

1 Comparative Brain-Wide Mapping of 2 Isoflurane and Ketamine-Activated 3 Nuclei and Functional Networks

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8 **Abstract** Ketamine (KET) and isoflurane (ISO) are two widely used general anesthetics, yet their
9 distinct and shared neurophysiological mechanisms remain elusive. In this study, we conducted a
10 comparative analysis of KET and ISO effects on c-Fos expression across the brain, utilizing
11 hierarchical clustering and c-Fos-based functional network analysis to evaluate the responses of
12 individual brain regions to each anesthetic. Our findings demonstrate that KET significantly
13 activates cortical and subcortical arousal-promoting nuclei, with the temporal association areas
14 (TEa) serving as a hub node, corroborating the top-down general anesthesia theory for
15 dissociative anesthesia. In contrast, ISO activates the nuclei in the hypothalamus and brainstem,
16 with the locus coeruleus (LC) as a hub node, implying a bottom-up mechanism for
17 anesthetic-induced unconsciousness. Notably, the coactivation of arousal-related nuclei,
18 analgesia-related, neuroendocrine-related nuclei (e.g., prelimbic area (PL) and infralimbic areas
19 (ILA), and the anterior paraventricular nucleus (aPVT), Edinger-Westphal nucleus (EW), locus
20 coeruleus (LC), parabrachial nucleus (PB), solitary tract nucleus (NTS) by both anesthetics
21 underscores shared features such as unconsciousness, analgesia, and autonomic regulation,
22 irrespective of their specific molecular targets. In conclusion, our results emphasize the distinct
23 actions of KET and ISO while also uncovering the commonly activated brain regions, thus
24 contributing to the advancement of our understanding of the mechanisms underlying general
25 anesthesia.

27 Introduction

28 Despite considerable investigation into the molecular targets, neural circuits, and functional con-
29 nectivity associated with various anesthetics, our comprehension of their effects on overall brain
30 activity continues to be limited and incomplete (*Hemmings et al., 2019*). At the molecular level, ke-
31 tamine (KET) and isoflurane (ISO) interact with N-methyl-D-aspartate (NMDA) and gamma-aminobutyric
32 acid type A (GABA_A) receptors, respectively, modulating neuronal excitability and ultimately lead-
33 ing to a loss of consciousness (*Franks, 2008*). In systems neuroscience, the neural mechanisms of
34 anesthetic induced unconsciousness involve both top-down and bottom-up processes (*Mashour,*
35 *2014; Mashour and Hudetz, 2017*). As evidenced by in vivo electrophysiology or functional mag-
36 netic resonance imaging (fMRI) studies, the top-down paradigm illustrates that anesthetics induce
37 unconsciousness by disrupting corticocortical and corticothalamic circuits responsible for neural
38 information integration, while peripheral sensory information can still be conveyed to the primary
39 sensory cortex (*Schroeder et al., 2016; Lee et al., 2013*). The bottom-up approach, exemplified by
40 ISO, involves the activation of sleep-promoting nuclei like ventral lateral preoptic nucleus (VLPO)

41 and inhibition of arousal centers in the brainstem and diencephalon, supporting the shared cir-
42 cuits of sleep and anesthesia (*Moore et al., 2012; Nelson et al., 2002*). However, the limited spatial
43 resolution of fMRI studies and the inability of EEG to capture specific brainstem nuclei hinder the
44 acquisition of comprehensive whole-brain information. Although a substantial body of knowledge
45 has been amassed, our understanding of the reciprocal responses among different brain regions
46 during general anesthesia remains relatively sparse and fragmented. To bridge these gaps, further
47 investigation using advanced techniques that can capture the whole-brain dynamics is needed to
48 elucidate the complex interactions and shared mechanisms between various anesthetics.

49 Neuronal extracellular stimulation typically results in the elevation of adenosine 3',5'-cyclic monophos-
50 phate (cAMP) levels and calcium influx, ultimately leading to the upregulation of immediate early
51 genes (IEGs) such as c-fos (*Yap and Greenberg, 2018; Morgan and Curran, 1989*). The transla-
52 tion product of c-fos, c-Fos protein, offers single-cell spatial resolution and has been utilized as
53 a biomarker to identify anesthetic-activated brain regions (*Zhang et al., 2022*). Previous investi-
54 gations of c-Fos expression throughout the brain demonstrated that GABAergic agents inhibited
55 cortical activity while concurrently activating subcortical brain regions, including the VLPO, median
56 preoptic nucleus (MnPO), lateral septal nucleus (LS), Edinger-Westphal nucleus (EW), and locus
57 coeruleus (LC) (*Smith et al., 2016; Lu et al., 2008; Yatziv et al., 2020; Han et al., 2014*). In con-
58 trast, KET was shown to provoke wake-like c-Fos expression and intense augmentation of c-Fos
59 expression in various brain regions at clinical dosages (75-100 mg/kg) (*Lu et al., 2008*). It is impor-
60 tant to note that these experiments administered KET at lights-on and GABA_A receptor agonists at
61 lights-off, potentially introducing circadian influences for direct comparison of ISO and KET. More-
62 over, it has been revealed that state of general anesthesia is not determined by activity in individual
63 brain areas, but emerges as a global change within the brain. This change involves the activation
64 of lateral habenular nucleus (LHb), VLPO, supraoptic nucleus (SO), and central amygdaloid nucleus
65 (CeA), which are essential for anesthetics induced sedation, unconsciousness, or analgesia (*Moore*
66 *et al., 2012; Gelegen et al., 2018; Hua et al., 2020; Jiang-Xie et al., 2019*). However, brain-wide map-
67 ping and comparison of distinct anesthetic (KET and ISO) activated nuclei at a cellular level have
68 not been fully elucidated.

69 In this study, we examined the distribution of nuclei activated by ISO and KET in 984 brain
70 regions using immunochemical labeling and a customized MATLAB software package, which facil-
71 itated signal detection and registration to the Allen Mouse Brain Atlas reference (*Ma et al., 2021*).
72 We compared whole-brain c-Fos expression induced by KET and ISO through hierarchical cluster-
73 ing and calculated inter-regional correlations by determining the covariance across subjects for
74 each brain region pair. Significantly positively correlated regions were then extracted to construct
75 functional networks and graph theory-based analyses were performed to identify hub nodes. Our
76 results revealed distinct yet overlapping whole-brain activation patterns for KET and ISO.

77 Results

78 A comparison of the activation patterns of c-Fos in 53 brain areas in response to 79 ISO and KET

80 To examine the pattern of c-Fos expression throughout the brain, 1.5% ISO was continuously ven-
81 tilated, or 100 mg/kg KET was administered 90 minutes before harvesting (Figure 1A). Sample col-
82 lection for all mice was uniformly conducted at 14:30 (ZT7.5), and the c-Fos labeling and imaging
83 were performed using consistent parameters throughout all experiments. To quantitatively assess
84 brain states across the four groups, we utilized electroencephalography (EEG) and electromyogra-
85 phy (EMG) recordings (Figure 1—figure supplement 1). The depth of anesthesia was evaluated
86 based on the power ratio of the EEG within the delta (0.5–4 Hz) and theta (6–10 Hz) frequency
87 bands, combined with the changes in the EMG (*Luo et al., 2023*). Our findings showed that ISO and
88 KET elevated EEG power ratio while reducing EMG power, suggesting both drugs induced a loss of
89 consciousness. The raw images of brain slices were aligned to the Allen Mouse Brain Atlas (Figure

90 1B). Based on the standard mouse atlas available at <http://atlas.brain-map.org/>, the mouse brain
91 was segmented into nine hierarchical levels, totaling 984 regions. The primary level consists of
92 grey matter, the secondary of the cerebrum, brainstem, and cerebellum, and the tertiary includes
93 regions like the cerebral cortex and cerebellar nuclei, among others, with some regions extending
94 to the 8th and 9th levels. The fifth level comprises 53 subregions, with detailed expression levels
95 and their respective abbreviations presented in Figure 2—figure supplement (*Do et al., 2016*).

96 To determine the effects of ISO and KET on neural activity, we normalized c-Fos densities across
97 brain regions to their control group averages region by region and log-transformed the data. Hier-
98 archical clustering of these values revealed distinct patterns of drug-induced changes (Figure 2A),
99 with both ISO and KET showing optimal clustering at a 0.5 cut-off value, as confirmed by high sil-
100 houette coefficients (Figure 2B). Concurrently, we computed the number of clusters formed at each
101 potential cut-off value and found that a 0.5 threshold resulted in four distinct clusters for both ISO
102 and KET treatments (Figure 2C). After hierarchical clustering, we maintained the sequence of brain
103 regions and substituted log relative c-Fos densities with Z-scores for both ISO and KET (Figure 2E).
104 While no significantly inhibited brain regions were detected in the ISO group (as no Z scores fell
105 below -2), clusters 3 and 4 showed expression levels similar to or lower than those in the control
106 group. These clusters are involved in sensory integration, motor coordination, and higher-order
107 cognitive processing, with cluster 3 having a mean Z score of 0.27 (n=17) and cluster 4 a mean
108 Z score of -1.1 (n=2), as shown in Figures 2D and 2E. The most pronounced upregulation of ISO
109 induced c-Fos expression was observed in the second cluster (n=13, mean Z score=4.7), which in-
110 cludes brain regions responsible for a variety of functions: the periventricular zone (PVZ) and lateral
111 zone (LZ) of the hypothalamus, vital for endocrine and autonomic system regulation; the ventral
112 striatum (STRv), pallidum (PAL), and lateral septal complex (LSX) of the striatum, key in reward and
113 motivation; the tenia tecta (TT) and piriform area (PIR), essential for olfactory processing; and the
114 infralimbic area (ILA) of the cortex, significant for emotional and cognitive functions. This collective
115 upregulation in cluster 2 underlines ISO's impact on hypothalamic regulation, reward mechanisms,
116 and olfactory processing. KET administration elicited a significant activation in a broad array of
117 brain regions, with Cluster 4 displaying the most substantial upregulation in expression. This clus-
118 ter incorporates cortical areas central to sensory processing and emotional regulation, such as the
119 visceral area (VISC), anterior cingulate area (ACA), and primary somatosensory cortex (SS) — as
120 well as cerebral nuclei integral for motor coordination and motivational behaviors — specifically,
121 the dorsolateral and ventrolateral pallidum (PALd, PALv) and striatum (STRd, STRv) (Figures 2D and
122 2E). ISO predominantly modulates brain regions associated with hormonal regulation, reward pro-
123 cessing, and olfaction, with marked activation in the hypothalamic and striatal regions. In contrast,
124 KET induces widespread activation across the brain, with heightened activity in a key cluster that
125 includes cortical and cerebral nuclei governing complex sensory, emotional, and motor processes,
126 thus highlighting its notable influence on higher-order functions.

127 **Similarities and differences in ISO and KET activated c-Fos brain areas**

128 To enhance our analysis of c-Fos expression patterns induced by ISO and KET, we expanded our
129 study to 201 subregions. After log-transforming the c-Fos densities relative to controls, we applied
130 hierarchical clustering to uncover patterns of brain activity (Figure 3A), with detailed expression lev-
131 els in Figure 3—figure supplement 1. ISO demonstrates a more concentrated influence on brain
132 activity with consistently higher clustering quality and fewer clusters than KET, suggesting a fo-
133 cused impact on specific brain functions (Figures 3B and 3C). Figure 3E maintains the order from
134 hierarchical clustering and marks both the most activated brain regions in the top 10% of Z-scores
135 with red boxes and areas of potential suppression with Z-scores below -2 with white boxes. ISO
136 hierarchical clustering identified three distinct clusters, with cluster 3 exhibiting the highest mean
137 Z-score of 7.27. This cluster encompasses regions like the supraoptic (SO), ventrolateral preoptic
138 (VLPO), tuberal (TU), and central amygdala (CEA). Notably, the SO, VLPO, and CEA have been previ-
139 ously reported to play critical roles in the mechanisms of anesthesia (*Moore et al., 2012; Hua et al.,*

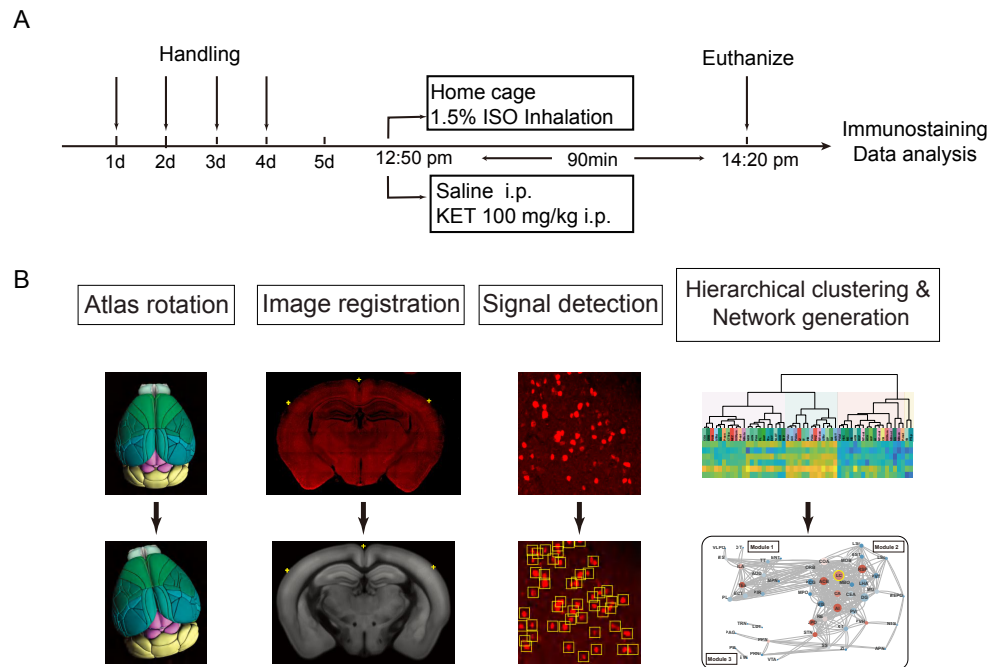


Figure 1. Brain-wide quantification of c-Fos expression. (A) Schematic representation of the habituation protocol typically used to acclimate mice. After being exposed to anesthetics for 90 minutes, the mice were euthanized. (B) Steps for data processing. Example of brain section registration to a corresponding coronal section from the Allen Brain Atlas. For Atlas rotation, the Allen reference atlas was rotated to mimic the slice angle of the experimental brain. Image registration maps the original coronal image (upper panel) to the corresponding Allen mouse brain atlas (lower panel). The registration module applies several geometric transformations (translation, rotation, and scaling) to optimize the matching of the original image to the anatomical structures. Fluorescence signals detected in the original image were projected onto the Allen Mouse Brain Atlas for quantification. Finally, the processed data undergo hierarchical clustering and network analysis to investigate the patterns of c-Fos expression and central network nodes.

Figure 1—figure supplement 1. EEG and EMG power change after each treatment. The box represents the 25th-75th percentiles; the central line denotes the median; whiskers indicate maximum and minimum values. $n = 6, 6, 8, 6$ for the home cage, ISO, saline, and KET, respectively. (A) Normalized change in EEG power: ISO vs KET, $P > 0.99$; Saline vs KET, $P = 0.01$; Home cage vs ISO, $P = 0.11$. (B) Normalized change in EMG power: ISO vs KET, $P = 0.36$; Saline vs KET, $P = 0.30$; Home cage vs ISO, $P = 0.02$. Analyses were conducted using the Kruskal-Wallis test, followed by Dunn's multiple comparisons tests.

