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# 1      **Encoding social preference by interhemispheric neurons in the Insula**

2

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11

12      **Keywords:** Insula interhemispheric neurons, social preference, anxiety,  
13      connectivity, network

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## 17      **Abstract**

18      The Insula is a multisensory relay that participates in socio-emotional processing  
19      through multiple projections to sensory, cognitive, emotional, and motivational  
20      regions. Interestingly, the Insula interhemispheric projection to the contralateral  
21      Insula is a strong but understudied projection. Using cutting-edge neuroanatomy,  
22      *ex vivo* and *in vivo* electrophysiology associated with specific circuit manipulation,  
23      we unraveled the nature and role of Insula interhemispheric communication in  
24      social and anxiety processing in mice. In this study, we 1) characterized the  
25      anatomical and molecular profile of the interhemispheric neurons of the Insula, 2)  
26      highlighted that stimulation of this neuronal subpopulation triggers excitation in  
27      the Insula interhemispheric circuit 3) uncovered their engagement in social  
28      processing. In conclusion, this study demonstrates that interhemispheric neurons  
29      of the Insula constitute a unique class of Insula neurons and proposes new  
30      meaningful insights into the neuronal mechanisms underlying social behavior.

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## 1 Introduction

2 The Insular Cortex is classically described as an integrator of multimodal sensory  
3 signals coming from external cues (the environment) and internal cues (the body  
4 changes). For example, Insula responds to auditory or tactile cues<sup>1</sup> and to cardiac  
5 interoceptive signals<sup>2</sup>. Interacting with novel individuals is an experience that leads  
6 to the integration of signals from both interoceptive and exteroceptive sources.  
7 Recently, it has been shown that some Insula cells respond to social interaction<sup>3</sup>.  
8 Interestingly these “social-on” cells solely represent a subset of Insula neurons  
9 that remained unexplored. In physiological situations, Insula neurons are engaged  
10 in social interaction and notably in social affective behaviors<sup>4-9</sup>. For example, it has  
11 been highlighted that Insula neurons projecting to the nucleus accumbens core  
12 regulate the social approach to stressed juvenile rats<sup>8</sup>. Autism spectrum Disorders  
13 (ASD) and Anxiety Disorders are pathologies with sensory integration defects that  
14 have been associated with dysfunction of the Insula<sup>1,1,10-13</sup>. An Insula maturation  
15 deficit was detected in a mouse model of ASD which is notably characterized by  
16 social interaction deficits, leading to an alteration in the integration of sensory  
17 information within the Insula<sup>1</sup>. Moreover, clinical studies show an Insula  
18 overactivation in anxious patients<sup>10,14</sup>. Altogether, these studies suggest that  
19 Insula is well-positioned to integrate and participate in regulating of socio-  
20 emotional processing. Indeed, the Insula shares multiple projections with sensory  
21 and interoceptive regions (sensory cortex, thalamus, olfactory bulb), with  
22 cognitive regions (medial prefrontal cortex, orbitofrontal cortex), emotional  
23 territories (amygdala, bed nucleus of the stria terminalis), and motivation-  
24 associated structures (ventral tegmental area, nucleus accumbens)<sup>15</sup>.

25 A strong but understudied projection is the Insula interhemispheric projection to  
26 the contralateral Insula<sup>16</sup>. As alteration in interhemispheric communication is  
27 associated with a social deficit<sup>17-20</sup>, we postulated that Insula interhemispheric  
28 communication is essential to develop adaptive reactions when facing novel social  
29 cues or threatening situations.

30 Recent evidence points toward a crucial role of cortical interhemispheric  
31 communication in complex cognitive and emotional processing. For example,  
32 individuals with high anxiety levels present an altered interhemispheric  
33 communication in reaction to the presentation of emotional images<sup>21</sup>. Across  
34 mammalian evolution, cortical interhemispheric communication occurs notably  
35 through the corpus callosum<sup>22</sup>. Alterations in callosal fiber integrity have been  
36 observed in several pathological conditions as in patients with strokes, multiple  
37 sclerosis, schizophrenia, or ASD<sup>23,24</sup>. Despite recent advances in Insula  
38 participation in social behavior, the anatomical and molecular profile and the role  
39 of the Insula interhemispheric circuit in this socio-emotional processing remained  
40 poorly understood.

41 We hypothesized that Insula interhemispheric communication is essential to  
42 regulate social interactions and anxiety phenotype, and alteration in this  
43 communication would lead to social impairments and maladaptive anxiety

1 behavior. To define the critical, yet unknown role of the Insula interhemispheric  
2 circuit, we used a combination of innovative neurotechniques, *in vivo*  
3 electrophysiology, and behavioral assays coupled with selective genetic neuron  
4 ablation and circuit manipulation in mice. This study was developed around 3  
5 specific objectives: **i)** Anatomical and molecular characterization of Insula  
6 interhemispheric neurons, **ii)** Synaptic and circuit properties of Insula  
7 interhemispheric communication **iii)** Role of Insula interhemispheric  
8 communication in social interaction and anxiety-related behaviors in mice.

9

## 10 **Results**

### 11 **Interhemispheric Insula neurons represent a unique subpopulation of** 12 **the Insula.**

13 We first confirmed that Insula project to multiple brain regions by using an  
14 anterograde monosynaptic viral approach (Supp. Fig 1a). Interestingly, we  
15 observed a strong bilateral innervation to the dorsolateral part of the bed nucleus  
16 of the stria terminalis (dBNST), the Central Amygdala (CeA), and contralateral  
17 labeling to the Insula (Supp. Fig 1b-f). To identify the projection targets of this  
18 Insula to-Insula circuit, we first mapped Insula interhemispheric neuron outputs,  
19 by injecting a retrograde monosynaptic virus (rAAV2-retro-CAG-Cre) in the  
20 contralateral Insula coupled with an anterograde monosynaptic virus (AAV2-DIO-  
21 eif1a-eYFP) injection in the ipsilateral Insula (Fig 1a). We showed that Insula  
22 interhemispheric neurons also projected massively to the dBNST and the CeA (Fig  
23 1b-g). In addition, we targeted the same interhemispheric Insula neuron  
24 population by injecting the retrograde monosynaptic virus in the CeA and the  
25 anterograde monosynaptic virus in the ipsilateral Insula. We confirmed that CeA-  
26 projecting Insula neurons also innervate both the dBNST and the contralateral  
27 Insula (Supp Fig 1g-j). Next, we injected a retrograde monosynaptic virus into the  
28 Insula of AI9 dTomato mice (Fig 1h). We observed tomato-positive neurons in the  
29 contralateral Insula which are thus interhemispheric Insula neurons and represent  
30 homotopic labeling (Fig 1i-j). We quantified that  $85.92 \pm 2.64$  % of cortical  
31 contralateral labeling was located in the homotopic cortical region and  $14.08 \pm$   
32  $2.64$  % in heterotopic cortical regions (Fig 1k-l). More precisely, we noted 44 % of  
33 homotopic labeling in the intermediate Insula, 43% in the posterior Insula, and 13  
34 % in the anterior Insula (Supp Fig 1l). Insula interhemispheric neurons were  
35 mainly located in layer II/III (Supp Fig1m-n). We found 70.93 % of Insula  
36 interhemispheric neurons in layer II/III and 29.07 % in layer V/VI.

