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Article

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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 puberty, 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.

Introduction

Most psychiatric illnesses occur in adolescents and adults. They are often triggered by physical or psychological trauma in individuals presenting a susceptibility \(^1\). However, most of the population exposed to similar traumas will not develop symptoms \(^2\). 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 \(^3,4\). Key contributing environmental factors include chronic stresses experienced early in life \(^5\), which are predisposing factors for depression and anxiety \(^5\) and are widely used in animal models to identify the circuits and mechanisms involved.
Among the validated forms of chronic stress that can induce long-term anxiety-like and depression-related behaviors in rodents, restraint has been shown to be very effective. The psychological and physiological changes induced by the restraint result from the distress associated with movement deprivation. This procedure is painless and therefore 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. 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 peri-pubertal period.

Among brain circuits, the reward system is considered 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 circuits and reward circuits and, in fact, considerable overlap between the two. 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. 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 studies link HIPS dysfunction to major depressive disorder and bipolar disorder. Studies on animal models have shown that the HIPS notably contributes to the pathological mechanism of anxiety- and depressive-like disorders. 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, research on the effect of early stress on HIPS plasticity and function is still scarce. Studies of this circuit focus primarily on its physiology in adults, and the few studies on HIPS development have not addressed the possibility of a direct relationship between HIPS developmental abnormalities and stress-induced
pathologies\textsuperscript{21,22}. Furthermore, although the HIPS is involved in many aspects of behavioral control and is known to contain heterogeneous neuronal populations with different developmental origins, molecular phenotypes, and outputs\textsuperscript{23-25}, which function is associated with which population is still unclear.

We recently characterized a circuit within the HIPS\textsuperscript{26}, which critically depends on $Otx2$ expression to develop normally. The habenular neurons of this subcircuit are located in the most medial part of both the dorsal MHB (dMHB) and ventral MHB (vMHB) and have the highest expression of $Otx2$ ($Otx2^{High}$). They preferentially target regions of the IPN that contain a high density of $Otx2^+$ neurons, with which they make synaptic contacts\textsuperscript{26}. Importantly, habenular and IPN neurons in this circuit retain $Otx2$ expression throughout development and into adulthood. 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 during development and in adulthood\textsuperscript{27,28}. Its expression is highly conserved throughout evolution\textsuperscript{29,30}, 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, $Otx2$ has recently been shown to play a major role during specific critical periods of postnatal development of the ventral tegmental area (VTA)\textsuperscript{17}. Early life stress (ELS) reduces $Otx2$ expression in the VTA, only during the stress period, and this reduction has been shown to be the main factor that induced long-term susceptibility to depression-like behavior. Thus, $Otx2$ is an important player in critical periods of neuronal development and any disruption of its expression could lead to long-term effects on brain functions related to the reward system. The fact that $Otx2$ is linked to human pathologies involving the reward system reinforces this idea. $Otx2$ has indeed been shown to be a susceptibility gene for bipolar diseases and stress-related disorders\textsuperscript{31,32}. Because HIPS dysfunctions may be involved in these diseases, it is therefore possible that the role of $Otx2$ in critical periods of HIPOPS development is important in this process.

In this study, we developed a new paradigm to investigate the neurodevelopmental basis of risk/resistance to specific psychiatric disorders and focused on the HIPOPS. We used restraint, as a
form of psychological and physical stress. 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 in later life \(^3\). To demonstrate the sensitivity of the HIPOPS to stress, rather than direct recording of electrical activity, we measured 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 may in turn regulate the transcriptional program related to neuronal activity and synaptic plasticity \(^3\). Therefore, *Egr1* represents a key integrating factor between environmental perception and its translation into an appropriate response. It is thus not surprising that *Egr1* is associated with neuropsychiatric disorders in which neuronal plasticity and activity are altered, such as stress-induced anxiety \(^3\). 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 they do induce long-term changes in the circuitry that make animals more prone to develop anxiety-like behaviors when faced with new periods of stress later on. 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.

**Material and methods**

*See supplemental material and methods for details*

**Animals**

*Otx2\(^{CreERT2/+}\)* and *Otx2\(^{floox/floox}\)* mice were generated as described previously \(^3\). The *Ngn1\(^{CreERT2/+}\);Ai14/Ai14* line was kindly provided by Lisa V. Goodrich \(^3\).
DREADDs microinjections

\( Otx2^{CreERT2/+} \) or \( Otx2^{lox/lox} \) and \( Otx2^{+/+} \) control mice were subjected to stereotactic injection of AAVs expressing the activating Gq-coupled DREADDs hM3D receptor (pAAV9-hSyn-DIO-hM3D(Gq)-mCherry).

