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Article

Keywords: habenulo-interpeduncular system, anxiety, stress, brain development

Posted Date: August 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-823079/v1>

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

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This article contains:

- pages; 10 main figures, 3 Supplemental figures; 1 Supplemental table
- Title: 16 words; Abstract: 150 words; Main text (not including abstract, Methods, References and figure legends): 4990 words; Methods: 1832 words; Figure legends: No more than 310 words each.

Conflict of interest: The authors of this manuscript declare no competing financial interests.

Author contributions. ¹These authors contributed equally to the work. F.D wrote the first draft of the paper. T.L, M.R and F.D wrote the paper. M.R and F.D designed the research. N.R.-R, N.F, M.R and F.D performed the research. T.L contributed unpublished reagents/tools and most of the financial support. T.L, M.R, N.F and F.D analyzed the data.

Aknowledgements: We would like to particularly thank Benjamin Lapijover and Erwann Touzet for preliminary data, Aurélie Biancardini for mouse husbandry, Mouna Sekoni Khemiri for genotyping. We would like to thank Olivier Deschaux and Michèle Studer for sharing behavioral equipment and software. We would like to thank Lisa V. Goodrich for the *Ngn1^{CreERT2}* line and Silvia Arber for the Er81 antibodies. We would like to thank the iBV PRISM and Histo-pathology platforms and especially Simon Lachambre and Samah Rekima. This work was supported by the University of Nice (CSI fundings, Programme de Recherches Avancées UCA^{JEDI}), the National Center for Scientific Research (CNRS), the French ministry of Research (MESR fellowship). The present address of N.R.-R is at the Institute of Neuroscience of the Universite Catholique de Louvain, 1200 Brussels, Belgium.

Abstract

Experiencing stress during sensitive periods of brain development has a major impact on how individuals cope with later stress. Although many become more prone to develop anxiety or depression, some appear resilient. The mechanisms underlying these differences are unknown. Key answers may lie in how genetic and environmental stressors interact to shape the circuits controlling emotions. Here we studied the role of the habenulo-interpeduncular system (HIPS), a critical node of reward circuits, in early stress-induced anxiety. We found that a subcircuit of this system, characterized by *Otx2* expression, is particularly responsive to chronic stress during puberty, which induces HIPS hypersensitivity to later stress and susceptibility to develop anxiety. We further show that *Otx2* deletion restricted to the HIPS counteracts these effects of stress. Together, these results demonstrate that *Otx2* and stress interact, around puberty, to shape the HIPS stress-response, revealed here as a key modulator of susceptibility/resilience to develop anxiety.

Introduction

Most psychiatric illnesses occur in adolescents and adults. They are often triggered by physical or psychological, trauma in individuals presenting a susceptibility that was latent (Bale, Baram et al. 2010). On the contrary, a fraction of the population appears resistance (Hegde and Mitra 2020). A major challenge is to understand how susceptibility or resilience to psychiatric disorders is established.

Since many psychiatric diseases are now considered to have a neurodevelopmental origin, caused by an interplay between genes and environment during specific sensitive periods of development (Assary, Vincent et al. 2018, Lopatina, Panina et al. 2021), susceptibility or resilience should have their roots at these periods. While this hypothesis is widely accepted, the neural circuits and the modifications involved remain to be discovered. Major contributing factors include chronic stresses endured during early life (Ross, Foster et al. 2017), especially during periods termed “sensitive” or “critical”, when environmental inputs combined with genetic or epigenetic factors shape neural circuits and influence their long-term functioning. It is therefore presumed that a sensitization or steeling effect of the stress would then set vulnerability or resilience to diseases, respectively (Rutter 2012). The nature of these opposite effects, which should be observed prior to detectable disease, remains mysterious.

Considering that chronic stress is a predisposing factor for depression and anxiety (Ross, Foster et al. 2017), investigators have applied it to animal models to identify the circuits and mechanisms involved. Among the validated form of chronic stress that can induce long-term anxiety-like and depression-related behaviors in rodents, restraint has been shown to be very efficient (Chiba, Numakawa et al. 2012) (Seewoo, Hennessy et al. 2020), and has many advantages. The psychological and physiological changes induced by the restraint result from the distress of the deprivation of movements. This procedure is thus painless and does not have the confounding effects of pain or injury associated with other physical stresses. It can be applied at any stage of postnatal development including pre- and post-weaning unlike maternal separation or social defeat, and its intensity can be modulated over a wide range. Depending on the developmental stages when the stress is applied, different important processes can be affected ranging from neurogenesis to synaptic plasticity. Among critical periods, stress endured during pubertal period increases risk for anxiety and depression throughout adolescence and into adulthood (Horovitz, Tsoory et al. 2012, Holder and Blaustein 2014, Winer, Parent et al. 2016). Regarding the brain circuits involved, the reward system is considered a prime candidate to be the substrate for stress induced anxiety-like and depression-like behaviors. Indeed, there is a strong

