Role of the habenulo-interpeduncular system in the neurodevelopmental basis of susceptibility and resilience to stress-induced anxiety

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**Title: Central role of the habenulo-interpeduncular system
in the neurodevelopmental basis of susceptibility and resilience to
anxiety in mice**

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Abstract

Having experienced stress during sensitive periods of brain development strongly impacts how individuals cope with later stress. Many become more prone to develop anxiety or depression, but some appear resilient. The as-yet-unknown mechanisms underlying these differences may lie in how genes and environmental stressors interact to shape the circuits controlling emotions. Here, we investigated the role of the habenulo-interpeduncular system (HIPS), a critical node of reward circuits, in early stress-induced anxiety in mice. Based on immediate early gene expression, we found that a subcircuit of this system, characterized by *Otx2* expression, is particularly sensitive to chronic restraint stress during the peripubertal period, and that this induces hypersensitivity of the HIPS to later stress and susceptibility to develop anxiety. We also show that conditional knockout of *Otx2* restricted to the HIPS in mice counteracts these effects of stress. Together, these results demonstrate that a genetic factor, *Otx2*, and stress interact around puberty to shape the stress sensitivity of the HIPS, revealing this sensitivity as a key modulator of susceptibility/resilience to develop anxiety.

Keywords: habenula, interpeduncular nucleus, psychiatric disorders, reward circuits, *Otx2*, homeobox gene, early life stress, pubertal period

1. Introduction

Most psychiatric illnesses occur in adolescents and adults. They are often triggered by physical or psychological trauma in susceptible individuals (Bale *et al.*, 2010). However, most people exposed to similar traumas will not develop symptoms (Hegde and Mitra, 2020). Understanding how susceptibility or resilience to psychiatric disorders is established is therefore an important health issue. Susceptibility or resilience may have a neurodevelopmental origin, caused by an interaction between genes and environment (G x E interaction) during

specific sensitive periods of development (Assary *et al.*, 2018; Lopatina *et al.*, 2021). Key contributing environmental factors include chronic stresses experienced early in life, which are predisposing factors for depression and anxiety (Ross *et al.*, 2017) and are widely used in animal models to identify the circuits and mechanisms involved. Among validated forms of chronic stress (CS) inducing anxiety-like and depression-related behaviors in rodents, restraint is very effective (Chiba *et al.*, 2012) (Seewoo *et al.*, 2020). This painless procedure induces a form a psychological stress, does not have the confounding effects of other physical stresses and can be applied at any stage of postnatal development including pre- and post-weaning. The developmental stages to which stress is applied, is indeed another critical parameter. Depending on it, different processes may be affected, and behavioral outcomes may vary considerably. Regarding anxiety and depression, the risk of their occurrence during adolescence and adulthood is increased by stresses endured during the peripubertal period (Holder and Blaustein, 2014; Horovitz *et al.*, 2012; Winer *et al.*, 2016).

Among brain circuits, the reward system is a prime candidate to be the substrate of stress-induced anxiety-like and depression-like behaviors. Indeed, there is a strong connectivity between stress response and reward circuits and, in fact, considerable overlap between the two (Bouarab *et al.*, 2019; Metzger *et al.*, 2021). Recently recognized as a component of reward circuits, the habenulo-interpeduncular system (HIPS), is also involved in the regulation of mood, novelty attraction, motivation, and memory (McLaughlin *et al.*, 2017; Viswanath *et al.*, 2013). This system is composed of the medial habenula (MHb) and its unique output, the interpeduncular nucleus (IPN). There is growing evidence that the HIPS plays a critical role in feedback from cortical and subcortical regions to the brainstem to elicit adapted emotional responses. Clinical and animal models studies link HIPS dysfunction to mood disorders (McLaughlin *et al.*, 2017; Ranft *et al.*, 2010). Thus, according to the neurodevelopmental

hypothesis, developmental changes in the HIPS during periods when this circuit is plastic could be at the origin of these disorders.

While the plasticity of other components of reward circuits, such as the amygdala and the ventral tegmental area (VTA), in response to early adversity has begun to be studied (Birnie *et al.*, 2020; Pena *et al.*, 2017), research on the effect of early stress on HIPS plasticity and function is still scarce. These studies are primarily focusing on its physiology in adults (Lee *et al.*, 2019; Vickstrom *et al.*, 2020; Yoo *et al.*, 2021), and the few studies on HIPS development have not addressed the direct relationship between HIPS developmental abnormalities and stress-induced pathologies (Aizawa *et al.*, 2011; Hsu *et al.*, 2016). Furthermore, the HIPS is involved in many aspects of behavioral control and is known to contain heterogenous neuronal populations with different developmental origins, identities and outputs (Lorente-Canovas *et al.*, 2012; Quina *et al.*, 2017; Ruiz-Reig *et al.*, 2019). However, which function is associated with which population is unclear.

We recently characterized a circuit within the HIPS (Ruiz-Reig *et al.*, 2019), which requires *Otx2* to develop normally. The habenular neurons of this subcircuit are in the most medial part of both the dorsal MHb (dMHb) and ventral MHb (vMHb) with highest expression of *Otx2* (*Otx2*^{High}). They preferentially target regions of the IPN that contain *Otx2*⁺ neurons, with which they contact synaptically. Importantly, habenular and IPN neurons in this circuit retain *Otx2* expression throughout development and adulthood. We named this subcircuit the HIPOPS (Habenulo-InterPeduncular-*Otx2*-Positive-System). *Otx2* is a developmental gene that encodes a homeoprotein with important brain functions during development and in adulthood (Beby and Lamonerie, 2013; Gat-Yablonski, 2011). *Otx2* is highly conserved throughout evolution (Ranade *et al.*, 2008; Sen *et al.*, 2013), making it a good marker of conserved brain circuits. Based on the known functions of *Otx2* and the HIPS, we hypothesize that the HIPOPS plays important and conserved functions inside the reward system.

Otx2 marks several other interconnected brain regions that also belong to the reward circuits. For example, *Otx2* plays a major role during specific critical periods of VTA postnatal development (Pena *et al.*, 2017) reducing its expression through Early life stress (ELS) induces long-term susceptibility to depression-like behavior. Thus, *Otx2* is an important player in critical periods of neuronal development and modulating its expression can lead to long-term effects on the reward system. The fact that *Otx2* is linked to human pathologies involving the reward system reinforces this idea. Indeed, it is a susceptibility gene for bipolar diseases and stress-related disorders (Maheu and Ressler, 2017; Sabunciyan *et al.*, 2007). Because HIPS dysfunctions are also involved in these diseases, *Otx2* roles in critical periods of HIPOPS development may be important in this process.

In this study, we developed a new paradigm to investigate the neurodevelopmental basis of risk/resistance to specific psychiatric disorders linked to the HIPOPS. We used restraint, as a stressor. We set up a two-hit restraint protocol in which the first stress applied during puberty is meant to generate susceptibility or resilience that translate into a pathological state only following a second period or “hit” of stress later (Daskalakis *et al.*, 2013). To demonstrate the sensitivity of the HIPOPS to stress, rather than direct recording of electrical activity, we measured the expression of immediate early genes that integrate more cellular activities. For instance, *Egr1* is regulated by a wide variety of environmental stimuli, including stress, and may in turn regulate the transcriptional program related to neuronal activity and synaptic plasticity (Gallo *et al.*, 2018). Furthermore, *Egr1* is associated with neuropsychiatric disorders in which neuronal plasticity and activity are altered, such as stress-induced anxiety. Using our stress paradigm and monitoring *Egr1* activation, we have highlighted the existence of a critical period of HIPOPS development under the influence of environmental stressors. Individuals subjected to restraint stress during this period exhibit specific HIPOPS hyperactivity relative to other circuits of the HIPS. These changes do not in themselves cause an anxiety state, but

induce long-term changes in the circuitry that make animals more prone to develop anxiety-like behaviors. Furthermore, we demonstrate that *Otx2* is a contributing factor since reducing its expression specifically in the HIPOPS alters the stress response and abolishes this long-term susceptibility.

