RESEARCH ARTICLE SUMMARY

NEUROSCIENCE

A cell type–specific cortico-subcortical brain circuit for investigatory and novelty-seeking behavior

Mehran Ahmadlou*, Janou H. W. Houba, Jacqueline F. M. van Vierbergen, Maria Giannouli, Geoffrey-Alexander Gimenez, Christiaan van Weeghel, Maryam Darbanfouladi, Maryam Yasamin Shirazi, Julia Dziubek, Mejdy Kacem, Fred de Winter, J. Alexander Heimel*

INTRODUCTION: Motivational drives are internal states that can be different even in similar interactions with external stimuli. Curiosity as the motivational drive for novelty-seeking and investigating the surrounding environment is for survival as essential and intrinsic as hunger. Curiosity, hunger, and appetitive aggression drive three different goal-directed behaviors—novelty seeking, food eating, and hunting—but these behaviors are composed of similar actions in animals. This similarity of actions has made it challenging to study novelty seeking and distinguish it from eating and hunting in nonarticulating animals. The brain mechanisms underlying this basic survival drive, curiosity, and novelty-seeking behavior have remained unclear.

RATIONALE: In spite of having well-developed techniques to study mouse brain circuits, there are many controversial and different results in the field of motivational behavior. This has left the functions of motivational brain regions such as the zona incerta (ZI) still uncertain. Not having a transparent, nonreinforced, and easily replicable paradigm is one of the main causes of this uncertainty. Therefore, we chose a simple solution to conduct our research: giving the mouse freedom to choose what it wants—double free-access choice. By examining mice in an experimental battery of object free-access double-choice (FADC) and social interaction tests—using optogenetics, chemogenetics, calcium fiber photometry, multichannel recording electrophysiology, and multicolor mRNA in situ hybridization—we uncovered a cell type–specific cortico-subcortical brain circuit of the curiosity and novelty-seeking behavior.

RESULTS: We analyzed the transitions within action sequences in object FADC and social interaction tests. Frequency and hidden Markov model analyses showed that mice choose different action sequences in interaction with novel objects and in early periods of interaction with novel conspecifics compared with interaction with familiar objects or later periods of interaction with conspecifics, which we categorized as deep and shallow investigation, respectively. This finding helped us to define a measure of depth of investigation that indicates how much a mouse prefers deep over shallow investigation and reflects the mouse's motivational level to investigate, regardless of total duration of investigation.

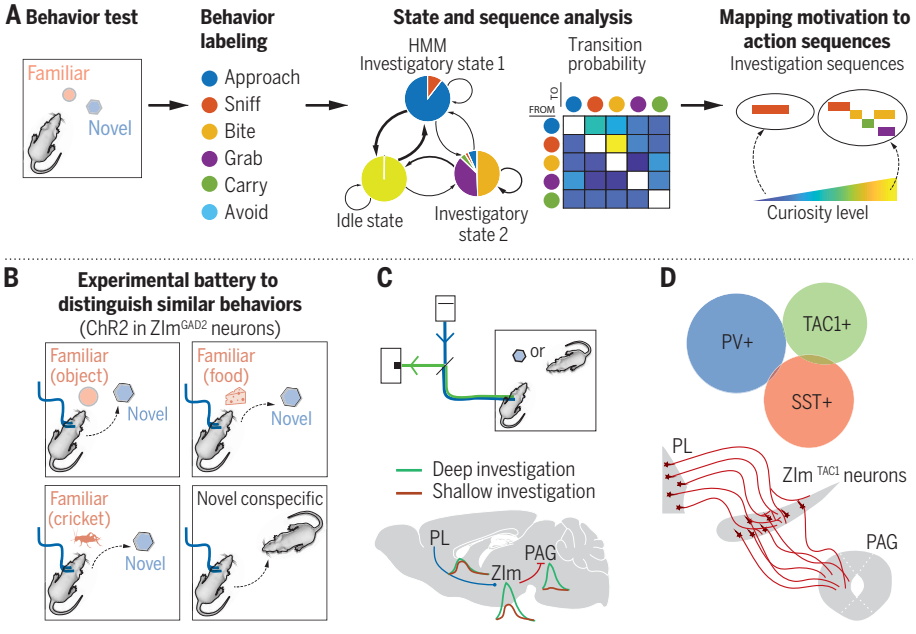
Optogenetic activation of inhibitory neurons in medial ZI (ZIm), ZIm^{GAD2} neurons, showed a dramatic increase in positive arousal level, depth of investigation, and duration of interaction with conspecifics and novel objects compared with familiar objects, crickets, and food. Optogenetic or chemogenetic deactivation of these neurons decreased depth and duration of investigation. Moreover, we found that ZIm^{GAD2} neurons are more active during deep investigation as compared with during shallow investigation.

We found that activation of prelimbic cortex (PL) axons into ZIm increases arousal level, and chemogenetic deactivation of these axons decreases the duration and depth of investigation. Calcium fiber photometry of these axons showed no difference in activity between shallow and deep investigation, suggesting a nonspecific motivation.

Optogenetic activation of ZIm^{GAD2} axons into lateral periaqueductal gray (lPAG) increases the arousal level, whereas chemogenetic deactivation of these axons decreases duration and depth of investigation. Calcium fiber photometry of these axons showed high activity during deep investigation and no significant activity during shallow investigation, suggesting a thresholding mechanism.

Last, we found a new subpopulation of inhibitory neurons in ZIm expressing tachykinin 1 (TAC1) that monosynaptically receive PL inputs and project to lPAG. Optogenetic activation and deactivation of these neurons, respectively, increased and decreased depth and duration of investigation.

CONCLUSION: Our experiments revealed different action sequences based on the motivational level of novelty seeking. Moreover, we uncovered a new brain circuit underlying curiosity and novelty-seeking behavior, connecting excitatory neurons of PL to lPAG through TAC1^{+} inhibitory neurons of ZIm. ■



Brain mechanism of curiosity. (**A**) How we mapped motivational level to action sequences. (**B**) Experimental battery to distinguish novelty-seeking behavior from food eating and hunting in mice with photoactivation of ZIm^{GAD2} neurons. (**C**) Schematic of calcium activity in PL→ZIm, ZIm, and ZIm→PAG during shallow and deep investigation. (**D**) TAC1^{+} neurons as a subpopulation of ZIm^{GAD2} neurons receive input from PL and project to PAG. HMM, hidden Markov model.

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RESEARCH ARTICLE

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A cell type–specific cortico-subcortical brain circuit for investigatory and novelty-seeking behavior

Mehran Ahmadlou[1]*†, Janou H. W. Houba[1], Jacqueline F. M. van Vierbergen[1], Maria Giannouli[1], Geoffrey-Alexander Gimenez[1], Christiaan van Weeghel[1], Maryam Darbanfouladi[1], Maryam Yasamin Shirazi[1], Julia Dziubek[1]†, Mejdy Kacem[1], Fred de Winter[2], J. Alexander Heimel[1]*

Exploring the physical and social environment is essential for understanding the surrounding world. We do not know how novelty-seeking motivation initiates the complex sequence of actions that make up investigatory behavior. We found in mice that inhibitory neurons in the medial zona incerta (ZIm), a subthalamic brain region, are essential for the decision to investigate an object or a conspecific. These neurons receive excitatory input from the prelimbic cortex to signal the initiation of exploration. This signal is modulated in the ZIm by the level of investigatory motivation. Increased activity in the ZIm instigates deep investigative action by inhibiting the periaqueductal gray region. A subpopulation of inhibitory ZIm neurons expressing tachykinin 1 (TAC1) modulates the investigatory behavior.

Investigating the physical and social environment and novelty-seeking behavior is essential for finding new food resources, assessing possible dangers, and for better understanding of the surrounding world. Novelty-seeking behavior can be dissected into the motivational drive: curiosity and the investigatory actions. Curiosity, the motivational drive behind this investigating, is considered as intrinsic as hunger and thirst (*1*, *2*). Work on neural mechanisms of curiosity focused on centers involved in reward-prediction in tasks with variable, but immediate, rewards (*3*). Curiosity, however, also drives exploration when there is no expectation of immediate reward, such as in the case of novelty-seeking behavior (*4*). It is unknown which part of the brain drives this novelty-seeking behavior. An area that drives approach and reduces fear in the mouse is the zona incerta (*5*–*10*). Activity in the rostral zona incerta induces eating (*11*), but activity in the medial zona incerta (ZIm) does not induce consummatory behavior and was reported to induce hunting (*7*, *8*). In mice, however, hunting, foraging, and object investigation overlap in both their action sequences (approaching, sniffing, grabbing, and biting) and in their modulatory sources, such as hunger and stress. This has complicated the analysis and interpretation of the experiments that have investigated these behaviors and consequently understanding of the underlying brain circuits. Lacking double-choice tests, it was difficult to determine whether the ZIm is involved in investigation and could be essential in novelty-seeking behavior.