Stress protocol

Mice were placed in perforated syringes to allow comfortable breathing, adapted to 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 of the last chronic stress.

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.

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.

Immunohistochemistry and histological analysis
Brains were perfused or directly frozen. Perfused sections were unmasked when Otx2 was to be revealed and sections were postfixed when the brain was directly frozen. Slides were incubated in a blocking solution for 1h. at RT, then with primary antibodies overnight at 4°C, then with secondary antibodies for 2hrs at RT, and a final incubation in DAPI.

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.

Statistical analysis

Statistical analyses were made using the R software. Comparison of the mean of two groups was done by using the Student t-test. Comparison of four 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.

Results

1. HIOPPS circuits are interconnected and Otx2\textsuperscript{High} MHb region drives Otx2\textsuperscript{+} cIPN activity

Previous results showed that MHb Otx2\textsuperscript{High} neurons preferentially make synapses with Otx2\textsuperscript{+} IPN neurons, all located in the caudal IPN (cIPN), suggesting that Otx2 marks a subcircuit inside the HIOPS that may be preferentially interconnected \textsuperscript{25}. To test this hypothesis further, we asked whether
chemogenetic activation of MHb Otx2\textsuperscript{High} neurons that are glutamatergic would preferentially drive the activation of their targets in the cIPN of mice. We forced activation of Otx2\textsuperscript{high} MHb neurons in Otx2\textsuperscript{CreERT2/+} mice through a DREADD-hM3D-approach (Fig1a), consisting of CreERT2-dependent expression and CNO-dependent activation of the DREADDs, and monitored neuronal activation (Fig1b) by examining the expression of immediate-early genes, strongly and specifically induced after neuronal activation \textsuperscript{37}. The approach was specific because in tamoxifen-injected control mice that did not express the CreERT2 (Otx2\textsuperscript{+/+}) or that expressed the CreERT2 (Otx2\textsuperscript{CreERT2/+}) but did not receive CNO, no evidence of neuronal activation could be detected in the MHb and IPN (Supplemental Fig.S1). In contrast, upon tamoxifen and CNO injections in Otx2\textsuperscript{CreERT2/+} mice, a strong Fos-immunoreactivity was found in dMHb neurons expressing hM3D in the Otx2\textsuperscript{High} region (Fig.1d-f). In cIPN targets, neuronal activity could be detected in the two Otx2\textsuperscript{+} subnuclei, the med-cIPN (medio-caudal IPN) and lat-cIPN (latero-caudal IPN) (Fig.1g,h). Moreover, only cells that were surrounded by hM3D-mCherry and expressing Otx2\textsuperscript{+} were strongly Fos-immunoreactive (Fig.h1-h2').

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

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. After the last stress, expression of c-Fos and Egr1 (Krox-24, Zif-268), another immediate early gene \textsuperscript{37}, was strongly induced in the dMHb compared to control in Otx2\textsuperscript{High} neurons (Fig.2a-h). 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 particularly evident post-
weaning into the pre- and adolescence stages (Fig.2c-d’, graph Fig.2j and Supplemental Fig.S2). Following the same trend, activation of the IPN was strong and more evident at these stages (Fig.2h-i’, graph Fig.2k and Supplemental Fig.S3). Interestingly, the neurons particularly activated belonged to Otx2⁺ neurons of the med- and lat-cIPN (Fig.2h-i’, graph Fig.2l and Supplemental Fig.S3). In the lat-cIPN, 2 different Otx2⁺ populations could be distinguished based on their precise location, reaction to stress and molecular profile. Neurons in the M-lat-cIPN but not in the L-lat-cIPN were activated by CS (Fig.2g-i). Only Otx2⁺ neurons of the L-lat-cIPN strongly expressed parvalbumin and were surrounded by perineuronal nets (PNNs), a specialized extracellular matrix marked by the lectin WFA (Supplemental Fig.S4). We then tested the effect of an acute stress (AS) of 2hrs restraint which was unable to activate neurons of the MHb (Supplemental Fig.S5a-c). In the med-cIPN, mainly neurons that were Otx2⁻ were strongly activated (Supplemental Fig.S5d-f). In the lat-cIPN activated neurons were both Otx2⁻ and Otx2⁺ neurons. These Otx2⁺ neurons were located in the L-lat-cIPN and thus belonged to the PV⁺ population.

