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► **To cite this version:**

Margherita Zaupa, Seyedeh Maryam Alavi Naini, Maroun Abi Younes, Erika Bullier, Erik R Duboué, et al.. Trans-inhibition of axon terminals underlies competition in the habenulo-interpeduncular pathway. *Current Biology - CB*, 2021, 31 (21), pp.4762-4772.e5. 10.1016/j.cub.2021.08.051 . hal-03367472

HAL Id: hal-03367472

<https://hal.science/hal-03367472v1>

Submitted on 13 Oct 2021

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Accepted Manuscript

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PII: S0960-9822(21)01187-8
DOI: doi:[10.1016/j.cub.2021.08.051](https://doi.org/10.1016/j.cub.2021.08.051)
Reference: CURBIO 17823

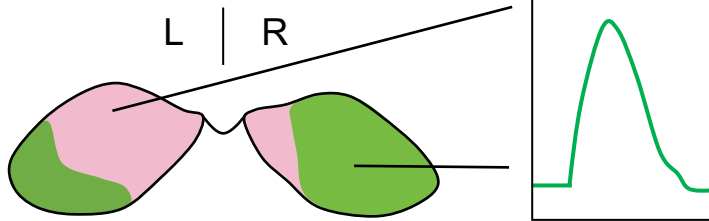
Published in: *Current Biology*

Received date: 22 April 2021
Revised date: 12 July 2021
Accepted date: 18 August 2021

Cite this article as: Zaupa M, Alavi Naini SM, Younes MA, Bullier E, Dubou ER, Le Corrnc H, Soula H, Wolf S, Candelier R, Legendre P, Halpern ME, Mangin J-M, Hong E, Trans-inhibition of axon terminals underlies competition in the habenulo-interpeduncular pathway, *Current Biology*, doi:[10.1016/j.cub.2021.08.051](https://doi.org/10.1016/j.cub.2021.08.051)

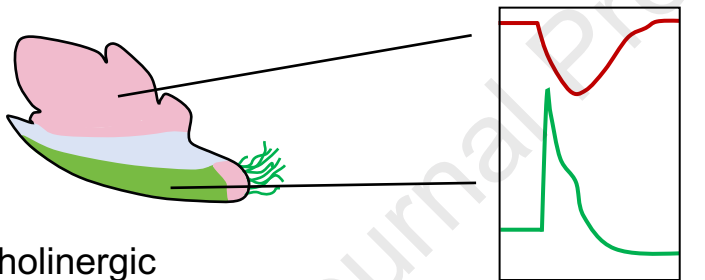
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dHb soma



Calcium signals are decoupled between dHb soma and their terminals

dHb terminals



Ca²⁺ activity

Negatively correlated event



ex vivo

spontaneous activity
nicotine

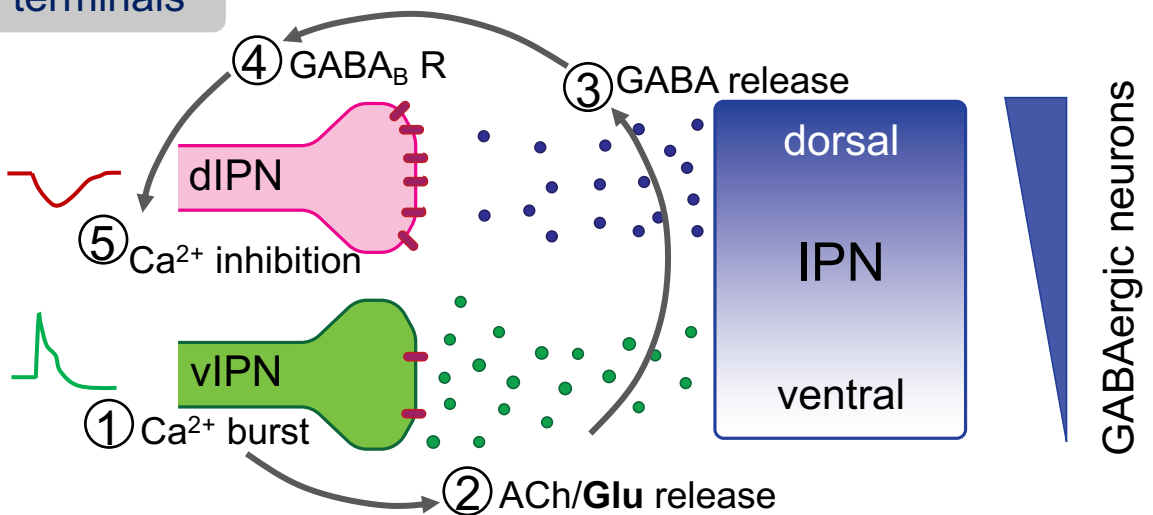


in vivo

electric shock

cholinergic
non-cholinergic

dHb terminals



Title

Trans-inhibition of axon terminals underlies competition in the habenulo-interpeduncular pathway

Authors

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Summary

Survival of animals is dependent on the correct selection of an appropriate behavioral response to competing external stimuli. Theoretical models have been proposed and underlying mechanisms are emerging to explain how one circuit is selected among competing neural circuits. The evolutionarily conserved forebrain to midbrain habenulo-interpeduncular nucleus (Hb-IPN) pathway consists of cholinergic and non-cholinergic neurons, which mediate different aversive behaviors. Simultaneous calcium imaging of neuronal cell bodies and of the population dynamics of their axon terminals reveals that signals in the cell bodies are not reflective of terminal activity. We find that axon terminals of cholinergic and non-cholinergic habenular neurons exhibit stereotypic patterns of spontaneous activity that are negatively correlated and localize to discrete subregions of the target IPN. Patch-clamp recordings show that calcium bursts in cholinergic terminals at the ventral IPN trigger excitatory currents in IPN neurons, which precede inhibition of non-cholinergic terminals at the adjacent dorsal IPN. Inhibition is mediated through presynaptic GABA_B receptors activated in non-cholinergic habenular neurons upon GABA release from the target IPN. Together, the results reveal a hardwired mode of competition at the terminals of two excitatory neuronal populations, providing a physiological framework to explore the relationship between different aversive responses.

Keywords

Habenula; interpeduncular nucleus; nicotine; GABA_B receptor; calcium imaging; electrophysiological recording; zebrafish

Introduction

The bilaterally paired habenular nuclei (Hb) are part of the highly conserved dorsal diencephalic conduction system connecting the limbic forebrain with monoaminergic brainstem areas^{1,2}. Anatomically, they consist of medial (MHb) and lateral (LHb) nuclei in mammals that are homologous to the dorsal (dHb) and ventral (vHb) nuclei of teleosts. The LHb/vHb send projections through the fasciculus retroflexus (FR) directly to serotonergic raphe neurons and dopaminergic neurons in the ventral tegmental area. Axons from MHb/dHb neurons course through the FR and terminate at an unpaired midbrain target, the interpeduncular nucleus (IPN). The Hb-IPN pathway is critical for understanding addiction and mood disorders due to its involvement in regulating behaviors such as stress, anxiety and fear learning^{1,3-6}.