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Mice use a different action sequence to investigate a novel object

Mice interact with objects in the surrounding environment for different purposes, such as collecting new information to test edibility or hazardousness. Mice interact less with a familiar object compared with a novel object (*12*–*14*). However, whether the actions taken to investigate a novel object are different from the actions during interaction with familiar objects is not so clear. Using a free-access double-choice (FADC) test (Fig. 1A and movie S1), we first tested how mice interact with familiar and novel objects. The number of approaches and duration of all the actions taken to interact—sniff, carry, grab, and bite—were higher in interaction with the novel object than with the familiar object (Fig. 1A). An unsupervised hidden Markov model (HMM) showed two states of investigatory behavior: (i) approach and sniff without any other interactions and (ii) approach and sniff with further interactions with the highest probability to bite (fig. S1A). Further analysis showed that there was also a much higher probability that after sniffing an object, mice continued their investigation by biting when the object was novel compared with when it was familiar, whereas none of the other transitions was significantly different (Fig. 1B and fig. S1B). Our data show that mice, after sniffing, decide to leave the investigation (with sniff to leave probability of 86 and 65% for familiar and novel objects, respectively) or continue the investigation with other sequences of actions, which mostly start with biting (with sniff to bite probability of 9 and 30% for familiar and novel objects, respectively) (Fig. 1B and fig. S1B). Therefore, we categorized the object investigation sequences into shallow investigation (in which sniffing is not followed by biting) and deep investigation (in which sniffing is followed by biting). In both cases, the investigatory event starts with sniff and ends when no investigatory action (sniff, bite, grab, and carry) is taken anymore for at least 100 ms (fig. S1C). We introduced the deep versus shallow investigation preference (DSP) using the relative time a mouse carries out deep investigation compared with the shallow investigation. DSP varies between $-\pi/2$ and $\pi/2$, where $-\pi/2$ and $\pi/2$ indicate the absolute preference for shallow and deep investigation, respectively, and 0 indicates equal preference for deep and shallow investigation. This depth of investigation was much higher for novel objects than it was for familiar objects (Fig. 1C).

γ-aminobutyric acid (GABA)–ergic neurons in ZIm play key role in object investigation

To investigate whether ZIm plays a role in driving object-investigation behavior, we expressed ChR2 by means of adeno-associated virus (AAV) to optogenetically activate inhibitory ($GAD2^+$) neurons in the ZIm (Fig. 1D and fig. S2A). Activation of the ZIm^{GAD2} neurons in a 2-min FADC test with a familiar and a novel object showed an increase in interaction with novel objects (sniff, bite, grab, and carry) and no significant change in interaction with familiar objects (Fig. 1E, fig. S2B, and movie S2). Activation of the ZIm^{GAD2} neurons in a more complex FADC test, in which we put four familiar objects and one novel object, gave the same results (fig. S3, A and B, and movie S3). In novel-object investigation compared with familiar-object investigation, there was a much higher probability of transition from sniffing to biting, whereas none of the other transitions was significantly different (fig. S2C), such as in the investigatory sequences of actions in wild-type mice. Furthermore, the DSP in novel-object interaction under ChR2 activation was much higher than in tdTomato (tdTOM) control mice (Fig. 1F), implying that there was a higher increase in deep than in shallow investigation.

To further understand whether the observed increased behavior by activation of the ZIm^{GAD2} neurons is investigatory behavior or food-eating and hunting as well, we used a FADC test with one familiarized food pellet and one novel static object and a FADC with one familiarized living cricket and one novel object moving in parallel with the cricket, respectively. Activation of the ZIm inhibitory neurons in both tests resulted in increased interaction with the novel object compared with the food (Fig. 1G and movie S4) or the cricket (Fig. 1H and movie S5). Moreover, considering movement and shape, odor, or flavor as the main components of the cricket that