connectivity between stress response and reward circuits and, in fact, a considerable overlap between the two (Bouarab, Thompson et al. 2019, Metzger, Souza et al. 2021). This highly interconnected system is considered important not only for reward value, but also for overall adaptive behavior in the face of stressful events. Moreover, a central component of reward circuits, the habenulo-interpeduncular system (HIPS), is also involved in the regulation of mood, novelty attraction, motivation, and memory (Viswanath, Carter et al. 2013, McLaughlin, Dani et al. 2017). This network is composed of the medial habenula (MHb) and its unique output the interpeduncular nucleus (IPN). Clinical studies are linking HIPS dysfunction to major depressive and bipolar disorders (Boulos, Darq et al. 2017) and animal model studies have shown that the HIPS contributes to the pathological mechanism of notably anxiety- and depressive-like disorders (McLaughlin, Dani et al. 2017). Thus, following the neurodevelopmental hypothesis, HIPS developmental abnormalities may 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 started to be studied (Pena, Kronman et al. 2017, Birnie, Kooiker et al. 2020), this has never been investigated for the HIPS so far: studies of this circuit are mainly focusing on adult physiology (Lee, Yang et al. 2019, Vickstrom, Liu et al. 2020, Yoo, Yang et al. 2021), and the few developmental studies of the HIPS have not addressed the direct relation between HIPS developmental abnormalities and stress-induced pathologies (Aizawa, Amo et al. 2011, Hsu, Morton et al. 2016). Importantly, although the HIPS is implicated in many aspects of behavioral control and is known to contain heterogeneous neuronal populations, it is unclear which function is associated with which population. This complexity may be at the origin of the discrepancies found in the literature. For example, one study in mice concluded that the dorsal part of the MHb (dMHb) plays a role in controlling fear responses and learning (Yamaguchi, Danjo et al. 2013). Another study found no effect on these parameters (Hsu, Wang et al. 2014), but showed that this circuit is involved in motivation and hedonic state. In any cases, what is clear is that the HIPS represents a critical and integrative node of the feedback from cortical and subcortical regions to the brainstem in order to elicit adapted emotional responses. Regarding clinical research, the precise defects underlying psychiatric status has been difficult to address because of the genetic heterogeneity and the unique and complex patient's trauma history. It is therefore difficult to isolate the parameters that condition vulnerability or resilience. Lifting these scientific barriers requires a model designed to identify the type of alteration leading to vulnerability or resilience.

We have recently characterized a genetically-recognizable circuit within the HIPS (Ruiz-Reig, Rakotobe et al. 2018). The habenula- and interpeduncular-parts of this subcircuit both critically require the expression of *Otx2* to develop and are marked by it, throughout development and into adulthood. The part where *Otx2* is highly expressed in the medial habenula (*Otx2*^{High}) corresponds to the central-most part of both the dMHb and ventral MHb (vMHb). It also corresponds to the latest born habenular neurons and preferentially targets IPN regions that contain high density of *Otx2*+ IPN neurons with which it establishes synaptic contacts (Ruiz-Reig, Rakotobe et al. 2018). Accordingly, we named this subcircuit the HIPOPS (Habenulo-InterPeduncular-*Otx2*-Positive-System). *Otx2* is a developmental gene that encodes a homeoprotein with many important brain functions. Its expression is highly conserved throughout evolution (Ranade, Yang-Zhou et al. 2008, Sen, Reichert et al. 2013), making it a good marker of conserved brain circuits. Based on all these evidences regarding *Otx2* and HIPS functions, we hypothesize that the HIPOPS plays important and conserved functions inside the reward system.

Beyond the HIPOPS, *Otx2* marks several other interconnected brain regions that also belong to the reward circuits. For example, *Otx2* has recently been shown to play major roles during specific critical periods of postnatal development of the ventral tegmental area (VTA) (Pena, Kronman et al. 2017).

Early life stress (ELS) reduced *Otx2* expression in the VTA only during the stress period and this reduction was the main factor that induced long-term susceptibility to depression-like behavior. Thus, *Otx2* is an important player of critical periods of neuronal development and disturbing its expression may lead to long-term effects on brain functions related to the reward system. In these particular periods, an association between *Otx2* expression and the maturation of one type of cells, the parvalbumin cells (PV cells), appears to be one of the driving forces that makes these periods, critical windows of development (Lee, Bernard et al. 2017). During these developmental phases, circuits are more plastic and are subjected to long term changes, under the influence of environmental cues (Hensch 2005). One such change is the appearance of perineuronal nets (PNNs), notably, around PV cells. PNNs may lock and stabilize existing synaptic contacts. Coincidentally, *Otx2* is linked to human pathologies involving the reward system. It was found to be a susceptibility gene for bipolar diseases and stress-related disorders (Sabunciyan, Yolken et al. 2007, Maheu and Ressler 2017). Because HIPS dysfunctions may be involved in these diseases, it is possible that the role of *Otx2* in critical periods of HIPOPS development is important in the process.

In this study, we have developed a new paradigm to investigate the neurodevelopmental basis of risk/resistance to specific psychiatric diseases and focused on the HIPOPS. We used restraint, as a form of psychological and physical stress. We employed a two-hit restraint stress protocol in which the first stress applied during development is meant to generate susceptibility or resilience that translate into a pathological state only following a second period or “hit” of stress in later life (Daskalakis, Bagot et al. 2013). That way, we uncovered the existence of a critical period of HIPOPS development under the influence of environmental stressors. Individuals undergoing restraint stress during this period exhibited a specific hyperactivity of the HIPOPS compared to other circuits of the HIPS. These changes did not by themselves provoke any state of anxiety, but they induced long-term modifications in the circuit that made animals more prone to develop anxiety-like behaviors when facing new periods of stress, later. Furthermore, *Otx2* is a contributing factor since reducing its expression specifically in the HIPOPS altered the stress response and abolished this long-term susceptibility.