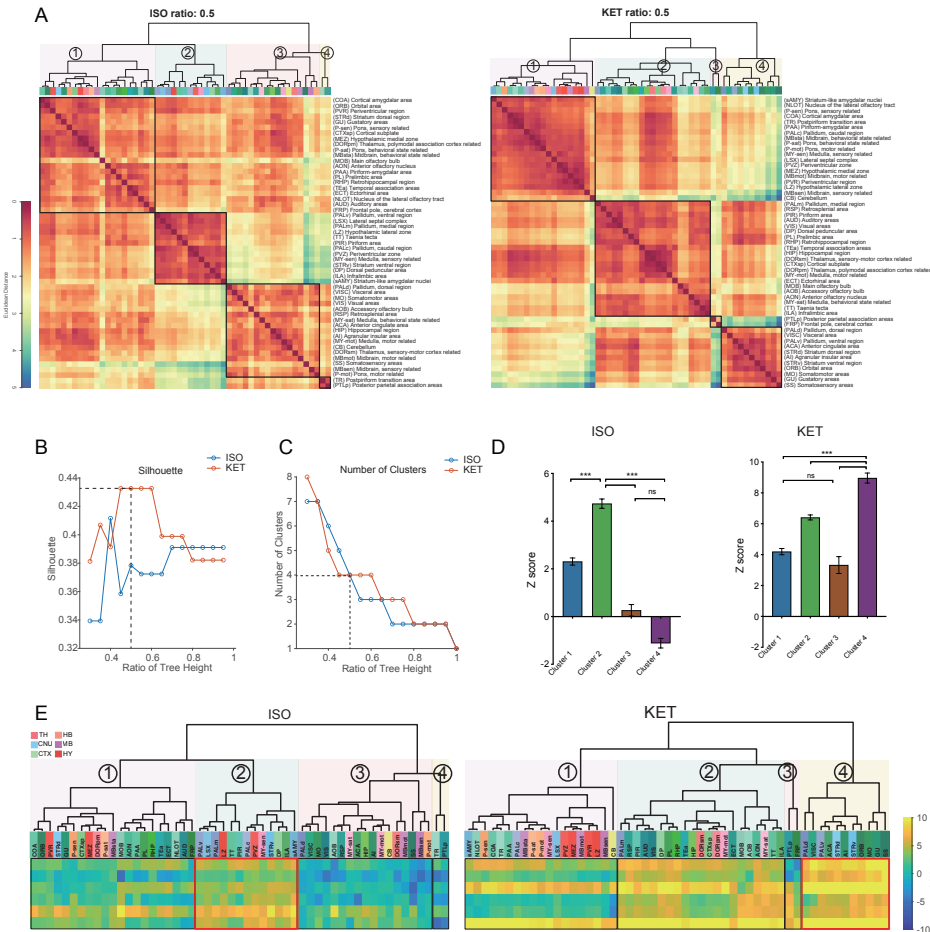


Figure 2. Whole-brain distributions of c-Fos+ cells induced by ISO and KET. (A) Hierarchical clustering was performed on the log relative c-Fos density data for ISO and KET using the complete linkage method based on the Euclidean distance matrix, with clusters identified by a dendrogram cut-off ratio of 0.5. Numerical labels correspond to distinct clusters within the dendrogram. (B) Silhouette values plotted against the ratio of tree height for ISO and KET, indicating relatively higher Silhouette values at 0.5 (dashed line), which is associated with optimal clustering. (C) The number of clusters identified in each treatment condition at different ratios of the dendrogram tree height, with a cut-off level of 0.5 corresponding to 4 clusters for both ISO and KET (indicated by the dashed line). (D) The bar graph depicts Z scores for clusters in ISO and KET conditions, represented with mean values and standard errors. One-way ANOVA with Tukey's post hoc multiple comparisons. ns: no significance; *** $P < 0.001$. (E) Z-scored log relative density of c-Fos expression in the clustered brain regions. The order and abbreviations of the brain regions and the numerical labels correspond to those in Figure 2A. The red box denotes the cluster with the highest mean Z score in comparison to other clusters. CTX: cortex; TH: thalamus; HY: hypothalamus; MB: midbrain; HB: hindbrain.

Figure 2—figure supplement 1. The c-Fos density in 53 brain areas for different conditions. (home cage, $n = 6$; ISO, $n = 6$ mice; saline, $n = 8$; KET, $n = 6$). Each point represents the c-Fos density in a specific brain region, denoted on the y-axis with both abbreviations and full names. Data are shown as mean \pm SEM. Brain regions are categorized into 12 brain structures, as indicated on the right side of the graph.

140 **2020; Jiang-Xie et al., 2019**). In contrast, cluster 1 showed a relative decrease in expression (n= 91,
141 mean Z-score: -0.54), indicating that nearly half of the brain areas had reduced c-Fos expression
142 compared to the control group. Cluster 2 was overall moderately activated, with significant activ-
143 ity in the top 10% of regions including the anterior hypothalamic (ARH) and paraventricular (PVH)
144 nuclei—key for hypothalamic functions—and cortical areas such as the infralimbic (ILA), dorsal pe-
145 duncular (DP), and temporal (TT) cortices, which are involved in emotional and cognitive processing.
146 Striatal and pallidal regions, particularly the olfactory tubercle (OT) and nucleus accumbens (ACB),
147 as well as midbrain and hindbrain structures like the area postrema (AP) and nucleus of the soli-
148 tary tract (NTS), showed the most pronounced activation. In the hierarchical clustering analysis of
149 KET exposure, Cluster 2 with a mean Z-score of -0.01 was similar to the control group in terms of
150 activation, while the other four clusters showed increased activation. Cluster 5, showing the most
151 significant activation, is composed mainly of cortical regions critical for sensory processing, spatial
152 memory, navigation, emotional regulation, and cognitive functions. It also includes cerebral nuclei
153 involved in motor control and reward pathways, alongside essential thalamic and hindbrain areas
154 for memory and sensory transmission. This composition, with a predominant focus on cortical re-
155 gions, suggests that KET chiefly modulates higher-order brain functions, while also affecting basic
156 neurological processes through its influence on cerebral nuclei, the thalamus, and the hindbrain.
157 In conclusion, while ISO's activation of specific areas like SO, VLPO, TU, and CeA in cluster 3 indi-
158 cates a targeted influence, KET's extensive engagement with cortical areas in cluster 5, along with
159 its effects on striatal and thalamic regions, underscores its profound and widespread modulation
160 of both higher-order and fundamental neural functions.

161 **Identification of brain regions activated by KET**

162 We further employed quantitative methods to identify the brain regions activated by KET across the
163 entire brain. Our findings concur with previous studies: numerous cortical regions associated with
164 somatosensory, auditory, visual, and movement were activated (Figure 4A and C, Figure 4—figure
165 supplement 1, Figure 4—source data 1). Additionally, we identified several innovative observations
166 that enrich the current understanding in this field. To provide a clearer overview of our findings,
167 we categorized the activated brain areas into different functional groups: (1) Arousal and REM
168 sleep regulation: Several nuclei associated with arousal were activated, including the prelimbic
169 area (PL), infralimbic cortex (ILA), paraventricular nucleus of the thalamus (PVT), dorsal raphe (DR),
170 and sublaterodorsal nucleus (SLD). Prior evidence indicates that the PL/ILA and PVT regions play
171 a role in regulating arousal in both cortical and subcortical areas (*Mashour et al., 2022; Gao et al.,*
172 *2020*). Additionally, dopaminergic neurons within the DR have been identified as vital components
173 of wake-promoting pathways (*Cho et al., 2017*). In the context of REM sleep, the sublaterodorsal
174 nucleus (SLD) stands out for its role in stabilization (*Feng et al., 2020*). (2) Pain modulation: KET
175 significantly activated pain-related areas such as the anterior cingulate cortex (ACA) in the cortex
176 (*Bliss et al., 2016*), the anterior pretectal nucleus (APN) in the thalamus, which is known to be in-
177 volved in managing chronic pain (*Villarreal et al., 2003*), and the anterior periaqueductal gray (PAG)
178 region and medial prefrontal cortex (mPFC), both part of the endogenous pain inhibitory pathway
179 (*Cheriyian and Sheets, 2018; Yeung et al., 1977*). Additionally, the activation of the locus coeruleus
180 (LC) in the midbrain may contribute to KET's analgesic effects (*Llorca-Torralba et al., 2021*). (3) Neu-
181 roendocrine regulation: The paraventricular hypothalamic nucleus (PVH) and supraoptic nucleus
182 (SO), which are neuroendocrine-related regions, were also activated (*Jiang-Xie et al., 2019; Duncan*
183 *et al., 1998*). (4) Movement: Subcortical nuclei associated with movement, such as the subtha-
184 lamic nucleus (STN) and nucleus incertus (NI), were prominently activated by KET administration
185 (*Ma et al., 2017; Musacchio et al., 2017*). (5) Connectivity: We observed significant activation of
186 the nucleus reuniens (Re) located in the thalamus, which receives substantial afferent input from
187 limbic structures and serves as a connector linking the hippocampus to the medial prefrontal cor-
188 tex (*Hauer et al., 2021*). While our functional categorization offers a simplified overview, it's worth
189 noting that each activated brain region may have multifaceted roles beyond this classification. In

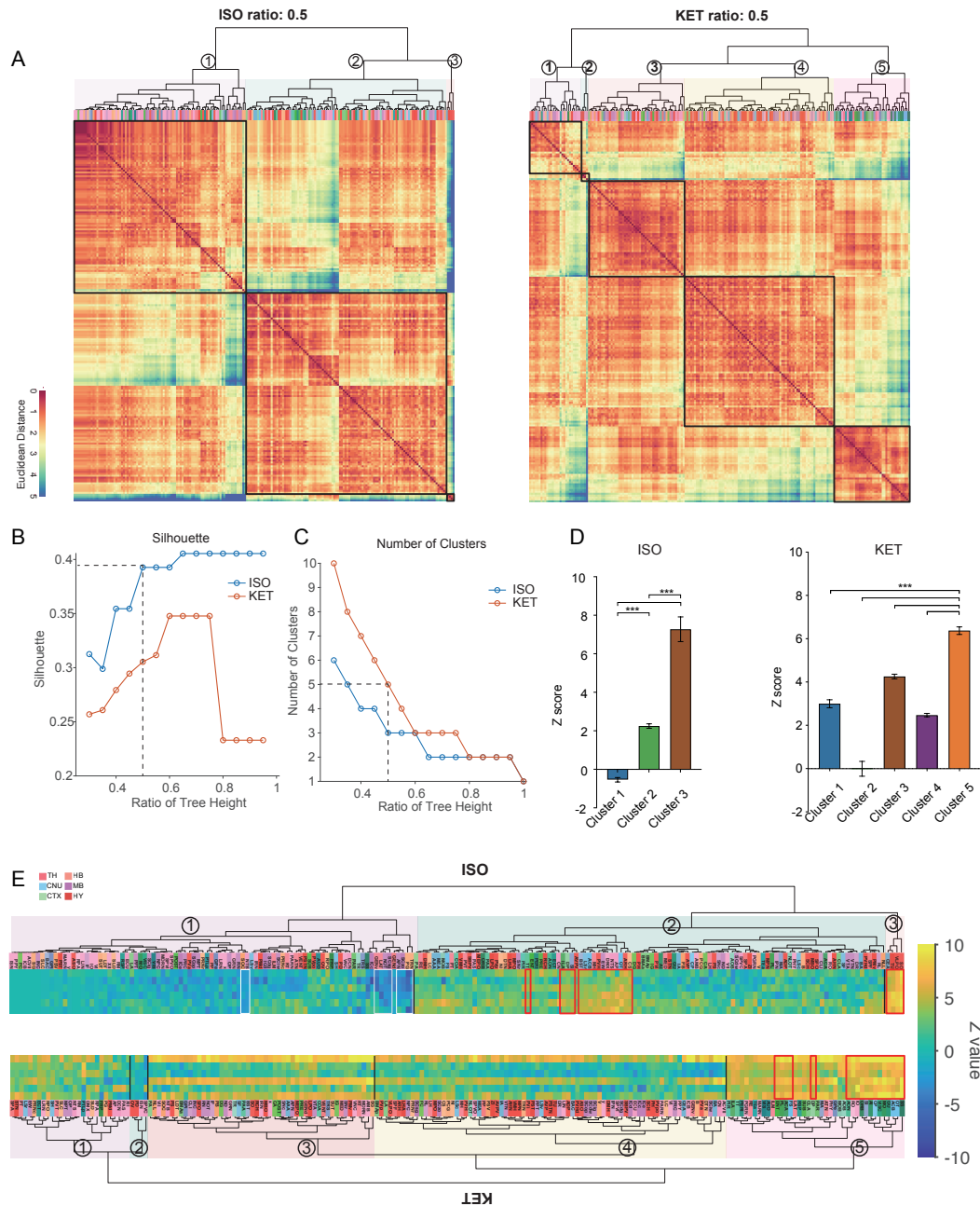


Figure 3. Similarities and differences in ISO and KET activated c-Fos brain areas. (A) Hierarchical clustering was performed on the log-transformed relative c-Fos density data for ISO and KET using the complete linkage method based on the Euclidean distance matrix, with clusters identified by a dendrogram cut-off ratio of 0.5. Brain region labels are provided in Supplementary Figures 4 and 5. (B) Silhouette values are plotted against the ratio of tree height from the hierarchical clustered dendrogram in Figure 3A. (C) The relationship between the number of clusters and the tree height ratio of the dendrogram for ISO and KET, with a cut-off ratio of 0.5 resulting in 3 clusters for ISO and 5 for KET (indicated by the dashed line). (D) The bar graph depicts Z scores for clusters in ISO and KET conditions, represented with mean values and standard errors. One-way ANOVA with Tukey's post hoc multiple comparisons. ns: no significance; *** $P < 0.001$. (E) Z-scored log relative density of c-Fos expression within the identified brain region clusters. The arrangement, abbreviations of the brain regions, and the numerical labels are in accordance with Figure 3A. The red boxes highlight brain regions that rank within the top 10 percent of Z score values. The white boxes denote brain regions with an Z score less than -2.

Figure 3—figure supplement 1. c-Fos density visualization across 201 distinct brain regions under various conditions. The graph depicts the c-Fos density levels for each condition, with data presented as mean and standard error. Brain regions with statistically significant differences are featured in Figures 4 and 5. Brain regions are organized into major anatomical subdivisions, as indicated on the left side of the graph.

Figure 3—figure supplement 2. Region labels for the hierarchical clustering of the ISO group in Figure 3A.

Figure 3—figure supplement 3. Region labels for the hierarchical clustering of the KET group in Figure 3A.

190 summary, our study identified extensive activation of cortical and subcortical nuclei during KET
191 anesthesia, encompassing regions related to arousal, pain modulation, neuroendocrine regula-
192 tion, movement, and connectivity.