37 We next molecularly characterized these Insula interhemispheric neurons that  
38 project to dBNST and CeA. Interhemispheric neurons identified with tomato  
39 labeling specifically colocalized with *Satb2* molecular marker without any  
40 colocalization with *Ctip2*, two transcriptional factors implicated in cortical  
41 development and maturation (Tomato<sup>+</sup>/*Satb2*<sup>+</sup> colocalization:  $96.13 \pm 1.91\%$ , Fig  
42 1m-q). We next determined whether Insula interhemispheric neurons are

1 exclusively pyramidal neurons or if they could be GABAergic projection neurons.  
2 No colocalization of Insula interhemispheric neurons with parvalbumin (PV) or  
3 glutamic acid decarboxylase (GAD67) staining was detected (Supp Fig 1o-q),  
4 thereby confirming that these Insula interhemispheric neurons belong to the  
5 category of excitatory pyramidal neurons.

6 By using *in vivo* electrophysiology in anesthetized mice, we functionally  
7 confirmed the reciprocal connectivity between both Insula (Fig 1r-s). Indeed, we  
8 recorded typical antidromic responses characterized by a collision test and, or  
9 high-frequency stimulation tests evoked by the electrical stimulation of their  
10 terminals in the contralateral Insula (Fig 1r, s). Interestingly, we observed a large  
11 variability in the latencies of antidromic responses (ranging from 3 to 30 ms; Fig  
12 1t). One parameter influencing the action potential velocity conduction is the  
13 degree of myelination. Intriguingly, the Insula is a unique and specific cortical  
14 region that poorly expresses the myelin basic protein (MBP), an oligodendrocyte  
15 protein essential for myelin wrapping of axons in adult mice (Fig 1u-v). By using  
16 the double viral approach to identify Insula interhemispheric neurons (with GFP  
17 labeling) coupled with electron microscopy preparation, we showed that all Insula  
18 interhemispheric neurons observed had unmyelinated axons passing through the  
19 corpus callosum or the anterior commissure (Fig 1w-y; 0 GFP<sup>+</sup> myelinated axons  
20 out of n=59 GFP<sup>+</sup> neurons, N=4 mice).

21 Thus, we highlight a novel neuronal subpopulation in the Insula that is the Insula  
22 interhemispheric pyramidal subpopulation characterized by bilateral projections to  
23 both dIBNST and CeA, mainly located in layer II/III, with a specific expression of  
24 the molecular marker Satb2<sup>+</sup> and unmyelinated axons.

## 25 **Insula interhemispheric neurons provide a synaptic-excitatory drive on** 26 **Insula interhemispheric circuit**

27 We demonstrated that Insula interhemispheric neurons make asymmetric  
28 synapses, which are excitatory in function, with the contralateral Insula, the CeA,  
29 and the dIBNST (Fig2 a-d). Insula to CeA and to dIBNST synapses have been  
30 previously described<sup>25-29</sup>. However, the Insula to Insula synapses remained poorly  
31 characterized. Studies that have functionally studied interhemispheric synaptic  
32 transmission have mainly studied the motor cortex and have demonstrated the  
33 importance of inhibition of the contralateral cortex in the execution of lateralized  
34 movements<sup>30-32</sup>. The Insula is involved in integrating of exteroceptive and  
35 interoceptive signals, contralateral inhibition does not seem necessary for the  
36 execution of emotional tasks. To complete our anatomical data, we tested the  
37 hypothesis that Insula stimulation may trigger an excitation in the contralateral  
38 Insula side by using *ex vivo* and *in vivo* electrophysiology in mice. First, we  
39 recorded contralateral Insula pyramidal neuron responses evoked by ipsilateral  
40 Insula optogenetic stimulation by using *ex vivo* electrophysiology (Fig 2e-g). We  
41 found that 87.5 % of the total recorded Insula pyramidal neurons respond by an  
42 excitation followed by inhibition to the ipsilateral Insula fiber optogenetic  
43 stimulation while 12.5% of these pyramidal neurons respond only by an excitation

1 (Fig 2h-j; EPSC amplitude  $-267.9 \pm 41.64$  pA; IPSC amplitude  $582.1 \pm 125.9$  pA).  
2 In addition, only inhibitory current is blocked by TTX+4AP pharmacological cocktail  
3 bath application suggesting that excitatory transmission is monosynaptic while  
4 inhibitory current is polysynaptic (Fig 2k; EPSC amplitude in aCSF:  $-242.8 \pm 59.29$   
5 pA; EPSC amplitude in TTX+4AP:  $-247.3 \pm 101.8$  pA; IPSC amplitude in aCSF:  
6  $508.2 \pm 123.4$  pA; IPSC amplitude in TTX + 4AP:  $5.167 \pm 5.02$  pA, IPSC amplitude  
7 aCSF vs TTX + 4AP: Wilcoxon test  $W=21, p=0.0313$ ; EPSC amplitude aCSF vs  
8 TTX+4AP: Wilcoxon test  $W=1; p>0.05$ ). IPSC response latency is also delayed  
9 compared to EPSC response latency (EPSC latency:  $1.88 \pm 0.22$  ms; IPSC latency:  
10  $4.46 \pm 0.25$  ms, Mann-Whitney test  $U=2, p<0.0001$ ). These data suggest that  
11 activation of Insula interhemispheric neurons drives monosynaptic excitation on  
12 Insula contralateral pyramidal neurons followed by polysynaptic feedforward  
13 inhibition.

14 Secondly, to decipher the net *in vivo* integrative effect of Insula interhemispheric  
15 transmission, we performed *in vivo* electrophysiology in anesthetized mice (Fig  
16 2m). We observed that 32.73 % of all contralateral Insula recorded neurons  
17 respond to ipsilateral Insula electrical stimulation (Fig 2p). These insula-responsive  
18 neurons are characterized by a half-action potential width of  $1.08 \pm 0.03$  ms and  
19 a spontaneous firing frequency of  $0.78 \pm 0.13$  Hz (Fig 2n-o). Insula  
20 interhemispheric neuronal stimulation triggered excitatory responses on the  
21 contralateral Insula neurons with  $10.61 \pm 1.715$  ms response latency (Fig 2q-s).

22 Together, these results suggest that interhemispheric neurons contact both  
23 excitatory and inhibitory contralateral insula neurons, and feed-forward inhibition  
24 was activated within  $\sim 2.5$  ms after the onset of excitation in both cell types,  
25 creating a precise temporal excitation in the Insula network.

26

## 27 **Genetic selective ablation of Insula interhemispheric communication** 28 **disrupts social preference following acute social isolation**

29 Lastly, to determine whether Insula interhemispheric communication plays  
30 a role in social interaction and anxiety processing, we measured mouse social  
31 interaction with a three-chamber social test in two different housing conditions  
32 associated with a caspase viral approach strategy to selectively lesion Insula  
33 interhemispheric neurons, leading to split Insula mice (Fig 3a-b). Since rodents  
34 are innately pro-social species, social isolation represents an aversive experience.  
35 Previous studies have shown that structures involved in the Insula  
36 interhemispheric network are recruited and display plastic adaptive neuronal  
37 responses after acute isolation such as the dorsal raphe nucleus or the dBNST<sup>33,34</sup>.  
38 To elucidate whether Insula interhemispheric neurons are crucial to developing  
39 adaptive social behavior after this aversive event, we assessed social preference  
40 in group-housed conditions and 24 h after acute social isolation in the control group  
41 mice and the caspase group. We injected a retrograde monosynaptic virus (rAAV2-  
42 retro-CAG-Cre) in two main outputs of the insula interhemispheric neurons