Results

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

Previous results showed that MHb *Otx2*^{High} neurons preferentially make synapses with IPN neurons that express *Otx2*, located in two subnuclei of the caudal IPN, the cIPN (centro-caudal IPN) and lat-IPN (latero-caudal IPN). From these results we hypothesized that *Otx2* marks a subcircuit inside the HIPS that may be preferentially interconnected (Ruiz-Reig, Rakotobe et al. 2018). To test this hypothesis further, we asked whether chemogenetic activation of MHb *Otx2*^{High} neurons that are glutamatergic would preferentially drive the activation of their hypothesized targets in the cIPN. We performed stereotactic injection of AAVs expressing a floxed Gq-coupled hM3D DREADD fused with mCherry under the control of human *synapsin* promoter into the MHb of *Otx2*^{CreERT2/+} and wild type male mice (Fig.1a,b). To monitor neuronal activation, we examined the expression of immediate-early genes that are strongly and specifically induced after neuronal activation (Zangenehpour and Chaudhuri 2002). As expected, in wild type mice, after tamoxifen induced recombination and CNO mediated activation of the DREADDs, no evidence of neuronal activation could be detected in the MHb or in the IPN (Supplemental Fig.S1). The absence of activation was also found in *Otx2*^{CreERT2/+} mice when CNO was omitted, despite the presence of DREADDs visualized by mCherry decorated MHb *Otx2*^{High}

neurons (Supplemental Fig.S1). In contrast, upon CNO injection in *Otx2^{CreERT2/+}* mice, a strong Fos-immunoreactivity was found in dMHb neurons expressing hM3D in the *Otx2^{High}* region (Fig.1c-f). In the IPN target, neuronal activity could be detected in neurons of the cIPN and lat-IPN (Fig.1g,h). Moreover, only cells that were *Otx2⁺* were strongly labeled (Fig.1h1-h2,'arrows'). In the lat-IPN, only cells in the central-most part of it (C-lat-IPN) were Fos-immunoreactive (Fig.1c; green cells). Thus, forced activation of *Otx2^{High}* neurons in the MHb triggers activity specifically in *Otx2⁺* neurons in the IPN.

These results show that MHb *Otx2^{High}* neurons are able to modulate the activity of *Otx2⁺* IPN neurons and also confirm that these neurons form a sub-circuit within the HIPS that can be marked by the expression of *Otx2*, which we therefore called the HIPOPS.

Interestingly, in the MHb, Fos-immunoreactivity was not only found in DREADD⁺ neurons. Many neurons within the MHb and some in the lateral habenula were also labelled (Fig.1f; arrows) suggesting that *Otx2^{High}* neurons can activate local habenular neurons in addition to the neurons in the cIPN that express *Otx2*.

2. HIPOPS critical period of response to stress

We then wanted to investigate the response of habenular neurons and their IPN targets upon stress-induced neuronal activation. As we wanted to test different developmental stages, we selected physical restraint.

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. After the last stress, *c-Fos* and *Egr1* (*Krox-24*, *Zif-268*), another immediate early gene (Zangenehpour and Chaudhuri 2002), were strongly induced in the dMHb compared to control in a part that corresponded to the *Otx2^{High}* region (Fig.2a-h). *Egr1* appeared an even more robust marker of stress-induced activity (Fig.2c,d versus Fig.2c',d'); and was, thus, used thereafter. *Egr1* induction was particularly evident post-weaning into the pre-adolescence and adolescence stages (Fig.2c-d', diagram Fig.2g and graph Fig.2h). Following the same trend, activation of the IPN was strong and more evident at these stages (Fig.3 and graph Fig.3k). Interestingly, the neurons particularly activated belonged to *Otx2⁺* neurons of the cIPN and of the C-lat-IPN (Fig.3a-h and graph 3k). We then tested the effect of an acute stress (1 session of 2 hours restraint) which did not significantly activate neurons of the MHb (Supplemental Fig.S3) nor *Otx2⁺* neurons of the cIPN. Such stress, however, activated IPN neurons that were *Otx2⁻* or *Otx2⁺* confined to the L-lat-IPN (Fig.3a purple cells and Fig.3i). Those *Otx2⁺* neurons displayed a different molecular identity than the ones activated by a chronic stress. They expressed parvalbumin (Fig.4a) and were surrounded by perineuronal nets (PNN), a particular form of the extracellular matrix (Fig.4b). PNN structures were particularly evident from the pre-adolescence stage suggesting that these neurons were immature prior to that (Fig.4c). We therefore called them, *Otx2^{PNN+}* neurons.

To verify that this activation was due to the stress and not to any other type of event that the animals would experience, we tested the response of the HIPOPS following an exposure to a pleasurable event such as pleasant-tasting sucrose containing water. In contrast to stress, this condition elicited very little response (Fig.3j) confirming the specificity of the IPN response towards unpleasant stressful event.

Thus, the HIPS responds to stressful events. Chronic stress activates MHb neurons in the *Otx2^{High}* region and consequently, *Otx2⁺* neurons in the cIPN and C-lat-IPN. The peak of activation is around the pre-adolescent period (P30-P36) (Fig.2g). By contrast, acute stress only activates *Otx2⁺* neurons in the L-

lat-IPN characterized by PV expression and surrounded by PNNs ($Otx2^{PNN+}$ neurons) as well as some $Otx2^{-}$ neurons. The maturation of $Otx2^{PNN+}$ neurons coincides with the time window when the HIPOPS is strongly activated by stress.