193 **Identification of brain regions activated by ISO**

194 To identify brain regions activated by ISO and compare these with those activated by KET. We began
195 by summarizing previously reported ISO activated nuclei, including PIR, LSd/LSv, CeA in the cortex
196 and striatum, and VLPO, MnPO, EW, NTS, LC, ventral group of the dorsal thalamus (VeN), and area
197 postrema (AP) in the hypothalamus and midbrain (Figure 5—source data 1). We subsequently
198 conducted a comprehensive MATLAB-based analysis of c-Fos expression throughout the entire
199 brain, uncovering previously undetected activated nuclei (Figure 5A, Figure 5—figure supplement
200 1). Newly identified activated nuclei in the CTX and the CNU included PL/ILA and ENT, aPVT in the
201 thalamus, TU, ARH, PVi, and PVH in the hypothalamus, and PB in the hindbrain. All nuclei activated
202 by ISO in this study were functionally classified and depicted in Figure 5C. Our results confirmed the
203 activation of several brain regions involved in arousal-related nuclei, such as the prelimbic area (PL)
204 and infralimbic areas (ILA), and the paraventricular nucleus (PVT) (*Mashour et al., 2022; Gao et al.,*
205 *2020*). Additionally, we observed activation in previously reported analgesia-related nuclei, includ-
206 ing CeA and LC, as well as the parabrachial nucleus (PB) (*Jiang-Xie et al., 2019; Llorca-Torralba et al.,*
207 *2021; Deng et al., 2020*). We also found activation in neuroendocrine function-related nuclei of the
208 hypothalamus, such as TU, PVi, ARH, PVH, and SO. Moreover, we identified activations related to
209 ISO induced side effects, such as in the piriform cortex (PIR) and ENT (*Bekkers and Suzuki, 2013;*
210 *Xu and Wilson, 2012*), which may be stimulated by ISO odor, and the solitary tract nucleus (NTS),
211 potentially responsible for ISO induced vomiting (*Gupta et al., 2017*). The only activated nucleus
212 in the midbrain was the Edinger-Westphal nucleus (EW). Recent research has found that sevoflu-
213 rane activates EW and is involved in sleep induction and maintenance of anesthesia, suggesting
214 its crucial role in general anesthesia (*Yi et al., 2023*). By comparing the ISO and KET induced c-Fos
215 expression, we summarized the brain regions activated by both anesthetics in Figure 5D. Despite
216 variations in molecular targets, the coactivation of regions such as PL/ILA, aPVT, CeA, PVH, SO, EW,
217 PB, LC, and NTS by both ISO and KET suggests an overlapping neuronal circuitry that influences
218 sleep-wake regulation, analgesia, and neuroendocrine functions. This shared neural circuitry may
219 potentially offer a common mechanism across the two anesthetics for the maintenance of general
220 anesthesia.

221 **Network generation and Hub identification**

222 Previous research has established that general anesthesia is mediated by various brain regions
223 (*Moore et al., 2012; Gelegen et al., 2018; Hua et al., 2020; Jiang-Xie et al., 2019*). C-Fos expres-
224 sion serves as an indicator of neuronal activity, providing a single index of activation per region
225 per animal. By examining the covariance of c-Fos expression across animals within each group,
226 we can infer interactions between brain regions and identify functional networks engaged during
227 general anesthesia (*Wheeler et al., 2013*). We initially incorporated a total of 63 brain regions, in-
228 cluding nuclei that were significantly activated by ISO and KET (as indicated by positive findings
229 in Figures 4 and 5) and those previously reported to be associated with the mechanisms of gen-
230 eral anesthesia and sleep-wakefulness (*Leung et al., 2014*). We first calculated a comprehensive
231 set of interregional correlations for four groups (Figure 6A). Correlation analysis revealed similar
232 network connectivity and densities between the ISO and home cage groups (mean interregional
233 correlations: Fisher Z = -0.018, P = 0.98; network densities: 0.10 vs. 0.09). In contrast, KET signifi-
234 cantly increased network density (0.46 vs. 0.06) and mean interregional correlations compared to
235 the saline group (Fisher Z = 3.54, P < 0.001; Figure 6—figure supplement 1D), indicating enhanced
236 interregional correlation with KET administration. Additionally, we segmented the functional net-
237 work into modules based on the hierarchical clustering of correlation coefficients (Figure 6—figure
238 supplement 1A). We chose to cut the tree at a value of 0.7 for the relatively high value of Silhouette

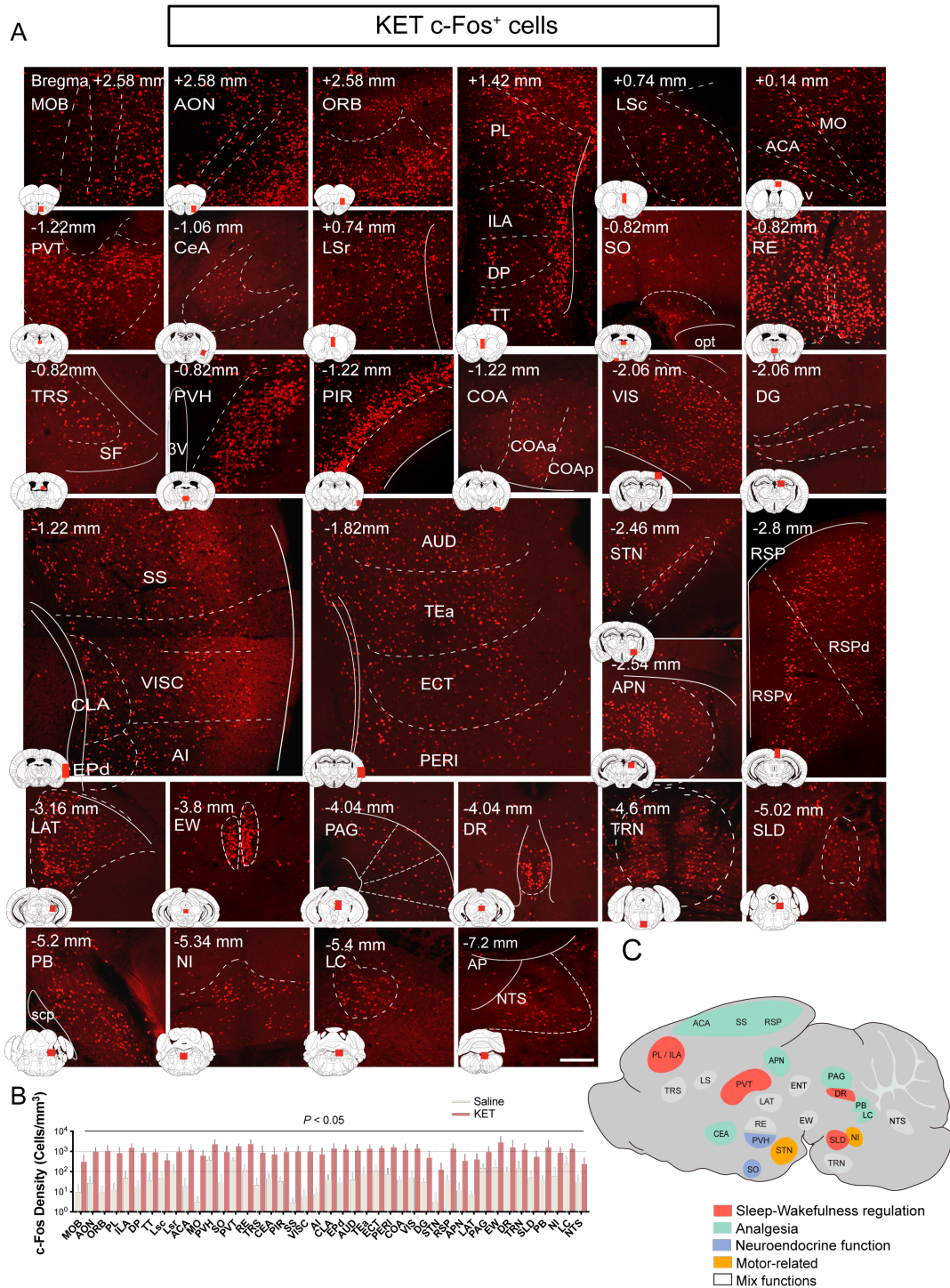


Figure 4. c-Fos expression in distinct brain regions after exposure to KET. (A) Representative immunohistochemical staining of MOB, AON, ORB, MPO, ACA, MO, TRS, PL, ILA, DP, LS, PVT, SO, PVH, RE, VISC, AI, CLA, EPd, PIR, COA, AUD, TEa, ECT, PERI, CeA, SS, DG, STN, RSP, APN, LAT, EW, DR, PAG, SLD, PB, TRN, NI, LC, NTS, and NI c-Fos⁺ cells from the indicated mice. Scale bar, 200 μ m. (B) Representation of c-Fos density in brain regions exhibiting significant statistical differences. Data are shown as mean \pm SEM. P values < 0.05. (C) Schematic cross-section of the mouse brain showing activated brain regions by KET. Different colors indicate distinct functional nuclei. The red nuclei are associated with the regulation of sleep-wakefulness, the blue-green nuclei are linked to analgesia, the yellow nuclei are associated with motor function, and the white nuclei are a composite of various functional nuclei.

Figure 4—figure supplement 1. c-Fos expression in specific brain regions following normal saline administration. (A) Representative immunohistochemical staining of c-Fos⁺ cells in MOB, AON, ORB, MPO, ACA, MO, TRS, PL, ILA, DP, LS, PVT, SO, PVH, RE, VISC, AI, CLA, EPd, PIR, COA, AUD, TEa, ECT, PERI, CeA, SS, DG, STN, RSP, APN, LAT, EW, DR, PAG, SLD, PB, TRN, NI, LC, and NTS. Scale bar represents 200 μ m.

Table 1. Figure 4—source data 1. Summary of prior studies on ketamine activated brain regions identified via c-Fos immunostaining.

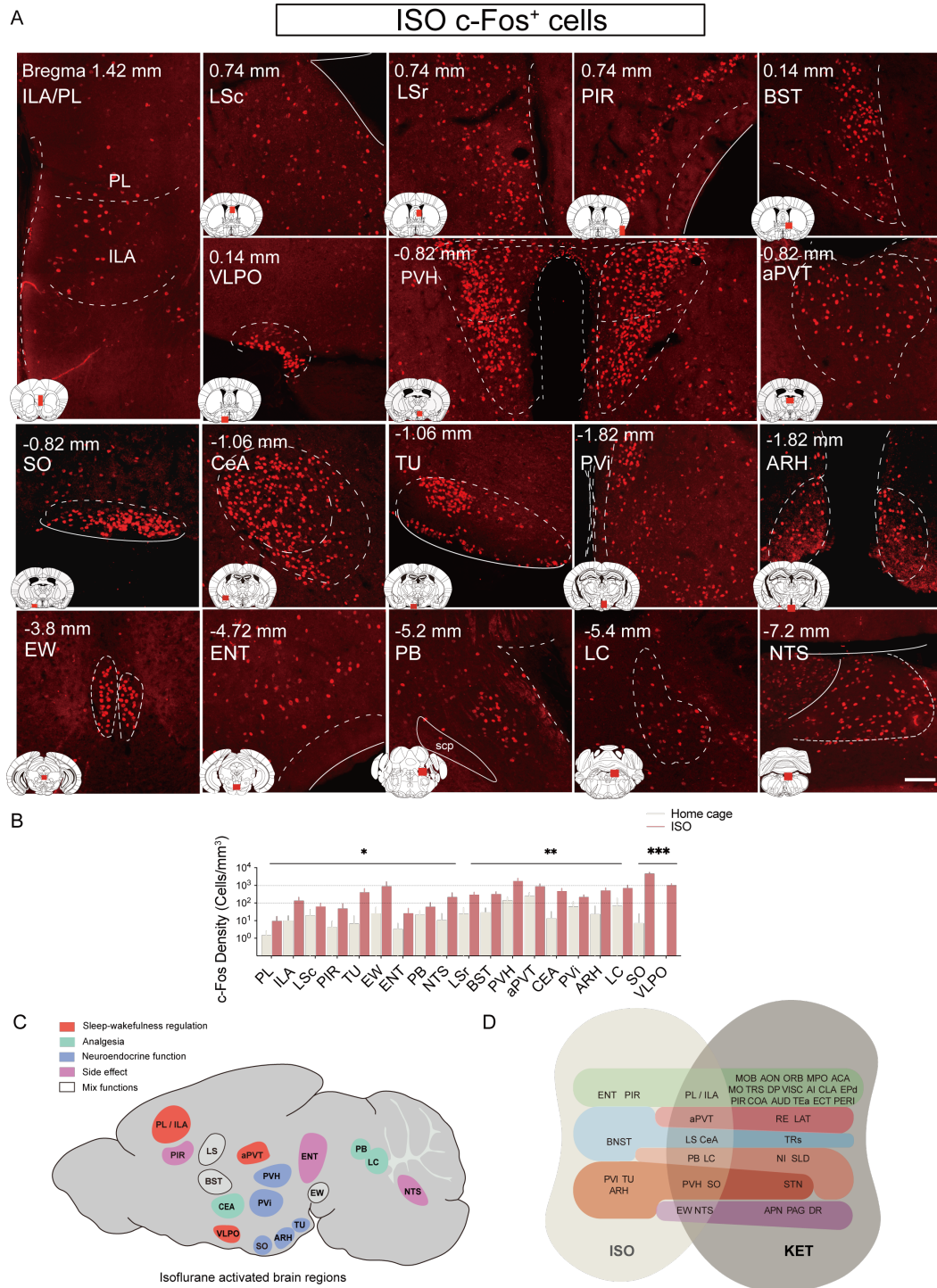


Figure 5. c-Fos expression in distinct brain regions after exposure to ISO. (A) Representative of brain regions with statistical differences c-Fos⁺ cells between the ISO group and home cage mice. Scale bar, 200 μ m. (B) Representation of cell counts in brain regions exhibiting significant statistical differences between home cage and ISO. Data are shown as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (C) Schematic cross-section of the mouse brain showing activated brain regions by ISO. Different colors indicate various functionally relevant nuclei. Red signifies nuclei involved in sleep-wake regulation, blue-green in pain management, blue in neuroendocrine function, pink in side-effect management, and white denotes nuclei exhibiting mixed functionalities. (D) The Venn diagram shows brain regions that are co-activated by ISO and KET and differentially activated brain regions.

Figure 5—figure supplement 1. c-Fos expression in home cage group. (A) Representative immunohistochemical staining of c-Fos⁺ cells in PL, ILA, LSc, LSr, PIR, BST, VLPO, PVH, aPVT, SO, CeA, TU, PVi, ARH, EW, ENT, PB, LC, and NTS c-Fos⁺ cells from the indicated mice. Scale bar, 200 μ m.

Table 2. Figure 5—source data 1. Summary of prior studies on brain regions activated by isoflurane, as detected through c-Fos immunostaining.

239 scores, resulting in two modules for the home cage and KET groups, and four for the saline and
240 ISO groups (Figure 6—figure supplement 1B and C). Networks were formed using Pearson's coef-
241 ficients over 0.82 and significant correlations ($P < 0.05$), leaving a single module for KET and three
242 modules for ISO (Figure 6B).

243 Hubs are nodes that occupy critical central positions within the network, enabling the network
244 to function properly. Due to the singular module structure of the KET network and the sparsity
245 of intermodular connections in the home cage and saline networks, the assessment of network
246 hub nodes did not employ within-module degree Z-score and participation coefficients, as these
247 measures predominantly underscore the importance of connections within and between modules
248 (*Kimbrough et al., 2020*). The analysis evaluated nodal importance using degree (number of di-
249 rect connections), betweenness centrality (proportion of shortest network paths passing through
250 a node), and eigenvector centrality (influence based on the centrality of connected nodes), iden-
251 tifying brain regions that scored highly in all three as hub nodes (Figure 6—figure supplement 2).
252 The locus coeruleus (LC), characterized by its high degree and eigenvector centrality, as well as
253 its notably higher betweenness centrality, underscores its pivotal role in propagating and integrat-
254 ing neural signals within the ISO-influenced network. In the KET group, the temporal association
255 areas (TEa) serve as a hub, emphasizing their integrative function in the manifestation of ketamine-
256 induced dissociative anesthesia. Meanwhile, the PAG and the lateral septal nucleus, rostral part
257 (LSr), function as central hubs in the home cage and saline groups, respectively.