1 (contralateral Insula and ipsilateral CeA) and an AAV-Flex-taCaspase-TEVp or the  
2 control virus (AAV-Flex-eGFP) in the ipsilateral Insula (Fig 3a). We first confirmed  
3 that the caspase viral strategy specifically lesioned Insula interhemispheric  
4 neurons as illustrated in the histological control example and quantified by NeuN  
5 fluorescence density in layer II/III of Insula (Fig 3c-e). NeuN fluorescence density  
6 is specifically decreased in the layer II/III of the Insula caspase injection site  
7 compared to its contralateral Insula control site (Insula control site NeuN density:  
8  $44.15 \pm 2.37$ ; Insula caspase injection site NeuN density:  $34.01 \pm 2.86$ , Two-tailed  
9 Paired-t-Test,  $t(8)=2.788$ ,  $p=0.0236$ ) without altering other proximal cortical  
10 regions as the somatosensory cortex (NeuN density in Somatosensory cortex  
11 control site:  $31.28 \pm 2.33$ ; NeuN density in the other Somatosensory cortex site:  
12  $33.96 \pm 0.91$ , Two-tailed paired t-Test  $t(8)=1.101$ ,  $p>0.05$ ). Under the group-  
13 housed condition, both control and caspase mice spent more time around the social  
14 enclosure compared to the object enclosure without differences in the three-  
15 chamber test (Fig 3f, time spent around for ctrl: object  $65.2 \pm 7.4$  s vs social  $149.4$   
16  $\pm 9.6$  s; caspase: object  $57.14 \pm 5.9$  s vs social  $144.9 \pm 10.72$  s; Two Way  
17 repeated measure Anova, zone x virus interaction effect  $F(1,18)=0.02973$ ,  
18  $p>0.05$ ; zone main effect  $F(1,18)=67.17$ ,  $p<0.0001$ ; virus main effect,  $F$   
19  $(1,18)=0.9348$ ,  $p>0.05$ ). Control and caspase mice developed a social preference  
20 in the group-housed condition indicated by a social preference ratio higher than  
21 0.5 (Fig 3g; ctrl social preference ratio:  $0.69 \pm 0.04$  and caspase social preference  
22 ratio  $0.72 \pm 0.02$ ; ctrl vs caspase social preference ratio, Mann-Whitney  $U=47$ ,  
23  $p>0.05$ ; One sample Wilcoxon test for ctrl:  $W=66$ ,  $p=0.001$ , and caspase:  $W=45$ ,  
24  $p=0.0039$ ). They spent a similar amount of time in the social zone (Fig 3h-i; time  
25 in the social zone for ctrl:  $49.79 \pm 3.2$  %; for caspase:  $48.31 \pm 3.57$  %; two-tailed  
26 unpaired t-test,  $t(18)=0.3089$ ,  $p>0.05$ ; mean social bout duration for ctrl:  $9.4 \pm$   
27  $0.71$ %; for caspase:  $8.13 \pm 0.78$  %; two-tailed unpaired t-test,  $t(18)=1.197$ ).

28 We next tested how social isolation affects attention to social stimuli in split  
29 Insula mice. After acute social isolation, ctrl mice spent more time around the  
30 social enclosure compared to the object enclosure while caspase mice spent the  
31 same time around both enclosures (Fig 3j, time spent around for ctrl: object  $80.49$   
32  $\pm 5.25$  s vs social  $133.7 \pm 7$  s; caspase: object  $92.91 \pm 9.31$ s vs social  $105 \pm 8.51$   
33 s; Two-Way repeated measure Anova, Zone x virus interaction effect,  
34  $F(1,18)=5.011$ ,  $p=0.0381$ ; zone main effect  $F(1,18)=12.65$ ,  $p=0.0023$ ; virus  
35 main effect  $F(1,18)=0.3213$ ,  $p>0.05$ ; Bonferroni *post hoc* test ctrl social caspase  
36 social  $p=0.02$ ; ctrl social vs control object:  $p=0.0008$ ). Control group mice still  
37 presented social preference after acute isolation whereas caspase mice did not,  
38 despite significant differences between groups (Fig 3k; ctrl social preference ratio:  
39  $0.62 \pm 0.03$  and caspase social preference ratio  $0.53 \pm 0.04$ ; Two-tailed unpaired  
40 t-test,  $t(18)=1.956$ ,  $p=0.0662$ ; One sample t-test for ctrl:  $t(10)=4.69$ ,  $p=0.0009$ ;  
41 for caspase:  $t(8)=0.8273$ ,  $p>0.05$ ). Caspase mice spent less time in the social  
42 zone compared to control mice only after acute social isolation (Fig 3l; time in the  
43 social zone for ctrl:  $44.58 \pm 2.33$  %; for caspase:  $35.01 \pm 2.84$  %; Two-tailed  
44 Unpaired t-Test,  $t(18)=2.632$ ,  $p=0.0169$ ). There was no difference in the mean  
45 social bout duration between groups (Fig 3m; mean social bout duration for ctrl:

1 7.8 ± 0.72 s; for caspase: 6.17 ± 0.58 s; Two-tailed unpaired t-test,  $t(18)=1.707$ ,  
2  $p>0.05$ ).

3 Insula is activated during anxious situations and Insula overactivation has  
4 been detected in patients with Anxiety disorders <sup>11,14,35-39</sup>. Since Insula  
5 interhemispheric neurons project to CeA and dBNST, we next investigated the  
6 impact of Insula interhemispheric communication split on unconditioned anxiety  
7 tests in mice. In rodents, anxiety can be measured based on the innate  
8 approach/avoidance behaviour in a novel environment. We didn't detect a change  
9 in the time spent and the number of visits in the center of the open field, nor in  
10 the total distance travelled (Supp Fig 2 a-d, Time spent in the center for ctrl: 16.65  
11 ± 2.06 %; caspase: 17.47 ± 1.45 %, two-tailed unpaired t-test,  $t(18)=0.3088$ ,  
12  $p>0.05$ ; the number of visits in the center for ctrl: 57.45 ± 5.42 visits; caspase:  
13 62.89 ± 4.26 visits, two-tailed unpaired t-test,  $t(18)=0.7612$ ,  $p>0.05$ ; total  
14 distance travelled for ctrl: 4367 ± 309.3 cm; caspase: 4526 ± 214.2 cm, two-  
15 tailed unpaired t-test,  $t(18)=0.404$ ,  $p>0.05$  ). In addition, we didn't observe a  
16 difference in the time spent and the number of entries in the open arms in the  
17 elevated plus maze nor in the total distance travelled between control and caspase  
18 mice (Supp Fig 2 e-h, Time spent in the open arms, ctrl: 4.47 ± 1.01 %; caspase:  
19 5.24 ± 1.37 %, Mann-Whitney,  $u=41.5$ ,  $p>0.05$ ; the number of entries in the OA  
20 for ctrl: 4.091 ± 0.72 visits; caspase: 3.89 ± 0.89 visits, Two-tailed Unpaired-t-  
21 test,  $t(18)=0.1788$ ,  $p>0.05$ ; total distance travelled for ctrl: 967 ± 80.99 cm;  
22 caspase: 1117 ± 69.14 cm, Two-tailed unpaired t-test,  $t(18)=1.375$ ,  $p>0.05$  ).

23 These data show that Insula interhemispheric communication split leads to  
24 impairment of social preference only after acute social isolation without interfering  
25 with anxiety-like behaviors.

## 26 **Discussion**

27 We unraveled the anatomical and molecular phenotype of an  
28 interhemispheric neuronal subpopulation in the Insula that belongs to a restricted  
29 network enrolling both bilateral dBNST/CeA and contralateral Insula, mainly  
30 located in the layer II/III, characterized by unmyelinated axons and expressing  
31 the transcriptional factor *Satb2*<sup>+</sup>. Our findings enlightened the contribution of the  
32 Insula interhemispheric neurons in social processing. Selective ablation of Insula  
33 interhemispheric neurons leads to a reduced interest in social stimulus after acute  
34 social isolation which is a maladaptive behavior. This data suggests that the Insula  
35 interhemispheric communication split created an imbalance in social homeostasis  
36 processes.