2. Material and methods

2.1. Animals

All mice used were generated and maintained in the animal house of the Institut de Biologie Valrose, Nice, France. All mouse lines were kept in the 129/Sv background. Only males were used in this study to avoid having effects due to sexual dimorphism regarding sexual maturation period and stress response. *Otx2*^{CreERT2/+} and *Otx2*^{flox/flox} mice were generated as described previously (Fossat *et al.*, 2006). The *Ngn1*^{CreERT2/+}; *Ai14/Ai14* line was kindly provided by Lisa V. Goodrich (Koundakjian *et al.*, 2007). The Ai14 allele (Gt(ROSA)26Sor(tdTomato-WPRE)) was used as the reporter of *Ngn1*^{CreERT2/+} expression. Adult mice were used for viral injection of DREADDs. Behavioral tests were done during the day cycle of the 12h day/night cycle. Postnatal deletion *Otx2* was achieved by injecting intraperitoneally 30µl of tamoxifen in neonates at P0 at a concentration of 2,5mg/ml with 300µl insulin syringes. Brains from all the mice that went through behavioral testing were analyzed post-mortem and showed successful recombination. Care and handling of the animals prior or during the experimental procedures followed European Union rules and were approved by the French Animal Care and local ethic Committees.

2.2. DREADDs microinjections

Animals were anesthetized by intraperitoneal injection (IP injection) with a mix of Tiletamine-Buprenorphine-Zolazepam-Xylazine (TBZX) (60 mg/kg). Xylocaïne gel was applied on the skull prior to the incision and in the ear to lessen the pain perception. After disinfection of the

area of incision, each mouse was successively placed and maintained on the stereotaxic apparatus. The eyes were protected from drying with ocry-gel. The cannula was implanted unilaterally from the bregma based on Paxinos et Franklin (2004). The implantation was successively made on the left and then on the right side, in the MHb according to these coordinates : A/P= -1,5 ; D/V= -2,75 ; lateral= +/- 0,2. 500 nl of the activating Gq- coupled DREADDs hM3D receptor (pAAV9-hSyn-DIO-hM3D(Gq)-mCherry, 100 μ L from titer $\geq 1 \times 10^{13}$ vg/mL) (Cat No. 44361-AAV9; Addgene, USA) were injected at a speed of 100 nl/min in *Otx2^{CreERT2/+}* or *Otx2^{flox/flox}* and *Otx2^{+/+}* control mice bilaterally. At the end of the injection, the cannula was left in place for infusion for 5 min at the site of injection before withdrawal. At the end of the surgery, an anti-inflammatory (Metacam; ,5 mg/ml) was administered intraperitoneally. The anti-inflammatory treatment was repeated for 3 days post-surgery. Ten days after the transduction, tamoxifen (Sigma-Aldrich) diluted in corn oil at a concentration of 10mg/ml was IP injected at 5 μ l/g of body weight to induce the translocation into the nucleus of CreERT2 and, thus, the excision of the STOP-cassette that was preventing the expression of hM3D in the neurons of the MHb. Ten days after the tamoxifen injection, Clozapine N-oxide hydrochloride (CNO) (SML2304, Sigma-Aldrich) diluted in DMSO was IP injected at a concentration of 0,3 mg/kg to activate hM3D signaling pathway and thus depolarization of the neurons. The mice were left undisturbed, for 7h, to let *Egr1* expression return to baseline, as it is induced by the stress of the injection, and then they were rapidly sacrificed.

2.3. Stress protocol

Mice were placed in pierced syringes suited for their size at each stage studied, to restrain their movements as much as possible. The restraint was used acutely (one unique 2h-restraint) or chronically (2h/day for 7 days). For the analysis related to the study of neuronal activity at the different developmental stages, the mice were immediately sacrificed at the end of the acute stress or at the end of the last chronic stress. The control mice that did not undergo any protocol

of stress were quickly sacrificed at the proper age. At the end of the behavioral analysis, mice for each condition were divided into two groups: a group of mice that underwent a last restraint stress before the collection of the brains, and a group that was immediately sacrificed.

2.4. Chronic sucrose consumption

Food pellet containing 40% sucrose was given every day for 6 days. Mice were sacrificed the last day, 2 hours after consumption of the pellet.

2.5. Behavioral tests

The open field test and the elevated plus maze test were used to assess anxiety level during the Behavior 1 and Behavior 2 steps. We also assessed the avoidance of a new object level at both steps. Depression level was assessed using the forced swim test.

Mice were acclimated in the behavioral room 1h prior to the beginning of the tests. Videos were acquired and analyzed with the EthoVision XfT software. For all the tests, the arena was cleaned with 70% ethanol and dried at the end of each trial.

2.6. Open field test

The open field test was used to measure the anxiety levels and the ambulatory behaviors of the mice. The test was recorded in an arena in which the walls and the floor are made of black PVC. The box dimension is (40 W x 40 L x 30 H cm) and was divided into the border area (50% of the total arena) and into the center area (800 cm²). The field was moderately illuminated at 60 lux. Mice were individually placed in a corner of the arena and the behavior was recorded for 5 min. The time spent, the latency to enter, the number of entries, the mean speed and the total distance travelled in the center, which is an anxiogenic area for the mice, were measured.

2.7. Elevated plus maze test

The Elevated Plus Maze test (EPM test) was used to assess the anxiety level. This apparatus is 50 cm elevated from the ground and composed of the open arms (50 x 5 cm) which are anxiogenic for the mice, and two closed arms (50 W x 5 L x 16 H cm) which spread from the central platform (5 x 5 cm). Mice were placed at the center of the platform, head toward a closed arm and the video was recorded for 5 min. The latency to explore the open arms and the time spent in the open arms were measured.

2.8. Avoidance of a novel object test

This test is based on the conflict between exploratory and avoidance behavior in the presence of a new object (Powell *et al.*, 2004; Roy and Chapillon, 2004). Excessive avoidance is the hallmark of anxiety disorders (Pittig *et al.*, 2018). An exploratory zone, containing a transparent cylinder with holes, was designed in the middle of one side of the wall of the open field. Mice were placed facing that side and the video was recorded for 5 min (Habituation phase). Following the habituation phase, an object was placed inside the cylinder and the mouse was relocated at the opposite side of the object and the video was recorded for 5 more min (Exploration of the object phase). The percentage of time spent in the exploratory zone, during the exploration of the object phase, is calculated and the statistics were made on the values obtained. We also used these values to divide the mice into subgroups: mice that spent a percentage of time $\leq 40\%$ in the exploratory zone are classified as avoiding/anxious, those that spend a percentage of time $\geq 60\%$ are classified as exploratory/curious, the mice in between are classified as indifferent.

2.9. Forced swim test

The forced swim test was used to measure the depression level of mice. Mice were placed individually in a transparent cylinder (12 cm diameter; 30 cm of height) high enough to avoid escaping or touching the bottom filled at 25cm from the bottom with water (23-25°C) for 6

min. The time of immobility during the last 4 min was measured on the EthoVision software. The immobility was defined as the lack of movement, except those necessary to sustain the head outside of the water. The water was changed at each trial.