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

We then wanted to test the consequences of chronic stress applied during the pre-adolescent period on the susceptibility to develop anxiety-like behavior on the long term. In accordance with the two-hit stress model, we wanted to apply a mild stress such as the one we used above (2 hours of restraint for 7 consecutive days) during the HIPOPS hyper-responsive period (P30-36), which by itself would not generate detectable long-term consequences on anxiety-like behavior but would produce a susceptibility to develop anxiety-like behavior. This susceptibility would then require a second hit of chronic stress to become detectable at the behavioral level (Fig.5a).

We first verified that 7 days of chronic stress applied from P30 to P36 would not result in behavioral abnormalities by testing general motility and anxiety levels 2 weeks after the end of the period of stress. No differences could be found between controls and stressed animals (Fig.5b-e). In sharp contrast, several weeks after a second period of stress applied during early adulthood (P60-P66), animals that had received the early stress presented higher anxiety-like behavior based on distance travelled, number of entries and time spent in an anxiogenic zone (Fig.5f,h-i). These differences could not be explained by motor dysfunctions as evidenced by a similar mean speed of the animals. In contrast to anxiety, depressive-like behavior was not significantly affected (Fig.5j), as measured by the Porsolt swim test. Interestingly, though, curiosity toward a new object was reduced (Fig.5k). However, this reduction in curiosity could be explained by potential anxiety toward the new object. Indeed, stressed mice were not more indifferent to the new object but avoided it, compared to unstressed mice.

These results show how a chronic stress during the pre-adolescent period can induce susceptibility to anxiety-like behavior revealed at the adult stage upon stressful events. This time window may thus represent a critical period of HIPOPS development particularly involved in setting long-term anxiety levels.

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

To directly implicate the HIPOPS in the mechanisms leading to increased anxiety-like behavior, we tested whether interfering with the normal development of the HIPOPS would change the outcome of individuals that experienced the early stress. Since *Otx2* is a master gene of HIPOPS development, we designed a strategy to delete *Otx2*, specifically in these circuits. We used the *Ngn1^{CreERT2}* line, which expresses an activable form of Cre driven by the *Ngn1* promoter (Koundakjian, Appler et al. 2007, Kim, Hori et al. 2011). In this line, CreERT2 expression becomes gradually restricted to a few sites as mice age. The lineage of CreERT2 expressing cells was followed from P0 to P30. $Otx2^{+}$ cells belonging to this lineage were almost exclusively found in the dMHb (Fig.6 and Supplemental Fig.S3). Only one other site could be found; it corresponded to rare photoreceptors in the retina (Supplemental Fig.S3).

Otx2 invalidation was triggered by tamoxifen injection at P0. At P30, *Otx2^{flox/flox};Ngn1^{CreERT2}* animals were compared to *Otx2^{flox/flox}* or to *Otx2^{+/+};Ngn1^{CreERT2}* mice. All received tamoxifen. The habenula in these mice appeared normal. Projections from the neurons that had lost *Otx2* expression were

maintained, surrounding their lat-IPN targets (Fig.6b-e'), as in control mice. The size of the MHb was unchanged further supporting that the postnatal ablation of *Otx2* did not affect their survival (Fig.6f). We next assessed the behavior of these mice at the adult stage at P100. No abnormalities were detected in term of anxiety levels or motor parameters measured by the mean speed or distance travelled in the open field (Fig.7a-b).

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

The dMHb is particularly active during chronic stress and especially during the critical period of HIPOPS development. We therefore tested whether *Otx2* deletion would affect stress-induced anxiety-like behavior. 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.7c-e). We next analyzed behaviors after the two-hit stress protocol. As expected, in two different controls *Otx2^{flox/flox}* and *Otx2^{+/+};Ngn1^{CreERT2}*, the two-hit stress protocol increased anxiety levels; however, this stress effect could not be observed in the *Otx2^{flox/flox};Ngn1^{CreERT2}* mice (Fig.7f). In fact, all the parameters that were affected in control animals (see Fig.5), after the early + late stress compared to the late stress alone, were unaffected in the *Otx2 cKO* mice (Fig.7g-i). Comparison of a global score of anxiety level based on both Elevated Plus Maze and open field parameters confirmed that only control mice were statistically affected by the two-Hit stress protocol (Fig.7k). The time of inactivity in the FST test was similar in *Otx2^{flox/flox};Ngn1^{CreERT2}* mice with or without the early stress (Fig.7j). This result was expected as the stress-protocol did not induce depressive-like symptoms, even in the controls. In the curiosity test, the proportion of curious, anxious and indifferent individuals facing a new object were similar between the no stress and the two-hit stress protocol groups (Fig.7l). This is dramatically different from what we observed in control animals (see Fig5k and Fig.7l). Interestingly, the effect of adult stress alone followed the same trend in controls (Fig.5k and graph Fig.7m) and *Otx2 cKO* (Fig.7l and graph Fig.7m) with an increase in the proportion of indifferent and anxious mice. These indicates that the consequences of the stress are different between the two genotypes if only the early stress is considered.

Thus, *Otx2* deletion in the dMHb leads to a relative protection against stress effects on the long-term regarding anxiety and curiosity. This effect of resilience seems to be the consequence of an altered interaction between *Otx2* and stress specifically during the juvenile period.

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.