37 Pioneering studies including lesioning approaches and split-brain patient  
38 cases who presented surgical callosal incisions shed light on brain lateralized  
39 functions<sup>40,41</sup>. One of the most studied cases of cortical interhemispheric  
40 communication has been described in the motor cortex region. For instance, the  
41 execution of lateralized motor movement requires inhibition of the contralateral  
42 side induced by interhemispheric cortical inhibition<sup>30</sup>. The degree of myelination



1 of axons directly impacts the efficiency of interhemispheric communication.  
2 Decreased efficiency in interhemispheric inhibition has been observed in children  
3 who are characterized by a hypo-myelination of callosal neurons resulting in  
4 difficulties in generating unilateral motor movement and leading to non-lateralized  
5 mirror movements<sup>42</sup>. The nature and the recruitment of cortical interhemispheric  
6 communication may depend on the type and the complexity of the performed task  
7 as well as the cortex involved<sup>43</sup>. Despite mammalian evolution, communication  
8 between two brain hemispheres presents some similarities across species between  
9 rodents, non-human primates, and humans. Here, we found that stimulation of  
10 Insula interhemispheric neurons leads to excitation of the Insula contralateral side  
11 in mice (Fig 2h-s).

12 In general, myelination of axons which is a dynamic process ensures a fast  
13 and precise transfer of information to the targeted zone. Thus, the degree of  
14 myelination is one of the parameters that influence the conduction velocity and  
15 define the efficiency of neuronal communication. Unexpectedly, the entire  
16 population of interhemispheric neurons in the insular cortex are unmyelinated  
17 neurons in adult mice under physiological conditions (Fig 1x, y). We confirmed this  
18 phenomenon by a low density in MBP immunostaining in the Insula (Fig 1u,v)  
19 contrary to what has been described in other cortical regions<sup>44 45</sup>. This lack of  
20 myelination on Insula interhemispheric axons may explain the variability in their  
21 onset latency in response to axonal stimulation (Fig 1t). Future studies would be  
22 required to understand this atypical Insula signature and the potential implication  
23 of myelination process within Insula interhemispheric neurons at synaptic, circuit  
24 and behavioural levels in physiological and pathological states.

25 In this study, we found that selective ablation of Insula interhemispheric  
26 neurons impaired social preference only following acute social isolation (Fig3 j-l).  
27 After this aversive event, control mice developed adaptive behavior that favours  
28 social interactions compared to object interactions which restore social  
29 homeostasis. Caspase mice did not present this appropriate strategy after acute  
30 social isolation which can suggest reduced attention toward social stimulus and/or  
31 a lack of motivation for orienting to social cues. Interestingly, a clinical study  
32 monitoring the Insula interhemispheric communication evoked by the presentation  
33 of social versus non-social stimuli in children with neurotypical development or  
34 with ASD, reported that only children with ASD presented an hyperconnectivity of  
35 this pathway<sup>12</sup>. Together, these results suggest that Insula interhemispheric  
36 communication may be recruited when social homeostasis is unbalanced to  
37 promote adaptive and appropriately motivated behavior to seek for social contacts.  
38 Here, we demonstrated that the interhemispheric neurons occupy a privileged  
39 position in synchronizing the activity of the Insula/CeA/dBNST network in the two  
40 hemispheres. Interestingly, interhemispheric neurons of the Insula and  
41 dopaminergic neurons of the Dorsal Raphe Nucleus also project massively to the  
42 dBNST and the CeA<sup>46</sup>. Future studies need to elucidate how the  
43 dopamine/glutamate interplay controls the CeA/dBNST network. Thus, all of these  
44 data suggest that interhemispheric neurons in the Insula play a specific role for

1 social processing. This specificity is confirmed because we did not detect an anxiety  
2 phenotype after Insula interhemispheric deletion by using an open field or an  
3 elevated plus maze (Supp Fig2).

4 Here, we assessed a right unilateral lesion of Insula interhemispheric neurons by  
5 using a double viral approach (Fig 3a). One limitation of our study is that our viral  
6 strategy may minimize the effect observed by manipulating a smaller quantity of  
7 cells. However, to be selective of Insula interhemispheric neurons without  
8 targeting interneurons (Supp Fig 1o-q), we restricted our ablation to the right  
9 Insula interhemispheric neurons. Despite this limitation, our viral approach allowed  
10 a selective ablation of right interhemispheric neurons in the Insula which can be  
11 useful for future investigations of Insula lateralization. Indeed, a lateralization of  
12 Insula with a right dominance has been observed with cFos analysis in response  
13 to intraperitoneal injection of lithium chloride, an aversive visceral stimulus or in  
14 feeding behavior<sup>47,48</sup>. Additional studies would be necessary to define the Insula  
15 laterization processes. This study has been carried on adult male mice and future  
16 detailed analysis will be valuable to dissect age- and sex-dependent effects.

17 Our study, by demonstrating the role played by the interhemispheric  
18 neurons of the Insula in social processing, reinforces the concept of cellular  
19 diversity of the Insula<sup>27,37,45,48-52</sup>. Another avenue for future research motivated by  
20 the present study will be to examine the development, maturation and  
21 neuromodulation of this interhemispheric Insula circuit in physiological and  
22 pathological states where social processing is altered as in the ASD mouse model.  
23  
24  
25

## 26 **Author contributions**

27 C.G. and F.G conceived and designed the experiments. C.G. performed and  
28 analyzed the anatomical study with the participation of E.L. and M.G. E.D., E.B.  
29 performed the data with electron microscopy. C.G and A.G. performed and  
30 analyzed the *in vivo* electrophysiology in anesthetized mice. A.T and J.B. designed,  
31 performed, and analyzed the *ex vivo* electrophysiological experiments. C.G.  
32 performed and analyzed the behavioural study. C.G. and F.G. wrote the  
33 manuscript and C.G. prepared the figures.  
34

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4 Center a service unit of the CNRS-INSERM and Bordeaux University, member of  
5 the national infrastructure France BioImaging supported by the French National  
6 Research Agency (ANR-10-INBS-04). The help of Sebastien Marais is  
7 acknowledged.