The MHb/dHb contains glutamatergic neurons are divided into those that co-release acetylcholine (cholinergic) or neuropeptides, including substance P (non-cholinergic) or somatostatin⁷⁻⁹. In rodents, cholinergic and non-cholinergic neuronal populations project to central and peripheral IPN domains, respectively⁷. Gene expression analyses, fluorescent dye tracing and transgenic reporters have demonstrated left-right differences between the bilateral dHb of larval zebrafish^{5,10,11}. For example, cholinergic neurons are located mainly in the right dHb and project to the ventral IPN (vIPN), whereas non-cholinergic neurons are more abundant in the left dHb and innervate the dorsal IPN (dIPN)^{9,12} (Figure 1A). In rodents, cholinergic and non-cholinergic MHb neurons receive afferent input from neurons located in the triangular septum (TS) and the bed of the anterior commissure (BAC), respectively¹³. Toxin-induced ablation of TS-cholinergic or BAC-non-cholinergic MHb projections selectively impaired anxiety or fear learning behaviors³. Conditional genetic deletion

of Cerebellin (Cbln) genes in the MHb, Cbln4 in cholinergic or Cbln2 in non-cholinergic neurons, affects freezing behavior or spatial learning, respectively⁴.

The dHb responds differently to external factors such as light, odor and aversive stimuli in zebrafish larvae^{6,14,15}. Inhibition of neural transmission in the non-cholinergic (dHbL) versus cholinergic (dHbM) pathways in adult zebrafish is associated with winner or loser states in a social conflict paradigm¹⁶. Studies in rodents and zebrafish suggest that the cholinergic and non-cholinergic MHb/dHb neuronal populations form functionally distinct, parallel circuits. However, it is unknown whether the two pathways interact or directly influence the activity of one another.

To understand functional connectivity of the dHb-IPN pathway, we took advantage of the small size of the larval zebrafish brain to monitor neural activity in whole explant preparations. Calcium imaging revealed that dHb terminals at the IPN can be resolved into distinct domains based on their signaling dynamics. We discovered an inverse relationship between cholinergic and non-cholinergic neurons only at their axon terminals. Results from pharmacology, imaging and electrophysiology experiments show that synchronized activity of cholinergic dHb neurons results in GABA release from the IPN, leading to presynaptic inhibition of non-cholinergic axon terminals. We propose a hardwired mode of competition between two neuronal populations, whereby synchronized activation of one group inhibits the activity of the other.

Results

Cholinergic and non-cholinergic dHb terminals exhibit negatively correlated calcium events

The bilateral dHb on the dorsal surface of the diencephalon form connections with the IPN deeply embedded in the ventral midbrain, making it challenging to simultaneously monitor and manipulate both pre-and post-synaptic neuronal populations. To identify the hardwired mode of action of the dHb-IPN pathway, we analyzed spontaneous activity in both regions using brain explants from larval zebrafish. The entire brain was removed from *TgBAC(gng8:GAL4ff)^{c426};Tg(UAS:GCaMP7a)* larvae expressing GCaMP in dHb neuronal cell bodies and their terminals at the IPN⁶. We found that whereas most dHb neurons exhibit spontaneous activity (Video S1, Figure S1A), a large cluster of neurons on the right together with a small cluster on the left exhibited correlated calcium bursts (Figure 1B-E, Video S1). The neuronal clusters with highly correlated calcium bursts were consistently identified by *k*-means and hierarchical clustering methods (Figure 1B-E, Figure S1). Their location (Figure 1B, Figure S1, green and blue) corresponds to that previously described for cholinergic and somatostatinergic neuronal clusters in the dHb (Figure 1A)^{9,12}.

We examined whether similar calcium signals were present in dHb axon terminals at the IPN. As the left dHb contains cholinergic and non-cholinergic neurons, which form connections with the dorsal and ventral IPN (Figure 1A)^{9,12}, we carried out simultaneous calcium imaging of neurons in the left dHb and axon terminals at the IPN (Video S2). We used *k*-means clustering to identify domains in an unbiased manner since results were comparable with hierarchical clustering. Distinct domains of spontaneous activity defined stereotypic and correlated patterns of dHb neurons. The soma of the left dHb segregated into two (dorsal and ventral) and dHb axon terminals fell into three domains at the dorsal IPN (dIPN), ventral IPN (vIPN) and raphe nucleus (raphe) (Figure 1F). The activation pattern of ventral dHb

soma clustered together with that of the raphe area, suggesting that these regions are functionally connected (Figure 1F, green). Surprisingly, we found that calcium signals in the dIPN were negatively correlated with those in the ventral dHb soma and raphe (Figure 1F-I, red). When an increased calcium signal (burst) occurred in the soma of the ventral dHb and terminals at the raphe, a transient decrease from baseline calcium signaling (inhibition) was observed at the dIPN (Figure 1H,I). Calcium inhibition was limited to terminals at the dIPN and was not observed in the soma of dHb neurons (Figure 1F). Indeed, analysis of different dHb focal planes in multiple explants failed to reveal neuronal cell bodies exhibiting negatively correlated calcium signals during spontaneous activity (Figure 1C-E, Figure S1). The results indicate that calcium signals in dHb soma are not always reflective of the calcium dynamics at their terminals.

Calcium signaling data also revealed a local negative interaction between dHb terminals at the IPN. We analyzed activity in dHb terminals throughout the IPN (Video S3) and by applying *k*-means clustering and manual inspection of the calcium signals, we identified five spatially segregated domains of axon terminals with distinct patterns of activity: 1) dIPN showing mainly calcium inhibition with few calcium bursts (red); 2) dorsal half of the vIPN (dvIPN) displaying frequent calcium bursts of low and high amplitude, with the high amplitude bursts followed by calcium inhibition (grey); 3) ventral half of the vIPN (vvIPN), showing high amplitude calcium bursts followed by inhibition (blue); 4) caudal area of the vIPN (cvIPN) (magenta), which clustered together with the dIPN exhibiting calcium inhibition; and 5) raphe area exhibiting calcium bursts without inhibition (green) (Figure 1J-P).

Notably, terminals at the dIPN and raphe exhibited negatively correlated calcium signals (Figure 1L), which we refer to as negatively correlated events.