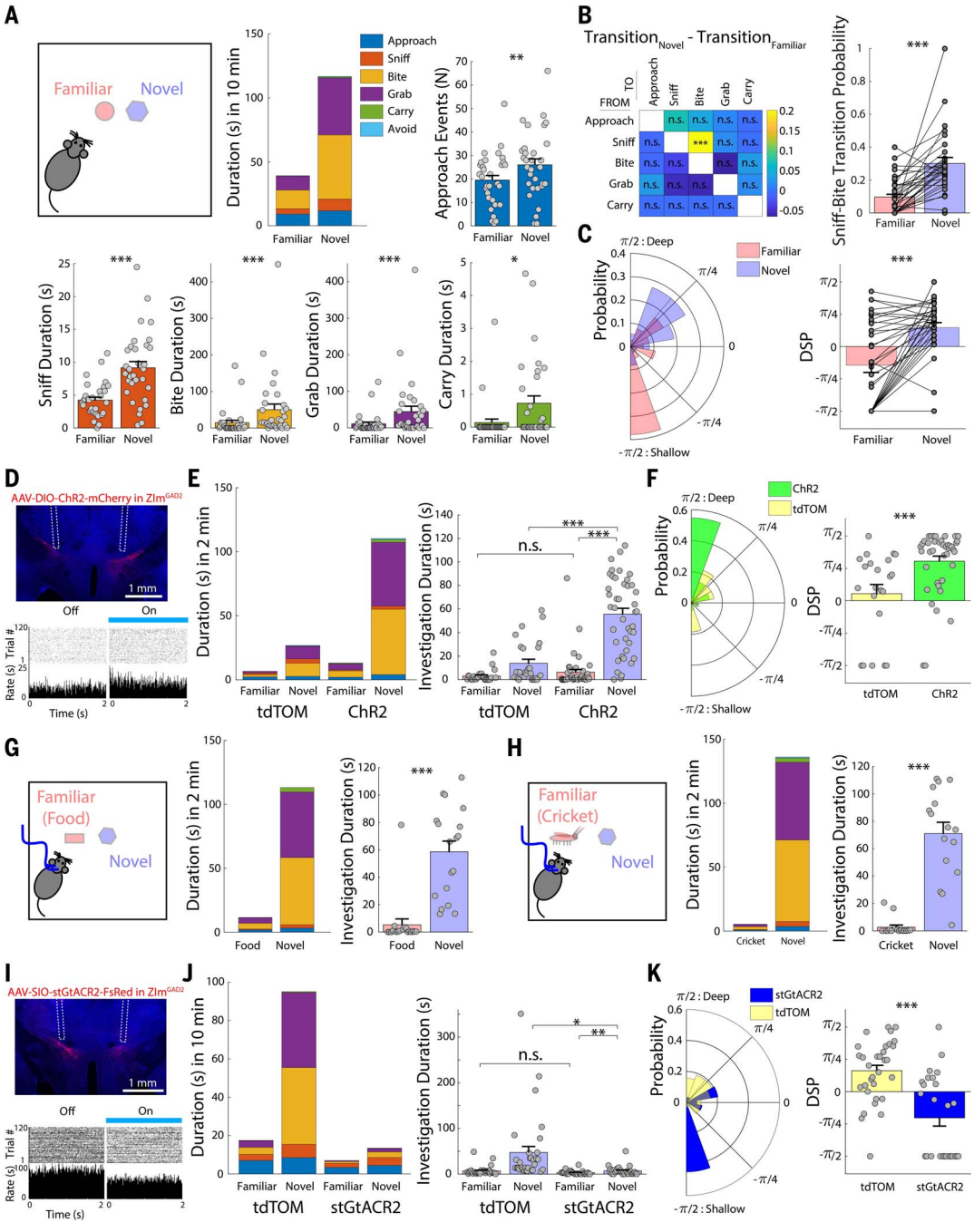

Fig. 1. Action sequences and role of ZIm GABAergic neurons in object investigation.
(**A**) Schematics of a FADC test with familiar and novel objects. Stacked bar graph shows duration for each action (approach, sniff, bite, grab, carry, and avoid) taken by C57BL/6 mice to interact with familiar and novel objects averaged over all 10-min tests (n = 37 tests from eight mice, four males). Bar graphs show quantification of individual actions: number of approaches and sniff, carry, grab, and bite durations in seconds. (**B**) Representation of the difference between transition matrices of actions taken in novel-object and familiar-object interactions by the animals in (A). Bar graph shows probability of sniff-to-bite transition in interaction with familiar and novel objects. (**C**) Probability histogram and bar graph of DSP index of mice in (A) in interaction with familiar and novel objects. DSP varies between $-\pi/2$ and $\pi/2$, where $-\pi/2$ and $\pi/2$ indicate the absolute preference for shallow and deep investigation, respectively, and 0 indicates equal preference for deep and shallow investigation. (**D**) Expression of AAV-ChR2-mCherry in ZIm of a GAD2-Cre mouse and scheme of location of the optic fibers (dashed lines). (Bottom) An example in vivo recording of a ZIm neuron with optogenetic light off and on. (**E**) Stacked bar graphs show average duration of actions taken by control mice with tdTomato (n = 27 tests from four mice, two males) and mice with ChR2-mCherry (n = 42 tests from seven mice, four males) in 2-min FADC tests with familiar and novel objects. Bar graphs show the investigation duration. (**F**) Probability histogram and bar graph of DSP index of mice in (E) in interaction with novel objects. (**G**) Schematics of a FADC test with familiar food and a novel object. The stacked bar graph and the bar graph show duration of the actions and duration of investigation with photoactivation of ZIm^{GAD2} neurons in a 2-min test (n = 16 tests from seven mice, four males). (**H**) Schematics of a FADC with familiar cricket and novel moving object. The stacked bar graph and the bar graph show duration of the actions and duration of investigation with photoactivation of ZIm^{GAD2} neurons in a 2-min test (n = 16 tests from seven mice, four males). (**I**) Example of expression of AAV-stGtACR2-FusionRed in ZIm of a GAD2-Cre mouse and scheme of location of the optic fibers (dashed lines). (Bottom) An example in vivo recording of a ZIm neuron with optogenetic light off and on. (**J**) Stacked bar graphs show average duration of actions taken by control mice with tdTomato (n = 32 tests from five mice, three males) and mice with stGtACR2-FusionRed (n = 29 tests from seven mice, four males) in 10-min FADC tests with familiar and novel objects. Bar graphs show the investigation duration. (**K**) Probability histogram and bar graph of DSP index of mice in (J) in interaction with novel objects. n.s.: not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

trigger hunting behavior in mice, we used a FADC test with one novel static object and one familiarized moving object and a FADC with one familiarized immobile cricket (dead). Activation of the ZIm^{GAD2} neurons in both tests showed an increase in the novel object interaction (fig. S4, B and C), which again shows that the underlying motivation is to investigate and not to hunt. However, in line with results of a previous study (*11*), activation of the inhibitory neurons in the rostral part of the ZI in a FADC test with one familiarized food and one novel object showed an increase in interaction with the food (binge-like eating) compared with the object (fig. S5).

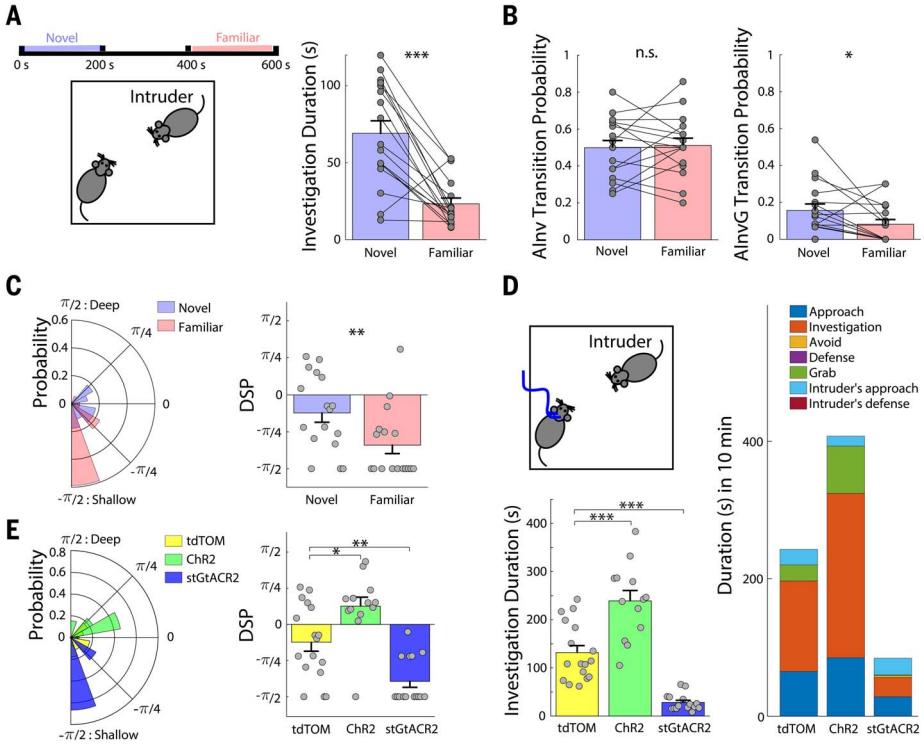

Fig. 2. ZIm plays a central role in social investigation. (**A**) Schematics of a 10-min social interaction test, in which the first third and the last third of the test are considered novel and familiar periods, respectively. Bar graph shows duration of investigation taken by control tdTomato mice in the familiar and novel periods (n = 17 tests from eight mice, four males). (**B**) Left and right bar graphs show transition from approach to investigation event without grab (AInv) and transition from approach to investigation event with grab (AInvG) in (A), respectively, in the familiar and novel periods. (**C**) Probability histogram and bar graph of DSP index of mice in (A) in familiar and novel periods. (**D**) Schematics shows optogenetic social interaction test. Bar graph shows investigation duration of tdTom (n = 17 tests from eight mice, four males), ChR2 (n = 13 tests from five mice, three males), and stGtACR2 (n = 15 tests from six mice, three males) mice in the social interaction test. Stacked bar graph shows duration for each action (approach, investigation, avoid, defense, and grab of the resident mouse; intruder's approach; and intruder's defense) taken by the tdTom, ChR2, and stGtACR2 mice. (**E**) Probability histogram and bar graph of DSP index of mice in (D) in the novel period. n.s., not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

To see whether the ZIm inhibitory neurons are essential in object-investigation behavior, we used an AAV virus to express stGtACR2 to suppress the ZIm^{GAD2} neurons (Fig. 1I and fig. S6A) in a 10-min FADC test with a familiar and a novel object. Suppression of the ZIm^{GAD2} neurons showed no significant change in interactions with the familiar objects (Fig. 1J and movie S6). However, in interactions with the novel objects, the number of approaches and duration of sniffing, which is a part of both deep and shallow investigation, stayed unchanged, whereas there was a significant decrease in duration of the investigatory actions that are involved in deep investigation (bite, grab, and carry) (Fig. 1J and fig. S6B). Furthermore, optogenetic deactivation of the ZIm^{GAD2} neurons decreased the transition probability from sniff to bite when the object was novel (fig. S6C) and, compared with the tdTOM control mice, showed a lower DSP in the novel-object investigation (Fig. 1K). Deactivation of ZIm^{GAD2} neurons in a familiar open field arena did not cause a significant change in mobility (fig. S6D). Chemogenetically silencing ZIm^{GAD2} neurons [by expressing hM4Di in ZIm^{GAD2} and injecting clozapine N-oxide (CNO) locally in ZIm] showed the same results: reduction of the investigation duration and the DSP (fig. S6, E and F).