To understand the mechanisms of resilience to stress observed in *Otx2^{flox/flox};Ngn1^{CreERT2}* animals, we analyzed the response of their HIPOPS to juvenile stress. Interestingly, in *Otx2^{flox/flox};Ngn1^{CreERT2}* mice, although the dMHb was even more active than in controls at the end of the juvenile chronic stress, IPN neurons did not respond (Fig.8a-d and graph Fig.8g). One possibility is that *Otx2* deletion leads to abnormal connectivity between the dMHb and its IPN output. However, at earlier time points during the protocol of stress, *Otx2⁺* cIPN and C-lat-IPN neurons were readily activated and this activation correlated with a relatively strong dMHb activation, even after an acute stress (Fig.8e-f). This was surprising as an acute stress is normally not strong enough to significantly activate the dMHb at any of the time points tested from P20 to adulthood (see supplemental Fig.S2).

Thus, deletion of *Otx2*, in *Otx2^{flx/flx};Ngn1^{CreERT2}* mice, makes the habenular part of the HIPOPS more activable by stress. This habenular hyper-sensitivity, paradoxically, desensitizes the IPN neurons after 5-7 days of stress.

We then tested whether this biphasic response to the early stress would have an impact on the long-term response of the circuit when confronted again to stress. We examined the status of the circuit at the adult stage, in controls and *Otx2* cKO that had or had not undergone the two-hit protocol, when subjected to a final acute stress, 2-hour before sacrifice. No activity was detected in the dMHb in any of the conditions (Fig.9a,c). Higher activity in IPN neurons could be detected in control animals with previous two-hit stress (Fig.9d), compared with the activity observed in *Otx2* cKO with prior two-hit stress (Fig.9e) or in controls with no previous stress (Fig.9f). As expected, following an acute stress in controls (see Fig3i), the most activated *Otx2*+ neurons corresponded to neurons located in the L-lat-IPN, including the PNN⁺ population (Fig.9f1,f1'). Many more cells were strongly Egr1-immunoreactive in control animals with previous two-hit stress protocol (Fig.9d and graph in Fig.9g). These cells belonged to both PNN⁺ (Fig.9d1, d1') and PNN⁻ populations (Fig.9d2). In sharp contrast, both populations were weakly Egr1-immunoreactive in *Otx2* cKO animals (Fig.9e-e2). Interestingly, many other regions known to be involved in stress response followed the same pattern as the HIPOPS. Indeed, regions such as the paraventricular nucleus of the thalamus (PV) or the LHb (Reviewed in(Hikosaka 2010, Rowson and Pleil 2021) and Fig.9a-c) presented a higher activity in control mice subjected to previous two-hit stress, compared to the activity found in both control and *Otx2* cKO animals without or with prior two-hit stress, respectively.

Thus, the deletion of *Otx2* in the dMHb changes the normal response of the HIPOPS to a biphasic response with hyperactivation in the beginning of the stress followed by inhibition of the IPN targets as the stress becomes more chronic. In the long term, *Otx2* ablation in the dMHb counteracts the hyper-responsiveness of HIPOPS neurons and stress-related circuits, correlated with apparent protection against the development of stress-induced anxiety. This suggests that genetic factors, such as *Otx2*, interact with environmental stress at the level of the HIPOPS, during a critical period of development. This also suggests that blocking this interaction, through deletion of *Otx2* in the dMHb, leads to a form of resilience to develop anxiety in adults. Finally, it suggests that HIPOPS neurons can drive this long-term anxiety and that the experience of stress by the HIPOPS, during the juvenile period, conditions the response of stress-related circuits to later stress-experiences (Fig.10).

Discussion

In this study, we have shown that chronic restraint stress during the P30-P36 period, corresponding to the sexual maturation period in mice (pre-adolescent period), specifically induces the HIPOPS circuit within the HIPS and makes animals more prone to develop high levels of anxiety but not depression. However, a role of the HIPOPS in the latter may have gone unnoticed because the stress used did not induce depression. Thus, while we cannot conclude on a potential role for the HIPOPS in depression, this study clearly shows its involvement in stress-induced anxiety. The two-hit stress protocol that we developed will certainly become a valuable tool to study mechanisms of susceptibility or resilience. Indeed, one can study neuronal circuit abnormalities that may be the basis of susceptibility or resilience without confounding effects of a pathological state only triggered after the second hit of stress. These early changes may be at the origin of the stress hypersensitivity that seemed to finally spread to the whole stress-related circuit. Also, we had previously shown that *Otx2* is important for HIPOPS early development and now suggest that *Otx2* is a master gene of HIPOPS development and function

We showed that from P36 onwards, HIPOPS neurons are not responsive to acute stress, except for PV⁺/PNN⁺ neurons. PNN structures revealed by WFA-immunoreactivity are rare before P30 (see Fig.4). Interestingly, before P30, neurons in the prospective PNN⁺ region are not activated upon an acute stress (Fjerdingsstad et al; unpublished observation). Thus, the maturation of PNN⁺ cells may well coincide with the maturation of acute stress response. We have no direct evidence that the maturation of these cells is related to the changes in circuit activity and behavior that we observed. However, this may be an interesting avenue to pursue. Indeed, PV⁺ cell maturation has been shown to control the opening and closing of critical periods in the brain and spinal cord (Wingert and Sorg 2021). It is therefore particularly interesting that this period corresponds also to the most sensitive period of HIPOPS activation. Environmental stress experienced at this period may, therefore, set the final sensitivity of the HIPOPS to stress in the long term (Fig.10), through habenula activation and PNN⁺ cell maturation. To explain what we observed, one possibility would be the existence of a bidirectional connectivity between the two HIPOPS sub-populations, PNN⁺/Otx2⁺ and PNN⁻/Otx2⁺ cells. When PNN⁺ neurons, become mature, they can inhibit other HIPOPS cells during an acute stress. If the stress becomes chronic, Otx2^{High} neurons of the habenula are strongly activated and overcome the inhibition from PNN⁺ cells on Otx2⁺/PNN⁻ neurons, leading to the activation of the latter. In turn, these newly activated Otx2⁺ cells, also GABAergic, inhibit the PNN⁺ cells. Although, still speculative this crosstalk deserves investigation as it would provide a straightforward explanation of our observations of the differential response to acute or chronic stress.