2.10. Immunohistochemistry and histological analysis

Mice used to identify critical periods of development were directly sacrificed at the end of the acute stress or at the end of the last stress in the two-hit stress paradigm. Brains were dissected and directly frozen in Tissue-Tek OCT compound at -80°C (Fisher Scientific, Waltham, MA, USA). In order to preserve endogenous fluorescence of the reporter, mice transduced with the DREADDs were subjected to intra-cardiac perfusion using PBS, then 4% paraformaldehyde (PFA). The same treatment was done to *Otx2*^{+/+};*Ngn1*^{CreERT2/+};*Ail4*/*Ail4*, *Otx2*^{flox/flox};*Ngn1*^{CreERT2/+};*Ail4*/*Ail4* and *Otx2*^{flox/flox} *Ail4*/*Ail4* mice used to study the anatomical effect of *Otx2* deletion in the MHb, the neuronal activity in response to stress between P30-36 and to mice that underwent the behavioral analysis. Brains were post-fixed overnight in 4% PFA at 4°C and cryoprotected in 30% PBS sucrose before freezing in Tissue-Tek OCT compound at -80°C. Eyes were collected from dislocated mice and placed in cold PBS. A small hole was done at the level of the ora serrata. The eyes were fixed in 4% PFA for 2h at room temperature, cryoprotected in 20% PBS sucrose overnight, then frozen in OCT and stored at -80°C.

Brains were cut in 16µm coronal sections using a Microm HM550 cryostat and sections were mounted on SuperFrost+ slides (Fisher Scientific). Eyes were cut in 14 µm sections collected on SuperFrost+ slides. The sections directly frozen were post-fixed in fresh 4% PFA. Before the blocking step, sections from perfused *Otx2*^{+/+};*Ngn1*^{CreERT2/+};*Ail4*/*Ail4*, *Otx2*^{flox/flox};*Ngn1*^{CreERT2/+};*Ail4*/*Ail4* and *Otx2*^{flox/flox} *Ail4*/*Ail4* mice were subjected to an unmasking step to better reveal the *Otx2* signal that is weaker in perfused brains. In this step,

the slides were incubated in Citrate Buffer, pH 6, for 8 min at 95°C. A second treatment was performed with PBS-Glycine (100 mM glycine) to limit background noise. The rest of the steps were similar for all the samples. Briefly, the slides were incubated in a blocking solution (PBS with 0,2% dTriton 10% FBS) for 1h. The primary antibodies were incubated overnight at 4°C in a solution of PBS (0,1% Triton-10%FBS). The sections were incubated 10 min in DAPI (1µg/ml).

The primary antibodies used were: mouse anti-Brn3a (Merck/Millipore, #MAB1585, 1:250), rabbit anti-Er81 (kind donation of Silvia Arber, 1:1000), goat anti-Otx (R&D Systems, #AF1979, 1 :500), rabbit anti c-fos (SySy, #226004 1 :2000), rabbit anti-egr1 (cell signaling, #15F7, 1 :500), guinea-pig anti-Parvalbumin (SySy, #195004, 1 :500).

The secondary antibodies used (Jackson ImmunoResearch, 1:500) were: anti-rabbit Alexa Fluor™ 488, anti-rabbit Alexa Fluor™ 647, anti-goat Cy3, anti-mouse Alexa Fluor™ 647, anti-guinea-pig Cy3.

2.11. Microscopy and immunoreactivity measures

Images of the retina were acquired on a confocal microscope Zeiss 780 (Zeiss, Oberkochen, Germany). All other images were acquired on the wide-field microscope AxioObserver - Zeiss (2011) with the sCMOS ANDOR Neo camera and the stereomicroscope coupled with the digital camera (Zeiss axioplan2). Images were processed with the ImageJ software and figures were mounted on Adobe Photoshop. For Egr1 immunoreactivity measurements, the mouse brain atlas, fourth edition was used to delineate the different regions. For the medial habenula, 8 sections of 16 µm thick per animal were selected every 5 sections that covered bregma -1,22 to -1,94 mm. Both sides were analyzed and averaged. For the caudal IPN, 3 sections of 16 µm thick per animal were selected every 5 sections that covered bregma -3,4 to -3,88 mm, covering the entire Otx2+ area of the IPN. The area of Otx2+ neurons was specifically delineated for the

analysis of Egr1 immunoreactivity in Otx2+ neurons. All medial habenula sections at all the stages and for all conditions were analyzed together and the same was done for all the interpeduncular nucleus sections considered. Analysis was made using imageJ plugins “Analyze particles” with a thresholding that was identical for all conditions.

2.12. Statistical analysis

Statistical analyses were made using the R software. Comparison was made within the same genotypes and between different stress conditions. Results are presented as the mean $\pm$ SEM. The comparison of the mean of two groups was done by using the Student t-test when the variance was equal and with a Welch correction when it was not. Comparison of three groups were made with a one-way ANOVA. The two parameters, genotype and stress condition, were compared with a two-way ANOVA, using ANOVA function of the package “car” and corrected for the type III error for unbalanced datasets. Statistical details can be found in figure legends and in supplemental materials (Supplemental table S1). To study the effect on anxiety levels of the interaction between stress and genotype, mean ranking was computed as follows. A rank was attributed to the mice according to the value of the variable (for example time spent in the open arms of the EPM), ordered increasingly among all mice independently of their genotype or stress condition. Global scoring of anxiety corresponded to the mean scoring between all parameters (time, distance, and number of entry) from both tests (EPM and Open Field).

3. Results

3.1. HIPOPS circuits are interconnected and Otx2^{High} MHb region drives Otx2⁺ cIPN activity

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278 Previous results showed that MHb neurons express Otx2 following a gradient (Ruiz-Reig *et*
279 *al.*, 2019). All neurons located in the Otx2^{High} region maintain high levels of Otx2 till
280 adulthood. Otx2^{High} neurons preferentially make synapses with Otx2⁺ IPN neurons, all located
281 in the caudal IPN (cIPN), suggesting that Otx2 marks a subcircuit inside the HIPS that may be
282 preferentially interconnected (Ruiz-Reig *et al.*, 2019). To test this hypothesis further, we asked
283 whether chemogenetic activation of MHb Otx2^{High} neurons that are glutamatergic would
284 preferentially drive the activation of their targets in the cIPN of mice. We forced activation of
285 Otx2^{high} MHb neurons in *Otx2*^{CreERT2/+} mice through a DREADD-hM3D-approach (Fig1a),
286 consisting of CreERT2-dependent expression and CNO-dependent activation of the
287 DREADDs, and monitored neuronal activation (Fig1b) by examining the expression of
288 immediate-early genes (IEGs), strongly and specifically induced after neuronal activation
289 (Zangenehpour and Chaudhuri, 2002). The approach was specific because in tamoxifen-
290 injected control mice that did not express the CreERT2 (*Otx2*^{+/+}) or that expressed the
291 CreERT2 (*Otx2*^{CreERT2/+}) but did not receive CNO, no evidence of neuronal activation could be
292 detected in the MHb and IPN (Supplemental Fig.S1 and graph Fig.1). In contrast, upon
293 tamoxifen and CNO injections in *Otx2*^{CreERT2/+} mice, a strong Fos-immunoreactivity was found
294 in dMHb neurons expressing hM3D in the Otx2^{High} region (Fig.1d-f). In cIPN targets, neuronal
295 activity could be detected in the two Otx2⁺ subnuclei, the med-cIPN (medio-caudal IPN) and
296 lat-cIPN (latero-caudal IPN) (Fig.1g,h). Moreover, the large majority of cells strongly Fos-
297 immunoreactive were surrounded by hM3D-mCherry and expressed Otx2 (86 ± 8% of total
298 Fos-immunoreactive cells) (Fig.1h1-h2' and Graph Fig.1).

299 Thus, activation of Otx2^{High} neurons in the MHb triggers activity mainly in Otx2⁺ neurons of
300 the IPN, confirming that these neurons form a sub-circuit within the HIPS that can be marked
301 by the expression of Otx2, the HIPOPS.