#### 8 **Conflict of interests**

9 The authors declare no conflict of interest.

- 1 STAR Methods
- 2 Key resource table

<b>REAGENT or RESOURCE</b>	<b>SOURCE</b>	<b>IDENTIFIER</b>
<b>Antibodies</b>		
a mouse anti-Satb2 primary antibody	Abcam	Cat#ab51502; RRID: AB_882455
a rat anti-Ctip2 primary antibody	Abcam	Cat#ab18465; RRID: AB_2064130
a rat anti-MBP primary antibody	Merckmillipore	Cat#MAB386; RRID: AB_94975
a rabbit anti-GFP primary antibody	Millipore	Cat#ab3080; RRID: AB_91337
a guinea pig anti-PV primary antibody	Synaptic system Merckmillipore	Cat #195004; RRID: AB_2156476
a mouse anti-GAD67 primary antibody	Life technologies	Cat#MAB5406; RRID:AB_2278725
a guinea pig anti-Neun/Fox3	Synaptic System	Cat#266004; RRID:AB_2619988
a donkey anti-mouse secondary antibody alexa 647	Life technologies	Cat#A31571; RRID: AB_162542
a donkey anti-rat secondary antibody alexa 488	Invitrogene	Cat# A21208; RRID: AB_141709
a goat anti-guinea pig secondary antibody alexa 488	Invitrogen	Cat#A11073;
a goat anti-rabbit secondary antibody conjugated with gold particle	Nanoprobe	1.4 nm
a donkey anti-rabbit secondary antibody alexa 488	life technologies	Cat#A21206;
streptavidin alexa 557	R&D system	Cat#NL999; RRID: AB_10175722
<b>Bacterial and viral strains</b>		
rAAV2-retro-CAG-Cre	UNC vector	Ed Boyden
AAV2.2-eif1a-DIO-eYFP	Addgene	Karl Deisseroth cat#27056-AAV2
AAV2.2-hSyn-eYFP	Addgene	Bryan Roth cat#50465-AAV2
AAV2.5-eif1a-DIO-eYFP	Addgene	Cat#27056-AAV5 RRID:Addgene_27056
AAV2.2-hSyn-ChR2(H134R)-eYFP	UNC vector core	
AAV5-flex-taCasp3-TEVp	Addgene	Cat# 45580-AAV5; RRID:Addgene_45580
<b>Chemicals</b>		
Isoflurane	virbac	
Lurocaine	centravet	
Buprenorphine	virbac	
Rimadyl	centravet	
Exagon	centravet	
Normal Donkey Serum	Sigma-aldrich	Cat#D9663;RRID: AB_2810235
Normal Goat Serum	Sigma-aldrich	Cat#G9023
Fluoromont-G	Southern Biotechn	Cat#0100-01
Skye blue pontamine	Sigma-aldrich	Cat#C8679-25g
CNO	Bio-techn	Cat#4936/50
TTX	ABCAM	Cat#ab120055
4AP	Ascent scientific	Cat#ASC-122-100mg
<b>Experimental models:</b>		
<b>Organisms/strains</b>		
C57BL/6JRj mice	Janvier-Labs	
Ai9 tdTomato; Gt(Rosa)26Sortm6(CAG-tdTomato)Hze	Jackson	Cat#007909

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## Software and algorithms

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Prism 9	GraphPad	RRID:SCR_002798
NDP.view2	Hamamatsu	
Fiji software	Schindelin et al 2012	<a href="https://imagej.net/software/fiji/">https://imagej.net/software/fiji/</a>
Ethovision XT 16		RRID:SCR_000441
Spike2		RRID:SCR_000903
PClamp		RRID:SCR_011323
Zotero		RRID:SCR_013784
Inskape		RRID : SCR_013784

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## Other

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Epifluorescent microscope	Olympus BX63
Confocal microscope	Leica TCS SP5
Slide scanner	Nanozoomer 2.0HT

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1

2 Resource availability

3 Lead contact

4 Further information and requests for resources and reagents should be directed  
5 and will be fulfilled by the lead contact, François Georges: [francois.georges@u-](mailto:francois.georges@u-bordeaux.fr)  
6 [bordeaux.fr](mailto:francois.georges@u-bordeaux.fr).

7

8 Data and code availability

9 This paper does not report original code.

10

11 Materials availability

12 This study did not generate new unique reagents.

13

14 Experimental model and subject details

15 Animals

16 Male C57BL/6JRj ( $\geq 10$  week old; Elevage Janvier, France) were used. Male Ai9  
17 tdTomato also called as Gt(Rosa)26Sortm6(CAG-tdTomato)Hze (stock number  
18 007909, from Jackson; C57BL6/j genetic background) were also used. Mice were  
19 housed three to five per cage under controlled conditions (22-23°C, 40 % relative  
20 humidity, 12 h light/dark illumination cycle; with lights on at 07:00). Mice were  
21 acclimatized to laboratory conditions at least one week prior to experiments, with  
22 food and water *ad libidum*. All procedures were conducted in accordance with  
23 European directive 2010-63-EU and with approval from the Bordeaux University  
24 Animal Care and Use Committee (license authorization 21134).

25

## 1 Methods details

### 2 Viruses and Drugs

3 rAAV2-retro-CAG-Cre (2,8x10<sup>12</sup> vg/mL ; UNC Vector Core, Boyden) ;AAV2.2-  
4 eif1a-DIO-eYFP (3x10<sup>12</sup> vg/mL ; Addgene); AAV2.5-eif1a-DIO-eYFP (1x10<sup>13</sup>  
5 vg/mL ; Addgene);AAV2.2-hSyn-eYFP (3x10<sup>12</sup> vg/mL ; 50465-  
6 AAV2,Addgene); AAV2.5—eif1a-DIO-eYFP (1x10<sup>13</sup> vg/mL ; 27056-AAV5,  
7 Addgene); AAV2.2-hSyn-ChR2(H134R)-eYFP (3.1x10<sup>12</sup> vg/mL ; UNC, AV4384G);  
8 AAV5-flex-taCasp3-TEVp (7x10<sup>12</sup> vg/mL; Addgene); Tetrodotoxin (TTX, 0.5 μM,  
9 abcam ab120055); 4 aminopyridine (4AP; 1 mM, ascent scientific, asc-122-  
10 100mg)

### 11 Surgery

12 Stereotaxic surgery for anatomy, *ex vivo* and *in vivo* electrophysiology  
13 experiments, and behavioral tests were performed under a mixture of isoflurane  
14 and oxygen as previously described<sup>53</sup>. Mice were placed on a stereotaxic frame  
15 and received a subcutaneous dose of buprenorphine (0.1 mg/kg, except for *in vivo*  
16 electrophysiology experiments) and local injection of an analgesic prior to skin  
17 incision (lurocaine, 7mg/kg). Single or bilateral craniotomy was made over the  
18 insular cortex at the following coordinates (+0.14 mm/bregma, ±3.8 mm/midline,  
19 2.2 mm/brain surface), the CeA (-1.58 mm/bregma, +2.4 mm/midline, 3.9  
20 mm/brain surface). Viruses were injected via a glass micropipette into the region  
21 of interest. Following injections, the incision was closed with sutures and mice were  
22 let to wake up on a heating plate. For all the experiments the virus was incubated  
23 at least four weeks before proceeding with further manipulation except for the  
24 experiment with the retrograde virus (rAAV2-retro-CAG-cre) injection in AI9  
25 dtTomato in which only two weeks were sufficient to clearly identify reporter  
26 protein expression.

27

### 28 Immunohistochemistry.

29 Mice were deeply anesthetized with a mixture of isoflurane and oxygen and  
30 received an i.p. lethal dose of exagon (300 mg/kg) and lidocaine (30mg/kg). Mice  
31 were perfused transcardially with phosphate-buffered saline (PBS 1X) and  
32 incubated (48h/4°C) in 4% paraformaldehyde. Coronal slices were cut at 50 μm  
33 and washed three times in PBS 1X before incubation in the blocking solution  
34 containing 0.03% Triton X-100 and 10% donkey serum or goat serum. Sections  
35 were incubated (overnight per 4°C) with a mouse anti-Satb2 primary antibody  
36 (1/300; abcam ab51502), a rat anti-Ctip2 primary antibody (1/500; Abcam  
37 ab18465), or with a rat anti-MBP (1/500,Merckmillipore), a guinea pig anti-  
38 NeuN/Fox3 (1/1000,cat 26604, Synaptic system), a rabbit anti-GFP primary  
39 antibody (1/1000; Millipore, AB3080), a mouse anti-GAD67 primary antibody  
40 (1/500; Millipore MAB5406), an guinea pig anti-PV primary antibody (1/1000,  
41 synaptic system, cat#195004). After washing sections were incubated overnight  
42 at 4° C with a donkey anti-mouse secondary antibody (labeling of Satb2, 1/500,  
43 life technologies A31571, alexa 647), a donkey anti-rat secondary antibody

1 (labeling of Ctip2 or labeling of MBP, 1/500, life technologies A21209, alexa 488),  
2 a goat anti-mouse secondary antibody (labeling of GAD67,1/500, Invitrogene  
3 A21202, alexa 488), a goat anti-guinea pig secondary antibody (labeling of PV or  
4 Neun/Fox3,1/500, Invitrogen A11073, alexa 488), a donkey anti-rabbit (labelling  
5 GFP,1/500,life technologies A21206, alexa 488), streptavidine (labeling of  
6 biocytin, R&D system NL 999, 1/500, alexa 557). Sections were washed and then  
7 mounted in Fluoromont-G medium (Southern Biotech), coverslipped, and imaged  
8 on a fluorescent microscope as a confocal microscope (Leica SP5) or a slide  
9 scanner (Nanozomeer 2.0HT), or an epifluorescent microscope (Olympus BX63).  
10 Photomicrographs were taken and displayed using image J to adjust the contrast  
11 and or perform Z stack images.