258 Discussion

259 In this study, a comparative analysis was conducted on the effects of two general anesthetics, ISO
260 and KET, on c-Fos expression throughout the brain. Hierarchical cluster analysis enabled a detailed
261 examination of individual brain region responses to each anesthetic. We found that KET primarily
262 activates the cerebral cortex, indicative of a top-down mechanism, whereas ISO chiefly stimulates
263 subcortical areas, particularly the hypothalamus, aligning with a bottom-up approach (*Mashour,*
264 *2014; Mashour and Hudetz, 2017*). Further analysis based on c-Fos expression revealed the TEa as
265 a key hub in the KET-induced network and the LC in the ISO network, underscoring their significant
266 roles in mediating the effects of KET and ISO, respectively.

267 Our hierarchical clustering method effectively identified brain regions impacted by ISO and KET,
268 aligning with multiple comparison results as shown in Figures 4 and 5. This approach facilitated
269 the efficient grouping of regions with similar expression patterns, enabling a comprehensive anal-
270 ysis of brain-wide c-Fos expression differences. Notably, certain brain regions such as SO, VLPO,
271 TU, and CeA were prominent under ISO treatment, with SO (*Jiang-Xie et al., 2019*), VLPO (*Moore*
272 *et al., 2012*), and CeA (*Hua et al., 2020*) previously reported to be involved in mechanisms of gen-
273 eral anesthesia. The LC was identified as a highly connected hub, underscoring its critical role in
274 the brainstem during ISO-induced general anesthesia. The LC is known for its diverse functions,
275 including arousal, pain modulation, attention, stress response, and neuroprotection. Studies indi-
276 cate that chemical activation of the LC enhances whole-brain functional connectivity, largely due
277 to its role as the primary source of norepinephrine (NE) and its extensive influence throughout
278 the brain (*Zerbi et al., 2019*). The significant activation of the LC and its pivotal position in the func-
279 tional network underlying ISO-induced unconsciousness indicate its crucial role in maintaining and
280 integrating the network. This finding highlights the LC's involvement in the bottom-up paradigm
281 of ISO-induced unconsciousness.

282 KET induces extensive activation across the brain, with a notable impact on cortical and cere-
283 bral nuclei, as indicated in Figures 3 and 4. This observation aligns with the proposal by Mashour
284 et al. that anesthesia-induced unconsciousness includes both the modulation of bottom-up and
285 top-down neural processing (*Mashour, 2014; Mashour and Hudetz, 2017*). In this framework, anes-
286 thetics like KET diminish consciousness by interfering with cortical and thalamocortical circuits,
287 which are crucial for neural information integration. Our study found that KET administration sig-
288 nificantly activates cortical and subcortical arousal-promoting nuclei. Graph theoretical analysis

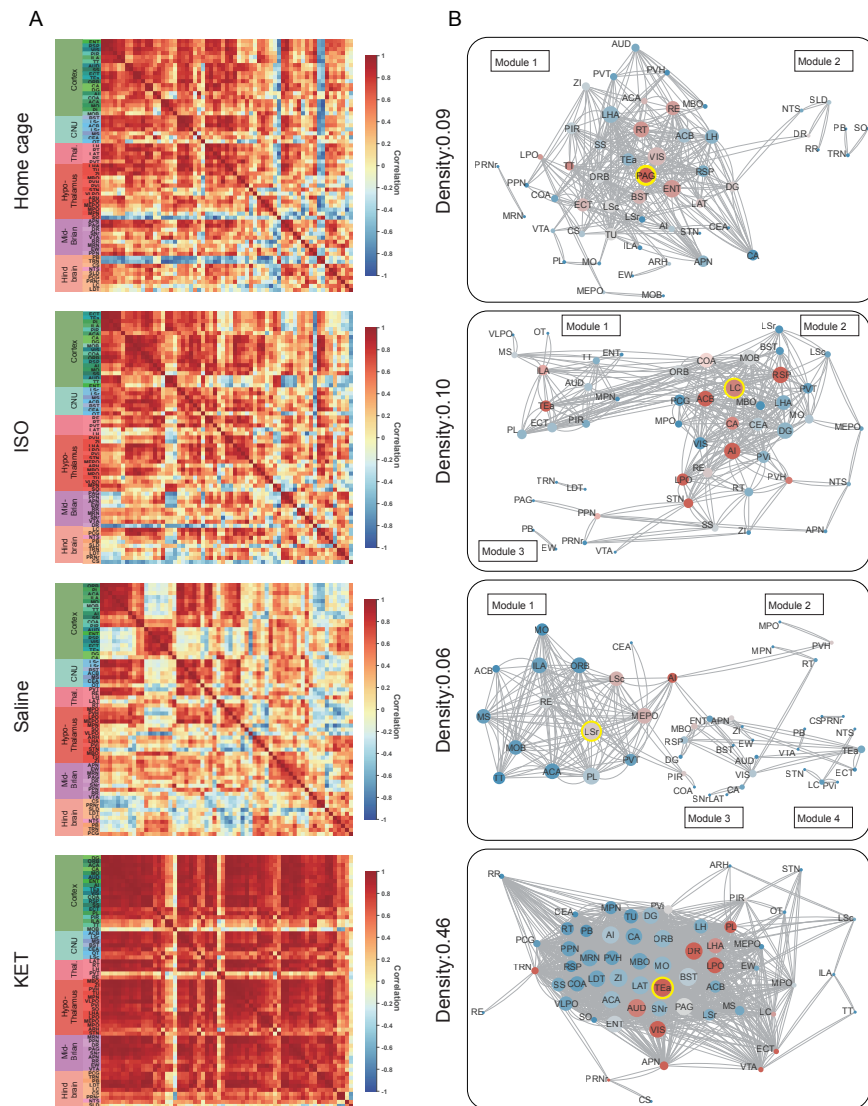


Figure 6. Generation of anesthetics-induced networks and identification of hub regions. (A) Heatmaps display the correlations of log c-Fos densities within brain regions (CTX, CNU, TH, HY, MB, and HB) for various states (home cage, ISO, saline, KET). Correlations are color-coded according to Pearson's coefficients. The brain regions within each anatomical category are organized by hierarchical clustering of their correlation coefficients. Full names and expression levels for each brain region are detailed in Supplementary Figure 3. (B) Network diagrams illustrate significant positive correlations ($P < 0.05$) between regions, with Pearson's r exceeding 0.82. Edge thickness indicates correlation magnitude, and node size reflects the number of connections (degree). Node color denotes betweenness centrality, with a spectrum ranging from dark blue (lowest) to dark red (highest). The networks are organized into modules consistent with the clustering depicted in Figure 6—figure supplement 1A.

Figure 6—figure supplement 1. Hierarchical clustering across different conditions. (A) Heatmaps depict the hierarchical clustering of brain regions within the home cage, ISO, saline, and KET groups, using Euclidean distance matrices calculated from correlation coefficients. Each brain region is represented by its abbreviation, with full names and expression levels detailed in Figure 3—figure supplement 1. Modules are demarcated within each dendrogram at a cut-off threshold of 0.7. (B) Silhouette scores are plotted against the dendrogram tree height ratio for each condition, with optimal cluster definition indicated by a dashed line at a 0.7 ratio. (C) The number of clusters formed at different cutoff levels. At a ratio of 0.7, ISO and saline treatments result in three clusters, whereas home cage and KET conditions yield two clusters. (D) The mean Pearson's correlation coefficient (r) was computed from interregional correlations displayed in Figure 6A. Data were analyzed using one-way ANOVA with Tukey's post hoc test, $***P < 0.001$.

Figure 6—figure supplement 2. Hub region characterization across different conditions: home cage (A), ISO (B), saline (C), and KET (D) treatments. Brain regions are sorted by degree, betweenness centrality, and eigenvector centrality, with each metric presented in separate bar graphs. Bars to the left of the dashed line indicate the top 20% of regions by rank, highlighting the most central nodes within the network. Red bars signify regions that consistently appear within the top rankings for both degree and betweenness centrality across the metrics.

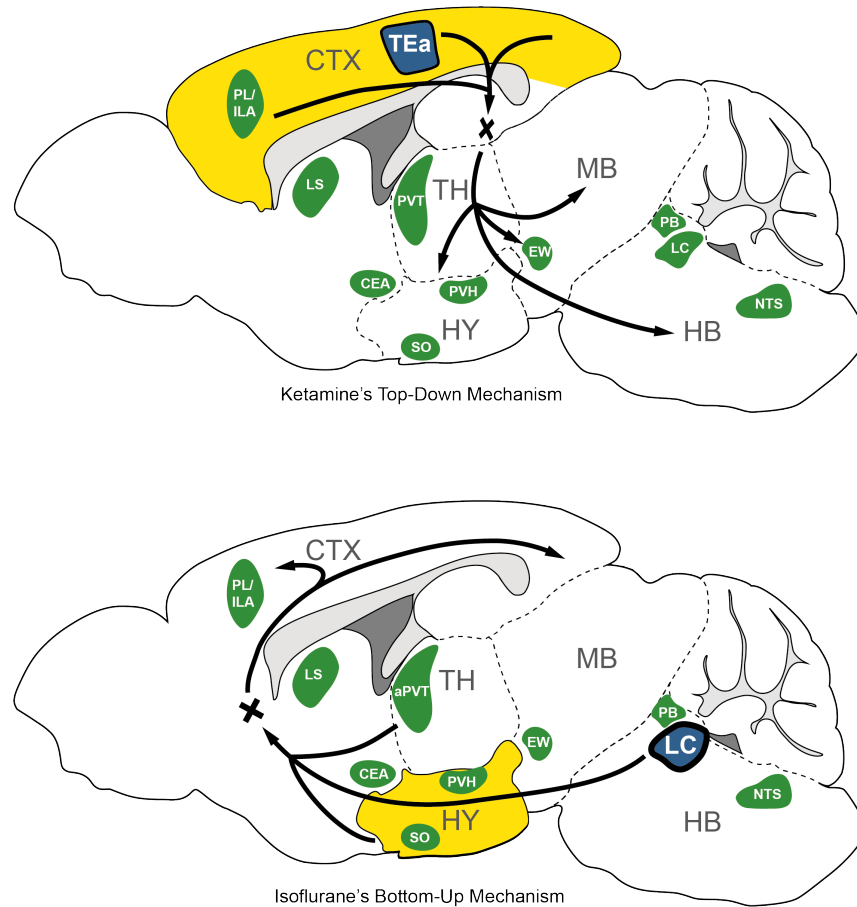


Figure 7. The possible framework for KET and ISO-induced unconsciousness. The distinct pathways of KET and ISO-induced unconsciousness can be explained by two contrasting mechanisms. The 'top-down' process attributes KET's effect to widespread cortical activation (represented in yellow), with the temporal association areas (TEa) acting as the central node in the functional network (depicted in blue). Conversely, the 'bottom-up' approach suggests that isoflurane-induced unconsciousness stems from the activation of certain hypothalamic regions (highlighted in yellow), with the locus coeruleus (LC) acting as the hub node within the isoflurane-induced functional network. Nuclei activated by both anesthetics are shown in green. Adapted from (*Mashour, 2014; Mashour and Hudetz, 2017; Reimann and Niendorf, 2020*). PL, prelimbic area; ILA, infralimbic areas; SO, supraoptic nucleus; PVH, paraventricular hypothalamic nucleus; LC, locus coeruleus; SS, somatosensory cortex; CTX: cortex; TH: thalamus; HY, hypothalamus; MB; midbrain; HB, hindbrain.

289 identified the TEa as a central hub in the KET-induced functional network. Given TEa's critical role
290 in integrating multisensory information, particularly auditory signals, and its involvement in com-
291 plex behaviors such as maternal responses as evidenced by its role in processing pup ultrasonic
292 vocalizations, our findings suggest that KET may influence the connectivity of TEa with other brain
293 regions, thus potentially altering the overall dynamics of the functional brain network (*Tasaka et al.,*
294 *2020*). This further supports the significance of cortical areas during KET anesthesia.

295 Identifying shared neural features between KET and ISO is crucial for understanding anesthetic-
296 induced unconsciousness. The coactivation of brain regions, such as PL/ILA and aPVT, alongside
297 analgesia-related nuclei like CeA, PB, and LC, suggests a shared mechanism for thalamocortical reg-
298 ulation and common pathways for pain relief. This observation provides valuable insights into the
299 fundamental mechanisms of anesthesia-induced hypnosis and analgesia. Additionally, the coac-
300 tivation of neuroendocrine-related nuclei, particularly PVH and SO in the hypothalamus, raises
301 questions about the potential influence of anesthetics on hormonal release and homeostatic reg-
302 ulation. Further investigation is warranted for other coactivated nuclei, such as EW and NTS, to
303 understand their roles in anesthesia. The coactivated nuclei suggest a potential shared neuronal
304 circuitry for general anesthesia, encompassing common features like unconsciousness, analgesia,
305 and autonomic regulation. Future research could focus on examining coactivated brain regions by
306 the two anesthetics or manipulating identified hub nodes to delve deeper into the mechanisms of
307 general anesthesia.

308 Our findings indicate that c-Fos expression in the KET group is significantly elevated compared
309 to the ISO group, and the saline group exhibits notably higher c-Fos expression than the home
310 cage group, as seen in Figure 3—figure supplement 1. Intraperitoneal saline injections in the
311 saline group, despite pre-experiment acclimation with handling and injections for four days, may
312 still evoke pain and stress responses in mice. Subtle yet measurable variations in brain states be-
313 tween the home cage and saline groups were observed, characterized by changes in normalized
314 EEG delta/theta power (home cage: 0.05 ± 0.09 ; saline: -0.03 ± 0.11) and EMG power (home cage:
315 -0.37 ± 0.34 ; saline: 0.04 ± 0.13), as shown in Figure 1—figure supplement 1. These changes suggest
316 a relative increase in overall brain activity in the saline group compared to the home cage group,
317 potentially contributing to the higher c-Fos expression. Although the difference in EEG power be-
318 tween the ISO group and the home cage control was not significant, the increase in EEG power
319 observed in the ISO group was similar to that of KET (0.47 ± 0.07 vs 0.59 ± 0.10), suggesting that
320 both agents may induce loss of consciousness in mice. Regarding EMG power, ISO showed a sig-
321 nificant decrease in EMG power compared to its control group. In contrast, the KET group showed
322 a lesser reduction in EMG power (ISO: -1.815 ± 0.10 ; KET: -0.96 ± 0.21), which may partly explain
323 the higher overall c-Fos expression levels in the KET group. This is consistent with previous studies
324 where ketamine doses up to 150 mg/kg increase delta power while eliciting a wakefulness-like pat-
325 tern of c-Fos expression across the brain (*Lu et al., 2008*). Furthermore, the observed differences
326 in c-Fos expression may arise in part from the dosages, routes of administration, and their dis-
327 tinct pharmacokinetic profiles. This variation is compounded by the lack of detailed physiological
328 monitoring, such as blood pressure, heart rate, and respiration, affecting our ability to precisely
329 assess anesthesia depth. Future studies incorporating comprehensive physiological monitoring
330 and controlled dosing regimens are essential to further elucidate these relationships and refine
331 our understanding of the effects of anesthetics on brain activity.