Another major result of this study is that the Otx2 protein, essential for the development of HIPOPS, also plays an important role during the P30-P36 period in setting the stress response of this circuit for the entire life. In control animals, the progressive activation of Otx2^{High} habenular neurons as the stress becomes chronic, leads to a strong activation of Otx2⁺ IPN neurons, until the end of the stress, correlated with a stress hyper-response of the HIPOPS in adults. In contrast, in Otx2 cKO animals, there is a relative stronger activation of Otx2^{High} habenular neurons, correlated with a paradoxical inhibition 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. These observations suggest that the level of HIPOPS activation, at the end of the stress period at P36, determines the intensity of HIPOPS response to further stress episodes after the closure of the hypothetical critical period (Fig.10). Speculating on reciprocal connection between Otx2⁺/PNN⁺ and Otx2⁺/PNN⁻ neurons of the IPN, a strong activation of Otx2⁺/PNN⁻ cells at the end of the stress period at P36 would lead to weak reciprocal inhibition in the long-term leading to HIPOPS hyperactivity. On the contrary, an inhibition of Otx2⁺/PNN⁻ cells at the end of the stress period at P36 would lead to a strong reciprocal inhibition in the long-term, leading to a hypo-reactive HIPOPS.

Whether or not reciprocal connections exist, hypo-responsivity due to a relative too early or too strong habenular activation could explain why individuals with a certain genetic background develop resistance rather than susceptibility to disease after trauma. Along this lines, lower levels of *Otx2* could be an important factor in promoting resilience to stress-induced anxiety, 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* is expressed at a twofold lower level than wild-type mice (Vincent, Gilabert-Juan et al. 2021). Such 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.; unpublished observation).

To demonstrate the sensitivity of the HIPOPS to stress, we did not directly record electrical activity, but monitored the expression of immediate early genes such as *Egr1* and *c-Fos* that integrate more

cellular activities. For instance, *Egr1* is regulated by a wide variety of environmental stimuli, including stress, and in turn can regulate transcriptional program related to neuronal activity and synaptic plasticity (Gallo, Kathe et al. 2018). Therefore, *Egr1* represents a key integrating factor between environmental perception and translation to the appropriate response. It is therefore not surprising to find *Egr1* associated with neuropsychiatric disorders in which neuronal plasticity and activity are altered, such as stress-induced anxiety (Gallo, Kathe et al. 2018). Whether or not they reflected direct neuronal activity, the changes in *Egr1* levels we found are significant and may be even more valuable than direct recording, as they reflect neuronal activity or plasticity of neurons in response to stress. A thorough electrophysiological correlate of these changes, however, would be helpful.

Animal models for reproducing stress-induced mood and anxiety disorders have largely focused on the pre-weaning or adult period. Here we found that the period that particularly involves the HIPOPS in the stress response is the period of sexual maturation. This period of development is a known 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, Compas 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 understanding susceptibility versus resilience to stress-induced anxiety.

Material and methods

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. *Otx2*^{CreERT2/+} and *Otx2*^{fllox/fllox} mice were generated as described previously (Fossat, Chatelain et al. 2006). The *Ngn1*^{CreERT2/+}; *Ai14/Ai14* line was kindly provided by Lisa V. Goodrich (Koundakjian, Appler et al. 2007). The *Ai14* allele 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 injecting intraperitoneally 15-30µl of tamoxifen in neonates at P0 at a concentration of 2,5mg/ml with 300µl insulin syringes. 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.

DREADDs microinjections

Animals were anesthetized by intraperitoneal injection (IP injection) with a mix of Tiletamine-Buprenorphine-Zolazepam-Xylazine (TBZX) (60 mg/kg). Xylocaine 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 ocri-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 µL from titer $\geq 1 \times 10^{13}$ vg/mL) (Cat No. 44361-AAV9; Addgene, USA) were injected at a speed of 100 nl/ms in *Otx2*^{CreERT2/+} or *Otx2*^{fllox/fllox} 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 µl/g of body weight to induce the activation of Cre and, thus, the expression of DREADDs 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. 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.

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.

Acute sucrose consumption

The water in the bottle of the cage was replaced by a 30% sucrose solution for the night. The sacrifice occurred the following day.

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 curiosity 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 XT software. For all the tests, the arena was cleaned with 70% ethanol and dried at the end of each trial.

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. 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.

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.

Curoosity test

This test allows the measurement of curiosity level of the mice, an aspect of anxiety. 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 anxious, those that spend a percentage of time $\geq 60\%$ are classified as curious, the mice in between are classified as indifferent.

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.