## 12 Electron microscopy sample preparation

### 13 Tissue preparation

14 Mice were deeply anesthetized and perfused transcardially with a mixture of 3%  
15 paraformaldehyde (PFA) and 0.5% glutaraldehyde in 0.1M phosphate buffer at pH  
16 7.4. Brains were quickly removed, left overnight in 3% PFA at 4°C. Coronal  
17 sections of the brain were cut on a vibrating microtome at 50 µm, collected in PBS,  
18 cryoprotected, freeze-thawed, and stored in PBS with 0.03% sodium azide until  
19 use.

### 20 Immunogold experiments

21 GFP was analysed at electron microscopic level in Insula, Corpus Callosum,  
22 Anterior Commissure,dIBNST and CeA. GFP was detected by the preembedding  
23 immunogold technique, sections were incubated in 4% NGS for 45 min and then  
24 in a mixture of a rabbit anti-GFP (1/5000) antibody supplemented with 1% NGS  
25 overnight at RT. After washing, in PBS and PBS-BSAc (aurion, the Netherlands),  
26 the sections were incubated for 3 hours at RT in Goat anti-rabbit IgG conjugated  
27 to ultrasmall gold particles (1.4nm; nanoprobes) diluted 1/100 in PBS-BSAc- gel.  
28 The sections were washed and post-fixed in 1% glutaraldehyde in PBS for 10 min.  
29 After washing in PBS and water distilled, the immunogold signal was intensified  
30 using a silver enhancement kit (HQ silver; Nanoprobes, Yaphank, NY) for 8 min at  
31 RT in the dark. After several washes in PBS, the sections were then processed for  
32 electron microscopy.

33 The sections were post-fixed in 0.5% osmium tetroxide and dehydrated in  
34 ascending series of ethanol dilutions that also included 70% ethanol containing 1%  
35 uranyl acetate. The sections were post-fixed, dehydrated, and included in resin (  
36 Durcupan ACM; Fluka). Serial ultrathin sections were cut with a Reichert Ultracut  
37 S, contrasted with lead citrate and imaged in a transmission electron microscope  
38 (H7650, Hitachi) equipped with a 467 SC1000 Orius camera (Gatan).

### 39 *Ex vivo* Electrophysiology

40 After allowing at least 4 weeks for viral vector expression acute coronal brain slices  
41 containing the Insula were cut on a vibratome (VT1200S; Leica microsystems).  
42 Mice were deeply anaesthetized by i.p. injection of a mixture of ketamine-xylazine  
43 (100mg/kg and 20mg/Kg, respectively). A thoracotomy followed by a transcardiac

1 perfusion with a saturated (95%O<sub>2</sub> / 5%CO<sub>2</sub>), iced-cold solution (cutting solution)  
2 containing 250 mM sucrose, 10 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 2.5 mM KCl, 1.25 mM  
3 NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.5 mM CaCl<sub>2</sub>·H<sub>2</sub>O, 1.3 mM MgCl<sub>2</sub>, 26 mM NaHCO<sub>3</sub>, and 10 mM D-  
4 glucose (pH 7.4) was performed. The brain was then quickly removed from the  
5 skull, blocked in the coronal plan, glued on the stage of the vibratome, submerged  
6 in iced-cold, saturated cutting solution and cut in 300-µm thick sections. Brain  
7 slices were transferred in a storage chamber at 34°C for 1 h in an artificial cerebral  
8 spinal solution (referred as « recording ACSF ») saturated by bubbling 95%O<sub>2</sub> /  
9 5%CO<sub>2</sub> and containing 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 2 mM  
10 CaCl<sub>2</sub>·H<sub>2</sub>O, 2 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 26 mM NaHCO<sub>3</sub>, and 10 mM D-glucose,  
11 supplemented with 5 mM glutathion and 1 mM sodium pyruvate (pH: 7.4 ;  
12 Osmolarity : 310-315 mOsm). They were then maintained at room temperature in  
13 the same solution until recording.

14 Whole-cell patch-clamp recordings were performed in a submerged chamber under  
15 an upright microscope (AxioExaminer Z1; Zeiss) equipped with IR-DIC  
16 illumination. Slices were bathed in recording solution. Recording pipettes (5-7 MΩ)  
17 were prepared from borosilicate glass capillaries (GC150F-10; Harvard Apparatus)  
18 with a horizontal puller (Sutter Instrument, Model P-97). They were filled an  
19 internal solution composed of 135 mM K-gluconate, 3.8 mM NaCl, 1 mM  
20 MgCl<sub>2</sub>·6H<sub>2</sub>O, 10 mM HEPES, 0.1 mM Na<sub>4</sub>EGTA, 0.4 mM Na<sub>2</sub>GTP, 2 mM Mg<sub>1.5</sub>ATP, 5  
21 mM QX-314 and 5 mM Biocytin (pH :7.25; Osmolarity: 290-295 mOsm).  
22 Experiments were conducted using a Multiclamp 700B amplifier and Digidata 1440  
23 digitizer controlled by Clampex 10.6 (Molecular Devices) at 34°C. Data were  
24 acquired at 20 kHz and low-pass filtered at 4 kHz. Insula pyramidal neurons were  
25 visualized under IR-DIC microscopy and recognized by the triangular shape of their  
26 soma. All the recordings were performed in voltage-clamp mode at -80 and 0 mV  
27 to record light-evoked glutamatergic EPSC and GABAergic IPSC, respectively.  
28 Voltages were corrected off line for liquid junction potentials. Optical stimulations  
29 were achieved using a 473 nm diode pumped solid state laser (Optotronics, USA)  
30 connected to a 800 µm diameter optical fiber (Errol, Paris, France) positioned just  
31 above the surface of the slice next to the recording site. At the end of the day,  
32 brain slices were fixed in 4% PFA overnight and stored in 0.2% sodium azide-PBS  
33 until histological processing.

34

35

### 36 *In vivo* electrophysiology

37 Electrical stimulation of the Insula. Bipolar electrical stimulation of the Insula was  
38 conducted with a concentric electrode (Phymep) and a stimulator isolator (800 µs,  
39 0.2-1.8 mA; Digitimer).