Calcium inhibition only occurred in terminals at the dIPN when high amplitude bursts were observed at the vvIPN and raphe (Figure 1N) but not when calcium bursts were restricted to the dvIPN (Figure 1O). This suggests that dHb neurons that target the dvIPN differ from those innervating the vvIPN and raphe. Moreover, prior to or following a negatively correlated event, terminals at the dIPN showed higher GCaMP signals compared to those observed in the vIPN (Figure 1K,N; Figure S2A,B). The higher signals were not merely due to greater terminal density at the dIPN relative to the vIPN because we found no difference in the ratio of axon terminals in the two regions when labeled with GFP (Figure S2A'-B).

Quantification of calcium signaling kinetics revealed that the time to reach maximum amplitude (peak time) occurred earlier in terminals at the vvIPN compared to the raphe (1.13 ± 0.42 s vs. 1.59 ± 0.72 s), and the duration at half peak time (half duration) of the calcium burst was shorter (2.72 ± 0.56 s vs. 3.64 ± 1.55 s) (Figure S3A-C). Peak inhibition in terminals at the dIPN occurred later (4.33 ± 1.4 s) and lasted longer (9.2 ± 2.9 s) than the calcium bursts (Figure S3D,E). Activation at the vvIPN was followed by a period of inhibition not observed in terminals at the raphe. Therefore, to resolve the relationship between the negatively correlated events, we measured activity in terminals at the dIPN and at the raphe as a proxy for the vvIPN. We found a 931 ± 50 ms delay between the onset of the calcium burst and inhibition (Figure 2A), suggesting that calcium activity at the vvIPN triggers calcium inhibition in the dIPN.

To identify the neurotransmitter phenotype of neurons that exhibit calcium bursts at the vvIPN and raphe, we assessed Vesicular Acetylcholine Transporter (VAcHT) expression by immunolabeling in *Tg(nptx2:Gal4-VP16)^{rw0143a};Tg(UAS:GFP)^{c354}* larvae. We previously found that this transgenic line

mainly labels non-cholinergic neurons in the left dHb and their projections to the dIPN¹². We confirmed that GFP expression does not colocalize with VACHT protein (Figure 2B) whereas dHb terminals at the vIPN and raphe are positive for VACHT staining in *TgBAC(gng8:GAL4ff)^{c426};Tg(UAS:GFP)^{c354}* larvae (Figure 2B). We therefore conclude that calcium bursts at these regions are due to the synchronized activation of dHb cholinergic neurons (Figure 2C). Negatively correlated events are already apparent in 3-day-old larvae (Figure S4) upon emergence of spontaneous activity in dHb neurons (data not shown), suggesting that it is a hardwired mode of activity, coincident with establishment of the dHb-IPN pathway.

To determine whether external stimuli provoke negatively correlated events in vivo, we applied a mild electric shock to larvae. Indeed, we observed negatively correlated events between dHb terminals at the dIPN and vIPN/raphe (Figure 2D). However, due to tissues surrounding the brain, the resolution of calcium activity was inferior in larvae compared with the brain explants. Nevertheless, this result indicates that negatively correlated events can be induced in vivo, suggesting it is a physiologically relevant mode of neuronal activation.

Nicotinic acetylcholine receptors do not mediate negatively correlated events

The MHb/dHb-IPN pathway shows the highest expression and greatest variety of nicotinic acetylcholine receptor (nAChR) subunits in the brain¹⁷. Since calcium inhibition in non-cholinergic dHb terminals is preceded by a calcium burst in cholinergic terminals, we investigated whether inhibition depends on the activation of nAChRs⁹. Perfusion of nicotine resulted in prolonged calcium inhibition of dHb terminals at the dIPN, which corresponded with an extended series of calcium bursts in dHb terminals at the raphe (Figure 3A,A', 260 ± 176 s). To determine the

contribution of nAChRs in the generation of negatively correlated events, we administered mecamylamine and d-tubocurarine, effective blockers of cholinergic transmission in the larval IPN⁹. nAChRs antagonists did not attenuate, but rather increased the frequency of negatively correlated events (Figure 3B-C'). Thus, activation of nAChRs is not required for negatively correlated events between cholinergic and non-cholinergic dHb terminals at the IPN.

dHb terminal calcium bursts correlate with GABAergic currents in IPN neurons

To understand the basis for negatively correlated events, we combined calcium imaging of dHb terminals with patch-clamp recordings of neurons at the interface between the dIPN and vIPN. Recording at -60 mV (equilibrium potential of Cl⁻ ions) revealed that calcium bursts correlated with a barrage of postsynaptic inward currents consisting of fast and slow events (Figure 4A,B), which is due to release of glutamate and acetylcholine⁹. Recording at 0 mV (reversal potential of cationic ions) revealed that calcium bursts also correlated with a barrage of outward currents (Figure 4A',B') indicative of GABAergic inhibitory postsynaptic currents. The cholinergic/glutamatergic postsynaptic barrage was relatively short (half duration = 262 ± 124 ms) and synchronized with the onset of the calcium burst (delay = 67 ± 69 ms). In contrast, the barrage of GABAergic synaptic currents had a delayed onset (delay = 162 ± 80 ms) and lasted longer (half duration = 387 ± 143 ms). These results suggest that calcium bursts correspond to the release of glutamate and acetylcholine from dHb terminals at the IPN, which is followed by local release of GABA from IPN neurons.

Presynaptic GABA_B receptors in dHb axon terminals mediate calcium inhibition

GABA has an inhibitory presynaptic action on transmitter release through metabotropic GABA_B receptors¹⁸. We asked whether GABA released by the IPN could activate presynaptic metabotropic GABA_B receptors, thereby repressing dHb terminal activity. Application of cgp55845, a GABA_B receptor antagonist, resulted in a 74 ± 9 % reduction in the amplitude of calcium inhibition (-0.16 ± 0.03 vs. -0.04 ± 0.01) without affecting the amplitude of calcium bursts (0.22 ± 0.09 vs. 0.26 ± 0.13) (Figure 5A,A'). To test the role of GABA_B receptors directly, we applied tetrodotoxin to inhibit the propagation of action potentials and eliminate all negatively correlated events. We then administered baclofen, a GABA_B receptor agonist, and observed a prolonged decrease in the calcium baseline, similar in amplitude to that observed during inhibition of dHb terminals at the dIPN (Figure 5B,B'). Calcium imaging of dHb terminals together with patch-clamp recordings of IPN neurons confirmed that prolonged inhibition corresponds with a decrease in the frequency of fast postsynaptic inward currents recorded in IPN neurons (Figure 5C,C').

In zebrafish, there are two GABA_B1 receptor genes, *GABA_B receptor 1a* (*gabbr1a*) and *1b* (*gabbr1b*)¹⁹. We found that *gabbr1a*, but not *gabbr1b*, is prominently expressed in the dHb in a larger domain of the left nucleus compared with the right, in the location of non-cholinergic neurons (Figure 5D,E). Strikingly, *gabbr1a* transcripts localized to the dHb nuclei and were not detected in other brain regions including the IPN (Figure 5D). Together, these results suggest that calcium inhibition is due to the activation of presynaptic GABA_B receptors in non-cholinergic terminals at the dIPN.

