 $^{-}$ is significantly increased in the experimental mice as compared to the control ones (SOM: elevated rod, 3.50 ± 0.35 cells versus home cage, 1.00 ± 0.18 cells; 3262 and 2458 cells in total, respectively, in 12 slices from three mice; CR: elevated rod, 7.40 ± 1.39 cells versus home cage, 1.00 ± 0.25 cells; 2074 and 1624 cells in total, respectively; Vglut2: elevated rod, 3.28 ± 0.30 versus home cage, 1.00 ± 0.17 cells; 2538 and 1551 cells in total, respectively; fig. S7, B to P). These data suggest that SOM-, CR-, and Vglut2-expressing cells are all activated by anxiety.

Last, considering that the intra-ZI infusion of diazepam induced anxiolysis, we sought of verifying the sensitivity of our three neuronal cell types to diazepam. This was done through in vitro recordings of pharmacologically isolated inhibitory postsynaptic currents (IPSCs) onto SOM-, CR-, and Vglut2-expressing cells. The neurons

Fig. 4. SOM-, CR-, and Vglut2-expressing ZI neurons display unique electrophysiological properties. (A) Example confocal images of ZI-containing coronal brain slices with the FISH labeling of the mRNAs for Vglut2 (green) and Vgat (cyan) and the merged image. Scale bars, 100 and 25 μ m. (B) Pie chart quantification of the proportion of cells in ZI expressing Vglut2, Vgat, or both (3390 cells total, three mice). (C) Example confocal images of the ZI-containing coronal brain sections with the FISH labeling of the mRNAs for SOM (red), CR (green), PV (cyan), and the merged. Scale bars, 100 and 25 μ m. (D) Pie chart representation of the percentage of expressing the mRNAs for SOM, CR, PV, or any combination of the three (1684 cells total, three mice). (E) Schematic of the experimental procedure. (F) Example bright-field (top) and fluorescent (bottom) images of patched cells: SOM (cyan), CR (magenta), and Vglut2 (maroon). (G) Bar graphs reporting the capacitance from SOM-, CR-, and Vglut2-expressing ZI cells. (H) Bar graphs depicting the resting membrane potential (Vm) from SOM-, CR-, and Vglut2-expressing ZI cells. (I) Bar graphs of the action potential width from SOM-, CR-, and Vglut2-expressing ZI cells. (J) Bar graphs presenting the rheobase from SOM-, CR-, and Vglut2-expressing ZI cells. (K) Bar graphs summarizing the maximum firing frequency from SOM-, CR-, and Vglut2-expressing ZI cells. (L) Example current clamp recordings from SOM-, CR-, and Vglut2-expressing ZI cells upon various current step injections. (M) Summary input/output curve for SOM-, CR-, and Vglut2-positive cells. Data are presented with means $\pm$ SEM. ** $P < 0.01$, **** $P < 0.0001$.

were identified through the expression of TdTomato/EGFP (Fig. 5A). Diazepam (1 μ M) potentiated the electrically evoked IPSCs (SOM: baseline, -523.9 ± 96.29 pA versus diazepam, -907.7 ± 175.3 pA; CR: baseline, -232.9 ± 63.65 pA versus diazepam, -500.3 ± 85.55 pA; Vglut2: baseline, -342.0 ± 71.26 pA versus diazepam, -669.0 ± 106.5 pA; 10 to 11 cells from four to five mice; Fig. 5B) without affecting the pair-pulse ratio in all three cell types (SOM: baseline, 1.11 ± 0.09 versus diazepam, 0.91 ± 0.07 ; CR: baseline, 0.95 ± 0.11 versus diazepam, 0.85 ± 0.08 ; Vglut2: baseline, 1.06 ± 0.07 versus diazepam, 0.98 ± 0.06 ; Fig. 5B). The amplitude (SOM: baseline, -41.78 ± 5.07 pA versus diazepam, -48.65 ± 6.27 pA; CR: baseline, -44.54 ± 3.05 pA versus diazepam, -50.08 ± 3.12 pA; Vglut2: baseline, 40.79 ± 3.22 pA versus diazepam, -47.54 ± 3.98 pA; 10 to 12 cells from four to five mice; Fig. 5C) and frequency (SOM: baseline, 0.05 ± 0.02 Hz versus diazepam, 0.09 ± 0.03 Hz; CR: baseline, 0.04 ± 0.01 Hz versus diazepam, 0.06 ± 0.01 Hz; Vglut2: baseline, 0.08 ± 0.02 Hz versus diazepam 0.09 ± 0.02 Hz;

Fig. 5C) of the spontaneous IPSCs were both increased. The IPSCs were blocked by PTX (100 μ M). These in vitro electrophysiological data reveal that all three neuronal subpopulations are sensitive to diazepam.

Together, these findings suggest that SOM-, CR-, and Vglut2-expressing cells of the ZI are distinct neuronal subpopulations with specific functional intrinsic properties. However, they are all similarly activated by anxious stimuli and are sensitive to diazepam.

SOM-, CR-, and Vglut2-expressing ZI neurons trigger distinct anxiety-related traits

Despite being activated by elevation and allosterically modulated by diazepam, SOM-, CR-, and Vglut2-expressing cells might still engage different components of anxiety-related behaviors. To verify this hypothesis, we bidirectionally manipulated the activity of these three subpopulations while mice performed the EOM task (Fig. 6).