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⁻ or Otx2⁺/PV⁺ neurons in the IPN, the HIPOPS specifically responds to CS. Repeated restraint activates MHb neurons in the Otx2²⁺ region and consequently, Otx2⁺ neurons in the med-cIPN and M-lat-cIPN. The peak of activation is around puberty (P30-P36).

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 on. We used a two-hit stress model, composed of a mild stress (2 hours of 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 general mobility and anxiety levels 2 weeks after the end of the stress. As shown in Fig.3b-e, the behavior of the stressed mice was indistinguishable from that of the control mice, 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 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.3f,h-i). These differences were not due to motor dysfunctions (Fig.3g). Depressive-like behavior was also not significantly affected (Fig.3j). Interestingly, though, curiosity toward a new object was reduced (Fig.3k). This reduction in curiosity could also be explained by increased anxiety as early stressed mice were not more indifferent to the new object but avoided it, compared to control mice.

These results show for the first time that a chronic stress, 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.

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 anxiety-like behavior, we tested whether interfering with the normal development of the HIPOPS would change the outcome of individuals experiencing the early stress. Since Otx2 is a master gene of HIPOPS development, we used the Ngn1CreERT2 line to delete Otx2 specifically in this circuit 36,38. In this line, CreERT2 expression becomes gradually restricted as mice age, overlapping Otx2 expressing sites only in the dMHb and in
rare photoreceptors in the retina, as shown by the lineage of CreERT2 expressing cells followed from P0 to P30 (Supplemental Fig.S6 and FigS7).

*Otx2* invalidation was triggered by tamoxifen injection at P0. At P30, *Otx2*<sub>flox/flox</sub>;*Ngn1<sub>CroERT2</sub> animals were compared to tamoxifen-treated *Otx2*<sub>flox</sub> or to *Otx2*<sub>+/-</sub>;*Ngn1<sub>CroERT2</sub> mice. The habenula in these mice appeared normal. Projections from the neurons that had lost *Otx2* expression were maintained, surrounding their IPN targets (Supplemental Fig.S6b-e'). The size of the MHb was unchanged further supporting lack of structural abnormalities in the circuit (Supplemental Fig.S6f). 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 such as mean speed or distance travelled in the open field (Fig.4a-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 conditions.

Because the dMHb is particularly activated by CS, especially during the critical period of HIPOPS development (Fig.2), and because the HIPOPS is closely dependent on *Otx2* gene function, we 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.4c-e). We next analyzed behaviors after the two-hit stress protocol. As expected, in controls, the two-hit stress protocol increased anxiety levels; in contrast, this effect of stress could not be observed in *Otx2*<sub>flox/flox</sub>;*Ngn1<sub>CroERT2</sub> mice (Fig.4f). In fact, all the parameters that were affected in control animals (see Fig.3), after the early + late stress compared to the late stress alone, were not affected in *Otx2*<sub>flox/flox</sub>;*Ngn1<sub>CroERT2</sub> mice (Fig.4g-i). Comparison of the global anxiety level score confirmed that only control mice were statistically affected by the two-Hit stress protocol (Fig.4k). The time of inactivity in the FST test was similar in *Otx2*<sub>flox/flox</sub>;*Ngn1<sub>CroERT2</sub> mice with or without the early stress (Fig.4j), as expected, since the stress-protocol does not induce depressive-like symptoms in controls. In the curiosity test, the proportion of curious, anxious and indifferent individuals to a new object was similar between cKO animals without stress and those with the two-hit stress protocol (Fig.4l). This result is dramatically different from what we observed in control animals (see Fig3k and...
Interestingly, the effect of adult stress alone followed the same trend as in controls (Fig.3k, Fig.4l and graph Fig.4m) with an increase in the proportion of indifferent and anxious mice.