Retrograde signaling from GABAergic IPN neurons to non-cholinergic habenular inputs

To determine that inhibition of dHb terminals at the dIPN results from GABA released upon IPN activation, we carried out electrical stimulation of the IPN with calcium imaging (Figure 6A, Video S4). Activation of the IPN effectively triggered negatively correlated events consisting of the vIPN/raphe calcium burst (likely due to the activation of the dHb terminals at the vIPN) and dIPN calcium inhibition (Figure 6B). We next perfused a GABA_B antagonist, cgp55845, during negatively correlated events induced by electrical stimulation and found a 90 ± 13 % decrease in calcium inhibition at the dIPN (-0.15 ± 0.07 vs. -0.02 ± 0.02) without any change in calcium bursts at the raphe (0.22 ± 0.08 vs 0.2 ± 0.06) (Figure 6B-E).

To verify the presence of GABAergic neurons in the IPN, we imaged the *TgBAC(gng8:GAL4ff)^{c426};Tg(UAS:GFP)^{c426};Tg(gad1b:|R|-GFP)* larvae in which dHb terminals are labeled with GFP and GABAergic neurons by RFP²⁰. We found that nearly half of IPN neurons were labeled by *gad1b:RFP* ($48 \pm 2\%$, $n = 3$ larvae, $N = 479$ cells, Figure 6F), with more located in the dIPN than vIPN ($79 \pm 11\%$ vs. $21 \pm 11\%$, $n = 3$ larvae, $N = 232$ *gad1b:RFP*⁺ cells). These results suggest that a higher concentration of GABA is released from neurons in the dIPN.

Discussion

The dHb of zebrafish larvae respond to external factors including light, odor and aversive stimuli^{6,14,15}. A prerequisite to investigating the innate hardwired activity of the dHb-IPN pathway was eliminating the influence of external factors. We achieved this by performing calcium imaging using a whole brain explant preparation, in which central neural circuits including the dHb-IPN pathway remain intact. We found a

stereotyped mode of spontaneous activity in dHb soma and axon terminals at the IPN.

Through pharmacological and electrophysiological manipulations, we determined that synchronized activation of dHb cholinergic neurons inhibit non-cholinergic neurons by an atypical mechanism of target-mediated, trans-inhibition at the level of axon terminals.

Negatively correlated events at axon terminals of cholinergic and non-cholinergic dHb neurons

Clustering algorithms are commonly used to segregate neurons into different functional groups during calcium imaging of neuronal soma²¹⁻²³. The k value and threshold for hierarchical clustering were determined from a previous study of the zebrafish dHb²³ and manual inspection of the activity of individual neurons assigned to different clusters. We obtained similar but not identical results from k -means and hierarchical clustering methods. Nevertheless, the minor difference resulting from the clustering algorithms does not alter the conclusion showing that most dHb neurons on the right and a small cluster on the left show high levels of synchronized spontaneous activity. The location of these neurons corresponds to different neurotransmitter phenotypes: cholinergic and somatostatin¹², suggesting that dHb neurons expressing the same neurotransmitter function together.

In analyzing group dynamics of axon terminals, we found it important to also carry out manual inspection of raw data. For example, only calcium peaks at the vvIPN and not the dvIPN correlated with dIPN inhibition. VACHT antibody labeling in transgenic lines confirmed that cholinergic dHb terminals innervate only the vvIPN, indicating that the dvIPN is innervated by different types of neurons. In addition, a

small domain in the caudal vIPN clustered together with the terminals at the dIPN. While there are currently no available molecular markers to differentiate between the dHb axon terminals at the cvIPN and dIPN, spatial segregation between the two areas suggests that they are targeted by different dHb neurons. In conclusion, cholinergic neurons exhibit synchronized activity and project to the vIPN/raphe, which displays negatively correlated events with axon terminals at the dIPN (Figure 2C).

Nicotine promotes negatively correlated events in the dHb-IPN pathway

We found that nicotine induces prolonged duration of the negatively correlated event. We previously showed that a cocktail of nAChR antagonists inhibits approximately 80% of cholinergic currents in IPN neurons⁹. However, application of antagonists failed to attenuate but, rather, increased the frequency of negatively correlated events. The cholinergic system can increase the excitability of GABAergic interneurons in the neocortex and the lateral habenula^{24,25}. Likewise, our data suggest that GABAergic neurons are activated by nAChRs in the IPN. We hypothesize that blocking nAChRs throughout the whole brain inhibits GABAergic signaling, resulting in a global increase in synchronized network activity.

While nAChR activation can generate a negatively correlated event, the release of GABA from IPN neurons to induce inhibition is predominantly triggered by glutamatergic rather than cholinergic transmission from the dHb terminals at the IPN during spontaneous activity,

Localization of the GABA_B receptor to non-cholinergic terminals at the dIPN mediates presynaptic inhibition

We found that *gabbr1a* is expressed asymmetrically in a large area in the left and smaller area in the right dHb, indicating that the GABA_B receptor is present in non-cholinergic neurons that innervate the dIPN in larvae. Moreover, *gad1b*-positive neurons predominate in the dIPN, suggesting that GABA released from dIPN neurons activates presynaptic GABA_B receptors on non-cholinergic terminals at the dIPN. Based on calcium imaging, pharmacology, electrical stimulation and molecular studies, we propose a model whereby 1) activation of cholinergic dHb neurons 2) activates GABAergic neurons in the IPN resulting in the 3) release of GABA that through retrograde signaling 4) activates inhibitory presynaptic GABA_B receptors on non-cholinergic dHb terminals at the dIPN, leading to 5) inhibition of glutamatergic transmission (Figure 6G).

How does activation of vIPN neurons induce GABA release by dIPN neurons? Single cell labeling experiments showed the diverse morphology of IPN neurons including those that appear to be interneurons extending their processes between the dorsal and vIPN²⁶. We hypothesize that communication between vIPN and dIPN neurons could be mediated by such interneurons connecting the dorsal and ventral IPN. Another possibility is that more vIPN neurons are GABAergic than visualized by the transgenic line used in this study, akin to what has been shown for the adult IPN²⁷. Thus, GABAergic vIPN neurons might form direct synaptic connections, or through non-synaptic volume transmission, release GABA to inhibit non-cholinergic terminal activity at the dIPN.

As the MHb/dHb-IPN pathway is evolutionarily conserved in all vertebrates, including rodents and zebrafish^{9,28}, it is likely that this mode of hardwired activity is not unique to zebrafish. Indeed, a recent study on mice reports that presynaptic

GABA_B receptors at non-cholinergic terminals at the IPN play an inhibitory role on plasticity²⁹, suggesting a conserved role for these receptors.