3.2. HIPOPS critical period of response to stress

We then investigated the response of habenular neurons and their IPN targets in mice facing stress stimuli. To explore different developmental stages (P14-20, P30-P37, P37-P43 or P60-66), we selected physical restraint, which is the only type of stress that can be applied identically at any stage. We tested the effect of a chronic stress (CS) on HIPS activity by applying the same restraint stress 2 hours a day for 7 consecutive days, on male mice, and looking at IEGs immediately after the last stress. Expression of *c-Fos* and *Egr1* (*Krox-24*, *Zif-268*), another IEG (Zangenehpour and Chaudhuri, 2002), was strongly induced in the dMHb compared to control in *Otx2*^{High} neurons (Fig.2a-f). *Egr1* appeared an even more robust marker of stress-induced activity (Fig.2c,d versus Fig.2c',d') and was used thereafter. *Egr1* induction was restricted to *Otx2*⁺ neurons (Fig.2e,f) and was statistically stronger than controls only post-weaning into the pre- (P30-36) and adolescence (P37-43) stages (Fig.2c-d', graph Fig.2j and Supplemental Fig.S2), but not before weaning or in the adult (P14-20 and P60-66, graph Fig.2j). Following the same trend, activation of the IPN was statistically stronger than controls at the same stages (Fig.2h-i', graph Fig.2k). The level of induction of *Egr1* expression was increased in both *Otx2*⁺ and *Otx2*⁻ neurons (Graph Fig.2l). The number of *Otx2*⁺ and *Otx2*⁻ neurons expressing *Egr1* tended to increase and reached significance for *Otx2*⁺ neurons (Graph Fig.2m). The ratio between CS and NS conditions for *Otx2*⁺ and *Otx2*⁻ populations revealed that *Otx2*⁺ neurons, upon CS, were preferentially induced based on the level of *Egr1*-immunoreactivity while the number of induced neurons were similar (graph Fig.2n-o). In the lat-cIPN, 2 different *Otx2*⁺ populations could be distinguished based on their precise location and molecular profile. Only L-lat-cIPN neurons strongly expressed parvalbumin and were surrounded by a perineuronal net (PNN), a specialized extracellular matrix marked by the lectin

WFA (Supplemental Fig.S3). Furthermore, only Otx2⁺ neurons of the med- and M-lat-cIPN, but not of the L-lat-cIPN, were strongly activated by CS (Supplemental Fig.S4). We then tested the effect of an acute stress (AS) of 2hrs restraint, immediately after, which was unable to activate neurons of the MHb (Supplemental Fig.S5a-c). In the med- and M-lat-cIPN, activity of Otx2⁺ neurons was not different from a non-stressed condition, whereas, it was statistically different in the L-lat-cIPN (Supplemental Fig.S5d-f, graph Fig.S5g). Accordingly, the ratio “stress/no stress” was statistically different between AS and CS conditions (Supplemental graph Fig.S5h).

To verify that this activation was due to stress and not to any other type of event, we tested the response of the HIPOPS following a chronic exposure to a pleasurable event such as pleasant-tasting food with high content of sucrose. This condition elicited no detectable response (Supplemental Fig.S3f).

Thus, while AS does not activate the MHb and involves Otx2⁺/PV⁺ neurons in the IPN, the HIPOPS specifically responds to CS. Repeated restraint activates MHb neurons in the Otx2^{high} region and consequently, Otx2⁺ neurons in the med-cIPN and M-lat-cIPN are preferentially induced. The peak of activation is around puberty (P30-P36).

3.3. Restraint Stress during hyper-responsive period precipitates anxiogenic effects of adult stress

We then examined the consequences of CS applied during puberty on the susceptibility to develop anxiety-like behavior later. We used a two-hit stress model, composed of a mild stress (same stress used above of 2 hours restraint for 7 consecutive days) during the HIPOPS

hyper-responsive period (P30-36), to generate the susceptibility, followed by a second hit of CS in the adult, to reveal this susceptibility at the behavioral level (Fig.3a).

Mice subjected to CS (P30-36) were tested for anxiety levels 2 weeks after the end of the stress. The behavior of the stressed mice was indistinguishable from that of the control mice (Fig.3b-g; “controls”) in two independent behavioral tests known to detect anxiety-like behavior in rodent, the open-field and the elevated plus maze tests (EPM), demonstrating the absence of a direct pathological consequence of the first stress. In sharp contrast, several weeks after a second period of stress applied during early adulthood (P60-P66), animals that also had received the early stress presented statistically higher anxiety-like behavior, based on distance travelled, time spent and number of entries in the open-field, and based on distance travelled and time spent in the open arm of the EPM (Fig.4a-c,e “Controls”). Difference in number of entries in the EPM did not reach significance, although it followed the same trend. Global scoring considering all the parameters of both tests (see material and methods) confirmed a statistical difference (Fig.4j “Controls”). These differences were not due to motor dysfunctions (Fig.4g-h “Controls”). Depressive-like behavior was also not significantly affected (Fig.4i “Controls”). Interestingly, avoidance of a novel object was increased (Fig.4k), in line with their increased anxiety in the other tests.

These results show for the first time that a CS, composed of repeated restraint sessions during puberty and able to strongly activate the HIPOPS, induces susceptibility to anxiety-like behavior, only revealed at the adult stage upon stressful events. This time window may thus represent a critical period of HIPOPS development involved in setting long-term anxiety levels.

3.4. Deletion of *Otx2* restricted to the HIPOPS protects from susceptibility to develop anxiety-like behavior on the long-term.

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375 To directly implicate the HIPOPS in the mechanisms leading to anxiety-like behavior, we
376 tested whether interfering with the normal development of the HIPOPS would change the
377 outcome of individuals experiencing the early stress. Since *Otx2* is a master gene of HIPOPS
378 development, we used the *Ngn1^{CreERT2}* line to delete *Otx2* specifically in this circuit (Kim *et al.*,
379 2011; Koundakjian *et al.*, 2007). In this strain, CreERT2 expression becomes gradually
380 restricted as mice age and overlaps *Otx2* expressing sites only in the dMHb and rare
381 photoreceptors in the retina, as shown by the lineage of CreERT2 expressing cells followed
382 from P0 to P30 (Supplemental Fig.S6 and FigS7).

383 *Otx2* deletion was triggered by tamoxifen injection at P0. To be able to compare the
384 experimental groups, all animals received tamoxifen. At P30, *Otx2^{lox/lox};Ngn1^{CreERT2}* animals
385 were compared to tamoxifen-treated *Otx2^{lox/lox}* or to *Otx2^{+/+};Ngn1^{CreERT2}* mice. The habenula
386 in these mice appeared normal. Projections from the neurons that had lost *Otx2* expression were
387 maintained, surrounding their IPN targets (Supplemental Fig.S6b-e'). The size of the MHb was
388 unchanged further supporting lack of structural abnormalities in the circuit (Supplemental
389 Fig.S6f). We next assessed the behavior of these mice at the adult stage of P100. No
390 abnormalities were detected in term of anxiety levels or motor parameters such as mean speed
391 or distance travelled in the open field (Supplemental Fig.S8a-b).

392 Thus, CreERT2 expression or postnatal *Otx2* deletion in the dMHb and in rare cells in the
393 retina does not result in anatomical or behavioral abnormalities under standard conditions.