Thus, deletion of Otx2 in the dMHb results in a relative protection against the effects of stress on the long-term regarding anxiety and curiosity. This resilience effect appears to be the consequence of an altered G x E 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 induced by Otx2 ablation in the dMHb, we analyzed the HIPOPS response to juvenile stress in Otx2\textsuperscript{lox/lox};Ngn1\textsuperscript{CreERT2} animals (Fig.5). Interestingly, in these mice, although the dMHb was even more active than in controls following juvenile CS (Fig.5a3-4’ and .5b and supplemental Fig.S8), this did not induce any activation of IPN neurons (Fig.5c-d and supplemental Fig.S8). This might suggest that Otx2 deletion results in abnormal connectivity between the dMHb and its IPN output. However, the strong activation of Otx2\textsuperscript{+} med-cIPN and M-lat-cIPN neurons by an acute stress (Fig.5c2-c2’), in correlation with a strong activation of the dMHb (Fig.5a2-a2’) ruled out this hypothesis. This was all the more surprising because an acute stress (AS) normally does not significantly activate the dMHb at all time points tested (Fig.5a1-a1’, c1-c1’ and supplemental Fig.S5 and S9).

Thus, deletion of Otx2 in Otx2\textsuperscript{lox/lox};Ngn1\textsuperscript{CreERT2} mice exacerbates the stress sensitivity of the habenular part of the HIPOPS circuit. This habenular hyper-sensitivity, paradoxically, renders IPN neurons insensitive after 5-7 days of stress.

We then tested whether this biphasic response to the early stress would have an impact on long-term response of the circuit to stress. We examined the status of the circuit at the adult stage (P130), in control and Otx2 cKO mice that had or had not undergone the two-hit protocol, when subjected to a final AS. No activity was detected in the dMHb in any of the conditions except rare cells in the dMHb of control
animals with previous two-hit stress (Supplemental Fig.S10a1,b1,c1). There was tendency of higher activity in IPN neurons in control animals with previous two-hit stress compared to all other conditions that only reach significance compared to the one in Otx2 cKO with prior two-hit stress (Fig.5e-g). Activity in Otx2 cKO appeared surprisingly low, particularly in the L-lat-cIPN, where PNN+ neurons are readily activated even in control animals without previous two-hit stress (f1-f2'). 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\textsuperscript{39,40} and supplemental Fig.S10) presented a higher activity in control mice than in Otx2 cKO animals subjected to previous two-hit stress.

\textit{Otx2} deletion of 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, \textit{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. This demonstrates the existence of a G x E interaction at the HIPOPS level between genetic factors, such as \textit{Otx2}, and chronic stress, during a critical period of development. This also suggests that blocking this interaction, through deletion of \textit{Otx2} in the dMHb, leads to a form of resilience to the development of anxiety. Finally, it suggests that HIPOPS neurons determine the susceptibility to anxiety conditioned by stress experiences during the juvenile period and the stress-response of other stress-related circuits (Supplemental Fig.S11).

**Discussion**

We showed that a chronic stress but not an acute stress 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 \textit{Otx2}\textsuperscript{+} 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 \textit{Otx2}\textsuperscript{high} neurons (\textsuperscript{25} and this study) project specifically and directly to \textit{Otx2}\textsuperscript{+} neurons of the IPN. Furthermore, DREADD-
mediated activation of Otx2<sup>High</sup> neurons, specifically triggers activity of Otx2<sup>+</sup> IPN neurons and only is those that are surrounded by DREADD<sup>+</sup> fibers in the med- and lat-cIPN. In Otx2 cKO animals, there is a relative stronger activation of Otx2<sup>High</sup> habenular neurons by a stressful experience, correlated with a paradoxical insensitivity of Otx2<sup>+</sup> 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<sup>high</sup> neurons, leading to stress hypo-response of Otx2<sup>+</sup> IPN neurons, in adults. According to these observations, we propose that the level of HIPOPS activity, at the end of the stress period at P36, determines the intensity of HIPOPS response to further stress episodes after the closure of a hypothetical critical period (Supplemental Fig.S11). This could explain why individuals of certain genetic background develop resistance rather that susceptibility to disease after trauma. 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<sup>41</sup>. These results also suggest that Otx2, which is known to be involved in critical periods of development<sup>42</sup>, may be involved 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<sup>43</sup>. Furthermore, the effect of the deletion in 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<sup>+</sup> cells through non-cell autonomous mechanisms that involves the binding of Otx2 to PNNs surrounding these neurons. In this study we found that the L-lat-cIPN is enriched in cells that are strongly PV<sup>+</sup> and surrounded by PNNs revealed by their high content in glycoconjugates that bind to Wisteria floribunda agglutinin (WFA). These Otx2<sup>+</sup> neurons are induced by acute but not chronic stress. Other Otx2<sup>+</sup> neurons in the IPN (M-lat-cIPN and med-cIPN), on the contrary, are only induced by repeated stress certainly due to strong activity of Otx2<sup>high</sup> neurons of the MHb. It is thus possible that a low activity of the MHb upon acute stress (below our detection threshold) is able to induce L-lat-cIPN neurons only, whereas, a strong activity is required to induce the other population. A hypothetical reciprocal inhibition would explain
why activation of these two different populations is mutually exclusive. Although this reciprocal inhibition is still hypothetical, it appears that an inhibitory mechanism is altered by chronic stress during the critical period. Indeed, an abnormal high activity of the M-lat- and med-cIPN upon an acute stress is found in anxious-like mice and an abnormal low activity of L-lat-, M-lat- and med-c-IPN upon an acute stress is found in resistant mice at the end of the two-hit stress protocol. The origin of this defect lies during the period of the juvenile stress and may be linked to a defect in the establishment and consolidation of the reciprocal inhibition between these two types of Otx2+ neurons and/or of MHb connections. This is an important lead to follow. In this context it is of particular interest that PNN structures are not fully mature until the end of the pre-adolescent period (Fjerdingstad et al.; unpublished observations). It is tempting to speculate that a non-cell autonomous mechanism of Otx2 operates here in the maturation and function of PV+ cells and that the source of extracellular Otx2 comes from the MHb projections.