Model for competitive selection between dHb circuits on behavior

It is crucial for an animal to identify the most salient stimulus amongst many concurrent external stimuli and select a single behavioral response. One example is the flight, freeze or fight response³⁰. During early stages of development, hardwired circuits to execute specific behavioral repertoires, such as escape (flight), are essential for survival³¹. The process of transforming multiple sensory inputs into a single behavioral outcome is termed competitive selection and includes selective attention and decision-making³². Computational formulations such as winner-take-all models have been proposed^{33,34} and recent studies have begun to elucidate the mechanisms for behavioral selection in competing circuits³⁵⁻³⁸.

Higher baseline GCaMP fluorescence in the terminals at the dIPN relative to the vIPN suggests that non-cholinergic neurons display high frequency oscillation³⁹. We propose that the synchronized burst in cholinergic terminals at the vIPN transiently inhibits this constitutive activity that occur in non-cholinergic neurons, ensuring only one pathway is active at any given time.

What could be the advantage of having local inhibition at the terminal level? Different external stimuli preferentially activate left or right dHb neurons^{6,14,15}. However, the ratio of asymmetric dHb activation depends on the intensity of the stimulus for both light and electric shock (personal observations), suggesting that the left and right dHb can be co-activated by the same stimulus. We suspect that this setup of local inhibition would ensure that 1) only one circuit is active at a time and 2) upon exposure to multiple stimuli a hierarchy is established between the stimuli.

Aversive behavior refers to emotional states with negative valence, such as fear or anxiety. Fear is defined as a response to a factual known threat while anxiety to an unknown, poorly defined threat. Brain areas that contribute to fear and anxiety exhibit great overlap including the amygdala, medial prefrontal cortex and the hippocampus⁴⁰. While recent optogenetic and in vivo studies have begun unraveling the microcircuits that participate in these behaviors^{35,41}, how they interact with other areas of the brain are unclear. The highly conserved MHb/dHb-IPN pathway-consists of two circuits composed of different neuronal populations, which mediate fear- or anxiety-related behaviors^{3-5,16}. We propose that the trans-inhibition mechanism between cholinergic and non-cholinergic dHb-IPN circuits underlies competitive selection of aversive behavioral responses.

A brief electric shock induces fast swim followed by freezing behavior before returning to baseline locomotion in larval zebrafish⁶. Here, we demonstrate that it can also promote negatively correlated events in the dHb terminals at the IPN. We hypothesize that activation of the cholinergic pathway by electric shock results in a stereotypical fast swim (flight) response, which is followed by inhibition of the non-cholinergic pathway allowing the animal to freeze. Prior to puberty, a flight/freeze response would be more beneficial than a fight response. In juveniles/adults, this dual circuit likely becomes more complex as different neuropeptides become expressed in the dHb-IPN pathway and with the maturation of behavioral repertoires from prior experience. For example, adult zebrafish exhibit a fight or flight response, manifested by a “winner” or “loser” phenotype in a social conflict paradigm, which is correlated with potentiation in non-cholinergic and cholinergic neuronal terminals, respectively¹⁶. We hypothesize that the “loser”/flight behavior corresponds to the default response to an aversive stimulus upon activation of the cholinergic pathway.

The “winner”/fight behavior could result from preferential hyper-activation of the non-cholinergic pathway, corresponding to a heightened level of anxiety.

In conclusion, investigating neuronal population dynamics within an intact pathway has revealed an atypical mode of trans-inhibition between two excitatory neuronal populations at their axon terminals. This hardwired mode of competition could underlie the mechanism for competitive selection between these neural circuits and provide a physiological framework to explore the relationship between anxiety and fear.

Acknowledgements

We thank Hitoshi Okamoto for insightful feedback on this project; Marco Diana and Nathalie Leresche for advice on pharmacology experiments; Eric Schwartz and Soumaiya Imarraine for helpful comments on the manuscript; Jean-Pierre Coutanceau for aid in zebrafish husbandry; and the IBPS aquatic animal facility and imaging facility. Hitoshi Okamoto, Koichi Kawakami and Shin-ichi Higashijima provided the *Tg(nptx2:Gal4-VP16)^{Y^{w0143a}}*, *Tg(UAS:GCaMP7a)* and *Tg(gad1b:|R|-GFP)* lines. This study was supported by a graduate fellowship from the Alexis and Anne Marie Habib Foundation (to M.A.Y), NIH grant R37HD091280 (to M.E.H.) and the ATIP-Avenir young investigator grant (INSERM/CNRS) and Emergence grant (Sorbonne Université) (to E.H).

Author Contributions

M.Z., S.W., R.C., E.R.D., P.L., M.E.H., J-M.M. and E.H. designed experiments. M.Z., E.B., M.A.Y., S.M.A.N., H.L., S.W., R.C., E.R.D., J-M.M. and E.H. conducted the

experiments. M.Z., S.M.A.N., S.W., H.S., J-M.M. and E.H. analyzed data. E.H. wrote the manuscript with input from all authors.

Declaration of Interests

The authors declare no competing interests.

Figure Legends

Figure 1. Identification of functional domains in dHb-IPN pathway during spontaneous activity

(A) Schematic of dHb-IPN pathway of larval zebrafish. Non-cholinergic (nCh, red) neurons are mainly in the left dHb and project to the dorsal IPN (d) whereas cholinergic (Ch, green) neurons are largely in the right dHb and project to the ventral IPN (v) and raphe (bracket). Somatostatinergic neurons are depicted in blue. Anterior (a), posterior (p), left (L) and right (R).

(B, F, J) Representative 6 day post fertilization (dpf) *Tg(gng8:GAL4ff)^{c426};Tg(UAS:GCaMP7a)* brain explants expressing GCaMP in dHb neurons (B), left dHb and their terminals (F) and terminals at the IPN and raphe (J), overlaid by colored circles representing functional domains identified by *k*-means clustering. Dotted line depicts the fasciculus retroflexus (FR) in (F).

(C, G, L) Correlation matrix of average $\Delta F/F$ signals between different clusters of dHb soma (C), dHb soma and terminals (G) and terminals at the IPN and raphe (L).

(D, H, M) Average $\Delta F/F$ traces from each cluster in 10-minute recordings. Colored boxed events in (D) correspond to single frame images in Figure S1.

(E, I, P) Average $\Delta F/F$ traces (black) of individual calcium events (grey).

(E) Traces of green/blue neurons exhibiting synchronized activity. n = 6 explants, 29 events. See Video S1.

(I) Traces of calcium bursts in the ventral dHb and dHb terminals at the vIPN and raphe (green, blue). Note that dHb terminals at the dIPN exhibit inhibition (trace 4). n = 5 explants, 24 events. See Video S2.

(P) Traces of negatively correlated events in dHb terminals at the IPN and raphe. n = 6 explants, 35 calcium events. See Video S3.