GABAergic neurons in ZIm have a major role in social investigation

We next asked whether ZIm's role in investigation is specific to objects or if it generalizes to conspecifics, in which the actions are different from the actions taken in object investigation. To answer this question, first we used tdTOM control mice in a social investigation test, in which we introduced a new conspecific (intruder) (Fig. 2A). The first and the last third period of the test were considered as novel and familiar periods, respectively (Fig. 2A). The significant reduction of the investigation duration in the familiar period compared with the novel period (Fig. 2A and fig. S7A) supports the reduction of novelty in the familiar period. An HMM analysis showed two states of investigatory behavior: (i) approach and investigation without grab and (ii) approach and investigation with grab (fig. S8A). We calculated the transition probability of approach to investigation without grab (AInv) and approach to investigation with grab (AInvG). The AInvG transition probability showed a significant reduction in the familiar period compared with the novel period, whereas the AInv transition probability did not show a significant difference. We categorized the social investigation sequences into shallow investigation (approach is continued by investigation without grab) and deep investigation (approach is continued by investigation with grab). In both cases, the investigatory event starts with approach and ends when no investigatory action (anogenital, facial, and body sniffing and grabbing) is taken anymore for at least 100 ms (fig. S8B). As before, we introduced the DSP using the relative time a mouse carries out deep investigation compared with the time spent in shallow investigation. This depth of investigation was much higher in the novel period than in the familiar period (Fig. 2C).

Next, we used AAV virus with ChR2 and stGtACR2 to activate and deactivate ZIm^{GAD2} neurons during the social investigation test. Compared with the tdTOM control mice, activation of the ZIm^{GAD2} neurons in a social investigation test showed a substantial increase in duration of the investigatory interaction with the intruder conspecifics—including the approach-chase, anogenital-body-facial investigation—and grabbing and did not induce any aggressive behavior or biting (Fig. 2D; fig. S7, B and C; and movie S7). Conversely, deactivation of the ZIm^{GAD2} neurons in the social investigation test showed a significant decrease in duration of the investigatory interaction with the intruder conspecific (Fig. 2D and fig. S7, B and C). The DSP in the novel period showed the same results as those in the novel-object investigation (Fig. 2E). Chemogenetically silencing ZIm^{GAD2} neurons (by expressing hM4Di in ZIm^{GAD2} and injecting CNO locally in ZIm) showed the same results: reduction of the investigation duration and the DSP (fig. S7, D, E, and F).

ZIm is active during investigation and in high arousal state

To examine whether inhibitory neurons in the ZIm are naturally active during investigatory behavior, we virally expressed GCaMP6s in the ZIm^{GAD2} neurons and recorded calcium photometry signal from freely moving mice during

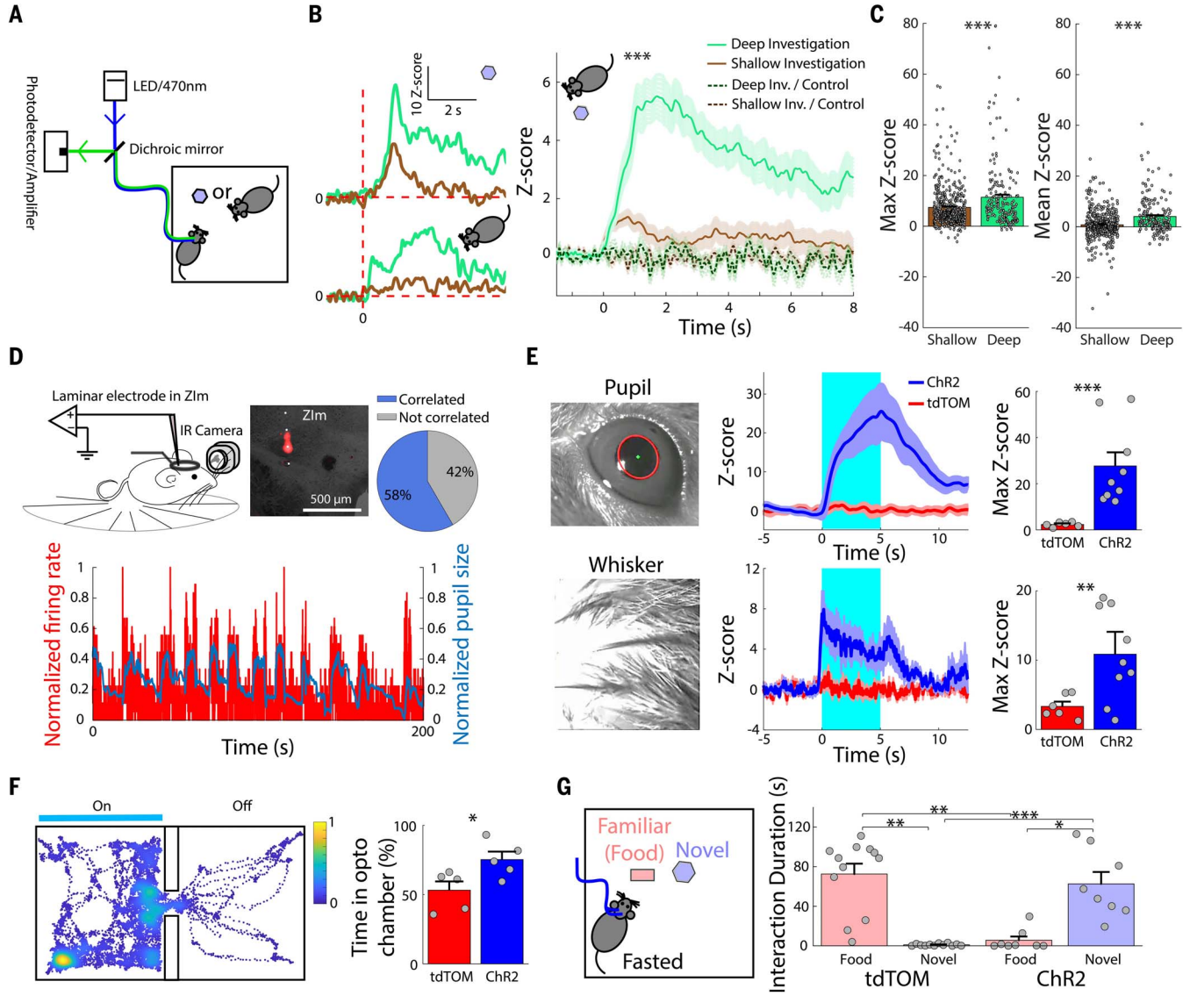

Fig. 3. ZIm is active during investigation and in high arousal state. (**A**) Schematics of calcium photometry during social or object interaction. (**B**) (Left) Example calcium photometry signals of AAV-GCaMP6s expressing ZIm^{GAD2} neurons during deep (green) and shallow (brown) object investigation (top) and social investigation (bottom). (Right) Calcium photometry signals of deep (n = 191 events) and shallow (n = 507 events) investigation averaged over all object and social investigation events (eight mice, four males). Signals of control mice with green fluorescent protein (GFP) expression in ZIm are indicated with dashed lines (n = 58 shallow and 91 deep investigation events from three mice, two males). Time 0 s indicates start of the investigation events: start of sniffing for object investigation and start of approaching the intruder conspecific for social investigation. Dark and the surrounding light colors indicate mean ± SEM. (**C**) Bar graphs show (left) maximum and (right) mean z scores of signals in (B). (**D**) Schematics of extracellular recording of ZIm units with laminar probe and pupil video capturing in awake head-fixed mice. DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and dashed line show trace of electrode in an example recording from ZIm. Pie diagram shows percentages of the recorded ZIm units that are and are not significantly correlated with the pupil size (n = 173 units from five mice, three males). (Bottom) Normalized pupil size (blue) and normalized firing rate (red) of an example ZIm unit correlated with the pupil size. (**E**) (Middle) z score and (right) maximum z score of (top) pupil size and (bottom) whisker activity of tdTom (red; n = 6 mice, three males) and ChR2 (blue; n = 9 mice, five males) mice with photo stimulation from 0 to 5 s. (**F**) An example heatmap of the track of a ChR2 mouse in a real-time place preference-aversion (RTPPA) test. Bar graph shows duration of time that control tdTom (n = 5 mice, three males) and ChR2 (n = 5 mice, three males) mice spent in the opto-linked chamber in the RTPPA test. (**G**) Schematics of a fasted mouse in a 2-min FADC test with familiar food and novel object. Bar graph shows duration of time that fasted tdTom (n = 12 tests from four mice, three males) and fasted ChR2 (n = 8 tests from six mice, four males) mice interact with the familiar food and the novel object. *P < 0.05, **P < 0.01, ***P < 0.001.

object and social investigation (Fig. 3A). In line with the optogenetic results, the calcium photometry showed a significant activity of ZIm^{GAD2} neurons during both deep (P < 0.0001) and shallow (P < 0.0001) investigations and a dramatic increase during deep investigation compared with the shallow investigation (Fig. 3, B and C, and fig. S9, A and B). While in interaction with the food, the same set of actions as the ones in deep investigation, ZIm^{GAD2} neurons show much less activity than during deep investigation (fig. S9C).

