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The habenula is crucial for experience-dependent modification of fear responses in zebrafish

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The zebrafish dorsal habenula (dHb) shows conspicuous asymmetry in its connection with the interpeduncular nucleus (IPN) and is equivalent to the mammalian medial habenula. Genetic inactivation of the lateral subnucleus of dHb (dHbL) biased fish towards freezing rather than the normal flight response to a conditioned fear stimulus, suggesting that the dHbL-IPN pathway is important for controlling experience-dependent modification of fear responses.

The habenula, an evolutionarily highly conserved diencephalic structure, is subdivided into medial and lateral regions (MHb and LHb, respectively)[1–3]. The LHb sends efferents to the monoaminergic neurons and has been implicated in various functions related to control of the aversive learning and emotional behaviors[1–3]. The MHb mainly projects to the interpeduncular nucleus (IPN)[1–3], and the previous study implicates the MHb as a possible regulator of fear responses[4]. However, the MHb still remains functionally ambiguous due mainly to a lack of suitable technology for manipulating MHb neurons reproducibly and with subdivision-specific precision. We recently found that the dorsal and ventral habenula (dHb and vHb) of zebrafish correspond to the MHb and LHb of mammals, respectively[5] (**Supplementary Fig. 1**). The dHb is further subdivided into the dHbL and the medial subnucleus (dHbM), which are anatomically left-right asymmetric in their relative size ratios and projection patterns to the IPN; the dHbM innervates the ventral (vIPN) and intermediate IPN (iIPN), whereas the dHbL is the predominant source of axons to the dorsal IPN (dIPN) and iIPN (**Supplementary Fig. 1**)[2,6].

To gain insight into the importance of these distinct neural pathways, we first examined projections from either dIPN or vIPN using anterograde tracers (**Fig. 1**). The labeled axons from the dIPN ($n = 6$; **Fig. 1a**) mainly projected bilaterally to the dorsal directions, through the region putatively corresponding to the dorsal raphe (**Fig. 1b**, see also **Supplementary Fig. 2**). They further extended laterally around the medial longitudinal fascicle (**Fig. 1b**), then turned caudally and elongated through the longitudinally extended region known as the griseum centrale[7], which underlies the rhombencephalic ventricle (**Fig. 1c**), forming a fasciculated fiber bundle (**Fig. 1d**). The griseum centrale is the periventricular structure that most likely includes the regions corresponding to mammalian periaqueductal gray (PAG), dorsal tegmental nucleus and nucleus incertus. The IPN, the PAG and the nucleus incertus have been implicated in control of behaviors under fear or stress conditions[8,9], suggesting that the dHbL-d/iIPN pathway might contribute to the modulation of fear behaviors.

We substantiated this projection by tract tracing in live fish ($n = 4$; **Fig. 1e**,**f** and **Supplementary Fig. 3**). Many neurons were also retrogradely labeled in the griseum centrale (**Supplementary Fig. 3**), suggesting that there is a reciprocal neural connection between the dIPN and the griseum centrale. In contrast, tract tracing in the vIPN confirmed efferent projections to the median raphe ($n = 4$; **Fig. 1g**,**h** and **Supplementary Fig. 3**) and revealed retrogradely labeled neurons in the median raphe (**Supplementary Fig. 3**), indicating that there is a reciprocal connection between the vIPN and the median raphe. The specific connection from vIPN to the median raphe was also verified by retrograde labeling from the median raphe ($n = 12$; **Supplementary Fig. 4**). These results suggest that there are subdivided parallel pathways, namely the dHbL-d/iIPN–griseum centrale pathway and the dHbM-v/iIPN–median raphe pathway, and that it is possible to use pathway-specific manipulations to examine their involvement in fear behaviors.

To achieve dHbL-specific control of gene expression, we carried out BAC-based transgenesis experiments using *Narp* (*neuronal activity regulated pentraxin*), an immediate early gene that is enriched in the rat habenula[10] (**Supplementary Methods**). We used a BAC clone carrying the zebrafish homolog of *Narp* to establish the transgenic line Tg(*narp:GAL4VP16*). Progeny obtained by crossing this line with two other lines, Tg(*UAS:DsRed2*) and Tg(*brn3a-hsp70:GFP*)[6], expressed DsRed2 in neurons in the dHbL that projected mainly to the dIPN, with some projecting to the iIPN (**Fig. 1i–l**). This expression pattern complemented the GFP expression of dHbM neurons that projected to the vIPN and the iIPN (**Fig. 1i–l**) and was similar to intrinsic *Narp* expression (**Supplementary Fig. 5**). The expression patterns of the transgenic lines confirmed previous observations of asymmetry in habenula-IPN projections[6] (**Supplementary Fig. 1**).