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*^{fllox/fllox}; *Ngn1*^{CreERT2/+}; *Ail4/Ail4* and *Otx2*^{fllox/fllox} *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, 1:250), rabbit anti-Er81 (kind donation of Silvia Arber, 1:1000), goat anti-Otx (R&D Systems, 1 :500), rabbit anti c-fos (SySy 1 :2000), rabbit anti-egr1 (cell signaling 1 :500), wfa biotinylated (vector laboratories, 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, Conjugated streptavidine Alexa Fluor™ 488.

Microscopy

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.

Statistical analysis

To study the interaction between anxiety phenotype with genotype, rankings were made by grouping the animals with the same genotype (stressed and non-stressed mice). A rank was attributed to the mice according to the value of the variable (time spent in the open arms of the EPM), ordered increasingly. Statistical analyses were made using the R software. Results are presented as the mean ± 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. Anova were calculated using the ANOVA function of the package “car” and corrected for the type III error for unbalanced datasets. Comparison of three groups were made with a one-way ANOVA. The interaction effects between the chronic stress and the Otx2 deletion were revealed by using a two-way ANOVA. Statistical details can be found in figure legends and in supplemental materials (Supplemental table S1).

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 green) (f). The arrows represent the Fos⁺ neurons outside of the $Otx2^{High}$ area of the MHb. **g-h**, Labelling of coronal sections of the IPN with anti- $Otx2$ antibodies (in red), the nuclear marker DAPI (in blue) (g), anti-Fos antibodies (in green), hM3D-cherry (in blue) (h). **h1-h2'**, Zoom on the c-IPN (h1, h1') and the lat-IPN (h2, h2') framed in (h) labeled with hM3D-cherry (in blue) and anti-Fos antibodies (in green). The arrows represent the overlap of hM3D, Fos and $Otx2$ labelling. Scale bar, 100 μm .

Figure 2. Neuronal activity of the MHb in response to a chronic stress endured at different postnatal periods. **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-f**, Area corresponding to the frame in (a) labeled with anti-Egr1 (b-d, e-f), anti-Fos (b'-d') antibodies and DAPI staining (in blue) in the absence of stress at P36 (b, b'), or in response to a chronic stress between P30-36 (c, c'), between P37-43 (d, d'), between P14-20 (e), and between P60-66 (f). **g**, Scheme comparing the different stages of a human life and the mice equivalent age. **h**, Quantification of neuronal activity based on density of Egr1-immunoreactivity in the Otx2^{High} region of the MHb, after 7 days of stress at different postnatal periods (chronic stress 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). The error bars represent the SEM. ** P < 0.01, bilateral t-test. Scale bar 50 μm .

Figure 3. Neuronal activity of the IPN in response to a stress endured at different postnatal periods. **a**, 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. The areas framed correspond to the central (cIPN) and lateral most part of the lat-IPN (L-lat-IPN) in the caudal IPN. **b-c'**, Immunolabelling of the caudal IPN coronal sections with anti-Egr1 (in red) and anti-Otx2 (in green) antibodies without stress at P36 (**b** and **b'**) and in response to a chronic stress between P30-36 (**c** and **c'**). **d-j**, Immunolabelling of coronal sections representing the area framed in (**a**) with anti-Egr1 (in red) and anti-Otx2 (in green) antibodies without stress P36 (**d**) and in response to a chronic stress between P30-36 (**e**), P37-43 (**f**), P14-20 (**g**), P60-66 (**h**) or an acute stress (**i**) or in response to sucrose consumption (**j**). The white arrows indicate a colocalization of Egr1 and Otx2 immunoreactivities. The magenta arrows indicate the same neurons as in Figure 4. **k**, Quantification of neuronal activity based on density of Egr1-immunoreactivity, after 7 days of stress or without stress as a control at different postnatal periods (chronic stress 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) in the caudal IPN region (left graph) or specifically in Otx2⁺ and Otx2⁻ neurons of the caudal IPN (chronic stress P30-36 n=3 and no stress P36 n=4) after 7 days of stress between P30-36 (right graph). Central most part of the lat-IPN (C-lat-IPN). The error bars represent the SEM. * P < 0.05, ** P < 0.01, bilateral t-test. Scale bar 100 μm.

Figure 4. 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 in an acute stress condition at P36. **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) in a condition of acute stress at P36. **b'-b''**, Zoom on the area framed on (b). The white arrows show the colocalization of WFA and anti-Otx2 antibodies. The magenta arrows show the same $Egr1^+$ neurons after the acute stress in the figure 3. **c**, Labelling of coronal sections in the Lat-IPN region with WFA, bound to PNNs, (in red) and anti-Otx2 antibodies (in green) at different postnatal stages (P20, 28 (n=3); P36 (n=5), P43 (n=3), P120 (n=5)). Scale bar 50 μ m.

Figure 5. 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 on mice that have not endured stress (n=6-7) or that have endured a juvenile stress (n=10-12) between P51-55. Parameters tested include total distance traveled (b), mean speed (c) and total number of entries (d) in the center of the open field test, and total time spent in the open arms of the elevated plus maze (e). **f-k**, Behavioral tests on the same mice from P100 after the adult chronic stress including measure of the total distance traveled (f), the mean speed (g) and the total number of entries (h) in the center of the open field test, the total time spent in the open arms of the elevated plus maze (i), the cumulated inactive time in the forced swim test (j), and the percentage of mice in the subgroups curious, indifferent and anxious in the curiosity test (k). The error bars represent the SEM. *P < 0.05, bilateral t-test.