394 Because the dMHb is particularly activated by CS, especially during the critical period of
395 HIPOPS development (Fig.2), and because the HIPOPS is closely dependent on *Otx2* gene
396 function, we tested whether *Otx2* deletion would affect stress-induced anxiety-like behavior.
397 We first assessed behavior after an initial stress hit. As in control mice, we did not detect

behavioral abnormalities induced by the first stress (Fig.3b-g “cKO”). We next analyzed behaviors after the two-hit stress protocol. As expected, in both controls, the two-hit stress protocol increased anxiety levels; in contrast, this effect of stress could not be observed in *Otx2^{flox/flox};Ngn1^{CreERT2}* mice (Supplemental Fig.S8f). In fact, all the parameters that were affected in control animals, after the early + late stress compared to the late stress alone, were not affected in *Otx2^{flox/flox};Ngn1^{CreERT2}* mice (Fig.4a-j). Comparison of the global anxiety level score confirmed that only control mice were statistically affected by the two-Hit stress protocol (Fig.4j). The time of inactivity in the FST test was similar in *Otx2^{flox/flox};Ngn1^{CreERT2}* mice with or without the early stress (Fig.4i), as expected, since the stress-protocol does not induce depressive-like symptoms in controls. Avoidance of a novel object test revealed that the proportion of individuals avoiding, exploring or indifferent to a new object was similar between cKO animals without stress or with the two-hit stress protocol (Fig.4l). This result is different from what we observed in control animals (Fig.4k). Interestingly, the effect of adult stress alone followed the same trend as in the controls with an increase in the proportion of mice indifferent or avoiding the new object (Fig.4k,l; middle histogram), and the time spent in the exploratory/curious zone (Fig.4m).

Thus, deletion of *Otx2* in the dMHB results in a relative protection against the effects of stress on the long-term regarding anxiety and avoidance of a new object. This resilience effect appears to be the consequence of an altered G x E interaction, between *Otx2* and stress specifically during the juvenile period.

3.5. Deletion of *Otx2* restricted to the HIPOPS leads to a bimodal response to juvenile chronic stress and protects from stress-hypersensitivity of the circuit later on.

422 To understand the mechanisms of resilience to stress induced by *Otx2* ablation in the dMHb,
 423 we analyzed the HIPOPS response to juvenile stress in *Otx2^{flox/flox};Ngn1^{CreERT2}* animals (Fig.5).
 424 Interestingly, in these mice, although the dMHb was even more active than in controls
 425 following juvenile CS (Fig.5a-b), this did not induce any activation of IPN neurons (Fig.5c-d).
 426 This might suggest that *Otx2* deletion results in abnormal connectivity between the dMHb and
 427 its IPN output. However, the strong activation of *Otx2*⁺ med-cIPN and M-lat-cIPN neurons by
 428 an acute stress (AS) (Fig.5c2-c2'), in correlation with a strong activation of the dMHb (Fig.5a2-
 429 a2') at P30, ruled out this hypothesis. This was all the more surprising because an AS normally
 430 does not significantly activate the dMHb at all time points tested (Fig.5a1-a1', c1-c1' and
 431 supplemental Fig.S5 and S9).
 432 Thus, deletion of *Otx2* in *Otx2^{flox/flox};Ngn1^{CreERT2}* mice exacerbates the stress sensitivity of the
 433 habenular part of the HIPOPS circuit. This habenular hyper-sensitivity, paradoxically, renders
 434 IPN neurons insensitive after 5-7 days of stress.
 435 We then tested whether this biphasic response to the early stress would have an impact on long-
 436 term response of the circuit to stress. We examined the status of the circuit at the adult stage
 437 (P130), in control and *Otx2* cKO mice that had or had not undergone the two-hit protocol, when
 438 subjected to a final AS. No activity was detected in the dMHb in any of the conditions except
 439 rare cells in the dMHb of control animals with previous two-hit stress (Supplemental
 440 Fig.S10a1,b1,c1). There was tendency of higher activity in IPN neurons in control animals with
 441 previous two-hit stress compared to all other conditions that only reach significance compared
 442 to the one in *Otx2* cKO with prior two-hit stress (Fig.5e-g). Activity in *Otx2* cKO appeared
 443 surprisingly low, particularly in the L-lat-cIPN, where PNN⁺ neurons are readily activated even
 444 in control animals without previous two-hit stress (Fig.5f1-f2'). Interestingly, many other
 445 regions known to be involved in stress response followed the same pattern as the HIPOPS.
 446 Indeed, regions such as the paraventricular nucleus of the thalamus (PV) or the LHb (Reviewed

in(Hikosaka, 2010; Rowson and Pleil, 2021) and supplemental Fig.S10) presented a higher activity in control mice than in *Otx2* cKO animals subjected to previous two-hit stress.

Otx2 deletion in the dMHb thus changes the normal HIPOPS response to a biphasic response with hyperactivation at the onset of stress followed by insensitivity of the IPN targets as stress becomes chronic. In the long term, *Otx2* ablation in the dMHb protects against the development of stress hyperreactivity in HIPOPS neurons and in stress-related circuits, correlating with apparent protection against anxiety.

4. Discussion

We showed that a CS but not an AS nor pleasurable stimuli during the preadolescent (P30-36) and early adolescent (P37-43) periods causes a strong activation of the dMHb that correlates with a strong activation of *Otx2*⁺ IPN neurons in the med-cIPN and M-lat-cIPN. This correlation supports a direct activation of IPN neurons by the MHb. Indeed, we showed that *Otx2*^{High} neurons ((Ruiz-Reig *et al.*, 2018) and this study) project specifically and directly to *Otx2*⁺ neurons of the IPN. Furthermore, DREADD-mediated activation of *Otx2*^{High} neurons, specifically triggers activity of *Otx2*⁺ IPN neurons and only is those that are surrounded by DREADD⁺ fibers in the med- and lat-cIPN. In animals with a specific deletion of *Otx2* in the dMHb, there is a relative stronger activation of *Otx2*^{High} habenular neurons by a stressful experience, correlated with a paradoxical insensitivity of *Otx2*⁺ IPN neurons towards the end of the stress period that may be due to long term depression upon overstimulation of the IPN or a gradual loss of function of *Otx2*^{high} neurons, leading to stress hypo-response of *Otx2*⁺ IPN neurons, in adults. Plasticity mechanisms has been shown recently between dMHb and Lat-cIPN neurons regulated through opposing actions of glycinergic and substance P transmission (Melani *et al.*, 2019). Whatever the mechanism engaged here, levels of IPN activity at the end

of the juvenile stress correlates with the activity observed on the long term, suggesting that stress experiences at this juvenile period sets the intensity of IPN response to further stress episodes. Puberty may therefore represent a critical period of HIPOPS development when environmental factors such as stress interact with genes of development to shape IPN functions involved in the control of anxiety (Supplemental Fig.S11). This could explain why individuals depending on their trauma history and genetic background develop resistance rather than susceptibility to disease. Lower levels of *Otx2* in the HIPOPS could be an important factor in promoting resilience, at least at these developmental periods. This fits well with the hypo-anxiety that has been recently described in *Otx2* heterozygote mice in which *Otx2* expression is twofold lower (Vincent *et al.*, 2021). This study and ours would seem contradictory to what was described in the VTA where *Otx2* deletion mimicks stress effect (Pena *et al.*, 2017), however, in that latter study the brain circuits and the critical period considered is different and depression-like symptoms are triggered. Furthermore, *Otx2* expression is only transiently decreased which may have very different effect on neuronal activity. In any case, collectively these results and many others (Bernard and Prochiantz, 2016) show that *Otx2* is involved in critical periods of development, and from our study in the opening and closure of a critical period of HIPOPS development regarding stress response. The protection against stress-induced anxiety observed in *Otx2* cKO mice cannot be explained by the few recombined photoreceptors. Indeed, deletion of *Otx2* in all photoreceptors does not affect anxiety levels (Pensieri *et al.*, 2021). Furthermore, the effect of the deletion is only revealed when combined with stress and only during HIPOPS critical period of stress response.