Fig. 5. Diazepam potentiates inhibitory drive onto SOM-, CR-, and Vglut2-expressing ZI neurons. (A) Schematic of the experimental setup. (B) Example IPSC from representative SOM-expressing (cyan), CR-expressing (magenta), and Vglut2-expressing (maroon) ZI cells during baseline (base) and following subsequent bath application of diazepam (Dz) and PTX. Scale bars, 100 pA (vertical) and 25 ms (horizontal). Bar graphs report the amplitude of the IPSCs and the paired-pulse ratio (PPR) of both conditions. (C) Example sIPSCs (spontaneous inhibitory post-synaptic currents) recorded from representative SOM-, CR-, and Vglut2-expressing ZI cells during baseline (base) and during bath application of diazepam (Dz) and PTX. Scale bars, 30 pA (vertical) and 500 ms (horizontal). Bar graphs of the amplitude and the frequency of sIPSCs in the baseline and following diazepam application are shown. Data are presented with means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 6. Optogenetic manipulation of SOM-, CR-, and Vglut2-expressing ZI neurons triggers distinct anxiety-related traits. (A) Schematic of the task. (B) Example confocal images of coronal brain slices with the expression of ChR2 in SOM-, CR-, and Vglut2-expressing ZI cells and optic fiber tract above (high-magnification inserts below the fiber). Scale bars, 25 μ m. Laser stimulation protocols for ChR2 (bottom left) and Arch (bottom right). (C) Example track plots of mice on the EOM (dashed lines denote the open arms; filled lines represent the closed arms) during laser OFF (top) and ON (bottom, shaded) periods with the speed color coded. (D) Bar graph depicting the delta (ON-OFF) open arm time in mice expressing EYFP, ChR2, and Arch in SOM-expressing ZI cells. (E) Bar graph depicting the delta body entries into the open arms [corresponding to D]. (F) Bar graph depicting the delta open arm time in CR-CRE mice. (G) Bar graph depicting the delta body entries [corresponding to F]. (H) Bar graph depicting the delta open arm time in Vglut2-CRE mice. (I) Bar graph depicting the delta body entries [corresponding to H]. (J) Schematic of the experimental procedure. (K) Schematic of setup (top, extracted from movie S3). Laser stimulation protocol for ChR2 (bottom). (L) Example minimum intensity projection of 0.5-s and 1-min windows before and after laser stimulation (blue shaded) in mice expressing ChR2 in the Vglut2-expressing ZI cells. (M) Bar graph presenting the cumulative jumps made by mice expressing ChR2 in the Vglut2-expressing ZI cells. (N) Example minimum intensity projection of 15-s and 1-min windows in the stimulation off and on period (blue shaded) in mice expressing ChR2 in the CR-expressing ZI cells. (O) Bar graph presenting the cumulative rears made by mice expressing ChR2 in the CR-expressing ZI cells. Data are presented with means $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001. Photo credit: Zhuoliang Li, University of Basel.

To do so, we injected AAV vectors containing either CRE-dependent ChR2 (AAV5-DIO-ChR2-EYFP) or Arch (AAV5-DIO-Arch-EYFP) in the ZI of SOM-, CR-, and Vglut2-CRE mice and implanted optic fibers above the injection site (Fig. 6, A and B). Our experimental preparations were validated through *in vitro* patch-clamp recordings (fig. S8, A and B). A short blue light illumination (400 ms, 473 nm) evoked desensitizing photocurrents in ChR2-positive SOM-, CR-, and Vglut2-positive ZI neurons (fig. S8C). Furthermore, either a 1-s continuous light pulse or a 10-s (40 Hz) stimulation elicited firing activity that was reversed as soon as the stimulation was turned off (fig. S8D). Such a 40-Hz blue light stimulation was efficient at significantly and sufficiently enhancing the firing activity of each three cell types (fig. S8D). In contrast, green light pulses (400 ms, 532 nm) triggered hyperpolarizing photocurrents in Arch expressing SOM-, CR-, and Vglut2-positive cells, preventing evoked or spontaneous firing (fig. S8, E and F).

At the behavioral level, mice performed the EOM task while the activity of SOM-, CR-, and Vglut2-expressing cells was manipulated (1 min off and 1 min on; three times; Fig. 6C). Behavioral changes induced by light stimulation were reported as the difference in the performance during ON and OFF light stimulation. Activation of ChR2 in SOM-expressing ZI neurons significantly reduced both the amount of time spent in the open arm (CTRL, 5.40 ± 5.47 s versus ChR2, -9.53 ± 3.16 s; 6 to 12 mice; Fig. 6D) and the number of entries made into the open arm (CTRL, 0.83 ± 1.01 entries versus ChR2, -3.26 ± 0.65 entries; Fig. 6E). In stark contrast, optogenetic activation of CR-expressing neurons induced an opposite behavioral phenotype, with a significant increase of both the time spent in the open arm (CTRL, 1.98 ± 4.47 s versus ChR2, 36.32 ± 13.83 s; five to eight mice; Fig. 6F) and the number of open arm entries (-0.67 ± 0.33 entries versus 8.40 ± 4.49 entries; Fig. 6G). The optogenetic inhibition of either SOM- or CR-positive ZI neurons had little effect on the EOM performance [Fig. 6D, SOM open arm time (CTRL, 5.40 ± 5.47 s versus Arch, 14.74 ± 2.89 s); Fig. 6E, SOM open arm entry (CTRL, 0.83 ± 1.01 entries versus Arch, 1.13 ± 0.44 entries); Fig. 6F, CR open arm time (CTRL, 1.98 ± 4.47 s versus Arch, 3.38 ± 3.74 s); Fig. 6G, CR open arm entry (-0.67 ± 0.33 entries versus 0.50 ± 0.78 entries)]. The light-induced inactivation of Vglut2-expressing cells increased the time mice spent in the open arms (CTRL, -6.64 ± 9.80 s versus Arch, 19.75 ± 5.14 s; five to six mice; Fig. 6H) without affecting the number of open arm entries (CTRL, 1.00 ± 0.32 entries versus Arch, 2.33 ± 0.62 entries; Fig. 6I).