It is known that investigatory behavior requires a high arousal level (*15*, *16*), which was confirmed with our pupil measurements by

using head-mounted cameras in freely moving mice (fig. S10). Therefore, we asked whether activity of ZIm neurons is correlated with the arousal level. We recorded from ZIm units in head-fixed mice using a laminar multichannel electrode during spontaneous arousal changes, which were quantified by the changes in pupil size and whisking. Of units in the ZIm, 58% were significantly correlated with the arousal level (Fig. 3D). We optogenetically activated ZIm^{GAD2} neurons in head-fixed mice and video recorded the pupil and whiskers. These measures revealed that activation of the ZIm^{GAD2} neurons increases the arousal level (Fig. 3E). Because curiosity-driven investigation has previously been associated with reward anticipation and positive valence (*17*), we examined whether the increased arousal level coincides with a positive or negative valence. We used a real-time place preference-avoidance test in a double chamber, where one of the chambers is linked to the optogenetic light (light-chamber). Compared with the tdTOM control mice, activation of ZIm^{GAD2} neurons caused an increase in time spent in the light-chamber (Fig. 3F). This result was confirmed by the increase of the number of returns to the nose poke linked to the photoactivation of the ZIm^{GAD2} neurons in a self-stimulation task (fig. S11).

To examine whether the activation of the ZIm^{GAD2} neurons led to a nonspecific and general increase in positive arousal and motivation, or if it specifically induced investigatory behavior, we fasted the mice for 24 hours to induce a strong preference for food-eating (familiar food) compared with the novel object investigation (in a FADC test). The control mice (fasted tdTOM mice) showed a strong preference for the food (Fig. 3G), whereas activation of ZIm^{GAD2} neurons in the fasted mice dramatically increased the novel object investigation and did not affect the food-eating behavior.

Suppressing prelimbic cortex to ZIm pathway reduces investigatory behavior

Next, we sought the upstream brain areas to the ZIm involved in the investigatory behavior. We injected a monosynaptic Rabies virus (with a previously injected Cre-dependent helper virus) in the ZIm of GAD2-Cre–positive mice. Microscopy revealed several brain areas projecting to the ZIm^{GAD2} neurons, among which prelimbic cortex (PL) (Fig. 4A) is well established in playing a key role in investigatory behavior (*18*, *19*).

Calcium photometry showed that PL→ZIm axons were active during investigation (deep, $P < 0.0001$; shallow, $P < 0.0001$), but there was no significant difference in their activity during deep and shallow investigations (Fig. 4, B and C). Moreover, electrophysiological recordings from PL of head-fixed mice showed that activity of 74% of PL units was significantly correlated with the arousal level (Fig. 4D). This raised the question whether the increase in arousal level that we had seen through activation of the ZIm^{GAD2} neurons could be inherited from the PL. To answer this, we first injected AAV-ChR2 in PL of C57BL/6 mice under control of a CaMKII excitatory promoter (Fig. 4E and fig. S12, A and B). Photo stimulation of the PL→ZIm axons caused an increase in firing rate of ZIm units (Fig. 4E) and a significant increase in pupil size and whisking (Fig. 4F).

Then we asked whether the direct projection from PL→ZIm is essential for the investigatory behavior. We expressed hM4Di in PL (and tdTomato as control), and through local injection of CNO in ZIm, we deactivated the PL→ZIm axons in the FADC object investigation and the social investigation tests. This deactivation of PL→ZIm axons significantly reduced the depth of investigation and suppressed object investigation (Fig. 4G) and social investigation (Fig. 4H). In vivo electrophysiology confirmed the high efficacy of the local injection of CNO in suppressing the PL input into the ZIm (Fig. 4I).

These results imply that the PL input into the ZIm contains a motivational signal, which is essential for investigation. At this processing stage, the shallow and deep investigations are not differentiated yet by the size of the signal.

ZIm-PAG projection plays a key role in investigation

To determine the investigatory pathway downstream from the ZIm, we first injected a Cre-dependent AAV tdTOM virus in the ZIm of GAD2-Cre–positive mice and found projections of the ZIm^{GAD2} neurons to several downstream brain areas, including the mesencephalic locomotor region (MLR), pontine reticular formation (PnO), and periaqueductal gray (PAG) (the lateral divisions; lPAG). Using optogenetics and multichannel extracellular recording in head-fixed mice, we examined to what extent activation of the ZIm^{GAD2} neurons affects the neuronal activity in these brain areas. We observed a significant decrease and increase of activity in portions of units in MLR, PnO, and lPAG, with the highest effect on suppressing lPAG units (Fig. 5A). To find out in which of these brain areas the inhibitory ZIm projection plays a role in the investigatory behavior, we virally expressed ChR2 in ZIm^{GAD2} neurons and optogenetically activated the axon terminals from ZIm into MLR, PnO, and lPAG in the FADC object investigation and the social investigation tests. The behavioral results revealed that activation of the ZIm→lPAG projection significantly increased both novel-object investigation (Fig. 5B) and social investigation (Fig. 5C) (compared with the control tdTOM mice) and that activation of the ZIm→MLR and ZIm→PnO did not have an equally strong effect. Activating the ZIm→lPAG axons also increased the depth of investigation (fig. S13). Calcium photometry from GCaMP6s expressed in ZIm→lPAG inhibitory axons showed that these axons were active during investigation. However, they were active only during deep investigation ($P = 0.0014$) and not significantly active during shallow investigation ($P = 0.3876$) (Fig. 5, D and E). Moreover, in line with our results from activation of the ZIm^{GAD2} neurons, the activation of the ZIm→lPAG (but not ZIm→MLR and ZIm→PnO) inhibitory projection significantly increased the arousal level (fig. S14). To further understand whether this projection is essential for the investigatory behavior, we first virally expressed hM4Di (and tdTomato as control) in the ZIm^{GAD2} neurons. Then, we injected CNO directly into the lPAG to deactivate the ZIm→lPAG inhibitory projection, and 30 min later, mice went through the FADC object investigation and social investigation tests. The deactivation of the ZIm→lPAG inhibitory axons significantly reduced the depth and duration of the object investigation (Fig. 5F) and social investigation (Fig. 5G).

ZIm inhibitory neurons expressing TAC1 are important for investigation

Diversity of the inhibitory subpopulations in the ZI is one of the reasons underlying the functional diversity of the ZI (*7*, *10*, *20*, *21*). Therefore, we sought to identify the inhibitory subpopulations of ZIm and examined their relevance in the investigatory behavior. Because tachykinin 1 (TAC1) in some thalamic regions [for example, in the areas where GABAergic neurons originate from the same lineage cells as the ZI: the thalamic reticular nucleus and lateral geniculate nucleus (*22*)] is exclusively expressed in inhibitory neurons (*23*) (https://portal.brain-map.org), we examined whether TAC1 is also expressed in ZIm inhibitory neurons. Using double fluorescence in situ hybridization (FISH), we found that the vast majority (92%) of TAC1$^+$ neurons in ZIm are inhibitory, and they make up ~11.5% of the inhibitory (VGAT$^+$) neurons (Fig. 6, A and B). Furthermore, multiple FISH showed that the TAC1$^+$ population is separate from the previously identified Somatostatin-positive (SST$^+$) and Parvalbumin-positive (PV$^+$) neuronal populations in the ZIm with less than 2% overlap (Fig. 6, C and D). This result was confirmed with immunohistochemistry experiments (fig. S15C). TAC1$^+$ neurons are more numerous in the medial part of the ZI than in the rostral and caudal parts (fig. S15, A and B).

Next, we optogenetically activated these three inhibitory subpopulations to see which inhibitory cell type in the ZIm is involved in the investigatory behavior. Activation of PV$^+$ neurons and SST$^+$ neurons during the FADC object investigation and social investigation tests did not cause a significant change in the investigatory behavior (Fig. 6, E and F).