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 this study, 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, that present at the circuit level stress hypersensitivity of the HIPOPS and of the whole stress-related circuit. These neuronal and behavioral effects are most likely 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. Clinical data point to puberty as, a window of vulnerability to anxiety, depression, schizophrenia, and substance abuse 44, 45, and exposure to stress plays an important role 46. 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<sup>High</sup> neurons chemoactivation in the MHb.**

**a**, Schematic representation of the activating DREADD induction in the Otx2<sup>High</sup> neurons of the MHb in Otx2<sup>CreERT2/+</sup> mice (n=3). **b**, Schematic illustration of an MHb coronal section representing the localization of substance P<sup>+</sup> (in red), ChaT<sup>+</sup> (in green), and Otx2<sup>+</sup> (in blue) neurons.

**c**, Projection map of the caudal IPN level showing the distribution of Otx2<sup>+</sup> neurons. **d**, Immunohistochemical staining for Otx2, Brn3a, and Er81 in the MHb. **e**, Immunohistochemical staining for Otx2 in the MHb. **f**, Immunohistochemical staining for hM3D and Fos in the caudal IPN. **g**, Immunohistochemical staining for Otx2 and DAPI in the cIPN. **h**, Immunohistochemical staining for Fos, Otx2, and hM3D in the IPN.
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-cIPN (h1,h1') and the lat-cIPN (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. Scale bar, 100 µm.

**Figure 2**

Neuronal activity of the MHb and the IPN in response to chronic stress. 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 ant-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, 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. 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 chronic stress 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 (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=4) (j) and in the caudal IPN (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) (k). l, Quantification of neuronal activity based on density of Egr1-immunoreactivity colocalizing or not with Otx2 immunoreactivity, after 7 days of stress between P30-36 (chronic stress P30-36 n=3 and no stress P36 n=4). M-lat-clPN = Medio-lateral clPN, L-lat-clPN = Latero-lateral clPN. The error bars represent the SEM. * P < 0.05, ** P < 0.01, bilateral t-test. Scale bar 50 µm b-f (shown in b), 100 µm h-i' (shown in h).
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 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 4**

**Effects of Otx2 deletion alone and in interaction with chronic stress.** a-b, Behavioral tests on Otx2<sup>lox/lox</sup>;Ai14/Ai14 (n=10), Otx2<sup>+/+</sup>;Ngn1Cre<sup>ERT2</sup>;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 5

Short and long term effects of Otx2 deletion combined to pre-adolescent stress. a, Schematic representation of an MHb coronal section showing the localization of substance P+ (in red), ChAT+ (in green), Otx2High (in blue) neurons. a1-a4’, Regions framed in (a) of coronal sections with anti-Egr1 antibodies (in red) and DAPI staining (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 of the caudal IPN regions in coronal sections where Otx2High substance P-ergic (in red) and cholinergic (in green) neurons of the MHb project and localization of the Otx2+ neurons of the IPN. 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 of the caudal IPN as in (c). e1-f4’, Regions of the med-cIPN framed in (d) (e1-e4) or of the L-lat-cIPN 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 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 < 0.05, **P < 0.01, bilateral t-test.
(b,d), one-way ANOVA with Holm-Šídák 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).

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