When mice were tested in the open field, the same light stimulations induced no significant change in locomotion (fig. S9), as reported by the distance traveled [fig. S9D, SOM (CTRL, -0.34 ± 0.35 m versus ChR2, -1.82 ± 0.63 m versus Arch, 0.40 ± 0.57 m); fig. S9F, CR (CTRL, -0.50 ± 0.67 m versus ChR2, -0.03 ± 1.15 m versus Arch, 0.78 ± 0.65 m); fig. S9H, Vglut2 (CTRL, -0.27 ± 0.65 m versus Arch, 1.42 ± -0.83 m)] and the immobility time [fig. S9E, SOM (CTRL, -6.22 ± 6.90 s versus ChR2, 26.2 ± 10.29 s versus Arch, -2.51 ± 9.26 s); fig. S9G, CR (CTRL, -7.22 ± 9.77 s versus ChR2, 8.28 ± 11.05 s versus Arch, -16.24 ± 8.11 s); fig. S9I, Vglut2 (CTRL, 11.18 ± 6.94 s versus Arch, -24.52 ± 14.03 s)]. Photoactivation of Vglut2-expressing neurons was also tested. Because of the phototriggered impressive jumps, testing mice in the EOM and the OFT was not possible. To measure this phenotype, mice were placed inside a closed transparent chamber (Fig. 6, J and K). Blue light stimulation (40 Hz) triggered time locked jumps that were immediately stopped when the light

stimulation was OFF (OFF, 0.00 ± 0.00 jumps versus ON, 242.4 ± 24.60 jumps; Fig. 6, L and M, and movie S3).

To verify that our light stimulation is restricted to Vglut2-expressing ZI cells, we conducted and reproduced the jumping phenotype in a separate cohort of Vglut2-CRE mice (off, 0.00 ± 0.00 jumps versus on, 179.7 ± 56.81 jumps; fig. S10, A to C) and post hoc stained slices containing the implant site against cfos. We observed that while cfos staining was sparsely observed in the ventral thalamus or the lateral hypothalamus, the majority of the cfos-positive neurons were concentrated within the ZI together with the ChR2-eYFP labeled cells (fig. S10D).

Last, considering that ZI CR-ChR2-expressing mice exhibited an increased exploration in the EOM, we also tested them in the closed transparent chamber. Light stimulation significantly increased the number of rears (OFF, 4.80 ± 1.72 rears versus ON, 27.60 ± 7.38 rears; Fig. 6, N and O, and movie S4). This exploratory rearing behavior (39, 40) provides an additional support that the activation of CR-expressing ZI neurons reduces anxiety. Furthermore, the activation of SOM-expressing neurons induced no clear-cut observable behavioral alterations (movie S5).

Together, our findings suggest that SOM-, CR-, and Vglut2-expressing ZI subpopulations differentially modulate anxiety traits and related behaviors. Specifically, activation of SOM-expressing ZI neurons induces anxiogenesis, while the activation of CR-expressing ZI neurons triggers anxiolysis and exploratory rears. Activation of Vglut2-expressing ZI neurons induces jumps and is contrasted by its inactivation, which is anxiolytic.

DISCUSSION

With this study, we aimed at providing experimental evidence that the ZI encodes anxiety, in light of the interesting recent clinical observations reporting that ZI DBS treatment in PD reduces anxiety (11, 12). Specifically, we showed that a powerful anxiety-provoking stimulus such as the forced exposure on a narrow elevated rod activates ZI neurons, as reported by their significant enhancement in cfos expression, adding to the growing literature that the ZI is sensitive to stressful experiences (20). Given the temporal limitations of such a biochemical investigation, we scrutinized the same question by examining the *in vivo* calcium dynamics of individual ZI neurons with a calcium indicator expressed under a CaMKII α promoter while mice explored an elevated platform. We revealed that a subset of ZI neurons is active in the open arm edges and become more active when mice enter a more anxiogenic area and less to a safer one. These attributes cannot be solely explained by changes in motion, as most movement speed-correlated ZI cells are not correlated with anxiety. Supplementing this, the local infusion of a classical anxiolytic drug, diazepam directly into the ZI, reduced anxiety without affecting locomotion on the EOM and OFT, respectively.

Note that not all ZI cells show potentiated cfos expression or increased calcium activity following exposure to anxiogenic cues. These observations hint toward some underlying heterogeneities. Past literature provided comprehensive insights into the diversity of biochemical cell type markers expressed in the ZI (29–31), without much further characterization into their physiological and functional properties. In line with previous studies (29, 31), we showed that cells expressing GABAergic markers are more prevalent over glutamatergic ones. We focused on two GABAergic subpopulations expressing Vgat with SOM or CR in addition to the rarer

Vglut2-expressing cells and reported their unique intrinsic properties, leading the way with the electrophysiological profiling of genetically defined ZI subpopulations. Each of them coexpress CaMKII α . SOM-expressing cells seem to not modulate local network activity, whereas CR- and Vglut2-expressing neurons do act as interneurons, providing local inhibitory and excitatory modulations onto other ZI cells, respectively. Anatomically, all three ZI subpopulations send long-range projections innervating brain structures involved in emotionally relevant behaviors (2, 41). Not only did we confirm the pathways ZI onto the LHb (42) and the PAG (27, 28), but we also delineate further that while CR-expressing cells innervate the dmPAG, Vglut2-positive cells project onto the l/vlPAG and SOM-expressing neurons project onto all three.