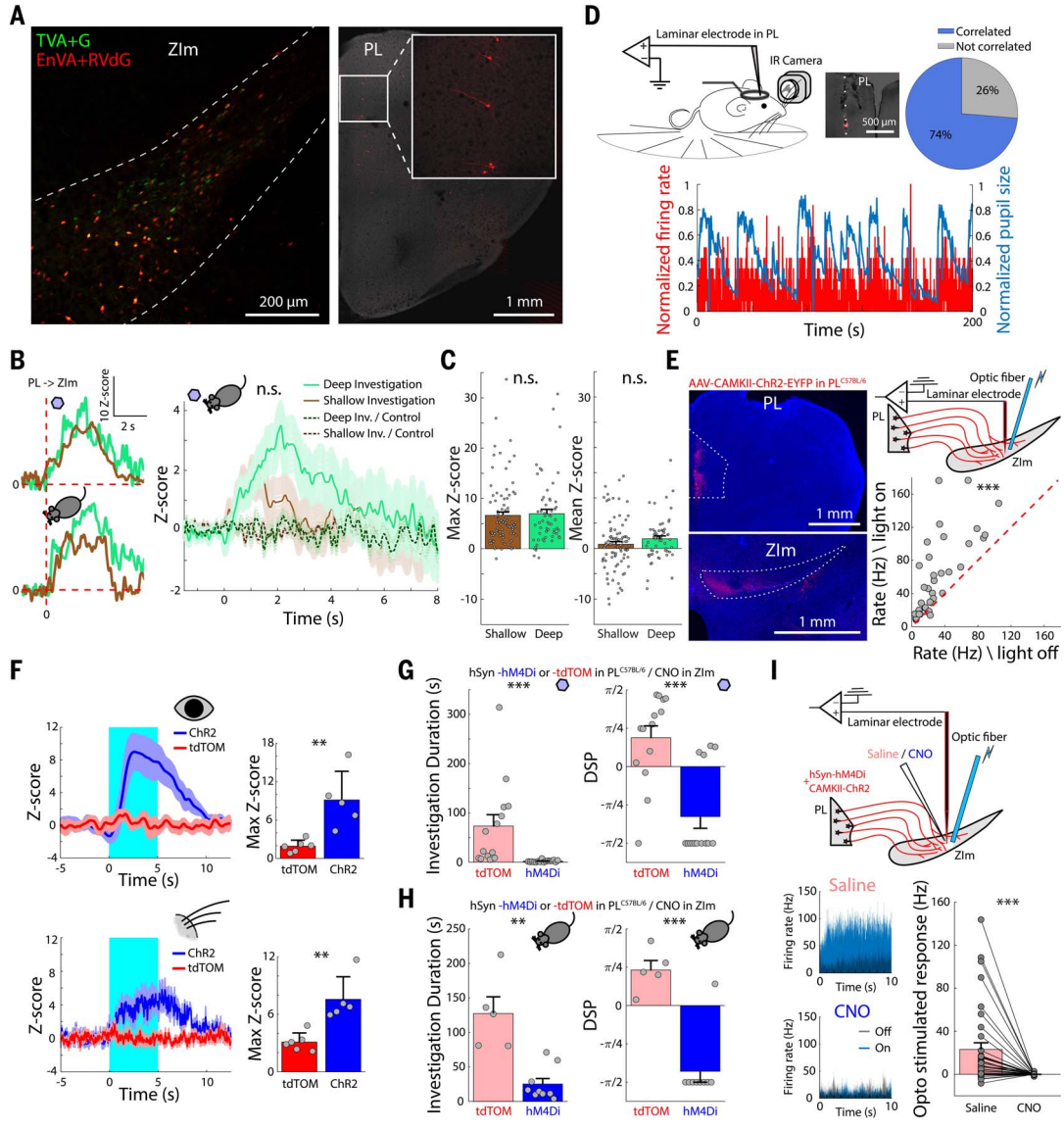

Fig. 4. Prelimbic cortex to ZIm pathway is a key factor in investigatory behavior.
(**A**) (Left) Retrograde mapping of presynaptic neurons to ZIm^{GAD2} neurons with TVA (GFP) and RVdg (tdTomato). TVA, the avian tumor virus receptor A; G, glycoprotein; EnVA, avian envelope; RVdg, glycoprotein-deleted rabies virus. (Right) Expression of RVdg in ZIm-projecting PL neurons. (**B**) (Left) Calcium photometry signals of PL→ZIm axons during (green) deep and (brown) shallow (top) object investigation and (bottom) social investigation. (Right) Calcium photometry signals of deep (n = 57 events) and shallow (n = 90 events) investigation averaged over all object and social investigation events (three mice, two males). Signals of control mice with GFP expression in ZIm are indicated with dashed lines. Time 0 s indicates start of the investigation events: start of sniffing for object investigation and start of approaching the intruder conspecific for social investigation. Dark and the surrounding light colors represent mean ± SEM. (**C**) Bar graphs show (left) maximum and (right) mean z scores of signals in (B). (**D**) Schematics of extracellular recording of PL units with laminar probe and pupil video capturing in awake head-fixed mice. DiI and dashed line show trace of electrode in an example PL recording. Pie diagram shows percentages of the recorded PL units that are and are not significantly correlated with the pupil size (n = 19 units from three mice, two males). (Bottom) A normalized pupil size (blue) and normalized firing rate (red) of an example PL unit correlated with the pupil size. (**E**) (Left) Expression of AAV-CAMKII-ChR2–enhanced yellow fluorescent protein (EYFP) in PL of a C57BL/6 mouse and (middle) the projections to ZIm. (Top right) Schematic of in vivo extracellular recording from ZIm while photostimulating the PL→ZIm axons and (bottom right) scatter plot of firing rate (Hz) of the ZIm neurons with the optogenetic light above ZIm being on versus off. (**F**) (Left) zscore and (right) maximum z score of (top) pupil size and (bottom) whisker activity of tdTom (red; n = six mice, three males) and ChR2 (blue; n = five mice, three males) mice. PL→ZIm axons are photo stimulated from 0 to 5 s. (**G**) Bar graphs show (left) novel-object investigation duration and (right) DSP of mice expressing tdTOM (n = 14 tests from five mice, two males) or hM4Di (n = 16 tests from five mice, two males) in PL while injecting CNO locally in ZIm. (**H**) Bar graphs show (left) social investigation duration and (right) DSP in the novel period in mice expressing tdTOM (n = 5 tests from five mice, two males) or hM4Di (n = 9 tests from five mice, two males) in PL while injecting CNO locally in ZIm. (**I**) (Top) Schematic of in vivo extracellular recording from ZIm while photostimulating the PL→ZIm axons and chemogenetically silencing them through local injection of CNO (top). (Bottom left) Firing rate of an example ZIm neuron in response to photo stimulation of PL→ZIm axons in presence of saline and CNO. (Bottom right) Bar graph represents responses of ZIm neurons to photostimulation of PL→ZIm axons in presence of saline and CNO (n = 35 units from three mice). n.s., not significant, *P <0.05, **P <0.01, ***P <0.001.

Although we cannot rule out an effect for PV and SST neurons because of the low sample size, activation of the TAC1$^+$ neurons was different and clearly increased both the object and the social investigation (Fig. 6, E and F). Activation of the TAC1$^+$ neurons increased the DSP (Fig. 6G and fig. S16), and the level of increase in the investigatory behavior by activating the TAC1$^+$ neurons was not different from that induced by activating the GAD2$^+$ neurons (fig. S17). Moreover, activation of the TAC1$^+$ neurons increased the arousal level to the same level that activation of GAD2$^+$ neurons did (fig. S18).