Roles of *Otx2* during the critical periods include the control of the maturation of PV⁺ cells through non-cell autonomous mechanisms that involves the binding of *Otx2* to PNNs surrounding these neurons (Vincent *et al.*, 2021). In this study we found that the L-lat-cIPN is enriched in cells that are strongly PV⁺ and surrounded by PNNs revealed by their high content

in glycoconjugates that bind to Wisteria floribunda agglutinin (WFA). These Otx2⁺ neurons are induced by acute but not CS as opposed to other Otx2⁺ neurons in the IPN (M-lat-cIPN and med-cIPN) and Otx2^{high} neurons of the MHb. It is thus possible that a low activity of the MHb upon AS (below our detection threshold) is able to induce L-lat-cIPN neurons only, whereas a stronger activity is required to induce the other populations. This difference of response may be linked to the presence of PNN structures, only surrounding L-lat-cIPN, that may increase synaptic efficiency. If this is the case, it is surprising that high activity of the MHb upon CS, is not followed by high activity of L-lat-cIPN. An inhibitory mechanism upon CS may thus prevent these cells to respond to MHb neurons. Alternatively, response to acute stress may be less dependent on habenular activity, although these cells are known to receive and to respond to habenular inputs (Melani *et al.*, 2019). Interestingly, we found that CS during the critical period leads to an abnormal response to AS on the long term. Anxious-like mice are characterized by high activity of the cIPN whereas resistant mice present an abnormally low activity of the cIPN. Knowing the important role of PNNs in restricting plasticity associated with learning and that PNN maturation is influenced by physiological stimuli, such as stress (Pesarico *et al.*, 2019), CS-induced activity of the MHb may thus modify PNN structures leading to increased or decreased stress-response of the HIPOPS in controls or *Otx2* cKO respectively. It is also tempting to speculate that a non-cell autonomous mechanism of Otx2 operates here in the maturation and function of PV⁺ cells, as described in other systems (Vincent *et al.*, 2021), and that the source of extracellular Otx2 comes from the MHb projections. The reduction of Otx2 in cKO animals may thus alter the maturation of PV⁺ cells and PNNs. However, in contrast to other systems described so far, L-lat-cIPN neurons express *Otx2* (Ruiz-Reig *et al.*, 2018) so they may not require external sources of Otx2, unless endogenous and exogenous Otx2 have different functions.

To monitor HIPOPS response to stress we relied on the expression of immediate early genes such as *Egr1* and *c-Fos* (see introduction). Whether or not they reflected direct neuronal activity, the changes in *Egr1* levels we found are significant and may be even more valuable to assess specific changes of the cells that may have gone unnoticed or difficult to find by direct recording. Nevertheless, a thorough electrophysiological study on brain slices *in vitro* would certainly help to understand which electrophysiological phenotype correlates with the change of *Egr1* expression.

In conclusion, we have shown that chronic restraint stress, during puberty, specifically induces the HIPOPS circuit within the HIPS and makes animals more prone to develop high levels of anxiety but not depression. At the circuit level, high anxiety is correlated to stress hypersensitivity of the HIPOPS and of the whole stress-related circuit. These neuronal and behavioral effects must be driven through the HIPOPS because *Otx2* deletion restricted to the dMHb interferes with juvenile stress only and renders the animals less prone to develop anxiety. This demonstrates the existence of a G x E interaction at the HIPOPS level between genetic factors, such as *Otx2*, and CS, during a critical period of development. Clinical data point to puberty as, a window of vulnerability to anxiety, depression, schizophrenia, and substance abuse (Dahl, 2004; Patton and Viner, 2007), and exposure to stress plays an important role (Grant *et al.*, 2004). It is therefore of utmost importance to further investigate the role of the HIPOPS during this period as it may hold the key to understand susceptibility versus resilience to stress-induced anxiety in human. The two-hit stress protocol that we used is a valuable tool to address this question. Indeed, one can study neuronal circuit abnormalities responsible for susceptibility or resilience without confounding effects of a pathological state only triggered after the second hit of stress, which would be extremely difficult to observe in clinic. Also, having previously shown that *Otx2* is important for HIPOPS early development, we now suggest that *Otx2* is a master gene of HIPOPS maturation and function. Also known

as a susceptibility gene for psychiatric disorders in human, *Otx2* may therefore act as such through the HIPOPS.

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Figure 1. Effects of $Otx2^{High}$ neurons chemoactivation in the MHb. **a**, Schematic representation of the activating DREADD induction in the $Otx2^{High}$ neurons of the MHb in $Otx2^{CreERT2/+}$ mice ($n=3$). **b**, Schematic illustration of an MHb coronal section representing the localization of substance P⁺ (in red), ChaT⁺ (in green), $Otx2^{High}$ (in blue) neurons. **c**, Schematic illustration of an IPN coronal section representing the $Otx2^{High}$ substance P-ergic (in red) and cholinergic (in green) neurons projections and localization of the $Otx2^{+}$ neurons of the IPN. **d-f**, Labelling of MHb coronal sections with anti- $Otx2$ (in blue), anti-Brn3a (in red), anti-Er81 (in green) antibodies (d-e), hM3D-cherry (in blue), anti-Fos antibodies (in red) (f). The yellow arrows point to cells that are co-labeled with hM3D-cherry and anti-Fos. **g-h**, Labelling of coronal sections of the IPN with anti- $Otx2$ antibodies (in green), the nuclear marker DAPI (in blue) (g), anti-Fos antibodies (in red), hM3D-cherry (in blue) (h). **h1-h2'**, Zoom on the med-clIPN (h1, h1') and the lat-clIPN (h2, h2') framed in (h) labeled with anti- $Otx2$ (in green), hM3D-cherry (in blue) and anti-Fos antibodies (in red). The arrows represent the overlap of hM3D, Fos and $Otx2$ labelling. **i**, Quantification of the total number of Fos⁺ cells (left graph), the number of $Otx2^{+}$ /Fos⁺ cells (middle graph) or the number of $Otx2$ /Fos⁺ cells in the IPN upon CNO injection in $Otx2^{CreERT2/+}$ ($n=3$) and $Otx2^{+/+}$ ($n=3$) mice. The error bars represent the SEM. ** $P < 0.01$, ns=non significant, bilateral t-test. Scale bar, 100 μm .

Figure 2. Neuronal activity of the MHb and the IPN in response to CS. **a**, Schematic representation of an MHb as described in Fig.1b. **b-d'**, Area corresponding to the frame in (a) labeled with anti-Egr1 (b-d), anti-Fos (b'-d') antibodies and DAPI staining (in blue) in the absence of stress at P36 (b, b'), or in response to a CS between P30-36 (c, c'), between P37-43 (d, d'). **e-f**, Area corresponding to the frame in (a) labeled with anti-Egr1 and anti-Otx2 antibodies in response to a CS between P30-36. White arrowheads show the cells doubly labeled with anti-Egr1 and -Otx2. **g**, Schematic illustration of the caudal IPN as described in Fig.1c. **h-i'**, Immunolabelling of the caudal IPN coronal sections with anti-Egr1 (in red) and anti-Otx2 (in green) antibodies without stress at P36 (h and h') and in response to a CS between P30-36 (i and i'). **j-k**, Quantification of neuronal activity based on density of Egr1-immunoreactivity, after 7 days of stress at different postnatal periods, in the Otx2^{High} region of the MHb (CS P14-20 n=3, P30-36 n=3, P37-43 n=3, P60-66 n=3 and no stress P20 n=4, P36 n=3, P43 n=4, P66 n=3) (j) and in the caudal IPN (CS P14-20 n=3, P30-36 n=3, P37-43 n=3, P60-66 n=4 and no stress P20 n=4, P36 n=4, P43 n=4, P66 n=3) (k). **l-m**, Quantification of neuronal activity based on density of Egr1-immunoreactivity colocalizing or not with Otx2 immunoreactivity (l) or based on the number of neurons doubly labeled with anti-Egr1 and anti-Otx2 antibodies (m), after 7 days of stress between P30-36 and P37-43 (CS P30-36 n=3 and no stress P36 n=4; CS P37-43 n=3 and no stress P43 n=4). **n-o**, ratio between stressed and non-stressed conditions of the same values in l and m calculated in n and o respectively. The error bars represent the SEM. * P < 0.05, ** P < 0.01, bilateral t-test between control

