Fig. S1 The RV monosynaptic retrograde tracing strategy is reliable. A AAV9-EF1α-DIO-RG (1) and AAV9-EF1α-DIO-DsRed-TVA (2) is injected into wild-type mice, and three weeks later RV-SAD-
ΔG-eGFP (EnvA) (3) is injected into the same ZI sector. B eGFP-positive cells in the ZI are all near the injection site. This verifies that the monosynaptic retrograde tracing strategy is Cre-dependent. Scale bar, 500 μm. C AAV9-EF1α-DIO-DsRed -TVA (1) is injected into Vgat-Cre mice, and three weeks later RV-SAD-ΔG-eGFP (EnvA) (2) is injected into the same ZI sector. D eGFP- and DsRed-positive cells in ZI, and no eGFP-positive cells outside the injection site. This verifies that the monosynaptic retrograde tracing strategy is RG-dependent. Without RG, RV could not spread retrogradely to presynaptic neurons. Scale bar, 500 μm. E AAV9-EF1α-DIO-RG (1) is injected into Vgat-Cre mice, and three weeks later RV-SAD-ΔG-eGFP (EnvA) (2) is injected into the same ZI sector. F No eGFP- or DsRed-positive cells in the whole brain, verifying that the monosynaptic retrograde tracing strategy is TVA-dependent. Modified RV only infects TVA-positive neurons. Scale bar, 500 μm.
**Fig. S2** Workflow and location of starter cells. 

**A** Distribution of starter cells in different ZI sectors in the input strategy. Scale bars, 500 μm (inset) and 250 μm (others). 

**B** Workflow of viral tracing, whole-brain imaging, registration, and quantification. 

**C** Location of the starter cells in the ZI. 

**D** Proportions of labeled starter neurons in injection regions (100%, all detected starter cells). Scale bar, 100 μm. 

**E** Schematic of the top view of anterograde tracing starter cells in distinct ZI sectors and heat map showing the fluorescence intensity distribution in each sample at each specific ZI target (lower panels). The density and brightness of injection regions are too high to distinguish the cell bodies. Since the
fluorescence brightness of neuron cell bodies was much greater than that of efferent axons, the
distribution of fluorescence brightness was used to show the distribution of infected neurons in the ZI.
Center left insert, visual aid representation the location of the ZI. Every row in each ZI sector is from
one sample, $n = 3$ mice per condition in the anterograde strategy.
Fig. S3 Representative images of ZI sector inputs and outputs. **A** Continuous coronal view of ZI sector inputs. **B** Continuous coronal view of ZI sector outputs. Slice thickness, 100 μm. Scale bars, 1 mm.
Fig. S4 Brain-wide input datasets for ZIr, Zlim, Zil, and ZIc. Whole-brain input regions are divided into 126 subregions for comparison. Values are presented as normalized percentage of total cells (RV). Data shown as the mean ± SEM. n = 4 mice per condition.
**Fig. S5** Brain-wide output datasets for ZIr, ZIm, ZIil, and ZIc. Whole-brain output regions are divided into 77 subregions for comparison. Values are presented as normalized percentage of total pixels (AAV). Data shown as the mean ± SEM. *n* = 3 mice per condition.
Fig. S6 Characteristics of ZI connectivity. A Cumulative input percentage, in the order of largest to
smallest. B Cumulative output percentage, in the order of largest to smallest (horizontal axis, cumulative percentage; vertical axis, average value of input strength of a single region). C Ipsilateral and contralateral input ratios. D Ipsilateral and contralateral output ratios. Area of a circle indicates strength of connection. E Input p-value heat map between the four ZI sectors. F Output p-value heat map between the four ZI sectors. Input data, \( n = 4 \) mice per condition in A, C, and E. Output data, \( n = 3 \) mice per condition in B, D, and F. Colors indicate data from each ZI sector: ZIr, blue; Zlim, orange; ZIIl, cyan; ZIc, purple.

**Fig. S7** Characteristics of isocortex projection to ZI. A Ratio of the number of eGFP-positive neurons in ipsilateral isocortex to the number of eGFP-positive neurons in the whole isocortex (100% indicates all cortical eGFP-positive cells). B Isocortex input p-value heat map between the four ZI sectors. \( n = 4 \) mice per condition in A and B.
Fig. S8 Characteristics of ZI connectivity. A Hypothalamic input p-value heat map between the four ZI sectors. B Hypothalamic output p-value heat map between the four ZI sectors. C, D Schematic coronal sections depicting the distribution of the eGFP signal in the hypothalamus (eGFP signals represent the output of ZI sectors; lower curves, output strengths to the hypothalamus; bin width, 100
μm). E Thalamic output p-value heat map between the four ZI sectors. F Output from ZII center neuron to the dorsal PO. G, H Enlarged view of the area indicated by the rectangle in F. G distribution of fibers in the PO, H distribution of somata in ZI. I Outputs from ZIil neurons to the ventral PO. J, K enlarged view of the area indicated by the rectangle in I. J distribution of fibers in the PO, K distribution of somata in the ZI. n = 4 mice per condition in A. n = 3 mice per condition in B and E. Slice thickness, 100 μm in C, D, and F–K). Scale bars, 500 μm (F, H, I, and K) and 250 μm (G and J).
**Fig. S9** Characteristics of connections between ZI and midbrain. 

