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► **To cite this version:**

Christelle Glangetas, Elodie Ladevèze, Adriane Guillaumin, Manon Gauthier, Evelyne Doudnikoff, et al.. Encoding social preference by interhemispheric neurons in the Insula. 2023. hal-04273139

HAL Id: hal-04273139

<https://hal.science/hal-04273139>

Preprint submitted on 7 Nov 2023

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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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16

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 cues¹ and to cardiac
5 interoceptive signals². 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 interaction³.
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 behaviors⁴⁻⁹. For example, it has
11 been highlighted that Insula neurons projecting to the nucleus accumbens core
12 regulate the social approach to stressed juvenile rats⁸. Autism spectrum Disorders
13 (ASD) and Anxiety Disorders are pathologies with sensory integration defects that
14 have been associated with dysfunction of the Insula^{1,1,10-13}. 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 Insula¹. Moreover, clinical studies show an Insula
18 overactivation in anxious patients^{10,14}. 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)¹⁵.

25 A strong but understudied projection is the Insula interhemispheric projection to
26 the contralateral Insula¹⁶. As alteration in interhemispheric communication is
27 associated with a social deficit¹⁷⁻²⁰, 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 images²¹. Across
34 mammalian evolution, cortical interhemispheric communication occurs notably
35 through the corpus callosum²². Alterations in callosal fiber integrity have been
36 observed in several pathological conditions as in patients with strokes, multiple
37 sclerosis, schizophrenia, or ASD^{23,24}. 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 ± 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⁺/*Satb2*⁺ 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⁺ myelinated axons
20 out of n=59 GFP⁺ 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⁺ 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 described²⁵⁻²⁹. 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 movements³⁰⁻³². 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 ± 41.64 pA; IPSC amplitude 582.1 ± 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 ± 59.29
5 pA; EPSC amplitude in TTX+4AP: -247.3 ± 101.8 pA; IPSC amplitude in aCSF:
6 508.2 ± 123.4 pA; IPSC amplitude in TTX + 4AP: 5.167 ± 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 ± 0.22 ms; IPSC latency:
10 4.46 ± 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 ± 0.03 ms and
19 a spontaneous firing frequency of 0.78 ± 0.13 Hz (Fig 2n-o). Insula
20 interhemispheric neuronal stimulation triggered excitatory responses on the
21 contralateral Insula neurons with 10.61 ± 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 ~ 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^{33,34}.
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 ± 2.37 ; Insula caspase injection site NeuN density: 34.01 ± 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 ± 2.33 ; NeuN density in the other Somatosensory cortex site:
12 33.96 ± 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 ± 7.4 s vs social 149.4
16 ± 9.6 s; caspase: object 57.14 ± 5.9 s vs social 144.9 ± 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 ± 0.04 and caspase social preference
22 ratio 0.72 ± 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 ± 3.2 %; for caspase: 48.31 ± 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 ± 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 ± 5.25 s vs social 133.7 ± 7 s; caspase: object 92.91 ± 9.31 s vs social 105 ± 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 ± 0.03 and caspase social preference ratio 0.53 ± 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 ± 2.33 %; for caspase: 35.01 ± 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 ^{11,14,35-39}. 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*⁺. 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^{40,41}. 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 inhibition³⁰. 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 movements⁴². 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 involved⁴³. 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^{44 45}. 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 pathway¹². 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 CeA⁴⁶. 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^{47,48}. Additional studies would be necessary to define the Insula
15 lateralization 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^{27,37,45,48-52}. 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

35 **Acknowledgments**

36 C.G. is supported by the Foundation for Medical Research: ARF20170938746. This
37 work was supported by recurrent funding from the University of Bordeaux and the

1 CNRS. This study also received financial support from the French government in
2 the framework of the University of Bordeaux's IdEx "Investments for the Future"
3 program / GPR BRAIN_2030. The microscopy was done in the Bordeaux Imaging
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

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
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
Bacterial and viral strains		
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
Chemicals		
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
Experimental models:		
Organisms/strains		
C57BL/6JRj mice	Janvier-Labs	
Ai9 tdTomato; Gt(Rosa)26Sortm6(CAG-tdTomato)Hze	Jackson	Cat#007909

Software and algorithms

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

Other

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

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 (≥ 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 (2,8x10¹² vg/mL ; UNC Vector Core, Boyden) ;AAV2.2-
4 eif1a-DIO-eYFP (3x10¹² vg/mL ; Addgene); AAV2.5-eif1a-DIO-eYFP (1x10¹³
5 vg/mL ; Addgene);AAV2.2-hSyn-eYFP (3x10¹² vg/mL ; 50465-
6 AAV2,Addgene); AAV2.5—eif1a-DIO-eYFP (1x10¹³ vg/mL ; 27056-AAV5,
7 Addgene); AAV2.2-hSyn-ChR2(H134R)-eYFP (3.1x10¹² vg/mL ; UNC, AV4384G);
8 AAV5-flex-taCasp3-TEVp (7x10¹² 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 described⁵³. 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%O₂ / 5%CO₂), iced-cold solution (cutting solution)
2 containing 250 mM sucrose, 10 mM MgSO₄·7H₂O, 2.5 mM KCl, 1.25 mM
3 NaH₂PO₄·H₂O, 0.5 mM CaCl₂·H₂O, 1.3 mM MgCl₂, 26 mM NaHCO₃, 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%O₂ /
9 5%CO₂ and containing 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄·H₂O, 2 mM
10 CaCl₂·H₂O, 2 mM MgSO₄·7H₂O, 26 mM NaHCO₃, 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 MgCl₂·6H₂O, 10 mM HEPES, 0.1 mM Na₄EGTA, 0.4 mM Na₂GTP, 2 mM Mg_{1.5}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 described⁵⁴. 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°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 × 40 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 cm x5cm)
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 (60x42x22 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 cm x 9 cm x 10 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⁺ neurons with Satb2⁺ and Ctip2⁺
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 μ m for b,d,e,f and 50 μ 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 μm) with a high magnification of contralateral labeling in Insula
3 (j, scale 25 μm). **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 μm) and high
8 magnification (bottom, scale 25 μm). 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 Insula⁵⁵. **t.** Histogram of the onset latency of Insula
13 antidromic responses. **u,v.** Representative epifluorescent image at low (left, scale
14 bar 500 μm) and high magnification (right, 150 μm) 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 μm) and high

1 magnification (scale bar: 25 μ 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 μ m) and
24 high magnification (right, scale bar: 100 μ 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 μ 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 μ m (h, i) and 25 μ 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 μ 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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