We also provide additional experimental evidence showing that they similarly respond to anxiety-related cues biochemically and to diazepam *ex vivo*; however, they drive specific and distinct actions. Optogenetic activation of SOM-expressing cells reduced exploration in the anxiogenic open arms, while the activation of CR-expressing neurons and inhibition of Vglut2-expressing ZI cells increased it. These findings differ from previous reports where manipulation of the ZI did not influence anxiety. Specifically, nonselective inhibition of ZI neuronal activity with either tetanus toxin or chemogenetic manipulations was reported to not significantly affect exploration in the EPM (27) and OFT (43). Moreover, chemogenetic inhibition of Vgat-expressing neurons did not reduce anxiogenic center exploration in the OFT (43). These differences could potentially derive from the compensatory mechanisms following permanent impairments of the ZI by the tetanus toxin in the former study and the mild nature of center in the OFT to provoke anxiety by the latter investigation or due to differences in the specificity of cells manipulated in both cases.

In addition, we observed that the photoactivation of Vglut2-expressing ZI cells induced time-locked jumps that were rapid, directed toward the walls of the chamber, and continuously changing in direction. It is tempting to associate these light-induced jumps with compulsive repetitive jumping behavior observed in a genetic model of autism spectrum disorder (44) or in mice undergoing kindling seizures (45) given the multitude of jumps within a small period of time in all three cases. However, the jumps that we observed following the stimulation of Vglut2-positive ZI cells were not stationary but rather directed as an attempt to escape and continuously change its direction once they fail to do so. The lack of additional motor disturbances before jumping further argues against the notion that these light-induced jumps were consequences of a seizure. Furthermore, the directed nature of these jumps resembles the active flight behavior observed in mice presented with an auditory tone that was previously paired with a strong aversive footshock (46). A recent report showed that the excitation of vlPAG promotes flight behavior (47). We show that ZI Vglut2-expressing cells do project onto the vlPAG; hence, we speculate that the observed flight-like jumps may be driven by the Vglut2-expressing ZI cells to vlPAG subpathway. On the other hand, the activation of CR-expressing ZI cells promoted rearing, a rodent-specific exploratory behavior (48, 49) that is reduced in anxious animals following stressful experiences while elevated in animals with low anxiety states (39, 40). Together with the anxiolytic effects observed in the EOM, the activation of CR-expressing ZI cells could provide the necessary foundations and partake in foraging (18) and hunting (50) behaviors, actions that have been previously attributed to the

ZI. Last, these behavioral alterations in anxiety-related behaviors that we observed with the optogenetic manipulations of its cell types are not concomitant with any significant changes in general locomotion, as is similarly reported by others (27, 28, 43).

While we bidirectionally manipulated the activity of SOM-, CR-, and Vglut2-expressing ZI neurons, the behavioral modifications were often unidirectional. The inability for the inhibition of these cell types to produce significant opposing modulations in anxiety may potentially be attributed to the insufficient drive by Arch to completely shut down ongoing activity for prolonged periods of time in face of strong excitatory inputs.

Photoactivation of SOM- and CR-expressing cells triggered opposite behavioral phenotype. This could potentially be due to the differences in their outgoing projections or underlying local connectivity. More specifically, it is tempting to speculate that CR-expressing cells may inhibit SOM-positive neurons, hence modulating and shifting the overall ZI dynamic to affect behavior. However, a much elaborated and systematic investigation into the ZI local connectivity including the identification of both the pre- and post-synaptic cells would be necessary to infer any circuit mechanisms.

Overall, our experimental findings support our hypothesis that the ZI plays an important role in anxiety. Through changes in the expression of activity-dependent biochemical markers and neuronal activity, subsets of ZI cells detect changes in the environment, triggering low and potentiated anxiety states. However, the ZI does not only have a sensory role but also actively influence anxiety-related behaviors, namely, the exploration of exposed and elevated areas, when pharmacologically targeted. Through specific anatomical connections, ZI neuronal subpopulations engage in distinct actions to potentially generate a global anxiety-related behavioral response. Specifically, the activation of SOM-expressing neurons promotes avoidance of anxiogenic areas, while the Vglut2-positive cells induce flight-like jumps. In stark contrast, the activation of CR-expressing cells drives exploration into anxiety-provoking environments and also encourages rearing. Together, these findings provide converging evidence that the ZI and its neuronal subpopulations can sense anxiety-related information and influence anxiety-related states and behavioral outcomes.

Building on the clinical observations, our findings provide important experimental evidence to show that the ZI and its individual subpopulations encode anxiety and guide related behaviors. Our study hence provides valuable knowledge regarding the role of the ZI in anxiety and extends a positive outlook for the ZI as a therapeutic target for the treatment of anxiety.

MATERIALS AND METHODS

Animals

Male and female SOM-CRE (B6-Sst<tm2.1(cre)Zjh>/J) (51), CR-CRE (B6(Cg)-Calb2<tm1(cre)Zjh>/J) (51), Vglut2-CRE (B6.Cg-Slc17a6<tm2(cre)Lowl>Unc/J, UNC) (52), and C57BL/6JRj mice were bred in house. SOM-TOM and CR-TOM reporter lines were generated by crossing the respective Cre lines with the Ai9 transgenic line [B6;129S6-Gt(ROSA)26Sor<tm9(CAG-tdTomato)Hze>/J] (53). All experimentation performed with transgenic mice involved mice of both sexes. No clear sex differences were observed, and the data were thus pooled (fig. S11). BALB/cByJRj mice, previously shown to have elevated levels of anxiety (36, 37), were purchased from Janvier Labs. All experimental procedures were approved by the

Institutional Animal Care Office of the University of Basel and the Cantonal Veterinary Office under the license number 2742.