(K) Lateral view of dHb terminals expressing GCaMP7a at the IPN and raphe (green arrowheads) before and during a calcium burst event.

(N,O) Single frame images showing calcium signal changes. Blue (N) and green (O) boxed events in (M) are shown. Areas with increased (white) or decreased (green) signals are outlined (dotted lines). Right panels show traces in the IPN and raphe during the calcium event on left. n = 21/23 explants show similar calcium signaling domains.. Scale bars: 20 μ m (B,J) or 50 μ m (F). See also Figures S1-S4.

Figure 2. Synchronized cholinergic terminal activity at vIPN/raphe precedes non-cholinergic terminal inhibition at dIPN

(A) Average $\Delta F/F$ trace (black) of negatively correlated events (grey) in dHb terminals at the dIPN (top) and raphe (bottom). Time of increase (green) and inhibition (red) are indicated (dotted lines). Note the delay between the two events (931 ± 50 ms, n= 5 explants, 62 events).

(B) Sagittal sections of anti-GFP (green) and anti-VACHT (magenta) labeling in 6 dpf *Tg(nptx2:Gal4-VP16)^{nw0143a};Tg(UAS:GFP)^{c426}* (left) and *Tg(gng8:GAL4ff)^{c426};Tg(UAS:GFP)^{c426}* (middle) larvae. White arrowheads indicate

VACHT labeling in vVIPN and raphe. White box (middle) corresponds to magnified panels on right. Scale bar: 20 μ m.

(C) Summary of dHb terminal domains. *nptx:Gal4;UAS:GFP*-positive dIPN domain is indicated (*nptx*⁺). The cvIPN is only identified based on calcium signals. Calcium bursts (green) and inhibition (red) are labeled with graph plots.

(D) Electric shock induces negatively correlated events. Left: Lateral view of *Tg(gng8:GAL4ff)^{c426};Tg(UAS:GCaMP7a)* larva expressing GCaMP in dHb terminals. dIPN, vIPN and raphe are outlined (dotted lines). Middle: Representative $\Delta F/F$ traces upon electric shock (black dotted line). Right: Average traces (black) of calcium events (grey) after electric shock. (n = 8 larvae, 16 events). Scale bar = 20 μ m.

Figure 3. Nicotine induces prolonged negatively correlated event

(A) Representative $\Delta F/F$ traces from dHb terminals in *Tg(gng8:GAL4ff)^{c426};Tg(UAS:GCaMP7a)* explant. 5 min nicotine (10 μ M) perfusion induces inhibition at the dIPN (top) and bursts at the raphe (bottom).

(A') Average $\Delta F/F$ traces (black) of nicotine-induced negatively correlated events (grey). Duration (cyan boxes) and initiation (red dotted lines) of nicotine application indicated. n = 8 explants.

(B,C) Representative raster plots of dIPN inhibition and raphe burst events. (B) Control and (C) nAChR antagonist (100 μ M mecamylamine and 10 μ M d-tubocurarine) application. Introduction of E3 solution (B) or antagonists (C) indicated (red lines).

(B',C') Ratio of average number of events in 15-minute windows (I, II, III) and baseline activity (b) after applying (B') E3 (n = 4) or (C') antagonists (n = 6). Bars indicate negatively correlated events in dHb terminals at the raphe (green, bursts)

and dIPN (red, inhibition). One-way ANOVA followed by Tukey's test, ** $p < 0.005$. b: 15 minutes of baseline, I: 0-15, II: 15-30, III: 30-45 min after application.

Figure 4. Calcium bursts correspond to acetylcholine/glutamate and GABAergic currents

(A-A') $\Delta F/F$ traces of dHb terminals (blue) aligned with simultaneous patch-clamp recording of IPN neurons (black) show correlation of calcium bursts with increase in glutamatergic/cholinergic inward currents (A, $V_h = -60$ mV) and GABAergic outward currents (A', $V_h = 0$ mV).

(B-B') Average traces (black) of calcium bursts (top) aligned with synaptic responses (bottom) at holding potentials of -60 mV (B, $n = 4$ explants, 42 events) and 0 mV (B', $n = 3$ explants, 23 events). Dotted lines indicate onset for inward (green) and outward (red) currents.

Figure 5. Calcium inhibition occurs via presynaptic GABA_B receptors

(A-B) Representative $\Delta F/F$ traces in terminals at dIPN (top) and raphe (bottom) after application of (A) $1 \mu\text{M}$ cgp55845 or (B) $3 \mu\text{M}$ tetrodotoxin (TTX) + $2 \mu\text{M}$ baclofen. Time of drug application indicated (vertical lines).

(A') Average peak amplitude before and after cgp55845 application in terminals at the dIPN (-0.16 ± 0.03 vs -0.04 ± 0.01) and raphe (0.22 ± 0.09 vs. 0.26 ± 0.13) during inhibition (top) and burst (bottom). $n = 6$ explants, 50 events. Red asterisks indicate attenuated calcium inhibition at the dIPN after cgp55845 application.

(B') Paired plots comparing average inhibition amplitude in control conditions (-0.22 ± 0.07) to the average baseline decrease after baclofen application (-0.24 ± 0.08). n

= 7 explants, 46 events. Note abrupt decrease (red arrowhead) in baseline at the dIPN following baclofen.

(C) $\Delta F/F$ traces of IPN (top) aligned with simultaneous whole-cell patch-clamp recording trace (middle) upon baclofen perfusion. Time of baclofen application indicated (black bar). Magnified traces of synaptic activity before (left) and after (right) baclofen (bottom panels).

(C') Paired plot of synaptic activity frequency before (4.29 ± 1.05 Hz) and after (0.47 ± 0.15 Hz) baclofen. $n = 6$ explants. Mean \pm SD indicated in (A', B', C').

(D,E) Dorsal (left) and sagittal (right) views of *gabbr1a* (D) and *gabbr1b* (E) expression at 6 dpf. dHb nuclei (red arrowheads) and absence of expression at IPN (white arrowhead) (D). Wilcoxon signed-rank test. * $p < 0.05$. Scale bars: 50 μm .

Figure 6. Direct stimulation of IPN induces GABA_B receptor-mediated inhibition in terminals at the dIPN

(A) Schematic of setup for simultaneous electrical stimulation and drug perfusion.

(B) Representative $\Delta F/F$ traces showing attenuation of dIPN inhibition by electrical stimulation after cgp55845 perfusion (top), with bursts unaffected in the raphe (bottom). Grey boxes represent 10 min after cgp55845 application. See also Video S4.

(C,D) Average traces (black) of calcium events (grey, 10 stimuli) before (c) and after (d) cgp55845.