**A** Midbrain input p-value heat map between the four ZI sectors ($n = 3$ mice per condition). 

**B** Midbrain output p-value heat map between the four ZI sectors ($n = 4$ mice per condition).

**C** Coronal section showing the injection sites of retrobeads and FG. Scale bar, 500 μm.
Fig. S10 Comparison of connection patterns between ZI sectors. A Pattern between ZIr input and ZIim input. B Pattern between ZIil input and ZIc input. Horizontal and vertical axes represent the average values of the inputs in A and B. C Pattern between ZIr output and ZIim output. D Pattern between ZIil output and ZIc output. The color of circles represents the main regions to which these circles belong (see illustration below). Circles (○, \( P > 0.05 \)) indicate no significant difference between inputs and outputs, while concentric circles (⊙, \( 0.01 < P < 0.05 \)) and solid circles (●, \( P < 0.01 \)) indicate a statistical difference between inputs and outputs. Lower right, number of regions and the number of regions with
significant differences between ZI sectors. Points on the horizontal axis represent regions with very little output (<0.03%), and points on the vertical axis represent regions with very little input (<0.03%). The difference in outputs between ZIr and ZIm is larger than the inputs; similarly for ZIil and ZIc.

**Abbreviations**

| Abbreviation | Description                        |
|--------------|------------------------------------|
| AAA          | Anterior amygdala area             |
| ACA          | Anterior cingulate area            |
| ACB          | Nucleus accumbens                  |
| AD           | Anterodorsal nucleus               |
| AHN          | Anterior hypothalamic nucleus      |
| AI           | Agranular insular area             |
| AM           | Anteromedial nucleus               |
| APN          | Anterior pretectal nucleus         |
| ARH          | Arcuate hypothalamic nucleus       |
| AUD          | Auditory areas                     |
| AV           | thalamus                           |
| AVP          | nucleus                            |
| CBN          | Cerebellar nuclei                  |
| CEA          | Central amygdala nucleus           |
| CL           | thalamus                           |
| CM           | thalamus                           |
| CP           | Caudatoputamen                     |
| CS           | Superior central nucleus raphe     |
| CTXpl        | Cortical plate                     |
| MS           | Medial septal nucleus              |
| MV           | Medial vestibular nucleus          |
| NDB          | Nucleus of the brachium of the     |
| NB           | inferior colliculus                |
| NI           | Nucleus insertus                   |
| NLL          | Nucleus of the lateral lemniscus   |
| NOT          | Nucleus of the optic tract         |
| NPC          | comissure                          |
| NTB          | Nucleus of the trigeminal body     |
| OP           | Olivary pretectal nucleus          |
| ORB          | Orbital area                       |
| OT           | Olfactory tubercle                 |
| PAG          | Periaqueductal gray                |
| PB           | Parabrachial nucleus               |
| PCG          | Pontine central gray               |
| PCN          | Paracentral nucleus                |
| PF           | Parafascicular nucleus             |
| PG           | Pontine gray                       |
| PH           | Posterior hypothalamic nucleus     |
| PL           | Prelimbic area                     |
| PM           | Premammillary nucleus              |
| Abbreviation | Full Name                                      | Abbreviation | Full Name                                      |
|--------------|-----------------------------------------------|--------------|-----------------------------------------------|
| CTXsp        | Cortical subplate                             | PO           | Posterior complex of the thalamus             |
|              |                                               |              | Posterior limiting nucleus of the thalamus    |
| CUN          | Cuneiform nucleus                             | POL          | Thalamus                                      |
|              | Dorsomedial nucleus of the hypothalamus       |              |                                               |
| DMH          | Hypothalamus                                   | POST         | Postsubiculum                                 |
| DN           | Dentate nucleus                                | PP           | Peripeduncular nucleus                        |
|              | Thalamus, polymodal association                |              |                                               |
| DORpm        | Cortex related                                | PPN          | Pedunculopontine nucleus                      |
|              | Thalamus, sensory-motor cortex                 |              |                                               |
| DORsm        | Related                                        | PPT          | Posterior pretectal nucleus                   |
| DR           | Dorsal raphe nucleus                           | PRC          | Precommissural nucleus                        |
| ECT          | Ectothalamic area                              | PRN          | Pontine reticular nucleus                     |
| ENT          | Entothalamic area                              | PSTN         | Parasubthalamic nucleus                       |
|              |                                               |              | Principal sensory nucleus of the              |
|              |                                               |              |                                               |
| EP           | Endothalamic nucleus                           | PSV          | Trigeminal                                    |
| EW           | Edinger-Westphal nucleus                       | PT           | Parataenial nucleus                           |
| FN           | Fastigial nucleus                              | PTLp         | Posterior parietal association areas          |