Next, by optogenetically deactivating the ZIm^{TAC1} neurons during the FADC object investigation and social investigation tests, we examined whether the ZIm^{TAC1} neurons are essential for the investigatory behavior. Deactivation of the ZIm^{TAC1} neurons suppressed the investigatory behavior (Fig. 6H

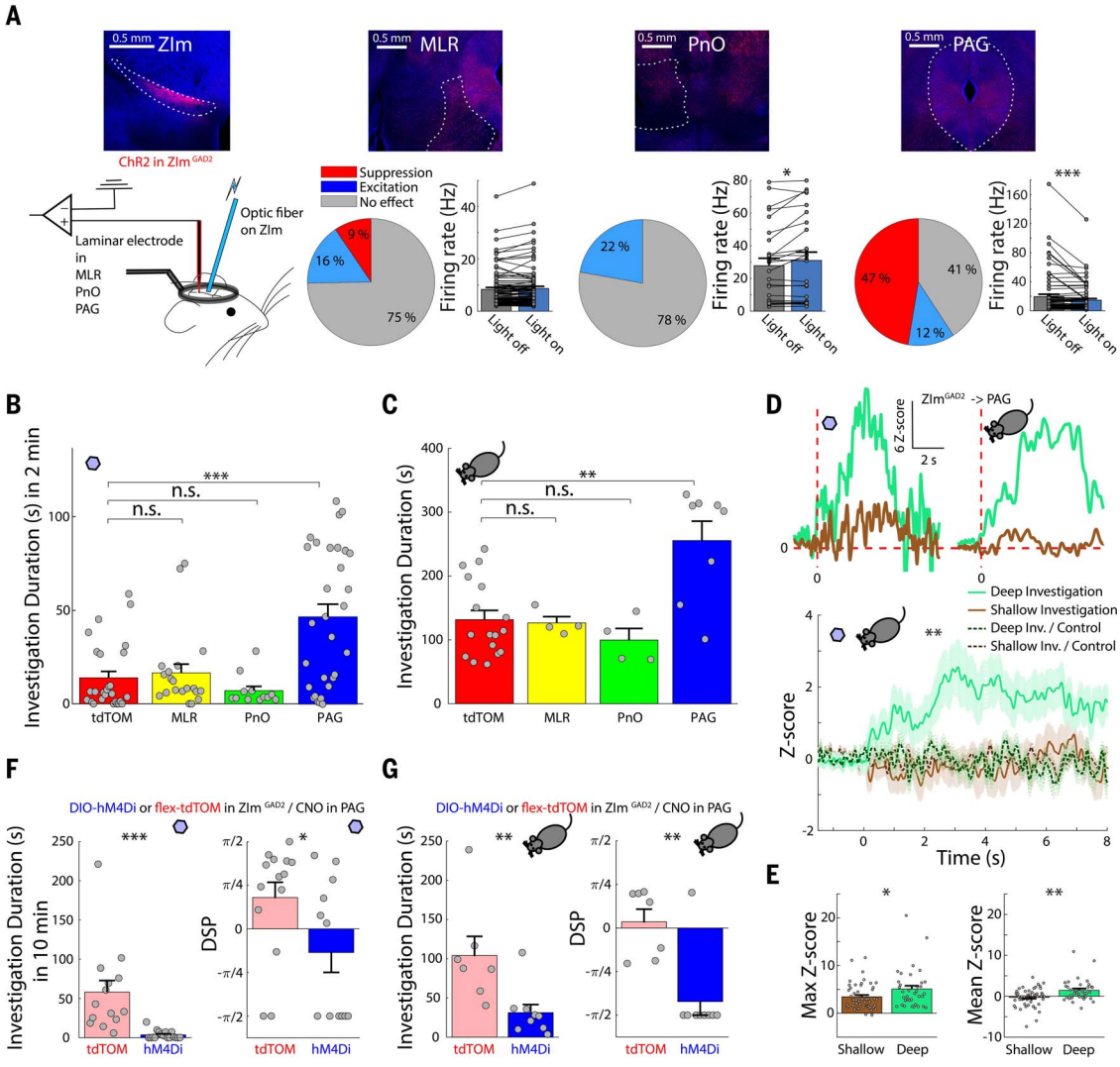

Fig. 5. ZIm→PAG projection plays a key role in investigation. (**A**) Expression of AAV-ChR2-mCherry in ZIm^{GAD2} neurons and axons in MLR, PnO, and PAG (top; left to right, respectively). The schematics represent the in vivo experiment of extracellularly recording from ZIm projection targets (MLR, PnO, and PAG) while photostimulating the ZIm^{GAD2}. Pie diagrams show percentage of units in MLR, PnO, and PAG (left to right, respectively) that are (red) significantly suppressed, (blue) excited, or (gray) not changed by photo stimulation of the ZIm^{GAD2}. Bar graphs show firing rate (hertz) of units in MLR (n = 95 units from four mice, three males), PnO (n = 27 units from two mice, two males), and PAG (n = 76 units from four mice, two males) (left to right, respectively) when photostimulation light above the ZIm^{GAD2} is off or on. (**B**) Bar graph shows the duration of novel-object investigation in control mice with tdTomato (n = 27 tests from four mice, two males) and mice with ChR2 photo stimulation of ZIm^{GAD2}→MLR (n = 19 tests from three mice, two males), ZIm^{GAD2}→PnO (n = 12 tests from three mice, two males) and ZIm^{GAD2}→PAG (n = 30 tests from 5 mice, 3 males) in 2-min FADC tests with familiar and novel objects. (**C**) Bar graph shows the duration of social investigation in control mice with tdTomato (n = 17 tests from eight mice, four males) and mice with ChR2 photo stimulation of ZIm^{GAD2}→MLR (n = 4 tests from three mice, two males), ZIm^{GAD2}→PnO (n = 4 tests from three mice, two males), and ZIm^{GAD2}→PAG (n = 8 tests from five mice, three males). (**D**) (Top) example calcium photometry signals of ZIm^{GAD2}→PAG axons during (green) deep and (brown) shallow (left) object investigation and (right) social investigation. (Bottom) Calcium photometry signals of deep (n = 36 events) and shallow (n = 50 events) investigation averaged over all object and social investigation events (three mice, one male). Signals of control mice with GFP expression in ZIm are indicated with dashed lines. Time 0 s indicates the start of the investigation events: start of sniffing for object investigation and start of approaching the intruder conspecific for social investigation. Dark and the surrounding light colors indicate mean ± SEM. (**E**) Bar graphs show (left) maximum and (right) mean z scores of signals in (D). (**F**) Bar graphs show (left) novel object investigation duration and (right) DSP after injecting CNO locally in PAG of mice expressing tdTOM (n = 14 tests from fvie mice, three males) or hM4Di (n = 17 tests from five mice, three males) in ZIm^{GAD2}. (**G**) Bar graphs show (left) social investigation duration and (right) DSP in the novel period after injecting CNO locally in PAG of mice expressing tdTOM (n = 7 tests from five mice, three males) or hM4Di (n = 9 tests from five mice, three males) in ZIm^{GAD2}. n.s., not significant, *P < 0.05, **P < 0.01, ***P < 0.001.

and fig. S13, A and B). Moreover, retrograde AAV injections in the lPAG (and anterograde AAV injections in the ZIm) and Rabies injections in the ZIm of TAC1-Cre–positive mice, respectively, showed that ZIm^{TAC1} neurons project to the lPAG (Fig. 6J and fig. S15E) and receive direct input from the PL (Fig. 6K and fig. S15D for other inputs), which may explain our behavioral results.

Together, our data demonstrate a brain circuit for driving and gating investigatory motivation and novelty-seeking behavior. We showed that using a simple approach of FADC, we can distinguish investigatory behavior from food-eating and hunting, providing us with a powerful strategy to study brain circuits that underlie investigatory behavior. Using this strategy—together with optogenetics, chemogenetics, and calcium fiber photometry—we showed that increasing the ZIm activity increases the motivation to investigate. Cortical excitatory input from PL into ZIm conveys nonspecific motivation and arousal level to investigate. Extra information (such as sensory inputs from midbrain) and processing selectively multiplies the resulting activity of ZIm^{GAD2} neurons. Next, a thresholding mechanism operates on a subpopulation of ZIm^{GAD2} neurons (likely to be ZIm^{TAC1} neurons) in such a way that only high ZIm activity causes an inhibitory signal to the PAG, leading to deep investigation (fig. S19). Although the inhibition of PAG can lead to action through disinhibition