We then expressed the tetanus toxin light chain (TeTxLC) in the dHbL neurons to prevent the release of synaptic vesicles and interrupt

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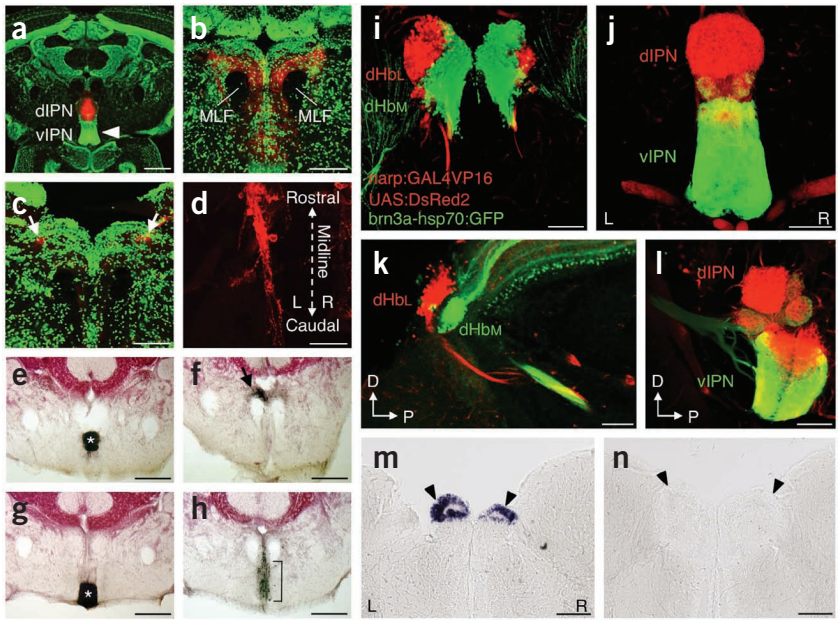

Figure 1 The habenula-IPN projection pattern and the genetic manipulation of dHbL-d/iIPN transmission. (**a**–**d**) Anterograde labeling of dIPN efferents by DiI (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate, red) in adult Tg(*brn3a-hsp70:GFP*), which expressed GFP specifically in the dHbM-v/iIPN pathway (arrowhead). Arrows indicate the cross-sections of the labeled tracts. Counter staining, SYTOX green. MLF, medial longitudinal fascicle. (**e**–**h**) Anterograde labeling from the IPN with Neurobiotin. Counter staining, neutral red. The efferents from the dIPN (**e**, asterisk) terminated in the griseum centrale (**f**, arrow). The efferents from the vIPN (**g**, asterisk) terminated in the median raphe (**h**, bracket). (**i**–**l**) Expression patterns of an adult Tg(*narp:GAL4VP16; UAS:DsRed2; brn3a-hsp70:GFP*) fish. (**m**,**n**) *TeTxLC* mRNA expression in adult Tg(*narp:GAL4VP16; UAS:TeTxLC*) (**m**) and control fish (**n**). Black arrowheads, dHbL. All panels are coronal sections except for **d** (horizontal) and **k** and **l** (sagittal). D, dorsal; L, left; P, posterior; R, right. Scale bars represent 200 μm (**a**,**e**–**h**), 100 μm (**b**,**c**,**i**–**n**) and 50 μm (**d**).

neural transmission[11]. *In situ* hybridization of the larval Tg(*narp:GAL4VP16; UAS:TeTxLC*) revealed that *TeTxLC* was specifically expressed in the dHbL (**Supplementary Fig. 6**), except for a small number of sparsely scattered cells near the otic vesicles (**Supplementary Fig. 6**) and olfactory bulbs (data not shown). In the adult fish, the expression area was highly restricted and there was no expression outside the habenula (**Fig. 1m**,**n** and **Supplementary Fig. 7**).