40 Insula recordings. A glass micropipette filled with 2% pontamine sky blue solution  
41 in 0.5 M sodium acetate was lowered in the insula. The *in vivo* single-unit  
42 recordings were performed as previously described (Glangetas et al 2015). Briefly,  
43 the extracellular potential was recorded with an Axoclamp-2B amplifier and filter



1 (300 Hz/0.5 kHz). Single neuron spikes were collected online (CED 1401, SPIKE  
2 2; Cambridge Electronic Design). During electrical stimulation of one insula,  
3 cumulative peristimulus time histograms (PSTH) (5 ms bin width) of the  
4 contralateral Insula were generated for each neuron recorded. Electrical  
5 stimulation of the contralateral Insula was also used to test for antidromic  
6 activation of ipsilateral insula neurons using high-frequency stimulation and  
7 collision methods as previously described<sup>54</sup>. Driven impulses were considered  
8 antidromic if they met the following criteria: (1) constant latency of spike response  
9 (fixed jitter), (2) driven by each of the paired stimulus pulses at frequencies of  
10 100 Hz or greater, and (3) collision of driven spikes by spontaneous impulses.  
11 Histological control. At the end of each recording experiment, the recording pipette  
12 placement was marked with an iontophoretic deposit of pontamine sky blue dye  
13 ( $-20 \mu\text{A}$ ; 30 min). To mark the electrical stimulation sites,  $+50 \mu\text{A}$  was passed  
14 through the stimulation electrode for 90 s. Then, mice were perfused with PBS 1x  
15 and stored for 48 h in PFA 4% at  $4^\circ\text{C}$ .

## 16 Behavioral procedures

17 One week prior behavioral experiment, mice were progressively handled by the  
18 experimenter. For each behavioral test, mice were acclimatized at least 30 min in  
19 the experimental room. Between each mouse, the behavioral apparatus was  
20 cleaned with 70% ethanol and then water and dried between each test.

## 21 Open Field test

22 Mice were placed in the corner of a square open field ( $40 \times 40 \text{ cm}$ ) and were  
23 allowed to freely explore the open field for a 10-min period in 70 lux illumination  
24 conditions. Total distance travelled, velocity, and time spent in the zone during the  
25 session were automatically reported (Ethovision, Noldus).

## 27 Elevated plus Maze

28 The elevated plus maze consisted of a platform of four opposite arms ( $30 \text{ cm} \times 5 \text{ cm}$ )  
29 two of them are open and two are closed arms (enclosed by 25 cm high walls).  
30 The apparatus was elevated from the floor. The task was analyzed with the  
31 software Ethovision (Noldus) and we measured the time spent in each arm in trials  
32 of 10 min. The luminosity of the open arms was around 120 lux.

## 34 Social preference test

35 A three-chamber rectangular plexiglas arena ( $60 \times 42 \times 22 \text{ cm}$ , Imetronic) divided  
36 into three chambers of the same dimension was used for this test. Briefly, each  
37 mouse was placed in the center of the arena and allow to freely explore the entire  
38 arena for a 10 min habituation period under approximately 90 lux illumination  
39 condition. At the end of the habituation, the mouse was placed in the center of the  
40 arena, and two metallic enclosures ( $9 \text{ cm} \times 9 \text{ cm} \times 10 \text{ cm}$ ) were positioned in the  
41 center of the two outer chambers. One enclosure contained a juvenile unfamiliar  
42 mouse whereas the other enclosure was empty (inanimate object) for group-  
43 housed condition or filled with lego toys for isolated condition. The position of the  
44 two enclosures was counterbalanced to avoid any bias. The juvenile mice were  
45

1 previously habituated to the enclosure and the arena for a brief period of 2 days  
2 preceding the experiment with a 10-min session per day. The experimental mouse  
3 was allowed to freely explore the three-chamber arena for a 5 min period session.  
4 The time spent around the enclosures were manually scored. The stimulus  
5 interaction was scored when the nose of the experimental mouse was in closed  
6 proximity to the enclosure (approximately around 2 cm).

7

#### 8 Data analysis

9 For *in vivo* electrophysiological experiments, cumulative PSTHs of insula activity  
10 were generated during stimulation of the contralateral insula. Excitatory  
11 magnitudes were normalized for different levels of baseline impulse activity.  
12 Baseline activity was calculated on each PSTH, during the 500 ms preceding the  
13 stimulation to generate a Z-score for each responding neuron.

14 For immunolabeling quantification. To quantify retrograde labelling (rAAV2-retro-  
15 CAG-cre/Ai9dtomato mouse), we acquired 3 slices for each Insula level (antero,  
16 intermediate and posterior level) per mouse, on a total of 4 mice with confocal  
17 microscope. For the co-localization of Tomato<sup>+</sup> neurons with Satb2<sup>+</sup> and Ctip2<sup>+</sup>  
18 labelling, we took 3 pictures with confocal microscope per slice, on 3 slices per  
19 mouse, with a total of 4 mice and analysed co-localization on focal plan. For  
20 caspase lesion, we took one picture in the mid-Insula level with a slide scanner  
21 per mouse and quantify with Image J, the fluorescence density between  
22 contralateral (ctrl side) and ipsilateral lesion side (caspase side).

23

#### 24 Statistical analysis

25 Statistical outliers were identified with the Rout Q method (Q=1%) and excluded  
26 from the analysis. Normality was checked with the Shapiro-Wilk criterion and when  
27 violated, non-parametric statistics were applied (Mann-Whitney and Kruskal-  
28 Wallis, Wilcoxon test). When samples were normally distributed, data were  
29 analyzed with independent or paired one or two-tailed samples t-tests, one-way,  
30 two-way, or repeated measures ANOVA followed if significant by Bonferroni *post*  
31 *hoc* tests. Data are represented as the mean  $\pm$  SEM, and the significance was set  
32 at  $p < 0.05$ . Data were analyzed using GraphPad Prism 9.

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## 20 **Figures legends**

21 **Figure1. Anatomical, molecular, and electrophysiological characterization**  
22 **of Insula interhemispheric neurons. a.,h.,r.,w.** Experimental design. **b-f.**  
23 Representative epifluorescent image of a coronal slice of brain injected with an  
24 AAV2-DIO-eif1a-eYFP anterograde virus in the Insula showing the injection site in  
25 the insula (b, c) and projections to the contralateral insula (d), the dIBNST (e), the  
26 CeA (f). Scale 100  $\mu\text{m}$  for b,d,e,f and 50  $\mu\text{m}$  for c. g. Quantification of the Insula  
27 interhemispheric projections in bilateral dIBNST, CeA, and contralateral Insula. **i,j.**  
28 Representative epifluorescent image of a coronal slice of brain injected with a

1 rAAV2-retro-CAG-Cre retrograde virus in the ipsilateral insula in AI9dTomato  
2 mouse (i, scale 500  $\mu\text{m}$ ) with a high magnification of contralateral labeling in Insula  
3 (j, scale 25  $\mu\text{m}$ ). **k,l.** Quantification of contralateral labeling in Insula (homotopic  
4 labeling) and other cortical regions (heterotopic labeling). **m-p.**  
5 Immunofluorescence confocal images showing Insula interhemispheric neurons  
6 (tomato labeling, m), insula Satb2 staining (yellow labeling, n), insula Ctip2  
7 labeling (green labeling, o), and the overlay at low (top, scale 100  $\mu\text{m}$ ) and high  
8 magnification (bottom, scale 25  $\mu\text{m}$ ). At the bottom, white arrows show examples  
9 of Tomato and Satb2 colocalizations. **q.** Quantification of Tomato, Satb2, and Ctip2  
10 colocalization in the Insula. **s.** Representative traces showing a collision test and a  
11 high-frequency stimulation protocol for an Insula interhemispheric neuron  
12 projecting to the other Insula<sup>55</sup>. **t.** Histogram of the onset latency of Insula  
13 antidromic responses. **u,v.** Representative epifluorescent image at low (left, scale  
14 bar 500  $\mu\text{m}$ ) and high magnification (right, 150  $\mu\text{m}$ ) of MBP staining (grey  
15 labeling) and Insula interhemispheric neurons (tomato labeling). **x,y.**  
16 Representative image obtained with electron microscopy showing immunogold GFP  
17 labeling of unmyelinated interhemispheric Insula axons passing through the corpus  
18 callosum (x) or the anterior commissure (y) (green arrow). White arrow shows an  
19 example of a myelinated axon (scale 500 nm). *dIBNST: dorsolateral bed nucleus*  
20 *of the stria terminalis; ovBNST: oval-BNST; juxta-BNST: juxtacapsular BNST; a.c.:*  
21 *anterior commissure; cc.corpus callosum; BLA: basolateral amygdala; CeA:*  
22 *central amygdala; Ins: Insula; Som: Somatosensory cortex; M1: primary Motor*  
23 *cortex; M2:secondary Motor cortex; mPFC: medial Prefrontal cortex; Rec:*  
24 *recording; MBP: myelin basic protein. n: number of neurons; N: number of mice.*