|              | Globus pallidus, external                      |              | Paraventricular hypothalamic                  |
| GPe          | Segment                                        | PVH          | Periventricular hypothalamic nucleus          |
|              | Globus pallidus, internal                      |              |                                               |
| GPI          | Segment                                        | PVi          | Nucleus, intermediate part                    |
| GRN          | Gigantocellular reticular nucleus              | PVp          | Nucleus, posterior part                       |
|              |                                               |              | Periventricular hypothalamic                  |
| GU           | Gustatory area                                 | PVR          | Periventricular region                        |
|              |                                               |              | Paraventricular nucleus of the                |
| HPF          | Hippocampal formation                          | PVT          | Thalamus                                      |
|              | Interanterodorsal nucleus of the thalamus      |              |                                               |
| IAD          | Thalamus                                       | PVZ          | Periventricular zone                          |
|              | Interanteromedial nucleus of the thalamus      |              |                                               |
| IAM          | Thalamus                                       | RCH          | Retrochiasmatic area                          |
| IC           | Inferior colliculus                            | RE           | Nucleus reuniens                             |
| ILA          | Infrahillic area                               | RH           | Rhomboid nucleus                              |
| Acronym | Full Name                                      | Acronym | Full Name                                      |
|---------|-----------------------------------------------|---------|-----------------------------------------------|
| IMD     | Intermediodorsal nucleus of the thalamus      | RHP     | Retrohippocampal region                        |
| INC     | Interstitial nucleus of Cajal                 | RL      | Rostral linear nucleus raphe                  |
| IP      | Interposed nucleus                            | RM      | Nucleus raphe magnus                          |
| IRN     | Intermediate reticular nucleus                | RN      | Red nucleus                                   |
| LAT     | Lateral group of dorsal thalamus              | RR      | Retrorubral area                              |
| LAV     | Lateral vestibular nucleus                    | RSP     | Retrosplenial area                            |
| LC      | Locus ceruleus                                | RT      | Reticular nuclei of dorsal thalamus           |
| LD      | Lateral dorsal nucleus of the thalamus        | SBPV    | Subparaventricular zone                       |
| LDT     | Laterodorsal tegmental nucleus                | SCH     | Subrachiasmatic zone                          |
| LG      | Lateral geniculate complex                    | SCm     | Superior colliculus, motor related            |
| LHB     | Lateral habenula                              | SI      | Substantia innominata                         |
| LHA     | Lateral hypothalamic area                     | SMT     | Submedial nucleus of the thalamus             |
| LM      | Lateral mammillary nucleus                    | SNc     | Substantia nigra, pars compacta               |
| LS      | Lateral posterior nucleus of the thalamus     |        |                                               |
| LP      | Lateral posterior nucleus of the thalamus     | SNr     | Substantia nigra, pars reticulata             |
| LPO     | Lateral preoptic area                         | SPA     | Subparafascicular area                        |
| LS      | Lateral septal nucleus                        | SPF     | Subparafascicular nucleus                     |
| LT      | Lateral terminal nucleus of the accessory optic tract | SPVI | Spinal nucleus of the trigeminal, interpolar part |
| LZ      | Hypothalamus lateral zone                     | SSp     | Primary somatosensory areas                   |
| MA      | Magnocellular nucleus                         | SSS     | Supplemental somatosensory area               |
| MARN    | Magnocellular reticular nucleus               | STN     | Subthalamic nucleus                           |
| MBmot   | Midbrain, motor related                       | SUB     | Subiculum                                     |
| MBsen   | Midbrain, sensory related                     | SubG    | Subgeniculate nucleus                         |
| MBsta   | Midbrain, behavior related                    | SUM     | Supramammillary nucleus                       |
| MD      | Mediodorsal nucleus of the thalamus           | SUV     | Superior vestibular nucleus                   |
| MEA     | Medial amygdala nucleus                       | TEa     | Temporal association areas                    |
| MEPO    | Median preoptic nucleus                       | TM      | Tuberomammillary nucleus                      |
| MEZ     | Hypothalamus medial zone                      | TRN     | Tegmental reticular nucleus                   |
| Abbreviation | Neuron | Description |
|--------------|--------|-------------|
| MG           | Medial geniculate complex | TU  | Tuberal nucleus |
| MM           | Medial mammillary nucleus | VIS | Visual areas |
|              |                    |     | Ventral medial nucleus of the |
| MOp          | Primary motor area | VM  | thalamus |
|              |                    |     | Ventromedial hypothalamic |
| MOs          | Secondary motor area | VMH | nucleus |
|              |                    |     | Ventral posterior complex of the |
| MPN          | Medial preoptic nucleus | VP  | thalamus |
| MPO          | Medial preoptic area | VTA | Ventral tegmental area |
| MPT          | Medial pretectal area | ZI  | Zona incerta |
| MRN          | Midbrain reticular nucleus | co-ZI | Contralateral ZI |