332 **Methods and Materials**

333 **Animals**

334 All animal experiments were conducted in accordance with the National Institutes of Health guide-
335 lines and were approved by the Chinese Academy of Sciences' Institute of Neuroscience. Adult
336 male wild-type (WT) mice (C57BL/6J) (8-10 weeks old, weight from 22 to 26 g) were purchased
337 from institute-approved vendors (LingChang Experiment Animal Co., China). Mice were individ-

338 ually housed and maintained on a 12 h:12 h light/dark cycle (lights on at 07:00 a.m. and off at
339 07:00 p.m.) with food and water available ad libitum.

340 **Drug administration**

341 All experiments were conducted between 13:00-14:30 (ZT6–ZT7.5). We adapted mice to handling
342 and the anesthesia chamber (10×15×15 cm) for four days to minimize experimental confound-
343 induced c-Fos induction. For KET administration, adult male mice were handled for 10 minutes
344 per day, and normal saline (NS) was injected intraperitoneally (i.p.) for four consecutive days at
345 13:00. On day five, a randomly chosen mouse received an injection of ketamine (Gutian Medicine,
346 H35020148) (n=6), and the control groups (n=8) received the same volume of saline. ISO group
347 (n=6) mice were handled and inhaled 1.5% isoflurane (RWD Life Science, 1903715) at 13:00 on day
348 four in the chamber. Meanwhile, the control groups (n=6) were left undisturbed in their home
349 cages prior to sampling. We confirmed the loss of righting reflex at 5 min after anesthetics expo-
350 sure. For 90 min after KET injection or ISO inhalation, mice were deeply anesthetized with 5% ISO
351 and transcardially perfused with 35 ml 0.1 M phosphate-buffered saline (PBS) followed by 35 ml
352 4% paraformaldehyde (PFA). The brains were then removed and postfixed overnight with 4% PFA.
353 Following fixation, the brains were dehydrated for 48 hours with 30% sucrose (wt/vol) in PBS. Coro-
354 nal sections (50 μ m) of the whole brain were cut using a cryostat (HM525 NX, Thermo Scientific)
355 after being embedded with OCT compound (NEG-50, Thermo Scientific) and freezing.

356 **Anesthesia depth measurement**

357 EEG and EMG recordings were conducted as previously described (*Luo et al., 2023*). Specifically,
358 a stainless steel electrode was positioned over the frontal cortex for EEG measurements, while
359 an insulated EMG electrode was inserted into the neck muscles. Additionally, a reference elec-
360 trode was placed above the cerebellum. The EEG/EMG data were captured using TDT system-3
361 amplifiers, specifically RZ5 + RA16PA and RZ2 + PZ5 configurations. To measure the depth of anes-
362 thesia using EEG/EMG signals, a fast Fourier transform spectral analysis was employed, featuring
363 a frequency resolution of 0.18 Hz. Data was noted every 5 seconds through a MATLAB tool and
364 subsequently verified manually by experts. The assessment was based on EMG power and the ra-
365 tio of EEG's δ power (0.5-4Hz) to θ power (6-10Hz). EEG and EMG power values within 30 minutes
366 post-administration were normalized to a 5-minute pre-administration baseline.

367 **Immunohistochemistry**

368 One out of every three brain slices (100 μ m intervals) of each whole brain was washed three times
369 with 0.1 M phosphate-buffered saline (PBS) for 10 min and permeabilized for 30 minutes at room
370 temperature with 0.3% Triton X-100 in PBS (PBST). Slices were incubated for 2 hours at room tem-
371 perature with 2% normal donkey serum (Sigma, G6767) in PBS overnight at 4°C with c-Fos pri-
372 mary antibodies (226003, Synaptic Systems; 1:500) diluted in PBS with 1% donkey serum. After
373 three washes with PBST, slices were incubated with the Cy3 donkey anti-rabbit (711165152, Jack-
374 son; 1:200) secondary antibody for 2 hours at room temperature. Immunostained slices were
375 mounted with VECTASHIELD mounting medium with DAPI and then scanned under a fluorescent
376 microscope equipped with a 10× objective (VS120, Olympus) or a confocal microscope with a 20×
377 objective (FV300, Olympus). Due to the limited scope of a single field of view, either 2 or 4 adja-
378 cent fields of view were stitched together to offer a comprehensive representation of specific brain
379 regions, including the PL, ILA, LSc, LSr, SS, and VISC.

380 **Quantification of c-Fos positive cells**

381 The procedures used for c-Fos analysis were based on previous research (*Ma et al., 2021*). A
382 custom-written software package was employed for cellular quantification within brain images.
383 The software consists of three components: atlas rotation, image registration, and signal detec-
384 tion.

385 Atlas rotation module

386 Utilizing the Allen Mouse Brain Atlas, this module allows rotations in both horizontal and vertical
387 dimensions to align with mouse brain samples. To determine the appropriate rotation angles, we
388 manually pinpointed anatomical landmarks corresponding to the samples. For the left-right axis
389 rotation, we chose points from the CA3 pyramidal layer in both hemispheres and the posterior slice
390 of the dentate gyrus, specifically the granule cell layer. For the dorsal-ventral axis rotation, we iden-
391 tified key anatomical landmarks. These include the initial connections of the anterior commissure
392 and corpus callosum between the hemispheres, as well as ventral regions like the interpeduncular
393 nucleus and the suprachiasmatic nucleus. After determining these rotation angles, we adjusted
394 the reference atlas to match our samples.

395 Image registration module

396 This module uses a tool that aligns brain slice images with a reference atlas, facilitating the align-
397 ment of overall brain region distribution. Registration starts by matching the coronal plane of the
398 sample sections to the atlas. After defining the external boundaries of the brain section, the system
399 performs geometric transformations on the section to optimize its fit with the atlas.

400 Signal detection module

401 The detection module is designed to automatically label c-Fos+ cells. Post detection, a manual veri-
402 fication was performed on each digitized brain section image to ensure the accuracy and precision
403 of the c-Fos+ cell markings.

404 Hierarchical clustering

405 Prior to hierarchical clustering in Figures 2 and 3, we calculated the relative c-Fos densities by divid-
406 ing the c-Fos densities of each brain region in the experimental groups by their respective controls
407 and then performed a log transformation on these values to obtain the log relative c-Fos densities.
408 These log ratios, which normalize the data and reduce variance, indicate differential expression
409 with a value of zero denoting no change compared to control. After normalizing the data, we per-
410 formed hierarchical clustering by first computing the pairwise Euclidean distances among brain
411 regions. Regions with the smallest distances, indicating high similarity, were grouped iteratively.
412 Cluster boundaries were defined using complete linkage, ensuring homogeneity within clusters by
413 considering the largest distance between members. In Figure 6, hierarchical clustering was per-
414 formed within each of the CTX, CNU, TH, HY, MB, and HB regions based on the log-transformed
415 c-Fos density correlations. In Figure 6—figure supplement 1, hierarchical clustering was performed
416 based on the interregional log c-Fos density correlations to identify modules of coactivation within
417 each treatment group, revealing underlying functional connectivity networks (*Kimbrough et al.,*
418 *2020*). To determine the statistical significance of c-Fos expression differences, we computed the
419 Z-score for each treatment condition—ISO and KET—by dividing the log relative c-Fos density by
420 the standard error. Positive z-scores indicate higher values than control, and negative z-scores
421 indicate lower values than control.

422 Network generation

423 To evaluate how functional connectivity changed under general anesthetics in WT mice, we ex-
424 tracted 63 brain regions from major brain subdivisions (cerebral cortex, cerebral nuclei, thalamus,
425 hypothalamus, midbrain, and hindbrain) listed in Figure 6A. Correlation matrices were generated
426 by computing Pearson correlation coefficients from interregional c-Fos expression in the 63 re-
427 gions. Mean correlations were calculated to assess changes in functional connectivity between
428 these major subdivisions of the brain. Weighted undirected networks were constructed by consid-
429 ering correlations with Pearson's $r \geq 0.82$, corresponding to a one-tailed significance level of $P < 0.05$
430 (uncorrected). The nodes in the networks represent brain regions, and the correlations that sur-
431 vived thresholding were considered connections. Theoretical graph analysis was performed using

432 Brain Connectivity Toolbox (<https://sites.google.com/site/bctnet/>, version 2019-03-03) in MATLAB
433 R2021 (The MathWorks Inc.) (*Rubinov and Sporns, 2010*). Network visualization was performed
434 using Cytoscape (version 3.2.1) (*Shannon et al., 2003*).

435 **Hub identification**

436 Network centrality was evaluated using degree, betweenness, and eigenvector centrality measures
437 to identify potential hub regions (*Guimera and Nunes Amaral, 2005*). Degree counts the number of
438 edges connected to a node, signifying its immediate influence. Betweenness centrality is gauged
439 by the number of shortest paths passing through a node, indicating its role as a connector or
440 'bridge' within the network. Eigenvector centrality measures a node's influence by the centrality
441 of its connections, valuing nodes linked to well-connected neighbors. High eigenvector centrality
442 indicates significant influence through these high-quality connections within the network.

443 **Statistical analysis**

444 Sample size was determined based on prior studies (*Lu et al., 2008; Yatziv et al., 2020*). Data are
445 presented as mean \pm SEM. In Figures 4 and 5, independent t-tests evaluating differences in c-Fos
446 expression for KET and ISO treatments were corrected for multiple comparisons using the Ben-
447 jamini, Krieger, and Yekutieli method, with a 5% false discovery rate (Q) (*Benjamini and Hochberg,*
448 *1995*). Pearson correlation coefficients (R) were transformed into Z-scores using Fisher's Z trans-
449 formation before computing group means and making statistical comparisons in Figure 6—figure
450 supplement 1D. All statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Soft-
451 ware, USA) and MATLAB R2021 (Mathworks Inc.).

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460 **Author contributions**

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462 original draft, Writing—review and editing; Jiangtao Qi, Conceptualization, Software, Formal anal-
463 ysis; Zhao Zhang, Resources, Investigation, Supervision, Writing—review and editing; Mengqiang
464 Luo, Resources, Supervision, Funding acquisition, Project administration, Writing—review and edit-
465 ing; Yingwei Wang, Conceptualization, Resources, Supervision, Funding acquisition, Project admin-
466 istration, Writing—review and editing.

467 **Competing Interest Statement**

468 All authors declare no competing financial interest.

469 **References**

- 470 **Bekkers JM**, Suzuki N. Neurons and circuits for odor processing in the piriform cortex. *Trends Neurosci.* 2013;
471 36(7):429–38. <https://www.ncbi.nlm.nih.gov/pubmed/23648377>, doi: 10.1016/j.tins.2013.04.005.
- 472 **Benjamini Y**, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple
473 Testing. *Journal of the Royal Statistical Society Series B (Methodological)*. 1995; 57(1):289–300. [http://www.](http://www.jstor.org/stable/2346101)
474 [jstor.org/stable/2346101](http://www.jstor.org/stable/2346101).

- 475 **Bliss TV**, Collingridge GL, Kaang BK, Zhuo M. Synaptic plasticity in the anterior cingulate cortex in acute and
476 chronic pain. *Nat Rev Neurosci*. 2016; 17(8):485–96. doi: [10.1038/nrn.2016.68](https://doi.org/10.1038/nrn.2016.68).
- 477 **Cheriyian J**, Sheets PL. Altered Excitability and Local Connectivity of mPFC-PAG Neurons in a Mouse Model of
478 Neuropathic Pain. *J Neurosci*. 2018; 38(20):4829–4839. doi: [10.1523/jneurosci.2731-17.2018](https://doi.org/10.1523/jneurosci.2731-17.2018).
- 479 **Cho JR**, Treweek JB, Robinson JE, Xiao C, Bremner LR, Greenbaum A, Gradinaru V. Dorsal Raphe Dopamine
480 Neurons Modulate Arousal and Promote Wakefulness by Salient Stimuli. *Neuron*. 2017; 94(6):1205–1219.e8.
481 doi: [10.1016/j.neuron.2017.05.020](https://doi.org/10.1016/j.neuron.2017.05.020).
- 482 **Deng J**, Zhou H, Lin JK, Shen ZX, Chen WZ, Wang LH, Li Q, Mu D, Wei YC, Xu XH, Sun YG. The
483 Parabrachial Nucleus Directly Channels Spinal Nociceptive Signals to the Intralaminar Thalamic Nuclei, but
484 Not the Amygdala. *Neuron*. 2020; 107(5):909–923 e6. <https://www.ncbi.nlm.nih.gov/pubmed/32649865>, doi:
485 [10.1016/j.neuron.2020.06.017](https://doi.org/10.1016/j.neuron.2020.06.017).
- 486 **Do JP**, Xu M, Lee SH, Chang WC, Zhang S, Chung S, Yung TJ, Fan JL, Miyamichi K, Luo L, Dan Y. Cell type-specific
487 long-range connections of basal forebrain circuit. *Elife*. 2016; 5. [https://www.ncbi.nlm.nih.gov/pubmed/](https://www.ncbi.nlm.nih.gov/pubmed/27642784)
488 [27642784](https://doi.org/10.7554/eLife.13214), doi: [10.7554/eLife.13214](https://doi.org/10.7554/eLife.13214).
- 489 **Duncan GE**, Moy SS, Knapp DJ, Mueller RA, Breese GR. Metabolic mapping of the rat brain after subanesthetic
490 doses of ketamine: potential relevance to schizophrenia. *Brain Res*. 1998; 787(2):181–90. <https://www.ncbi.nlm.nih.gov/pubmed/9518601>, doi: [10.1016/s0006-8993\(97\)01390-5](https://doi.org/10.1016/s0006-8993(97)01390-5).
- 492 **Feng H**, Wen SY, Qiao QC, Pang YJ, Wang SY, Li HY, Cai J, Zhang KX, Chen J, Hu ZA, Luo FL, Wang GZ, Yang N,
493 Zhang J. Orexin signaling modulates synchronized excitation in the sublateralodorsal tegmental nucleus to
494 stabilize REM sleep. *Nat Commun*. 2020; 11(1):3661. doi: [10.1038/s41467-020-17401-3](https://doi.org/10.1038/s41467-020-17401-3).
- 495 **Franks NP**. General anaesthesia: from molecular targets to neuronal pathways of sleep and arousal. *Nat Rev*
496 *Neurosci*. 2008; 9(5):370–86. <https://www.nature.com/articles/nrn2372.pdf>, doi: [10.1038/nrn2372](https://doi.org/10.1038/nrn2372).
- 497 **Gao C**, Leng Y, Ma J, Rooke V, Rodriguez-Gonzalez S, Ramakrishnan C, Deisseroth K, Penzo MA. Two genet-
498 ically, anatomically and functionally distinct cell types segregate across anteroposterior axis of paraven-
499 tricular thalamus. *Nat Neurosci*. 2020; 23(2):217–228. <https://www.ncbi.nlm.nih.gov/pubmed/31932767>, doi:
500 [10.1038/s41593-019-0572-3](https://doi.org/10.1038/s41593-019-0572-3).
- 501 **Gelegen C**, Miracca G, Ran MZ, Harding EC, Ye Z, Yu X, Tossell K, Houston CM, Yustos R, Hawkins ED, Vyssotski
502 AL, Dong HL, Wisden W, Franks NP. Excitatory Pathways from the Lateral Habenula Enable Propofol-Induced
503 Sedation. *Curr Biol*. 2018; 28(4):580–587.e5. doi: [10.1016/j.cub.2017.12.050](https://doi.org/10.1016/j.cub.2017.12.050).
- 504 **Guimera R**, Nunes Amaral LA. Functional cartography of complex metabolic networks. *Nature*. 2005;
505 433(7028):895–900. <https://www.ncbi.nlm.nih.gov/pubmed/15729348>, doi: [10.1038/nature03288](https://doi.org/10.1038/nature03288).
- 506 **Gupta RG**, Schafer C, Ramarosan Y, Sciuillo MG, Horn CC. Role of the abdominal vagus and hindbrain in in-
507 halational anesthesia-induced vomiting. *Auton Neurosci*. 2017; 202:114–121. [https://www.ncbi.nlm.nih.gov/](https://www.ncbi.nlm.nih.gov/pubmed/27396693)
508 [pubmed/27396693](https://doi.org/10.1016/j.autneu.2016.06.007), doi: [10.1016/j.autneu.2016.06.007](https://doi.org/10.1016/j.autneu.2016.06.007).
- 509 **Han B**, McCarren HS, O'Neill D, Kelz MB. Distinctive recruitment of endogenous sleep-promoting neu-
510 rons by volatile anesthetics and a nonimmobilizer. *Anesthesiology*. 2014; 121(5):999–1009. doi:
511 [10.1097/aln.000000000000383](https://doi.org/10.1097/aln.000000000000383).
- 512 **Hase T**, Hashimoto T, Saito H, Uchida Y, Kato R, Tsuruga K, Takita K, Morimoto Y. Isoflurane induces c-Fos
513 expression in the area postrema of the rat. *J Anesth*. 2019; 33(4):562–566. doi: [10.1007/s00540-019-02662-](https://doi.org/10.1007/s00540-019-02662-0)
514 [0](https://doi.org/10.1007/s00540-019-02662-0).
- 515 **Hauer BE**, Pagliardini S, Dickson CT. Prefrontal-Hippocampal Pathways Through the Nucleus Reunians Are
516 Functionally Biased by Brain State. *Front Neuroanat*. 2021; 15:804872. [https://www.ncbi.nlm.nih.gov/pmc/](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8842257/pdf/fnana-15-804872.pdf)
517 [articles/PMC8842257/pdf/fnana-15-804872.pdf](https://doi.org/10.3389/fnana.2021.804872), doi: [10.3389/fnana.2021.804872](https://doi.org/10.3389/fnana.2021.804872).
- 518 **Hemmings J H C**, Riegelhaupt PM, Kelz MB, Solt K, Eckenhoff RG, Orser BA, Goldstein PA. Towards a Compre-
519 hensive Understanding of Anesthetic Mechanisms of Action: A Decade of Discovery. *Trends Pharmacol Sci*.
520 2019; 40(7):464–481. doi: [10.1016/j.tips.2019.05.001](https://doi.org/10.1016/j.tips.2019.05.001).
- 521 **Hua T**, Chen B, Lu D, Sakurai K, Zhao S, Han BX, Kim J, Yin L, Chen Y, Lu J, Wang F. General anesthetics activate
522 a potent central pain-suppression circuit in the amygdala. *Nat Neurosci*. 2020; 23(7):854–868. [https://www.](https://www.nature.com/articles/s41593-020-0632-8.pdf)
523 [nature.com/articles/s41593-020-0632-8.pdf](https://doi.org/10.1038/s41593-020-0632-8), doi: [10.1038/s41593-020-0632-8](https://doi.org/10.1038/s41593-020-0632-8).