Surgery

General

Mice were anesthetized with gas isoflurane (5% for induction and 1.5% for maintenance) in O₂ (Provet/Primal Healthcare, EZ Anesthesia Systems) and placed onto the stereotaxic frame (World Precision Instruments). Lidocaine (0.2 mg/g) (Streuli Pharma) was injected subcutaneously above the skull. The skin was disinfected with 70% ethanol and Betadine (Mundipharma). A small incision along the anterior-posterior axis was made at the midline, and then, the skull was leveled. A small hole was drilled through the skull above the ZI, which consists of the regions ZI and subincertal nucleus in the mouse atlas (1.3 mm posteriorly to the bregma, $\pm$ 1.3 mm laterally to the midline, and -4.87 mm deep from the surface of the skull at a 10° angle away from the midline) (54). Glass micropipettes (Drummond) were pulled with a vertical puller (Narishige). Viral vectors were ejected into the ZI with a pressure injector system controlled by a pulse generator (A.M.P.I.; 10 to 100 ms, 20 psi, at 0.333 Hz). The wound was either stitched together with tissue absorptive silk sutures (SABANA) or continued with additional implantations (optic fiber, cannula, or GRIN lens). Buprenorphine (Bupaq P, Streuli Pharma AG) pain killers were administered (0.1 μ g/g) after surgery as needed.

Calcium imaging

Viruses (AAV1.Camk2a.GCaMP6m.WPRE.SV40, Ready-to-Image virus, Inscopix) were unilaterally (150 nl) injected in the ZI, and then, a GRIN lens (Inscopix; 600 μ m wide and 7.3 mm long) was placed at the same coordinates. The lens was fixed with an ultraviolet (UV)-light curable glue (Henkel). A custom-made head bar (2 cm long, 0.4 cm wide, and 0.1 cm tall) was placed for future handling. A fixed headcap was built from layers consisting of superglue (Cyberbond), UV-light curable glue (Loctite), and dental cement (Lang). Small screws were anchored into the skull to improve adhesion between the skull and the headcap. The headcap was secured to the skin with Vetbond tissue adhesive glue (3M). The implant was covered with temporary silicone gels (KauPo Smooth-On). The expression of the GCaMP6m and the clearing of the lens were assessed regularly starting from 2 weeks after surgery. Once the conditions were visually determined to be satisfactory, the mice were placed under isoflurane anesthesia on the stereotaxic frame and the baseplate was secured to the headcap using a UV-light curable flowable composite glue (Kerr).

Cannula implantation

Custom guide cannulas (460 μ m in diameter and 5.5 mm in length; P1 Technologies) were bilaterally placed above the ZI. The cannulas were filled with a dummy cannula of the same length and covered by temporary silicone gel (KauPo).

Diazepam infusion

Following surgical recovery (>2 weeks), internal cannulas (100 μ m in inner diameter and 5.5 mm in length; P1 Technologies) compatible with the guide cannulas were inserted. Freshly prepared diazepam (1 mg/kg; Fagron) in vehicle (50% propylene glycol in sterile deionized H₂O; Sigma-Aldrich) or vehicle were bilaterally infused into the ZI with the syringe pump (Harvard Apparatus) at 100 nl/min. The treatments were reversed after 2 weeks. The long gap is implemented to avoid potential acute reexposure effects on anxiety-related behaviors in the behavioral paradigms involving elevated mazes (55, 56).

Following the end of all behavioral experiments, Red RetroBeads (Lumafluor) were infused via the same injection system for anatomical validation. The mice were then sacrificed, and histological preparations (see the “Histology” section) were made.

Preparation of reporter mice for *cfos* screening or in vitro recordings

To identify Vglut2⁺ ZI neurons while a reporter line is lacking, adult male and female (8 to 10 weeks) Vglut2-CRE mice were bilaterally injected with a virus expressing a CRE-dependent fluorescent protein (AAV5-EF1 α -DIO-EYFP, UNC Vector Core; 300 nl per side). The virus was expressed for at least 2 weeks before histology or in vitro electrophysiology experiments.

Anatomical tracing

Mice were injected with viruses that contained a CRE-dependent tdTOM with a synaptophysin-tagged EGFP (AAV1-phSyn-FLEX-tdTom-T2A-SypEGFP, UNC Vector Core; 100 nl per side). The virus was expressed for at least 3 weeks before perfusion and histology.

Optogenetics and fiber implantation

Mice were injected with viruses that contained a CRE-dependent opsin construct (AAV5-EF1 α -DIO-eArch3.0-EYFP and AAV5-EF1 α -DIO-ChR2-H134R-EYFP; UNC/UZH Vector Cores) or a control fluorescent protein (AAV5-EF1 α -DIO-EYFP; UNC Vector Core) bilaterally in the 5ZI (150 nl per side). Optic fibers [>70% light transmittance, 200 μ m in diameter, and 0.39 numerical aperture (NA) optic fibers fixed by epoxy to 1.25-mm-wide 6.4-mm-long ceramic ferrules; Thorlabs] (57) were bilaterally placed above the ZI (250 μ m).