(E) Paired plot of average peak amplitudes before and after cgp55845 application during inhibition (top, -0.15 ± 0.07 vs. -0.02 ± 0.02) and burst (bottom, 0.22 ± 0.08 vs. 0.2 ± 0.06). $n = 6$ explants, 71 events. Purple dotted lines indicate time of electrical

stimulation (B-D). Purple line in (B) denotes duration of *cgp55845* application.

Wilcoxon signed-rank test, * $p < 0.05$, n.s. not significant. Mean \pm SD indicated.

(F) Transverse section of *Tg(gng8:GAL4ff)^{c426};Tg(UAS:GFP)^{c426};Tg(gad1b:|R|-GFP)* larval IPN surrounded by dHb terminals (green) and GABAergic neurons (magenta). Hoechst nuclear staining (blue). White asterisks indicate *gad1b:RFP*⁺ neurons in vIPN. White dotted line separates dIPN and vIPN (right panel).

(G) Model illustrating the mechanism underlying negatively correlated events in dHb terminals at the IPN (see discussion).

STAR Methods

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Elim Hong (elim.hong@inserm.fr).

Material availability

All plasmids generated in this study are available upon request to the Lead Contact.

Data and code availability

- All data reported in this paper will be shared by the Lead Contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

AB wild-type and transgenic zebrafish lines *TgBAC(gng8:GAL4ff)^{c426}*, *Tg(UAS:GCaMP7a)*, *Tg(nptx2:Gal4-VP16)^{rw0143a}*, *Tg(gad1b:|R|-GFP)*, *Tg(UAS:GFP)^{c354}* ^{5,9,20,42,43} were used. Fish were maintained at 28°C on a 14:10h light: dark cycle in a recirculating system. All experiments were carried out in agreement with the European Directive 210/63/EU on the protection of animals used for scientific purposes and the French application decree 'Décret 2013-118'. The projects of our research group have been approved by the ethical committee 'Comité d'éthique Charles Darwin' (APAFIS#15909-2018070912072530 v5). The fish facility has been approved by the French 'Service for animal protection and health' (A-75-05-25).

METHOD DETAILS

Explant Dissection

TgBAC(gng8:GAL4ff)^{c426};Tg(UAS:GCaMP7a) 3, 6 and 7 day-post-fertilization (dpf) larvae were used for experiments. Larvae were anesthetized in Tricaine (0.01%) and brains dissected using fine forceps in ice cold Ringer's solution (134mM NaCl, 2.1 KCl, 1.2mM MgCl₂, 10 mM HEPES, 10mM Glucose, 2.1 CaCl₂, pH = 7.6) and mounted in 1.2% low melting agarose for calcium imaging and electrophysiology experiments.

Pharmacology

Drugs were applied to the explants either directly using a pipette or by using a perfusion peristaltic pump (Ismatec) at a rate of 5-6 ml/min. Mecamylamine hydrochloride (100 µM), (+)-D-tubocurarine chloride (10 µM), nicotine ditartrate (10

μM, Acros Organics), tetrodotoxin (3 μM), baclofen (2 μM) and cgp55845 (1 μM) were used. The drugs were purchased from Tocris Bioscience unless otherwise noted.

Calcium Imaging

Calcium imaging was performed on a spinning disk microscope (Zeiss Axio Examiner.Z1) using a 40X water immersion objective (NA =0.95) or Leica SP5 laser scanning confocal microscope using a 25X water immersion objective (NA = 0.95). Images were acquired at a rate between 2.2-6.7Hz, depending on the experiment. Nicotine was perfused for 5 minutes followed by E3 medium to wash out the drug. For experiments with mecamylamine hydrochloride and D-tubocurarine chloride, as a short-term application did not show any changes in calcium signals, we carried out the following experiment to assay for long-term change: baseline calcium signals were recorded for 15 minutes before pipetting the drug directly to the explant. The calcium signals were recorded for an additional 45 minutes after drug application. For the cgp55845 and baclofen experiments, 10-15 minutes baseline activity was recorded followed by drug application using the perfusion system and imaged for another 15 minutes.

Patch-clamp recordings and simultaneous Ca²⁺ imaging

Explants from *TgBAC(gng8:GAL4ff)^{C426};Tg(UAS:GCaMP7a)* larvae were placed in a recording chamber and constantly perfused with Ringer's solution at a rate of 1–2 mL/min. The IPN was made accessible for patch-clamp recordings and Ca²⁺ imaging by orienting the explants with the ventral surface upward. dHb terminals and the IPN were located and imaged using a 63x water immersion objective mounted on an

epifluorescence microscope BX51W1 (Olympus) equipped with a 470 nm LED fluorescent excitation light. Patch electrodes were filled with an intracellular solution containing 130 mM CsMeSO₄, 4 mM MgCl₂, 4 mM Na-ATP, 0.3 mM Na-GTP, 10 mM Hepes, and 10 mM EGTA, 5 mM QX-314 adjusted to pH 7.2, 290 mOsm, for resistances between 5 and 8 MΩ. This intracellular solution allowed discrimination of cationic excitatory currents and chloride inhibitory currents based on their distinct reversal potentials ($E_{\text{cation}} = 0 \text{ mV}$; $E_{\text{Cl}} = -60 \text{ mV}$). By recording the cells at -60 mV , EPSCs appeared as inward currents, whereas IPSCs were barely detectable. Conversely, IPSCs appeared as outward currents when recording the cells at a holding potential of -0 mV , while EPSCs became undetectable. Whole-cell recordings were obtained using a Multiclamp 700B amplifier connected to a digidata 1440 acquisition system monitored using pClamp 10 software (Molecular Devices). Analyses were performed using Clampfit 10 (Molecular Devices). For all experiments, data were filtered at 4 kHz during recording and the traces were digitized at 20 kHz.

Simultaneous time-lapse imaging of GCaMP fluorescence in dHb terminals was performed using either an Orca Flash4.0 (Hamamatsu, Japan) or Orca 03G (Hamamatsu, Japan) camera and recorded using the HC Image Live software (Hamamatsu, Japan) at an acquisition frequency of 5 Hz or 10Hz with an exposure time of 200 ms and 100 ms, respectively. Time-lapse and patch-clamp acquisition were synchronized via a TTL signal sent to the camera via the pClamp 10 software. Time-lapse frames were encoded at a 256x256 pixel resolution using a 16-bit grey scale. Time-lapse images were analyzed using the ImageJ software (National Institute of Health, USA) and fluorescence signals were plotted using Clampfit 10.