- 578 **Mashour GA**, Hudetz AG. Bottom-Up and Top-Down Mechanisms of General Anesthetics Modulate Different
579 Dimensions of Consciousness. *Front Neural Circuits*. 2017; 11:44. [https://www.ncbi.nlm.nih.gov/pubmed/](https://www.ncbi.nlm.nih.gov/pubmed/28676745)
580 [28676745](https://www.ncbi.nlm.nih.gov/pubmed/28676745), doi: 10.3389/fncir.2017.00044.
- 581 **Mashour GA**, Pal D, Brown EN. Prefrontal cortex as a key node in arousal circuitry. *Trends Neurosci*. 2022;
582 45(10):722–732. doi: 10.1016/j.tins.2022.07.002.
- 583 **Moore JT**, Chen J, Han B, Meng QC, Veasey SC, Beck SG, Kelz MB. Direct Activation of
584 Sleep-Promoting VLPO Neurons by Volatile Anesthetics Contributes to Anesthetic Hypno-
585 sis. *Current Biology*. 2012; 22(21):2008–2016. <https://doi.org/10.1016/j.cub.2012.08.042>.
586 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
587 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
588 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
589 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
590 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
591 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
592 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
593 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
594 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
595 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
596 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
597 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
598 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
599 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
600 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
601 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
602 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
603 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
604 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
605 <https://www.ncbi.nlm.nih.gov/pubmed/2272099>, doi: 10.1016/j.cub.2012.08.042.
- 606 **Morgan JI**, Curran T. Stimulus-transcription coupling in neurons: role of cellular immediate-early genes. *Trends*
607 *Neurosci*. 1989; 12(11):459–62. doi: 10.1016/0166-2236(89)90096-9.
- 608 **Musacchio T**, Rebenstorff M, Fluri F, Brotchie JM, Volkmann J, Koprach JB, Ip CW. Subthalamic nucleus deep
609 brain stimulation is neuroprotective in the A53T α -synuclein Parkinson's disease rat model. *Ann Neurol*.
610 2017; 81(6):825–836. doi: 10.1002/ana.24947.
- 611 **Nagata A**, Nakao S, Miyamoto E, Inada T, Tooyama I, Kimura H, Shingu K. Propofol inhibits ketamine-induced
612 c-fos expression in the rat posterior cingulate cortex. *Anesth Analg*. 1998; 87(6):1416–20. <https://www.ncbi.nlm.nih.gov/pubmed/9842840>, doi: 10.1097/00000539-199812000-00040.
- 613 <https://www.ncbi.nlm.nih.gov/pubmed/9842840>, doi: 10.1097/00000539-199812000-00040.
- 614 **Nagata A**, Nakao S, Nishizawa N, Masuzawa M, Inada T, Murao K, Miyamoto E, Shingu K. Xenon inhibits but
615 N(2)O enhances ketamine-induced c-Fos expression in the rat posterior cingulate and retrosplenial cortices.
616 *Anesth Analg*. 2001; 92(2):362–8. doi: 10.1097/00000539-200102000-00016.
- 617 **Nakao S**, Arai T, Mori K, Yasuhara O, Tooyama I, Kimura H. High-dose ketamine does not induce c-Fos protein
618 expression in rat hippocampus. *Neurosci Lett*. 1993; 151(1):33–6. doi: 10.1016/0304-3940(93)90038-m.
- 619 **Nakao S**, Miyamoto E, Masuzawa M, Kambara T, Shingu K. Ketamine-induced c-Fos expression in the mouse
620 posterior cingulate and retrosplenial cortices is mediated not only via NMDA receptors but also via sigma
621 receptors. *Brain Res*. 2002; 926(1-2):191–6. doi: 10.1016/s0006-8993(01)03338-8.
- 622 **Nelson LE**, Guo TZ, Lu J, Saper CB, Franks NP, Maze M. The sedative component of anesthesia is mediated by
623 GABA(A) receptors in an endogenous sleep pathway. *Nat Neurosci*. 2002; 5(10):979–84. doi: 10.1038/nn913.
- 624 **Reimann HM**, Niendorf T. The (Un)Conscious Mouse as a Model for Human Brain Functions: Key Principles of
625 Anesthesia and Their Impact on Translational Neuroimaging. *Front Syst Neurosci*. 2020; 14:8. <https://www.ncbi.nlm.nih.gov/pubmed/32508601>, doi: 10.3389/fnsys.2020.00008.
- 626 <https://www.ncbi.nlm.nih.gov/pubmed/32508601>, doi: 10.3389/fnsys.2020.00008.
- 627 **Rubinov M**, Sporns O. Complex network measures of brain connectivity: uses and interpre-
628 tations. *Neuroimage*. 2010; 52(3):1059–69. <https://www.ncbi.nlm.nih.gov/pubmed/19819337>, doi:
629 [10.1016/j.neuroimage.2009.10.003](https://www.ncbi.nlm.nih.gov/pubmed/19819337).

- 630 **Schroeder KE**, Irwin ZT, Gaidica M, Nicole Bentley J, Patil PG, Mashour GA, Chestek CA. Disruption of corticocortical information transfer during ketamine anesthesia in the primate brain. *Neuroimage*. 2016; 134:459–465. doi: [10.1016/j.neuroimage.2016.04.039](https://doi.org/10.1016/j.neuroimage.2016.04.039).
- 633 **Shannon P**, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research*. 2003; 13(11):2498–2504. <https://pubmed.ncbi.nlm.nih.gov/14597658><https://www.ncbi.nlm.nih.gov/pmc/articles/PMC403769/>, doi: [10.1101/gr.1239303](https://doi.org/10.1101/gr.1239303).
- 637 **Smith ML**, Li J, Cote DM, Ryabinin AE. Effects of isoflurane and ethanol administration on c-Fos immunoreactivity in mice. *Neuroscience*. 2016; 316:337–43. <https://www.ncbi.nlm.nih.gov/pubmed/26742790>, doi: [10.1016/j.neuroscience.2015.12.047](https://doi.org/10.1016/j.neuroscience.2015.12.047).
- 640 **Tasaka GI**, Feigin L, Maor I, Groysman M, DeNardo LA, Schiavo JK, Froemke RC, Luo L, Mizrahi A. The Temporal Association Cortex Plays a Key Role in Auditory-Driven Maternal Plasticity. *Neuron*. 2020; 107(3):566–579 e7. <https://www.ncbi.nlm.nih.gov/pubmed/32473095>, doi: [10.1016/j.neuron.2020.05.004](https://doi.org/10.1016/j.neuron.2020.05.004).
- 643 **Villarreal CF**, Del Bel EA, Prado WA. Involvement of the anterior pretectal nucleus in the control of persistent pain: a behavioral and c-Fos expression study in the rat. *Pain*. 2003; 103(1-2):163–74. <https://www.ncbi.nlm.nih.gov/pubmed/12749971>, doi: [10.1016/s0304-3959\(02\)00449-9](https://doi.org/10.1016/s0304-3959(02)00449-9).
- 646 **Wheeler AL**, Teixeira CM, Wang AH, Xiong X, Kovacevic N, Lerch JP, McIntosh AR, Parkinson J, Frankland PW. Identification of a functional connectome for long-term fear memory in mice. *PLoS Comput Biol*. 2013; 9(1):e1002853. <https://www.ncbi.nlm.nih.gov/pubmed/23300432>, doi: [10.1371/journal.pcbi.1002853](https://doi.org/10.1371/journal.pcbi.1002853).
- 649 **Xu W**, Wilson DA. Odor-evoked activity in the mouse lateral entorhinal cortex. *Neuroscience*. 2012; 223:12–20. <https://www.sciencedirect.com/science/article/pii/S0306452212008159>, doi: <https://doi.org/10.1016/j.neuroscience.2012.07.067>.
- 652 **Yap EL**, Greenberg ME. Activity-Regulated Transcription: Bridging the Gap between Neural Activity and Behavior. *Neuron*. 2018; 100(2):330–348. <https://www.ncbi.nlm.nih.gov/pubmed/30359600>, doi: [10.1016/j.neuron.2018.10.013](https://doi.org/10.1016/j.neuron.2018.10.013).
- 655 **Yatziv SL**, Yudco O, Dickmann S, Devor M. Patterns of neural activity in the mouse brain: Wakefulness vs. General anesthesia. *Neurosci Lett*. 2020; 735:135212. <https://www.ncbi.nlm.nih.gov/pubmed/32593772>, doi: [10.1016/j.neulet.2020.135212](https://doi.org/10.1016/j.neulet.2020.135212).
- 658 **Yeung JC**, Yaksh TL, Rudy TA. Concurrent mapping of brain sites for sensitivity to the direct application of morphine and focal electrical stimulation in the production of antinociception in the rat. *Pain*. 1977; 4(1):23–40. <https://www.ncbi.nlm.nih.gov/pubmed/927879>, doi: [10.1016/0304-3959\(77\)90084-7](https://doi.org/10.1016/0304-3959(77)90084-7).
- 661 **Yi T**, Wang N, Huang J, Wang Y, Ren S, Hu Y, Xia J, Liao Y, Li X, Luo F, Ouyang Q, Li Y, Zheng Z, Xiao Q, Ren R, Yao Z, Tang X, Wang Y, Chen X, He C, et al. A Sleep-Specific Midbrain Target for Sevoflurane Anesthesia. *Adv Sci (Weinh)*. 2023; p. e2300189. <https://www.ncbi.nlm.nih.gov/pubmed/36961096>, doi: [10.1002/advs.202300189](https://doi.org/10.1002/advs.202300189).
- 664 **Zerbi V**, Floriou-Servou A, Markicevic M, Vermeiren Y, Sturman O, Privitera M, von Ziegler L, Ferrari KD, Weber B, De Deyn PP, Wenderoth N, Bohacek J. Rapid Reconfiguration of the Functional Connectome after Chemogenetic Locus Coeruleus Activation. *Neuron*. 2019; 103(4):702–718 e5. <https://www.ncbi.nlm.nih.gov/pubmed/31227310>, doi: [10.1016/j.neuron.2019.05.034](https://doi.org/10.1016/j.neuron.2019.05.034).
- 668 **Zhang D**, Liu J, Zhu T, Zhou C. Identifying c-fos Expression as a Strategy to Investigate the Actions of General Anesthetics on the Central Nervous System. *Curr Neuropharmacol*. 2022; 20(1):55–71. <https://www.ncbi.nlm.nih.gov/pubmed/34503426>, doi: [10.2174/1570159X19666210909150200](https://doi.org/10.2174/1570159X19666210909150200).

Table 1. Figure 4—source data 1. Summary of prior studies on ketamine activated brain regions identified via c-Fos immunostaining.

Brain regions	Function	Treatment Regimen	Duration	Time of day	Effect	References
CeA	Pain Suppression	100mg/kg and 10mg/kg xylazine i.p.	2h	/	Up-regulated	Hua T, et al.,(2022) (<i>Hua et al., 2020</i>)
SO	Natural sleep/General anesthesia	100mg/kg with or without xylazine(10mg/kg) i.p.	2h	/	Up-regulated	Jiang-Xie LF, et al.,(2019) (<i>Jiang-Xie et al., 2019</i>)
pCC, RSC	/	100mg/kg i.p.	2h	/	Up-regulated	Nagata A, et al.,(1998) (<i>Nagata et al., 1998</i>)
RSP, CeA, Midline thalamic nuclei	Antidepressant	10mg/kg i.p.	1.5h	/	Up-regulated	Inta D, et al.,(2009) (<i>Inta et al., 2009</i>)
Cg, BF, Orx, TMN, vPAG, LC, LHb, EW, midline thalamus	LC/vPAG engaged in analgesia	150mg/kg; 60mg/kg; 30mg/kg i.p.	2h	ZT3	Up-regulated	Lu J, et al.,(2008) (<i>Lu et al., 2008</i>)
CC, PVT	/	100mg/kg i.p.	/	/	/	Nakao S, et al.,(1993) (<i>Nakao et al., 1993</i>)
PC, RSP	/	50mg/kg i.p.	2h	ZT3-9	Up-regulated	Nakao S, et al.,(2002) (<i>Nakao et al., 2002</i>)

Table 2. Figure 5—source data 1. Summary of prior studies on brain regions activated by isoflurane, as detected through c-Fos immunostaining.