751 and stressed conditions (j-m), between Otx2⁺ and Otx2⁻ cells (n-o). Scale bar 50 μ m b-f (shown in b).,
752 100 μ m h-i' (shown in h).
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Figure 3. Effects of the pre-adolescent stress on the level of anxiety and depression in the long-term. **a**, Illustration of the experimental protocol. **b-e**, Behavioral tests in the open field (b-d) and the elevated plus maze (e-g) on control and *Otx2* cKO mice that have not endured stress (n=6 controls and n=5 *Otx2* cKO) or that have endured a juvenile stress (n=11 controls and 11 *Otx2* cKO for open field, 12 *Otx2* cKO for elevated plus maze) and that have all received tamoxifen injection at P0. Parameters tested include distance traveled (b,e), time spent (c,f), and total number of entries (d,g) in the center of the open field and in the open arms of the elevated plus maze. The error bars represent the SEM. *P < 0.05, bilateral t-test between no stress and stressed conditions.

Figure 4. Effects of *Otx2* deletion alone and in interaction with CS. **a-f**, Behavioral tests on control and *Otx2* *cKO* that have endured an adult stress but have (n=9 controls and n=12 *Otx2* *cKO*) or have not endured (n=7 controls and n=6 *Otx2* *cKO*) a juvenile stress and that have all received tamoxifen injection at P0. Parameters evaluated include the total distance traveled (a,d) the time spent (b,e) and the number of entries (c,f) in the center of the open field or in the open arms of the elevated plus maze. **g-h**, Measures of mean speed in the open field and the forced swim test to test for potential motor dysfunctions. **i**, Time spent inactive in the forced swim test for control and *Otx2* *cKO* mice that have endured an adult stress but have (n=10 controls and n=12 *Otx2* *cKO*) or have not (n=7 controls and n=5 *Otx2* *cKO*) endured a juvenile stress. **j**, Global scoring of anxiety based on mean ranking for all the parameters in both open field and elevated plus maze allowing to evaluate anxiety levels in each mouse more accurately. **k-l**, Percentage of control (k) or *Otx2* *cKO* (l) mice in the exploratory/curious, indifferent and avoiding/anxious subgroup in the avoidance of a novel object test. **m**, Interaction plot representing the effect of *Otx2* deletion and juvenile CS associated with the adult stress on the time spent in the exploratory/curious area in the presence of an object ($F(1,47)= 6,713$, Interaction effect

780 p=0, 014). The error bars represent the SEM. *P < 0.05, **P < 0.01, two-way ANOVA with TukeyHSD
781 post-hoc test (j, m), bilateral t-test between adult stressed and two-hit stressed conditions (a-i).

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Figure 5. Short- and long-term effects of *Otx2* deletion combined to pre-adolescent stress. **a**, Schematic representation as described in Fig.1b. **a1-a4'**, Regions framed in (a) of coronal sections stained with anti-Egr1 antibodies (in red) and DAPI (in blue) in control mice (n=3) (a1-a1') or in cKO animals (n=3) (a2-a2') that underwent an AS at P30 or in control mice (n=3) (a3-a3') or in cKO animals (n=3) (a4-a4') that underwent a CS between P30-36. **b**, Quantification of neuronal activity in the MHb based on density of Egr1-immunoreactivity in the animals that underwent a CS between P30-36. **c**, Schematic illustration as in Fig.1c. **c1-c4'**, Regions framed in (c) from coronal sections labeled with anti-Egr1 (in red) and anti-*Otx2* (green) antibodies at the level of the caudal IPN in control mice (n=3) (c1-c1') or in cKO animals (n=3) (c2-c2') that underwent an AS at P30 or in control mice (n=3) (c3-c3') or in cKO animals (n=3) (c4-c4') that underwent a CS between P30-36. **d**, Quantification of neuronal activity in the caudal IPN based on density of Egr1-immunoreactivity in the animals that underwent a CS between P30-36. **e**, Schematic illustration as in Fig.1c. **e1-f4'**, Regions of the med-clPN framed in (d) (e1-e4) or of the L-lat-clPN framed in (d) (f1-f4') from coronal sections labeled with anti-Egr1 (in red) and anti-WFA (green) antibodies at the level of the caudal IPN in controls (n=3) (e1-f1') and in cKO mice (n=3) (e2-f2') that underwent a final AS only or a 2-hit stress protocol + a final AS in controls (n=3) (e3-f3') and in cKO mice (n=3) (e4-f4'). **g**, Quantification of neuronal activity in the caudal IPN based on the density of Egr1-immunoreactivity after a final AS before sacrifice in the caudal IPN of controls and *Otx2* cKO that have or have not endured the two-hit stress protocol. The error bar represents the SEM. **P* < 0.05, ***P* < 0.01, bilateral t-test (b,d), two-way ANOVA with TukeyHSD post-hoc test (g). Scale bar 25 μm a1-a4' (shown in a1), 50 μm c1-c4' (shown in c1), e1-f4' (shown in e1).

Supplemental figures

Supplemental figure S1. Control experiments of *Otx2^{High}* neurons chemoactivation in the MHb.
a, coronal section at the level of the MHb labeled with hM3D-cherry (in red) and anti-*Otx2* antibodies (in green), in mice stereotactically injected in the bilateral MHb with viruses expressing the DREADDs.
b-e, labelling in the equivalent region framed in (a) with hM3D-cherry (in blue) and anti-Fos antibodies (in green), in mice stereotactically injected in one side of the MHb with viruses expressing the DREADDs. The injected side (a; same image as Fig.1f) and the contralateral side of the injection (b) in *Otx2^{CreERT2/+}* mice that have been injected with CNO (n=3) are shown. The injected side (c) in *Otx2^{CreERT2/+}* mice that did not receive CNO (n=3) is shown. The injected side in wild type mice that were injected with CNO (n=3) is shown. Scale bar : 100 μ m.

Supplemental figure S2. Scheme comparing the different stages of a human life and the mice equivalent age.

Supplemental figure S3. Molecular signature of the Otx2⁺ neurons in the lateral and central subnuclei of the IPN. **a**, Immunolabelling of IPN coronal sections with anti-PV (in red) and anti-Otx2 (in green) antibodies at P36 (n=5). **a'-a''**, Zoom on the area framed on (a). The white arrows show the colocalization of the immunoreactivities for PV and Otx2. **b**, Labelling of the IPN coronal sections with WFA, binding to PNNs, (in red) and anti-Otx2 antibodies (in green) at P36. **b'-b''**, Zoom on the area framed on (b). The white arrows show the colocalization of WFA and anti-Otx2 antibodies. Scale bar 50 μ m.