In vitro electrophysiology

Coronal mouse brain slices (180 μ m thick) containing the ZI were collected with a vibratome (Leica) in cooled artificial cerebrospinal fluid (ACSF) cutting solution (92 mM *N*-methyl-D-glucamine, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 30 mM NaHCO₃, 20 mM Hepes, 25 mM glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Na-pyruvate, 0.5 mM CaCl₂·2H₂O, 10 mM MgSO₄·7H₂O, and 10 mM NAC (*N*-Acetyl-L-cysteine); osmolarity of 290 to 300 mosM) bubbled with 95% O₂ and 5% CO₂. The slices were kept in the holding ACSF solution [92 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 30 mM NaHCO₃, 20 mM Hepes, 25 mM glucose, 2 mM thiourea, 5 mM Na-ascorbate, 3 mM Na-pyruvate, 2 mM CaCl₂·2H₂O, 2 mM MgSO₄·7H₂O, and 10 mM NAC (pH 7.3); osmolarity of 290 to 300 mosM] at 31°C before being transferred into the recording chamber containing the recording ACSF solution [119 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 24 mM NaHCO₃, 12.5 mM glucose, 2 mM CaCl₂·2H₂O, and 2 mM MgSO₄·7H₂O (pH 7.3); osmolarity of 290 to 300 mosM] bubbled with 95% O₂ and 5% CO₂. Visualized whole-cell patch recordings were performed to record cells from the ZI. We identified SOM-, CR-, and Vglut2-positive cells by the expression of TdTomato from reporter lines or virally introduced fluorescent proteins (AAV5-EF1 α -DIO-EYFP, AAV5-EF1 α -DIO-eArch3.0-EYFP, and AAV5-EF1 α -DIO-ChR2-H134R-EYFP) in each of the three cell type-specific CRE lines (SOM-CRE, CR-CRE, and Vglut2-CRE) visualized under an upright microscope (Olympus) through PLANFL N $\times$ 4 and $\times$ 40 (Olympus) objectives. The internal solution contained the following: for electrophysiological profiling and optogenetic tools validation, 130 mM K-gluconate, 10 mM creatine phosphate, 4 mM MgCl₂, 3.4 mM Na₂ATP, 0.1 mM Na₃GTP, 1.1 mM EGTA, and 5 mM Hepes (pH 7.3); osmolarity of 289 mosM; for IPSC recordings and recording inhibitory transmission, 30 mM K-gluconate, 100 mM KCl,

10 mM creatine phosphate, 4 mM MgCl_2 , 3.4 mM Na_2ATP , 0.1 mM Na_3GTP , 1.1 mM EGTA, and 5 mM Hepes (pH 7.3); osmolarity of 289 mosM; and for recording excitatory transmission, 130 mM CsCl, 4 mM NaCl, 5 mM creatine phosphate, 2 mM MgCl_2 , 2 mM Na_2ATP , 0.6 mM Na_3GTP , 1.1 mM EGTA, and 5 mM Hepes (pH 7.3); osmolarity of 290 mosM.

To investigate the *ex vivo* effect of diazepam, diazepam (1 μM ; Fagron) was subsequently washed in and blocked with PTX (100 μM). In separate slices, spontaneous inhibitory postsynaptic potentials were recorded and also subsequently bath applied with diazepam and blocked with PTX. For the validation of optogenetic tools, light flashes (400 ms) controlled by a pulse generator (A.M.P.I) were delivered from a microscope mounted light-emitting diode (CoolLED) to evoke synaptic currents in voltage clamp mode held at resting membrane potential. To study the local connectivity, brief (5 ms) light pulses (50 ms apart) were delivered to evoke postsynaptic currents in non-ChR2-expressing cells. The concentrations of the pharmacological agents were applied at 500 nM (TTX), 300 μM (4-AP), 100 μM (PTX), and 2 mM (KA). In current clamp mode, blue light flashes (1 s continuous and 10 s at 40 Hz) were used to evoke firing in ChR2-expressing cells and orange light flashes (1 s continuous) were used to reduce electrically evoked (current injected) firing.

Histology

Immunohistochemistry

Mice received a lethal dose of pentobarbital (0.3 mg/kg, intraperitoneally) (Streuli Pharma AG). Mice were fixed onto a Styrofoam board (with the back facing the board and the chest facing outward) with 25-gauge needles (Braun) by the skin of their paws. The fixed mice are then placed at approximately 45°. While clenching the chest skin with forceps, a vertical incision is made with surgical scissors (Fine Science Tools) to remove the outer skin. An incision is then made to expose, and another is made to break open the diaphragm. Fat deposits around the heart are carefully removed with forceps. A small cut is made with a finer scissor to tear open the right atrium. The animals were then transcardially perfused with 25 ml of phosphate-buffered saline (PBS) buffer (Sigma-Aldrich) followed by the same volume of 4% paraformaldehyde (PFA; Sigma-Aldrich) in PBS through the left ventricle. Brains were extracted and postfixed in 4% PFA overnight then immersed in 30% sucrose in PBS until the brains have completely sunk to the floor of the container (complete absorption of sucrose). Sections were prepared (50 μm) using a cryostat (Leica CM1950). Brain slices containing the ZI slices were first washed (three times for 3 min) with 1 $\times$ tris-buffered saline (TBS) (Sigma-Aldrich) with 0.1% Tween 20 (Sigma-Aldrich), permeabilized for 20 min with 1 $\times$ TBS with 0.1% Tween 20 and Triton X-100 (Sigma-Aldrich), and blocked for 120 min with 1% bovine serum albumin (Sigma-Aldrich) or 1% bovine serum albumin with 10% donkey serum (Sigma-Aldrich) in 1 $\times$ TBS with 0.1% Tween 20. The brain slices were stained overnight in the blocking solution with the primary antibody [rabbit anti-cfos (1:500; Abcam, ab7963) and/or goat anti-CR (1:1000; Swant CG1)]. In the following day, the brain slices were first washed and then stained for 120 min with the secondary antibody [goat anti-rabbit conjugated to Alexa Flour 555 (1:500; Invitrogen, A21429), donkey anti-rabbit conjugated to Alexa Flour 488 (1:500; Invitrogen, A21206), or donkey anti-goat conjugated to Alexa Flour 647 (1:500; Abcam, ab150131)]. The brain slices were again washed and then mounted on glass slides