Brain regions	Function	Concentration of ISO	Duration	Time of day	Effect	References
VLPO	Sleep-Promoting, Hypnosis	1.2%, 0.6%, 0.3%	2h	ZT12-14/ZT4-6	Up-regulated	Moore JT, et al.,(2012) (<i>Moore et al., 2012</i>)
PIR, LSD, LSV	/	4% 5mins+1%	2h	ZT2-7	Up-regulated	Smith ML, et al.,(2016) (<i>Smith et al., 2016</i>)
NTS, VeN	Vomiting	1% and 3%	1.5h	/	Up-regulated	Gupta RG, et al.,(2016) (<i>Gupta et al., 2017</i>)
CeA	Pain Suppression	1.5%	2h	/	Up-regulated	Hua T, et al.,(2022) (<i>Hua et al., 2020</i>)
SO	Natural sleep, General anesthesia	1%,1.2%	2h	/	Up-regulated	Jiang-Xie LF, et al.,(2019) (<i>Jiang-Xie et al., 2019</i>)
VLPO, MnPO	Sleep and unconsciousness	1.2%	2h	ZT14-17	Up-regulated	Han B, et al.,(2022) (<i>Han et al., 2014</i>)
EW, VLPO, LC	Sleep-Promoting, analgesia	2%	2h	ZT12	Up-regulated	Lu J, et al.,(2008) (<i>Lu et al., 2008</i>)
AP	Nausea, vomiting	1.3%,2.6%	2h	/	Up-regulated	Hase T, et al.,(2019) (<i>Hase et al., 2019</i>)

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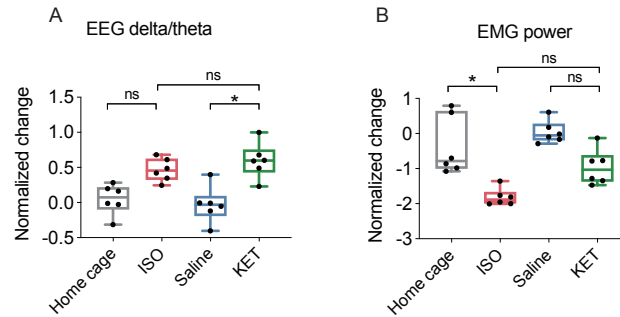


Figure 1—figure supplement 1. EEG and EMG power change after each treatment. The box represents the 25th-75th percentiles; the central line denotes the median; whiskers indicate maximum and minimum values. $n = 6, 6, 8, 6$ for the home cage, ISO, saline, and KET, respectively. (A) Normalized change in EEG power: ISO vs KET, $P > 0.99$; Saline vs KET, $P = 0.01$; Home cage vs ISO, $P = 0.11$. (B) Normalized change in EMG power: ISO vs KET, $P = 0.36$; Saline vs KET, $P = 0.30$; Home cage vs ISO, $P = 0.02$. Analyses were conducted using the Kruskal-Wallis test, followed by Dunn's multiple comparisons tests.

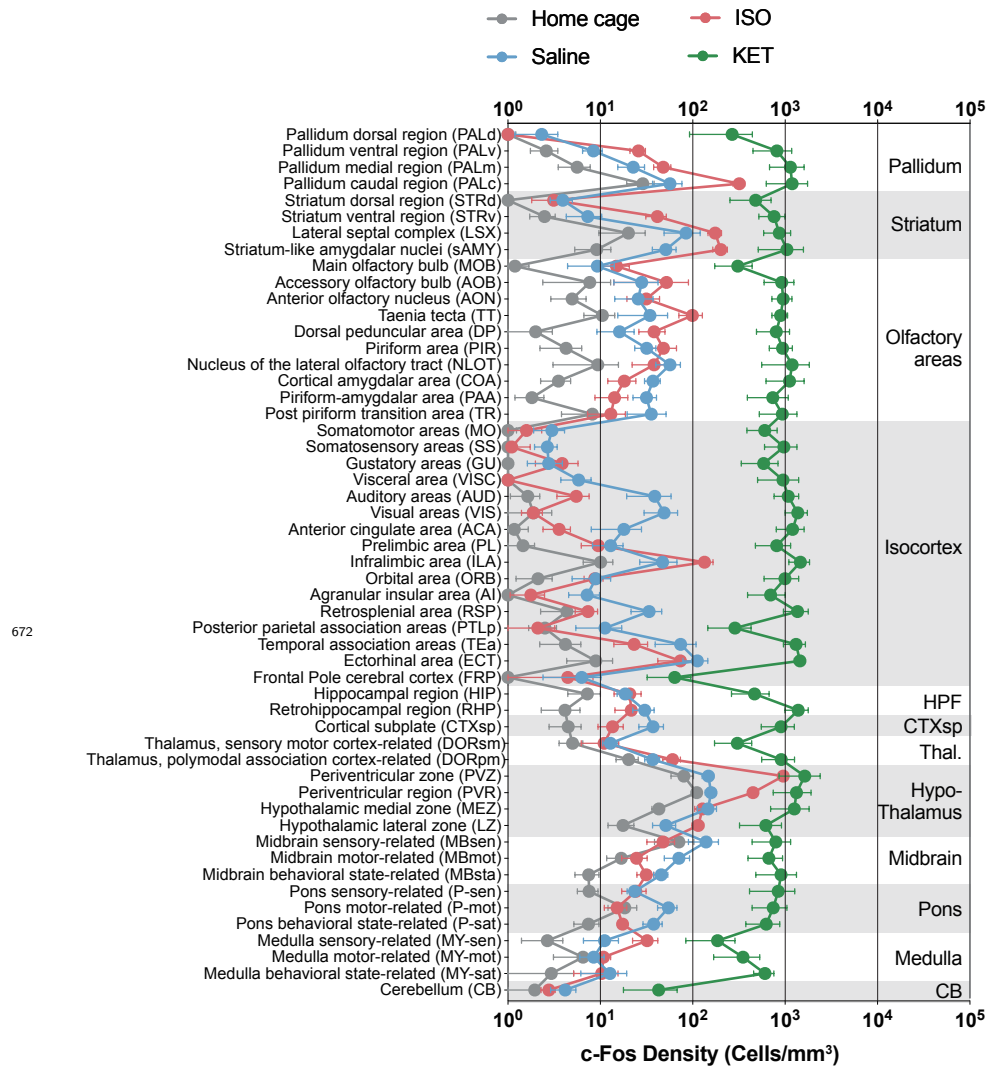


Figure 2—figure supplement 1. The c-Fos density in 53 brain areas for different conditions. (home cage, n = 6; ISO, n = 6 mice; saline, n = 8; KET, n = 6). Each point represents the c-Fos density in a specific brain region, denoted on the y-axis with both abbreviations and full names. Data are shown as mean ± SEM. Brain regions are categorized into 12 brain structures, as indicated on the right side of the graph.

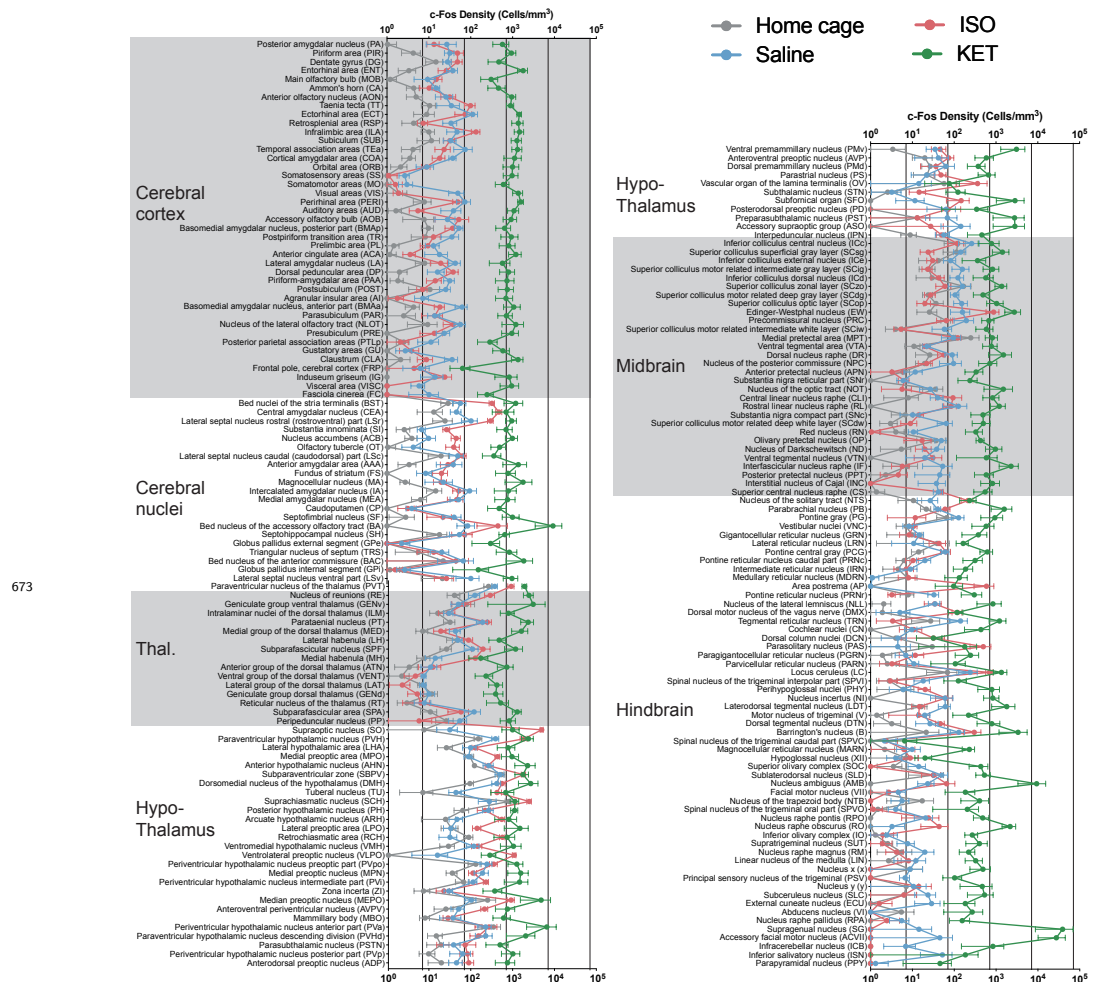
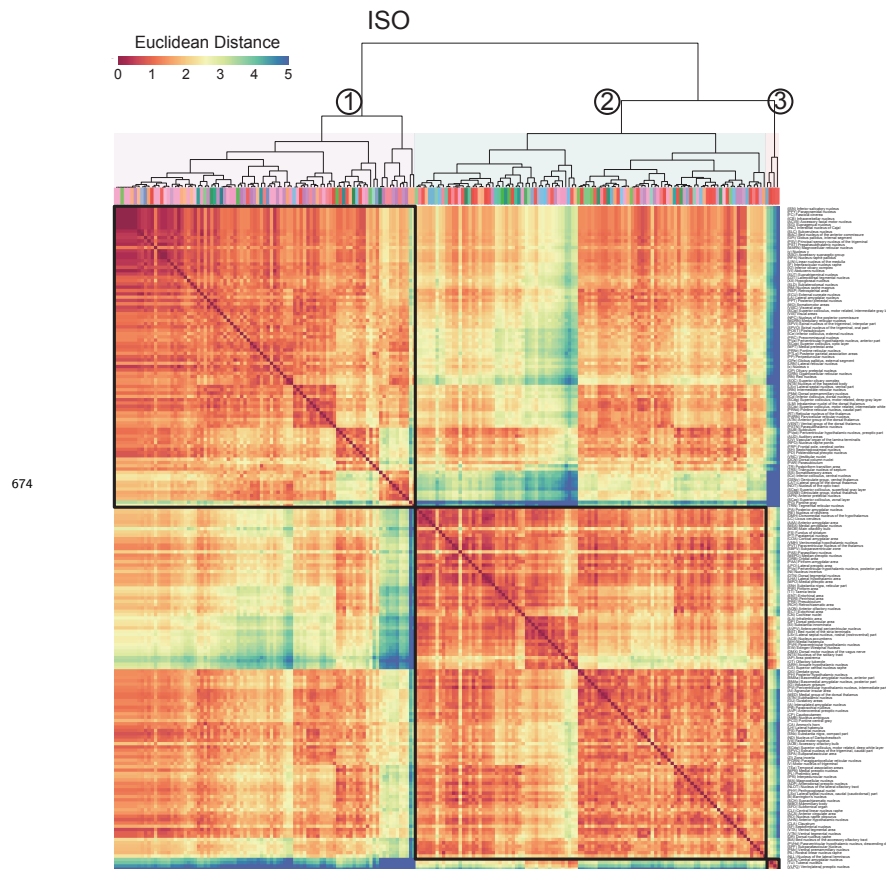


Figure 3—figure supplement 1. c-Fos density visualization across 201 distinct brain regions under various conditions. The graph depicts the c-Fos density levels for each condition, with data presented as mean and standard error. Brain regions with statistically significant differences are featured in Figures 4 and 5. Brain regions are organized into major anatomical subdivisions, as indicated on the left side of the graph.



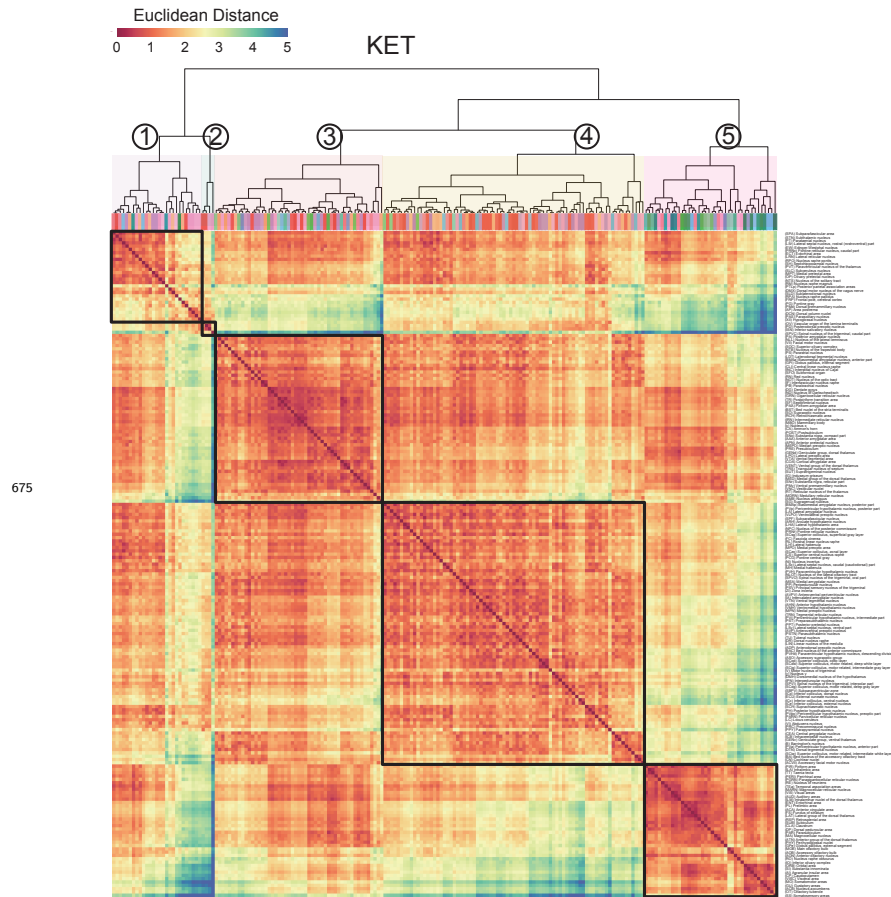


Figure 3—figure supplement 3. Region labels for the hierarchical clustering of the KET group in Figure 3A.