Supplemental figure S4. Neuronal activity in med-cIPN+M-latcIPN and L-lat-cIPN in response to chronic stress at different developmental periods. **a-f**, Regions of the med-cIPN and L-latcIPN in coronal sections at the level of the IPN labeled with anti-Egr1 and anti-Otx2 from mice that endured no stress at P36 (**a**), CS between P14-20 ($n=3$)(**b**), CS between P30-36 ($n=3$)(**c**), CS between P60-66 ($n=4$)(**d**), or chronic sucrose consumption between P30-36 ($n=3$)(**e**). **f**, Schematic illustration of the caudal IPN regions in coronal sections where Otx2^{High} substance P-ergic (in red) and cholinergic (in green) neurons of the MHb project and localization of the Otx2⁺ neurons of the IPN. **g**, Quantification of Egr1-immunoreactivity density in med-cIPN+M-lat-cIPN and in L-lat-cIPN regions in Otx2⁺ cells in mice that have endured NS ($n=4$) or CS ($n=3$). The error bar represents the SEM. * $P < 0.05$, ** $P < 0.01$, bilateral t-test between control (no stress) and stressed conditions. Scale bar : 25 μm

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Supplemental figure S5. Neuronal activity of the MHb and the IPN in response to chronic and acute stress in juvenile mice. **a**, Schematic representation of an MHb coronal section showing the localization of substance P+ (in red), ChAT+ (in green), Otx2^{High} (in blue) neurons. **b-c**, Coronal sections at the level of the MHb with the same region as in (a) showed. Labeling with anti-Egr1 antibodies and DAPI staining (in blue) in response to a chronic stress between P37-43 (n=3) (b) or to an acute stress at P43 (n=3) (c). The approximate same region is framed in (a), (b) and (c). **d**, Schematic illustration of the caudal IPN regions in coronal sections where Otx2^{High} substance P-ergic (in red) and cholinergic (in green) neurons of the MHb project and localization of the Otx2⁺ neurons of the IPN. **e-f**, Coronal sections at the level of the IPN with the same regions framed in (d) of the med-clIPN and L-lat-clIPN showed. Immunolabeling with anti-Egr1 (in red) and anti-Otx2 (in green) antibodies in response to a chronic stress between P37-43 (n=3) (e) or to an acute stress at P43 (n=3) (f). **g**, Quantification of Egr1-immunoreactivity density in Otx2+ cells between in the med-clIPN+M-lat-clIPN and the L-lat-clIPN at P43 upon AS (n=3), CS (n=3), or NS (n=4). **h**, same measurements shown in g for AS and CS relative to the mean NS value. The error bar represents the SEM. *P < 0.05, **P < 0.01, bilateral t-test between chronic stress and acute stress or no stress conditions. Scale bar 50 μm b-c (shown in b), 25 μm e-f (shown in e).

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878 **Supplemental figure S6. Expression of tdTomato and effects of Otx2 deletion on the HIPS. a,**
879 Schematic representing the strategy used to induce the recombination of *Otx2* and
880 *Gt(ROSA)26Sor(tdTomato-WPRE)* floxed alleles specifically in MHb neurons observed at P30. **b-c,**
881 Labelling of coronal sections at the level of the MHb (b-b') and the IPN (c-c') in control mice (n=3)
882 with DAPI staining (in blue), anti-Otx2 antibodies (in green) and tdTomato (in red). Arrowheads in b
883 and b' point to tdTomato⁺ cells that are not Otx2⁺, resembling glial cells. **b₁-b₁'**, Zoom on the area
884 framed in b and b'. The arrowheads in b₁ and b₁' point to cells co-expressing Otx2 and tdTomato. **c₁,**
885 Zoom on the area of Lat-IPN framed in c and c'. **c₂-c₂'**, Zoom on the area of rIPN framed in c and c'.
886 **d-e**, Labelling of coronal sections with anti-Otx2 antibodies (in green), tdTomato (in red) and DAPI
887 staining (in blue) at the level of the MHb (d-d') and the IPN (e-e') in *Otx2* cKO mice (n=3). **d₁-d₁'**,
888 Zoom on the area framed in d and d'. The arrowheads in d₁ and d₁' point to cells recombined and do
889 not present Otx2-immunoreactivity. **e₁**, Zoom on the area framed in e and e'. **f**, Quantification of the total
890 surface of the MHb in μm² in controls (n=3) and in *Otx2* cKO mice (n=3). The error bar represents the
891 SEM. P is not significant, bilateral t-test. Scale bars: b-e' = 100 μm (shown in b', c', d', and e'); c₁, e₁
892 = 50 μm; b₁, b₁', c₂, c₂', d₁, d₁' = 25 μm (shown in b₁', c₂').

Supplemental figure S7. Regions expressing Otx2 and TdTomato in *Otx2*^{+/+};*Ngn1Cre*^{ERT2};*Ai14/Ai14* mice. a-f, Labelling with anti- Otx2 antibodies (in green) and tdTomato (in red) of retina sections (a), coronal section of the choroid plexus (b), VTA (c), colliculi (d), septum (e) and LGN (f). a1-a1', b1-b1', c1-c1', d1-d1' Zoom on the area framed in a, b, c, and d, respectively. RPE: retinal pigmented epithelium. ONL: outer nuclear layer containing photoreceptors. INL: inner nuclear layer containing bipolar cells. LSD: lateral septum dorsal part. LSI: lateral septum intermediate part. LSV: lateral septum ventral part. LGN: lateral geniculate nucleus. Scale bars: a-f = 100 μ m; a1-d1' = 25 μ m (shown in a1'-d1').

Supplemental figure S8. Control for the effect of Ngn1CreERT2 genetic background in normal and stressed conditions on behavior. a-b, Behavioral tests on *Otx2*^{lox/lox} ;*Ai14/Ai14* (n=10), *Otx2*^{+/+};Ngn1CreERT2 ;*Ai14/Ai14* (n=7) and *Otx2* cKO (n=8) measuring the total distance traveled (a) and the mean speed (b) in the open field. These animal groups have not endured any previous stress. c, Behavioral tests evaluating anxiety-like phenotype by measuring the total time spent in the open arms of the elevated plus maze for Ctrl1, Ctrl2 and *Otx2* cKO mice that have endured juvenile (n=10, 7, 12, 626 respectively) or juvenile + adult stresses (n=10, 7, 11, respectively). The error bar represents the SEM. *P < 0.05, **P < 0.01, One-way ANOVA (a, b), bilateral t-test between juvenile stress and two-hit stress conditions (c).

Supplemental figure S9. Effect of acute stress on neuronal activation in the MHb. Coronal sections at the level of the MHb stained with anti-Egr1 antibodies (in magenta) and DAPI staining (in blue) from brains collected at different developmental stages (from P20 to Adulthood) after an acute restraint stress. (P20 n=3; P30 n=3; P36 n=5; Adult n=4). Scale bar : 100 μ m.

Adult - Egr1

Supplemental figure S10. Effects of Otx2 deletion in the dMHb on the neuronal activity of the habenula and other stress-related regions in the long-term in response to a last acute stress. a-d, Labelling of coronal sections with anti-Egr1 antibodies (in magenta) at the level of the MHb in control (n=3)(a) and in Otx2 cKO (n=3) (b) animals that only endured an acute stress before the sacrifice or after the “two-hit stress” protocol (juvenile+adult chronic restraint stress) followed by a last acute stress in controls (n=3)(c) and Otx2 cKO mice (n=3)(d). **a1-a3, b1-b3, c1-c3, d1-d3,** Zoom on the area framed in a, b, c, d, respectively. LHb = Lateral Habenula, PV = Paraventricular nucleus of the thalamus. Scale bar: 100 μ m in a, b, c, d (shown in a).

Supplemental figure S11. Proposed mechanism of susceptibility or resilience to-stress inducing anxiety taking roots during the peripubertal period. The maturation of the stress response in HIPOPS circuits is taking place during the prepubertal period. In control situation, no chronic stresses are endured, and the maturation is normal in the presence or absence of *Otx2* (green curve). If a chronic stress is experienced during the critical period, the activity is higher by the time of closure (red curve). In the absence of *Otx2* expression in the MHb, a chronic stress activates even more strongly the MHb, leading to abnormally low response of the IPN by the time of critical period closure (blue curve). In all cases, following closure of the critical period, the IPN activity in response to new stresses during later life is dependent on the activity it had at the end of the critical period. This activity reflects the susceptibility or resilient state of the individual to develop pathologies such as chronic anxiety.

972 **Supplemental table S1 (See file in Excel format)**

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