(Superfrost Plus, Thermo Scientific), immersed by 4',6-diamidino-2-phenylindole containing ProLong Gold Antifade mounting solution (Invitrogen), and covered with borosilicate cover glass (VWR).

Fluorescent *in situ* hybridization

Mice were anesthetized with gas isoflurane. The brains were extracted and quickly frozen on aluminum foil over dry ice. Brains were kept overnight at -80°C . On the next day, the brains were equilibrated at -20°C in the cryostat for at least 30 min. Fresh frozen brain sections (20 μm) containing the ZI were prepared and directly mounted onto glass slides. Selected slices were fixed for 30 min using 4% PFA in PBS, dehydrated with increasing concentrations of ethanol (50, 70, 100, and 100%), and surrounded by hydrophobic barriers (ImmEdge, Vector Laboratories), before proceeding with the RNAscope Fluorescent Multiplex Assay (ACD, Bio-Techne) to label the mRNAs for Vgat (Slc32a1, ref319191), Vglut2 (Slc17a6, ref319171), SOM (SST, ref482691), CR (CALB2, ref313641), PV (Pvalb, ref421931), and CaMKII α (ref445231). Processed slides were immersed in ProLong Diamond Antifade mounting solution (Invitrogen) and covered with borosilicate cover glass.

Imaging

To visualize fluorescent immunohistochemistry, FISH, or reporter protein labeling in brain sections, mounted sections were imaged with a Zeiss LSM700 upright confocal microscope controlled by the ZEN Black image acquisition software (v2010; Zeiss). PLAN APO $\times 20$ (0.8 NA, air) and $\times 40$ (1.3 NA, oil) objectives were used to take low- and high-magnification images, respectively. Fixed wavelength (405, 488, 555, and 639 nm) lasers were used to visualize specific fluorescent signals, while bright-field imaging was used for the anatomical features. For large throughput fluorescence imaging (anatomical tracing), mounted sections were imaged with a Zeiss Axio Scan.Z1 with a Fluor $\times 5$ (0.25 NA, air) objective and filter sets suitable for imaging EGFP and tdTomato signals (38 and 43HE). Images were stitched with the acquisition software and further post-processed in Fiji (v2.0.0, ImageJ).

Behavior

General

Mice were brought into the testing room from the housing facilities on the experimental day and acclimatized for at least an hour before starting any experimentation or experimental preparations, such as connecting optic fibers to fiber-implanted mice.

Elevated rod exposure for cfos screening

Control and experimental mice were group-housed in their home cages and were brought from the housing room into the testing room. A 60-min acclimation period was allowed before mice were tested or directly transcardially perfused. The remaining mice were placed on an elevated rod (100 cm tall and 3 cm in diameter) for 30 min and put back into the home cage for another 30 min before being perfused. It has been previously reported that increased cfos protein expression has been observed for as little as 30 min following stimulus exposure (58, 59). We chose a time frame that could detect changes in cfos expression and as close to the stimulus exposure as possible. Soft packing foam was placed around the bottom of the elevated rod. Mice that fell were placed back into the home cage and excluded.

Elevated platform exposure for *in vivo* calcium imaging

Mice were connected to the nVoke2 (Inscopix) system and recorded with the Inscopix data acquisition software with a 20-Hz sampling rate while behaviorally recorded simultaneously from above using a

camera (The Imaging Source) that is controlled by ANY-maze (v5.23, Stoelting) and sampled up to 20 Hz. The mice were then placed in the enclosed center of the elevated platform and recorded while freely moving for 5 min. The elevated platform is constructed by completely blocking the entrance of the closed arms of an EPM (arms with 7.5 cm in width, 30 cm in length separated by a center that is 7.5 cm in width and length and enclosed by 60-cm-tall cardboard pieces on the two sides not connected to the arms). This elevated platform maximizes and forces the recorded mice to explore the anxiogenic exposed open edges. The larger dimensions of this platform also provided more room for the recorded mice to maneuver.

Optogenetic stimulation

The light power for optogenetic stimulation (473 or 532 nm) was adjusted with a power meter (Thorlabs) such that the illumination at the tip of the optic fibers would be at around 10 mW. To sufficiently activate ZI neurons with ChR2, 5-ms pulses were provided at 40 Hz (473 nm). Constant light (532 nm) stimulation was used to drive the Arch3.0-mediated inhibition. The number of light stimulation periods was counterbalanced with periods where no light was provided in an alternating manner over 6 min with 1-min bins.