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26 **Figure2. Functional characterization of insula interhemispheric circuit. a-**  
27 **c.** Representative images of immunogold GFP labeling obtained with electron  
28 microscopy showing asymmetric synapses (at white arrows) for Insula to Insula  
29 synapses (a), Insula to CeA synapses (b), and Insula to dIBNST synapses (scale  
30 bar: 150 nm). **d.** Schematic representation of insula interhemispheric circuit. **e.**  
31 *Ex vivo* electrophysiological experimental design. **f,g.** Representative example of  
32 a histological control showing insula fibers expressing the Channelrhodopsin  
33 (green labeling) projecting to the contralateral insula and insula recorded neurons  
34 filled with biocytin (tomato labeling) at low (f, scale bar: 150  $\mu\text{m}$ ) and high



1 magnification (scale bar: 25  $\mu$ m). **h.** Quantification of contralateral Insula  
2 pyramidal neuron responses to ipsilateral insula optogenetic stimulation. **i.**  
3 Representative traces of evoked ESPC recorded at -80 mV and IPSC recorded at  
4 0mV in Insula pyramidal neuron before (top) and after TTX+4AP bath application  
5 (bottom). **j, k.** Group mean of evoked PSC amplitude of insula pyramidal neurons  
6 (j) and after TTX+4AP (k). **l.** Group mean of PSC response latency of insula  
7 pyramidal neurons. **m.** Experimental design (top) and cartography of stimulation  
8 and recording sites in the insula (bottom). **n,o.** Group mean of AP width (n) and  
9 spontaneous firing rate (o) of all the recorded insula neurons. **p.** Quantification of  
10 insula-responsive neurons to the electrical stimulation of the contralateral insula.  
11 **q.** Typical PSTH and raster show a contralateral Insula-evoked excitatory response  
12 of an Insula neuron. Electrical stimulus at 0 ms, with 5 ms bin width. **r.** Heatmap  
13 plot of Z-scored PSTH traces for each individual responsive Insula neuron to an  
14 Insula contralateral electrical stimulation. The electrical stimulus is represented by  
15 a vertical black line at 0 ms. **s.** Mean Z-score of PSTH over all responsive insula  
16 cells. Stim: stimulation; Rec: recording; PSC: postsynaptic current; EPSC:  
17 excitatory PSC; IPSC: inhibitory PSC; AP: action potential. n: number of neurons;  
18 N: number of mice.

19 **Figure 3. Split insula interhemispheric communication disrupts social**  
20 **preference only after acute social isolation. a,b.** Experimental design for the  
21 viral injection (a) and behavioral assay (b). **c.** Example of a histological control of  
22 caspase lesion in the insula identified by NeuN immunofluorescence labeling (green  
23 labeling) taken at epifluorescence microscope at low (left, scale bar: 500  $\mu$ m) and  
24 high magnification (right, scale bar: 100  $\mu$ m). **d,e.** Quantification of NeuN  
25 fluorescence density in the control side (contralateral side to the injected lesion  
26 side) compared to the caspase injection side in the Insula cortex (d) and in the  
27 Somatosensory cortex (e). **f,j.** Quantification of the time spent around the object  
28 and social enclosures in the three-chamber test in grouped housed condition (f) or  
29 after acute social isolation (j) in insula control and insula caspase groups. **g,k.**  
30 Social preference ratio in the control group and caspase group in group-housed  
31 mice (g) or isolated mice (k). **h,l.** Time spent in the social zone between control  
32 and caspase mice in group-housed (h) or acute isolated condition (l). **i,m.** Mean  
33 social bout duration in control and caspase group in group-housed (i) and acute

1 isolated housing condition (m). *ctrl*: control, *S*: time in the social zone; *O*: time in  
2 the object zone; *contra*: contralateral; *ipsi*: ipsilateral.

### 3 **Supplementary figure legends**

4 **Supplementary Figure1. a.** Experimental design. Representative epifluorescent  
5 image of a coronal slice of brain injected with an AAV2-hSyn-eYFP anterograde  
6 virus in the insula showing the injection site in the Insula (b) and projections to  
7 the dIBNST (c), the CeA (d), the contralateral insula (e). Scale bar: 1 mm for (b)  
8 and 500  $\mu$ m for (c-e). **f.** Quantification of the insula bilateral projections. **g.**  
9 Experimental design. **h-j.** Representative epifluorescent image of a coronal slice  
10 of brain injected with a rAAV2-Cre retrograde virus in the CeA coupled with an  
11 AAV5-DIO-eif1a-eYFP anterograde virus in the Insula showing the injection site in  
12 the insula (h) and projections to the dIBNST (h), the CeA (i), the contralateral  
13 insula (h,j). Scale bar: 500  $\mu$ m (h, i) and 25  $\mu$ m (j). **k.** Cartography of insula  
14 subregions. **l-n.** Quantification of contralateral labeling in the homotopic region in  
15 insula subregions (l), and layers (m,n) after rAAV2-retro-CAG-Cre retrograde virus  
16 injection in the insula cortex in Ai9 dTomato. **o-q.** Representative confocal images  
17 showing an absence of co-localization between PV (o, green) or GAD67 (p, green)  
18 immunofluorescence staining and interhemispheric insula neurons (tomato  
19 labeling and its quantification (q). scale bar: 25  $\mu$ m. *ovBNST*: oval-BNST; *juxta-*  
20 *BNST*: juxtacapsular BNST, *am-BNST*: anteromedial BNST; *dIBNST*: dorsolateral  
21 *BNST*, *CeA*: central amygdala, *BLA*: Basolateral amygdala; *a.c.*: anterior  
22 *commissure*; *cc*: corpus callosum; *Claust*: claustrum; *GI/DI*: granular/disgranular  
23 *insula*; *AI*: agranular insula; *Nac*: nucleus accumbens, *mPFC*: medial Prefrontal  
24 *cortex*; *M1*: primary Motor cortex; *M2*: secondary Motor cortex; *PAG*:  
25 *periaqueductal grey substance*; *d*: dorsal; *l*: lateral; *v*: ventral; *PV*: parvalbumin;  
26 *GAD67*: glutamic acid decarboxylase.

27 **Supplementary Figure2. Caspase insula lesion does not modify locomotion**  
28 **or anxiety phenotype. a., e.** Example heatmaps of insula control and caspase  
29 mice performance in the open field test (a) or in the elevated plus maze (e). **b-d.**  
30 Quantification of the time spent in the center of the open field (b), the number of  
31 visits to the center (c), and the total distance traveled (d) in control and caspase  
32 mice. **f-h.** Quantification of the time spent and the number of entries in the open

1 arms of the elevated plus maze, (f and g respectively) and the total distance  
2 traveled (h) in both groups. *Ctrl: control; OA: open arms; CA: closed arms.*

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