Immunohistochemistry

TgBAC(gng8:GAL4ff)^{c426};Tg(UAS:GFP)^{c354} 6 dpf larvae were fixed in BT fix (4% PFA, 0.15mM CaCl₂, 4% sucrose in 1x PBS) overnight in 4°C for rabbit anti-VACHT (1/200, Synaptic systems) labeling. Larvae were embedded in 4% low melt agarose and sectioned to 50µm using a vibratome (Leica, Inc). Floating sections were incubated in primary antibody in incubating solution (0.8% Triton-X in 1x PBS) for 3 days on a shaker in 4°C. The sections were washed and incubated in secondary anti-rabbit Alexa Fluor 594 (1:1000) with Hoechst 33342 (1:2000) overnight at 4°C on a shaker. *TgBAC(gng8:GAL4ff)^{c426};Tg(UAS:GFP)^{c354};Tg(gad1b:|R|-GFP)* larvae were fixed overnight in 4% PFA, sectioned to 50µm and incubated with Hoechst 33342 (1:2000) overnight at 4°C on a shaker. All sections were then mounted on slides using Mowiol (Sigma). They were imaged using the Leica TCS SP5 AOBS upright microscope using a 63x (N.A. = 1.4) objective.

RNA in situ Hybridization

PCR fragments for *gabbr1a* (ENSDARG00000018967) (553-bp) and *gabbr1b* (ENSDARG00000016667) (539-bp) were subcloned into pCRII-TOPO vector using the TOPO®TA cloning Kit (Invitrogen). Reverse primer sequences were from ¹⁹. The following forward primers were used: ***gabbr1a*** (ACGTATGGTTCCTCATCGGC) ***gabbr1b*** (AGACAAGAGAAAGCACCTGGA). Restriction enzymes and RNA polymerase used to synthesize the antisense RNA probes are as follows: *gabbr1a* (*KpnI/T7*), *gabbr1b* (*KpnI/T7*). Colorimetric in situ hybridization (ISH) assay was performed as described in ⁴⁵. Following ISH, the larvae were mounted in 100% glycerol and imaged on a Nikon Eclipse E800 microscope.

Electrical shock assay

Custom built electrical stimuli setup consisted of copper mesh electrodes that were mounted 6 cm apart on opposing sides and connected to a DS3 isolated constant current stimulator (Digitimer, Ltd.). Three electrical field stimulations (6mA, 20V, 500msec) were triggered every five minutes by MetaMorph (Molecular Devices). The larvae were paralyzed in 2% pancuronium bromide and mounted in the middle of 90mm diameter petri dishes filled with 30 ml E3 medium to ensure consistent stimulation. Shock assay was performed in 6-7 dpf agarose-restricted larvae, paralyzed with 2% pancuronium bromide.

Electrical stimulation The IPN neurons were stimulated using a low-resistance (<1M Ω) glass pipette filled with Ringer solution containing an AgCl electrode. Train stimulations (20 μ s duration 50Hz stimulations lasting 400ms) were triggered every 1 minute with an isolated current stimulator (DS3; Digitimer Ltd.) using a Master-8 pulse generator (A.M.P.I). Prior to each experiment, a dose-dependent curve was performed to select the optimal setting that induced negatively correlated events in the terminals at the IPN. Stimuli ranged between 100 μ A - 1.2 mA.

QUANTIFICATION AND STATISTICAL ANALYSIS

Calcium data analysis

Average fluorescence intensity of habenular soma was calculated by manually drawing regions of interest (ROI) in FIJI as described in⁶. To carry out a non-biased analysis of the dHb-IPN pathway and dHb terminals at the IPN, 2x2 μ m² grid ROIs were drawn over the entire recording using FIJI macro. Average fluorescence

intensity for each ROI was then extracted per frame and analyzed on MATLAB using custom written scripts.

Amplitude of calcium signal was calculated according to the formula:

$$\Delta F/F = (F_i - F_{\text{mean}})/F_{\text{mean}},$$

whereby, F_i is the mean intensity in a single ROI at a single time point while F_{mean} is the mean intensity in a single ROI throughout the entire recording time. Multiple clustering algorithms were tested including functional clustering algorithm⁴⁴ and spectral clustering on calcium traces and correlation coefficient values. After comparing the clusters generated using various algorithms, we found *k*-means and hierarchical clustering based on pairwise linear correlation matrix were the most representative of calcium signals in the spontaneous activity recordings. *K*-means and hierarchical clustering based on pairwise linear correlation matrix were carried out on MATLAB using *kmeans* and *linkage* functions ('euclidean' distance and 'complete' method), respectively. Different *k* values were tested and *k* = 5 was selected based on manual inspection of calcium signals in the time-lapse recordings and also using a heatmap. The correlation matrix displayed in figures was generated by calculating the correlation coefficients between the average calcium signal traces for each cluster.

The threshold for hierarchical clustering was determined by manual inspection of the calcium signals in the recording and heatmap. Once specific IPN domains were identified by *k*-means, larger ROIs were manually drawn containing the different IPN domains. Analysis of calcium burst frequency using 'findpeaks' function was performed in MATLAB (Mathworks). Peak of calcium burst, calcium inhibition and half duration were identified using *findpeaks* and *half prominence* functions in MATLAB and verified manually. Calcium burst peak was defined by the highest

value during a calcium burst. Peak of calcium inhibition was defined by the lowest value during a calcium inhibition event. Half duration was calculated as the width of the calcium signal at the half value of a peak during a calcium burst or inhibition. All graphs and plots were made on MATLAB using custom written scripts.

Statistical analysis

Statistical tests were carried out using a Wilcoxon signed rank test for averaged paired samples, Kruskal-Wallis test followed by a Dunn's test for comparison between more than 2 groups, Mann-Whitney test was used with independent samples and a linear mixed model with fixed effect was used to compare multiple values from repeated events. All statistical tests were performed on R or GraphPad Prism (GraphPad Software, Inc.). $p < 0.05$ was considered as statistically significant. All statistical details including statistical tests, exact values of n , value representation (mean \pm SD) are indicated in the figure legends.

Multimedia file titles

Video S1. Spontaneous activity in dHb neurons, Related to Figure 1.

Video S2. Spontaneous activity in the left dHb cell bodies and their terminals at the IPN and raphe, Related to Figure 1.

Video S3. Spontaneous activity in the dHb terminals at the IPN, Related to Figure 1.

Video S4. Electrical stimulation of the IPN induces GABA_B receptor-mediated inhibition in terminals at the dIPN, Related to Figure 6.

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Highlights

- Calcium signals in habenular (Hb) soma do not reflect axon terminal activity
- Synchronized Hb cholinergic activity inhibits non-cholinergic terminals
- Presynaptic inhibition of non-cholinergic terminals is mediated by GABA_B receptors
- GABA_B R activation by retrograde signaling from the interpeduncular nucleus

eToc (350 characters)

Zaupa et al., show that calcium signals in habenular soma are not reflective of activity in axon terminals at the interpeduncular nucleus. They uncover an atypical mode of lateral inhibition between two habenular circuits where the activation of cholinergic neurons inhibits non-cholinergic activity by retrograde signaling from their target site.