Elevated O-maze

Cannula-implanted mice that received intracranial infusion of either diazepam or vehicle (see the “Surgery” or “Diazepam infusion” section), or fiber-implanted mice that were attached to the laser cables (see the “Behavior” or “Optogenetic stimulation” section) were placed on the open arm of the EOM (arms with 5 cm in width; the maze is 55 cm in diameter and 60 cm above the ground) facing toward the closed arm (enclosed by two 15-cm-tall gray opaque plastic walls). The translucent floor allowed a camera mounted below facing upward to record the shadow of the tested mice as it traveled along the EOM. Videos were recorded and tracked with ANY-maze at a rate of up to 30 Hz for either 10 min (infusion animals) or 6 min (optogenetics animals).

Open field test

Mice were placed in the center of a rectangular OFT box (30 cm in width and 45 cm in length, with 30-cm-tall walls) for either 10 min (infusion animals) or 6 min (optogenetics animals). Videos were recorded with a downward-facing camera from above with ANY-maze in the same manner as mentioned previously.

Unique behaviors in the closed chamber

Mice were placed in the center of a transparent plexiglass rectangular box (25 cm in width and 20 cm in length, with 15-cm-tall walls) with a closed ceiling that had a circular (5 cm in diameter) hole that let out the fiber cables but not the mice for 6 min. Videos were recorded with a front-facing camera from the side with ANY-maze in the same manner as mentioned previously. To test the extent of activated neurons following laser stimulation, a cohort of bilaterally implanted Vglut2-ChR2-expressing mice were stimulated in the closed chamber as mentioned above, returned to the home cage, transcardially perfused after 60 min, and followed up with a post hoc cFos immunohistochemistry staining.

Analysis

Calcium imaging

The position of the center of the video recorded the mouse, and its movement speed was extracted from ANY-maze together with the digital boundaries of the center and the open arms of the elevated platform. The behavioral trace was then exported to MATLAB (v2018b, MathWorks) for further analysis.

Calcium transients recorded from the nVoke2 system were pre-processed in the Inscopix Data Processing Software (v1.3, Inscopix). The time-stamped calcium traces were also exported to MATLAB. Calcium transients and the behavioral traces were first aligned to each other. Calcium traces were denoised and standardized. Calcium and behavior traces were then both binned to 500-ms windows. To examine the relationship between anxiety and the neuronal activity in the ZI, correlational analyses were made between the distance of the recorded mice to the enclosed center (center distance) and the calcium transients of the recorded cells. Center distance is an assessment for anxiety because the further the animal is from the center, the more exposed the animal is and, thus, the higher the level of anxiety. The center distance is calculated by subtracting the lengthwise position of the center of the animal from that of the center of the platform and taking the absolute value. Pearson’s correlation coefficient was calculated from the distribution of the binned calcium activity and center distance values. To characterize cells as being positively, negatively, or not correlated between these two parameters, a bootstrap test was performed by randomly shifting the calcium traces 1000 times in a circular manner and then calculating the correlation coefficient. Cells whose real correlation coefficient was negative and below the 5th percentile or positive and above the 95th percentile of the random correlation coefficients were classified as negatively or positively correlated, respectively. Cells that did not fall into this range were classified as not correlated.

To further show that these correlated cells displayed activity with respect to the level of anxiety that the recorded mice experienced, the difference in activity between the time when the animals was in the center and the edges (defined as an area on extremities of the open arm with the same size as the center) was calculated for each cell and compared across groups. All recorded animals spent more time in the center as compared to the edges. To reduce sampling bias, the activity in the center was randomly sampled 1000 times to match the number of bins the animals spent in the edges, and the averaged difference was reported.

To examine whether the correlated cells displayed changes in activity as the recorded animal transitioned between areas that are less anxiogenic to more anxiogenic and vice versa, calcium transients surrounding (2.5 s before and after) the entry or the exit of the edge or the center were extracted, grouped, and compared. To visualize changes across cells and groups, transients were adjusted such that the average activity in the baseline window before the change in compartment is 0. The delta responses following each compartment change between positively, negatively, and not correlated cells were extracted and compared.

To show that the center distance–correlated cells encoded anxiety rather than movement speed, the same correlation analysis was applied between calcium transients and movement speed for each cell. For each categorized (positively, negatively, and not correlated) center distance–correlated cell groups, the proportion of each of the three categories of movement speed–correlated cells were shown.

Behavioral paradigms

All parameters were extracted from ANY-maze (EOM and OFT). Unique behaviors such as jumps and rears were visually identified and manually quantified.

Statistics

All statistical comparisons, unless otherwise noted, were analyzed with Prism (v8.4.3; GraphPad). The significance level was set at 5% ($\alpha = 0.05$) in all cases. Student’s *t* test was used to make comparisons

between two groups of data. The unpaired design was used when the samples were independent from one another (number of cfos-labeled ZI cells prepared from mice that were exposed to the elevated anxious experience or the control home conditions), while the paired design was applied in cases where repeated measures were made (activity of center distance cells before and after compartment change, effect of diazepam or vehicle on the performance in the EOM and OFT of the same mice, effect of diazepam on the recorded inhibitory currents onto ZI cells with respect to the baseline period before, and effect of photoactivation of CR- and Vglut2-ZI cells to respectively trigger rears and jumps as compared to periods in the same session where the lights were off). One-way analysis of variance (ANOVA) with Bonferroni's multiple comparison tests were used to compare the differences across multiple (greater than two) independent samples (differences in activity between the center and the edge across the different center distance–correlated groups; electrophysiological properties among the SOM-, CR-, and Vglut2-expressing ZI cells; and the differences in behavioral parameters among control, ChR2-expressing, and Arch-expressing groups).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <https://science.org/doi/10.1126/sciadv.abf6709>

[View/request a protocol for this paper from Bio-protocol.](#)

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