Figure 6. Expression of tdTomato and effects of Otx2 deletion on the HIPS. **a**, Schematic representing the strategy used to induce the recombination of Otx2 floxed allele specifically in MHb neurons observed at P30. **b-c**, Labelling of coronal sections at the level of the MHb (b-b') and the IPN (c-c') in control mice (n=3) with DAPI staining (in blue), anti-Otx2 antibodies (in green) and tdTomato (in red). Arrowheads in b and b' point to tdTomato⁺ cells that are not Otx2⁺, resembling glial cells. **b₁-b₁'**, Zoom on the area framed in b and b'. The arrowheads in b₁ and b₁' point to cells co-expressing Otx2 and tdTomato. **c₁**, 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'. **d-e**, Labelling of coronal sections with anti-Otx2 antibodies (in green), tdTomato (in red) and DAPI staining (in blue) at the level of the MHb (d-d') and the IPN (e-e') in Otx2 cKO mice (n=3). **d₁-d₁'**, Zoom on the area framed in d and d'. The arrowheads in d₁ and d₁' point to cells recombined and do not present Otx2-immunoreactivity. **e₁**, Zoom on the area framed in e and e'. **f**, Quantification of the total surface of the MHb in μm^2 in controls (n=3) and in Otx2 cKO mice (n=3). The error bar represents the SEM. P is not significant, bilateral t-test. Scale bars: b-e' = 100 μm (shown in b', c', d', and e'); c₁, e₁ = 50 μm ; b₁, b₁', c₂, c₂', d₁, d₁' = 25 μm (shown in b₁', c₂').

Figure 7. Effects of *Otx2* deletion alone and in interaction with chronic stress. **a-b**, Behavioral tests on *Otx2^{flax/flax};Ai14/Ai14* (n=10), *Otx2^{+/+};Ngn1Cre^{ERT2};Ai14/Ai14* (n=7) and *Otx2* cKO (n=10) 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-e**, Behavioral tests on *Otx2* cKO that have (n=11) or have not (n=5) endured a juvenile stress. Parameters evaluated include the total distance traveled (c) and number of entries (d) in the center of the open field, and total time spent in the open arms of the elevated plus maze (e). **f**, 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, 6, 12, respectively) or juvenile + adult stresses (n=10, 7, 11, respectively). **g-j**, Behavioral tests evaluating anxiety-like (g-i) and depression-like phenotype (j) by measuring the total distance traveled, the number of entries in the open field, the total time spent in the open arms of the elevated plus maze and the inactive time in the forced swim test for *Otx2* cKO mice that have endured adult (n=6) or juvenile + adult stress (n=12). **k**, global scoring for Ctrl1 and *Otx2* cKO of anxiety-like levels based on mean ranking in both open field and elevated plus maze. **l**, Percentage of mice in the curious, indifferent and anxious subgroup in the curiosity test. **m**, Interaction plot representing the effect of *Otx2* deletion and juvenile chronic stress associated with the adult stress on the time spent in the exploratory area in the presence of an object ($F(1,38)= 6,7127$, Interaction effect $p=0,01350$). The error bars represent the SEM. * $P < 0.05$, ** $P < 0.01$, one-way ANOVA with Holm-Šidák post-hoc test (a,b,k), bilateral t-test (c-j), two-way ANOVA (m).

Figure 8. Effects of *Otx2* deletion in the MHb on the response to pre-adolescent stress. a-f, Labelling of coronal sections with anti-Egr1 antibodies (in red) and DAPI staining (in blue) at the level of the MHb (a, c, e) and with anti-Egr1 (in red) and anti-*Otx2* (green) antibodies at the level of the caudal IPN (b, d, f) in controls (n=3) (a-b) and in cKO mice (n=3) that underwent a chronic stress between P30-36 (c-d) and an acute stress at P30 (e-f). a1-a2, b1-b2, c1-c2, d1-d2, e1-e2, f1-f2 Zoom of the area framed in a, b, c, d, e, f, respectively. g, Quantification of neuronal activity based on density of Egr1-immunoreactivity, after 7 days of stress between P30-36 (CS P30-36) in the MHb and the caudal IPN of control (n=3) and *Otx2* cKO mice (n=3). The error bar represents the SEM. *P < 0.05, **P < 0.01, bilateral t-test. Scale bars: 100 μm.

Figure 9. Effects of *Otx2* deletion in the dMHb on the neuronal activity of the HIPOPS in the long-term in response to a last acute stress. a-f, Labelling of coronal sections with anti-*Egr1* antibodies (in magenta) at the level of the MHb (a-c) and the caudal IPN (d-f) after the “two-hit stress” protocol (juvenile+adult chronic restraint stress) followed by a last acute stress in controls (a,d; n=3) and *Otx2* cKO mice (b,e; n=3), and in controls that only endured the acute stress before the sacrifice (c,f; n=3). a1-a2, b1-b2, c1-c2, d1-d1’-d2, e1-e1’-e2, f1-f1’-f2, Zoom on the area framed in a, b, c, d, e, f, respectively. PNN⁺ cells are labeled with WFA (in green), and anti-*Egr1* antibodies (in red) in d1’, e1’ and f1’. g, Quantification of neuronal activity based on the density of *Egr1*-immunoreactivity after a final acute stress 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 < 0.01, one-way ANOVA with Holm-Šidák post-hoc test. Scale bars: 100 μm.

Figure 10. Proposed mechanism of susceptibility or resilience to juvenile-stress-induced anxiety.

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.

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