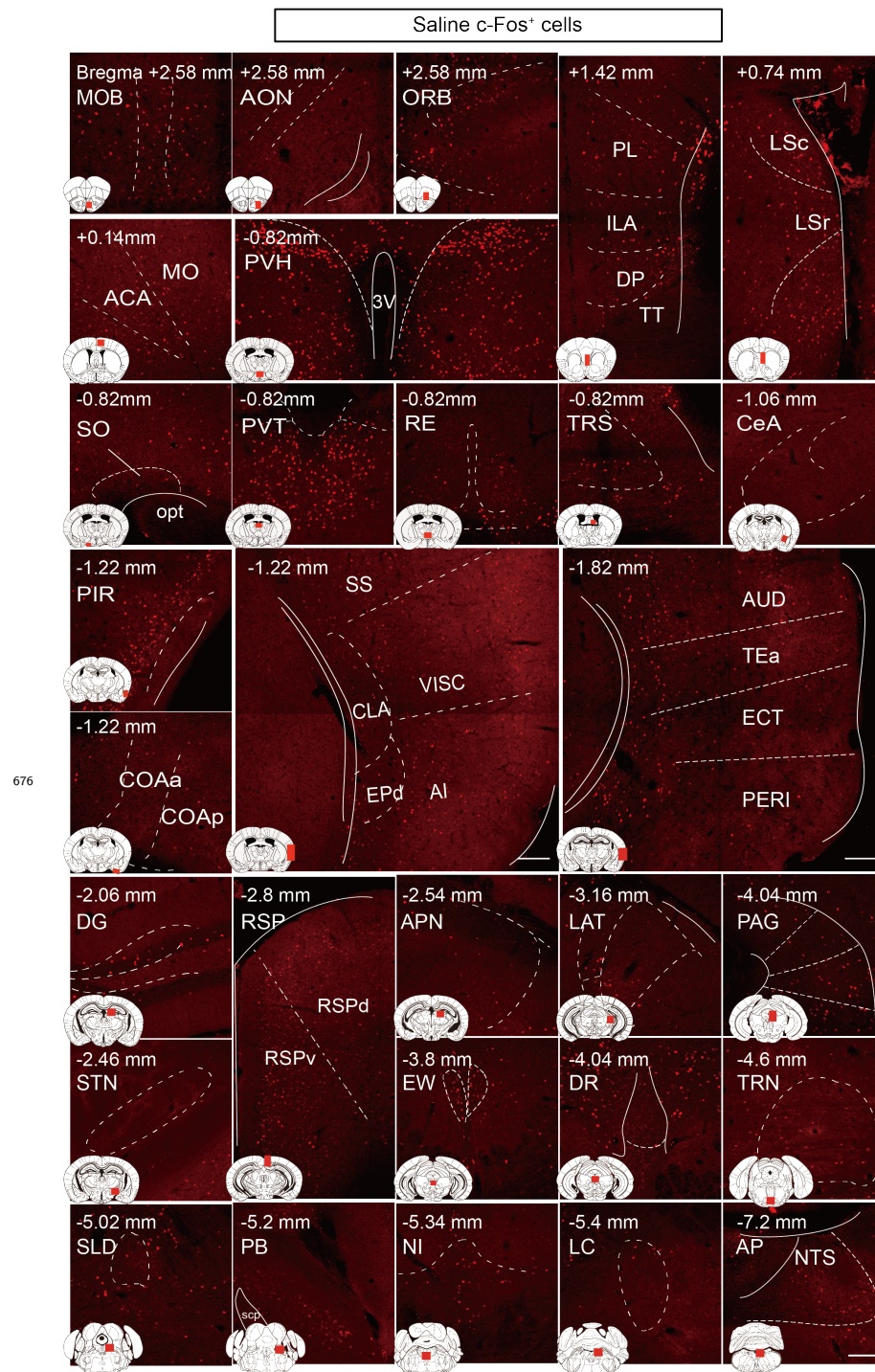


Figure 4—figure supplement 1. c-Fos expression in specific brain regions following normal saline administration. (A) Representative immunohistochemical staining of c-Fos⁺ cells in MOB, AON, ORB, MPO, ACA, MO, TRS, PL, ILA, DP, LS, PVT, SO, PVH, RE, VISC, AI, CLA, EPd, PIR, COA, AUD, TEa, ECT, PERI, CeA, SS, DG, STN, RSP, APN, LAT, EW, DR, PAG, SLD, PB, TRN, NI, LC, and NTS. Scale bar represents 200 μ m.

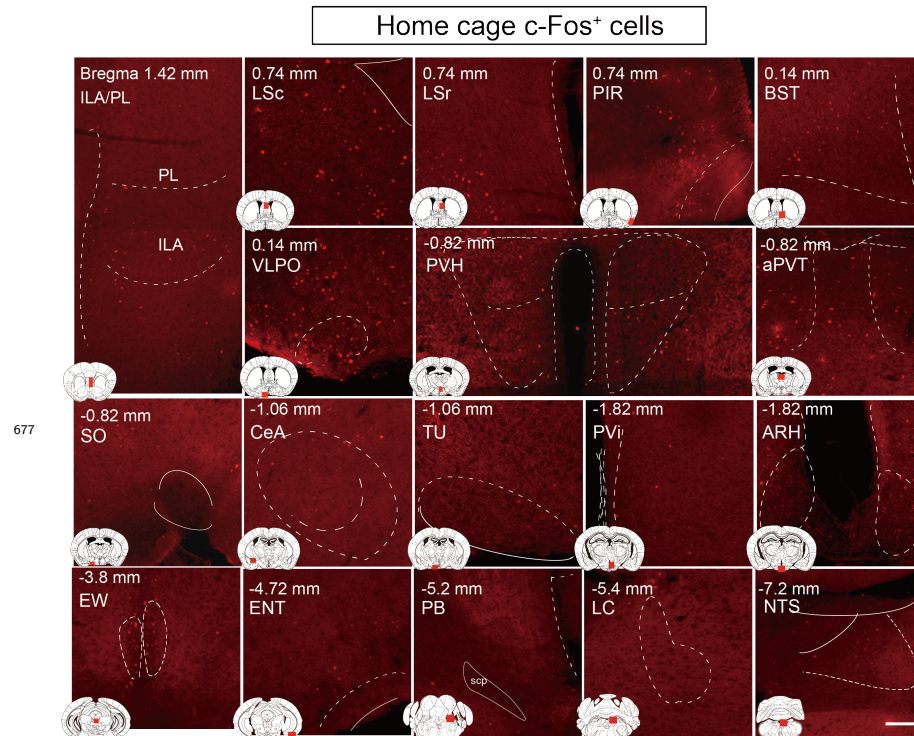


Figure 5—figure supplement 1. c-Fos expression in home cage group. (A) Representative immunohistochemical staining of c-Fos⁺ cells in PL, ILA, LSc, LSc, PIR, BST, VLPO, PVH, aPVT, SO, CeA, TU, PVi, ARH, EW, ENT, PB, LC, and NTS c-Fos⁺ cells from the indicated mice. Scale bar, 200 μ m.

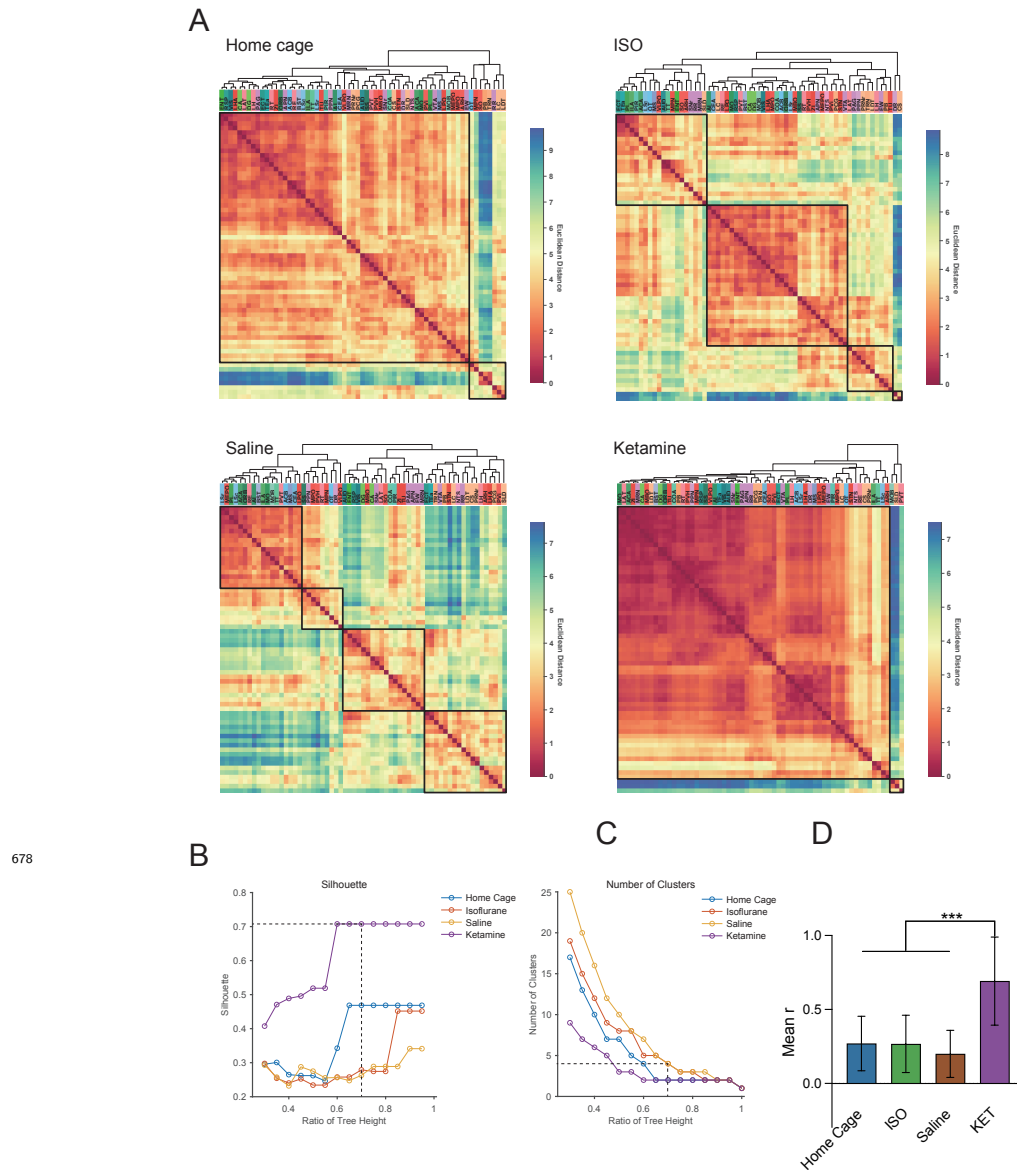


Figure 6—figure supplement 1. Hierarchical clustering across different conditions. (A) Heatmaps depict the hierarchical clustering of brain regions within the home cage, ISO, saline, and KET groups, using Euclidean distance matrices calculated from correlation coefficients. Each brain region is represented by its abbreviation, with full names and expression levels detailed in Figure 3—figure supplement 1. Modules are demarcated within each dendrogram at a cut-off threshold of 0.7. (B) Silhouette scores are plotted against the dendrogram tree height ratio for each condition, with optimal cluster definition indicated by a dashed line at a 0.7 ratio. (C) The number of clusters formed at different cutoff levels. At a ratio of 0.7, ISO and saline treatments result in three clusters, whereas home cage and KET conditions yield two clusters. (D) The mean Pearson's correlation coefficient (r) was computed from interregional correlations displayed in Figure 6A. Data were analyzed using one-way ANOVA with Tukey's post hoc test, *** $P < 0.001$.

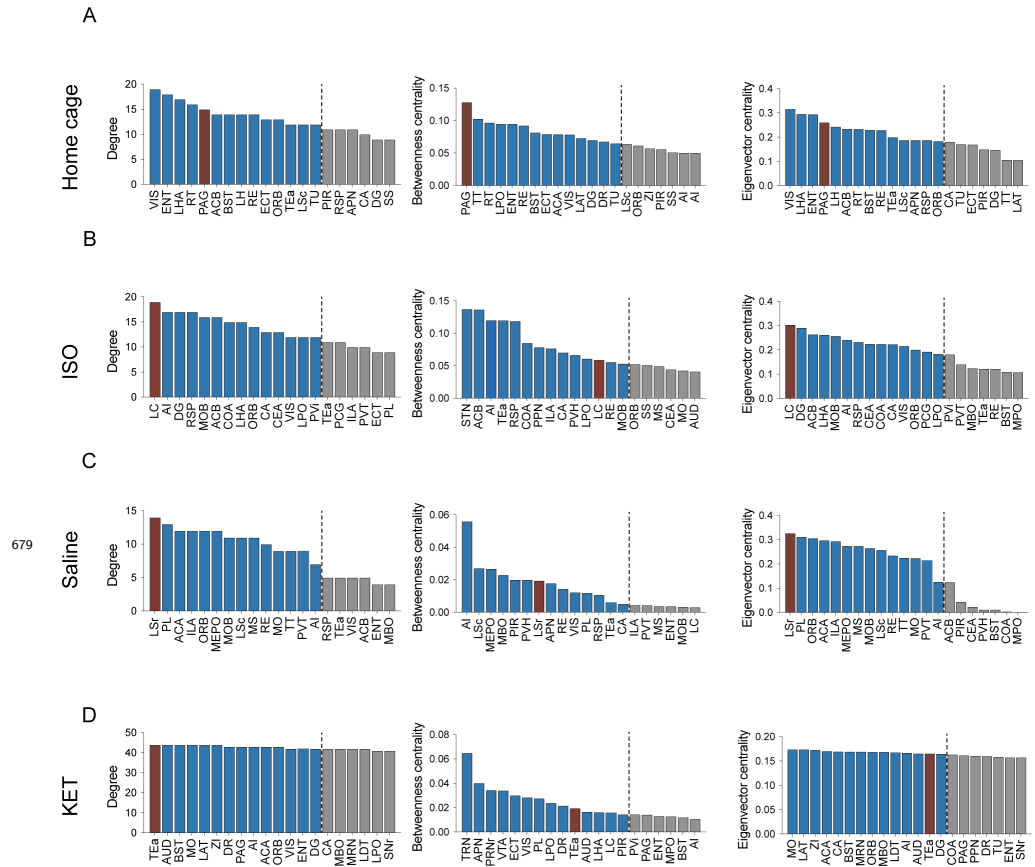


Figure 6—figure supplement 2. Hub region characterization across different conditions: home cage (A), ISO (B), saline (C), and KET (D) treatments. Brain regions are sorted by degree, betweenness centrality, and eigenvector centrality, with each metric presented in separate bar graphs. Bars to the left of the dashed line indicate the top 20% of regions by rank, highlighting the most central nodes within the network. Red bars signify regions that consistently appear within the top rankings for both degree and betweenness centrality across the metrics.