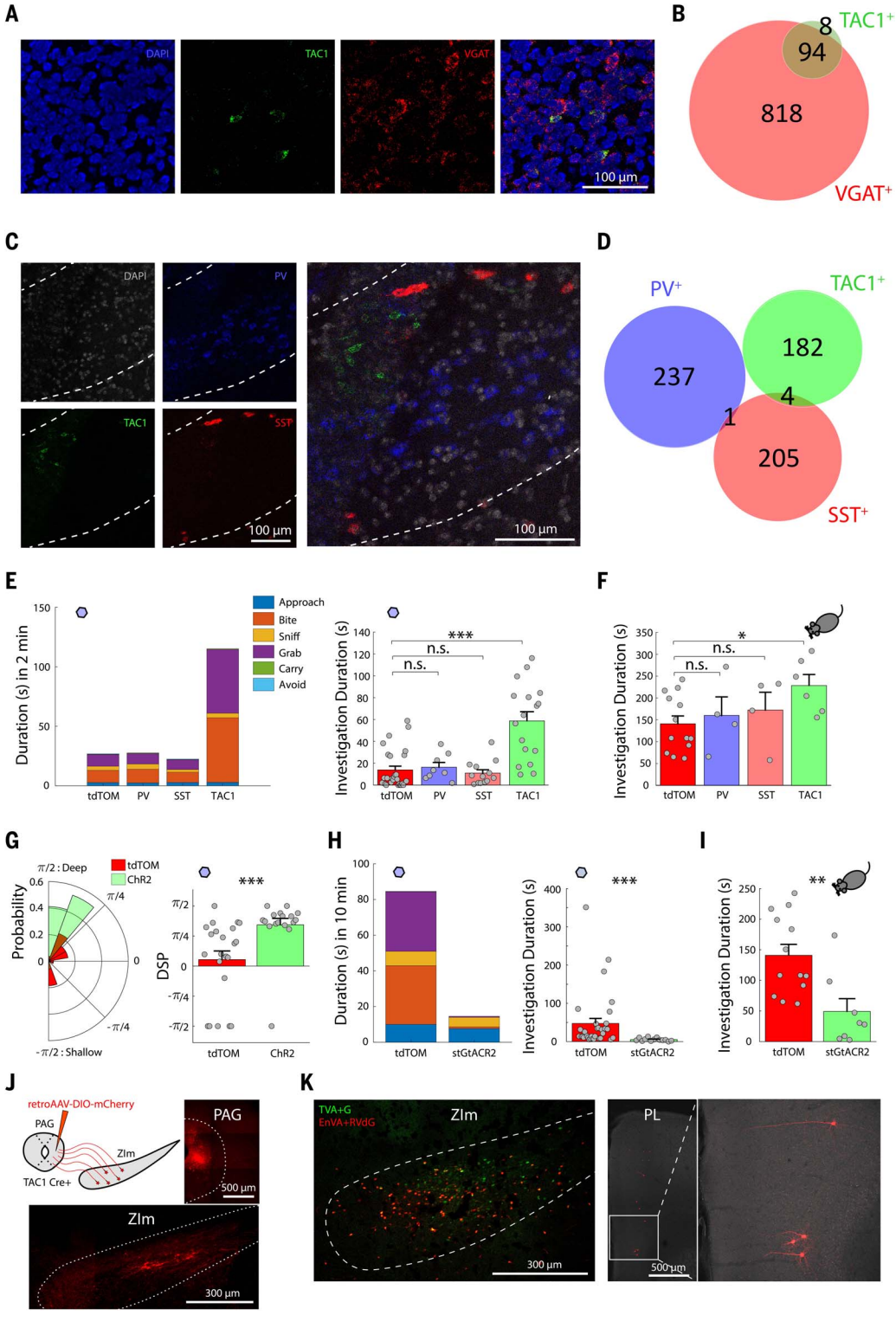

Fig. 6. The small subpopulation of ZIm inhibitory neurons expressing TAC1 is important for investigation. (**A**) Example of a double-color in situ mRNA hybridization in ZIm. DAPI (4′,6-diamidino-2-phenylindole) is shown in blue, and TAC1$^+$ and VGAT$^+$ cells are shown in green and red, respectively. (Right) The overlap between the three colors. (**B**) Venn diagram represents number of TAC1$^+$, VGAT$^+$, and TAC1$^+$/VGAT$^+$ cells in ZIm (from eight slices). (**C**) Example of a triple-color in situ mRNA hybridization in ZIm. DAPI is shown in gray, and PV$^+$, SST$^+$, and TAC1$^+$ cells are shown in blue, red, and green, respectively. (Right) The overlap between the four colors. (**D**) Venn diagram of ZIm cells expressing PV, SST, and TAC1 (from eight slices). (**E**) Stacked bar graphs show average duration of actions taken by control mice with tdTomato (n = 27 tests from four mice, two males) and mice with ChR2-mCherry expression in ZIm in PV$^+$ neurons (n = 8 tests from three mice, two males), in SST$^+$ neurons (n = 13 tests from three mice, two males), and in TAC1$^+$ neurons (n = 17 tests from five mice, three males) in 2-min FADC tests with familiar and novel objects. Bar graphs represent duration of the novel-object investigation. (**F**) Bar graphs represent duration of social investigation in control mice with tdTomato (n = 13 tests from four mice, two males), and mice with ChR2-mCherry expression in ZIm in PV$^+$ neurons (n = 4 tests from three mice, two males), in SST$^+$ neurons (n = 4 tests from three mice, two males), and in TAC1$^+$ neurons (n = 6 tests from five mice, three males) in social investigation test. (**G**) Probability histogram and bar graph of DSP index of control and TAC1-Cre mice in (E) in interaction with novel objects. (**H**) Stacked bar graph shows average duration of actions taken by control mice with tdTomato (n = 32 tests from five mice, three males) and mice with stGtACR2-FusionRed expression in ZIm^{TAC1} (n = 13 tests from five mice, three males) in 10-min FADC tests with familiar and novel objects. Bar graph shows duration of the novel object investigation. (**I**) Bar graph represents duration of social investigation in control mice with tdTomato (n = 13 tests from four mice, two males) and mice with stGtACR2-FusionRed expression in ZIm^{TAC1} (n = 8 tests from five mice, three males). (**J**) Schematic of a retrograde tracing experiment with injection of retroAAV-EYFP is PAG of TAC1-Cre mice (n = 2 mice, 2 males) and examples of the EYFP expression in the injection site (PAG) and in the PAG-projecting ZIm^{TAC1} neurons. (**K**) (Left) Rabies monosynaptic retrograde tracing experiment shows the expression of TVA and RVdG in neurons at the injection site (ZIm) in a TAC1-Cre mouse (n = 2 mice, one male). (Right) Expression of RVdG in the PL neurons projecting to ZIm^{TAC1} neurons. n.s., not significant; *P < 0.05, **P < 0.01 and ***P < 0.001.

of defensive actions within the PAG (*5*, *24*), we argue that the increased exploration is not due to decreased fear because we found that activation of ZIm^{GAD2} increased arousal level and specifically increased deep investigation. However, how the sensory information and motivational signals in ZIm integrate to increase the investigatory motivation and initiate this deep investigation remains to be uncovered. Moreover, because dorsal and ventral subdivisions of ZIm differ in their connectivity and neurochemical composition (*25*, *26*), a further subdivision of function may be discovered.

Methods summary

Mice were habituated to the experimental box for several days. The object-investigation test was implemented by using a familiar and a novel object in a FADC manner; for social investigation, test mice were exposed to one novel conspecific, and the test period was split into the first third and the last third as novel and familiar periods for further analysis. Mice were either wild type with no stimulation or they were optogenetically or chemogenetically stimulated or inhibited during the tests (with the corresponding control groups). HMM and transition probability analyses of the labeled behaviors categorized the investigatory behaviors to shallow and deep investigations, and investigation duration and depth of investigation were calculated.

Optogenetic effects on arousal level were measured by pupil size and whisker activity. Anatomical and functional connectivity between ZIm and its inputs and outputs was studied by using anterograde and retrograde viruses and in vivo electrophysiology. Calcium activity of ZIm and its input (PL→ZIm axons) and output (ZIm→lPAG axons) was measured during object and social investigation by using fiber photometry. Immunohistochemistry and single-molecule mRNA multifluoresent in situ hybridization were performed to examine ZIm^{TAC1} neurons, a subpopulation of ZIm inhibitory neurons. Furthermore, we photoactivated and photoinhibited the ZIm^{TAC1} neurons during object and social investigation tests and measured the effects on investigation duration and depth of investigation.

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SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/372/6543/eabe9681/suppl/DC1
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View/request a protocol for this paper from *Bio-protocol*.

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Science

A cell type–specific cortico-subcortical brain circuit for investigatory and novelty-seeking behavior

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A brain circuit that drives and gates curiosity

Curiosity is what drives organisms to investigate each other and their environment. It is considered by many to be as intrinsic as hunger and thirst, but the neurobiological mechanisms behind curiosity have remained elusive. In mice, Ahmadlou *et al.* found that a specific population of genetically identified γ-aminobutyric acid (GABA)—ergic neurons in a brain region called the zona incerta receive excitatory input in the form of novelty and/or arousal information from the prelimbic cortex, and these neurons send inhibitory projections to the periaqueductal gray region (see the Perspective by Farahbakhsh and Siciliano). This circuitry is necessary for the exploration of new objects and conspecifics.

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