We subjected these adult transgenic fish to the cued fear-conditioning tasks (dHbL-silenced fish, $n = 12$; control, $n = 15$; **Supplementary Fig. 8**). One adaptation session of five repeated presentations of conditioned stimulus (red light) alone was followed by two conditioning sessions of five repeated presentations of the conditioned stimulus paired with the unconditioned stimulus (electrical shock). We monitored the responses to the presentations of conditioned stimulus alone in the retrieval session (**Fig. 2**). The control sibling fish showed enhanced flight behaviors (conditioned stimulus-evoked change in turning frequency) during the conditioned stimulus presentations (**Fig. 2a**,**c**, **Supplementary Movie 1** and **Supplementary Fig. 9**), as observed previously with medaka, another teleost, in similar behavioral experiments[12]. Notably, most of the dHbL-silenced fish did not show a similar response to the conditioned stimulus (**Fig. 2c**), but instead showed persistent freezing (**Fig. 2b**,**d**,**e**, **Supplementary Movie 2**, **Supplementary Figs. 9** and **10**, and **Supplementary Table 1**). As an exception, one dHbL-silenced fish initiated persistent rotation on the conditioned stimulus presentation in the retrieval session (**Supplementary Movie 3** and **Supplementary Fig. 11**). These results suggest that silencing of the dHbL tends to elicit different responses from those of control fish when exposed to the same conditioned stimulus after the same fear experiences (see **Supplementary Data 1** for further statistical analyses).

The dHbL-silenced fish exhibited no substantial abnormalities in sensorimotor reactions measured by locomotor activities during 1-s periods of electrical shock (**Fig. 2f**) or exploratory behavior (tendency to prefer the center of the tank; **Fig. 2g**). They also showed similar levels of locomotor activity to those of control fish just before the first conditioned stimulus presentation in the retrieval session (**Fig. 2h**) and similar levels of sensitivity to the conditioned stimulus during the adaptation session (**Supplementary Fig. 12** and **Supplementary Data 2**).

Differences between the behaviors of the dHbL-silenced fish and the control fish were evident throughout the course of the fear conditioning. After the first encounter with the unconditioned stimulus paired with the conditioned stimulus, both fish frequently showed freezing at the same level (**Fig. 2i**). Such responses generally waned in the control fish as they experienced the paired conditioned stimulus and unconditioned stimulus repeatedly, although there were occasional fluctuations. In contrast, the freezing tendency of the dHbL-silenced fish became higher than that of the control fish during the second conditioning session (**Fig. 2i**,**j**). This indicates that the experience-dependent modification of the innately encoded initial response strategy is impaired in the dHbL-silenced fish.

Chronic inactivation of the dHbL by expression of TeTxLC did not impair the anatomical projection from the dHbL to the d/iIPN (**Supplementary Fig. 13**) and we found no *TeTxLC* expression outside of the habenula in the adult brain (**Fig. 1m**,**n** and **Supplementary Fig. 7**). However, it remains possible that the larval stage-specific expression in a small number of sparsely scattered cells near the otic vesicles and olfactory bulbs (**Supplementary Fig. 6**) might have long-term effects on the fear responses of adult fish. To exclude this possibility, we further examined the effect of the conditional elimination of the dHbL neurons using a Nitroreductase-metronidazole–based inducible cell ablation system[13]. We confirmed that the expression of a nitroreductase-mCherry fusion protein in the transgenic line was completely specific in the dHbL neurons at adult stages, the expressing cells were successfully eliminated by the metronidazole, and this acute and specific ablation of dHbL also similarly impaired the experience-dependent behavioral modification (**Supplementary Results**, **Supplementary Data 3** and **4** and **Supplementary Figs. 14**–**19**).

Although the switching of the active brain networks according to different behavioral choices has been described[8,14], the regulation of this switching remains unclear. Our results indicate that the dHbL-d/iIPN–griseum centrale pathway is crucial for the modification of the behavioral responses in an experience-dependent manner. Notably, this pathway was also implicated in the experience-dependent behavioral modification of the larval zebrafish in response to the repeated switching from light to dark environment (**Supplementary Results**, **Supplementary Data 5** and **Supplementary Figs. 20**–**24**). In mammals, the IPN is connected to the dorsal raphe and the nucleus incertus, which are hypothesized to comprise the brainstem network involved in controlling behavioral activation[9,15]. This makes it likely

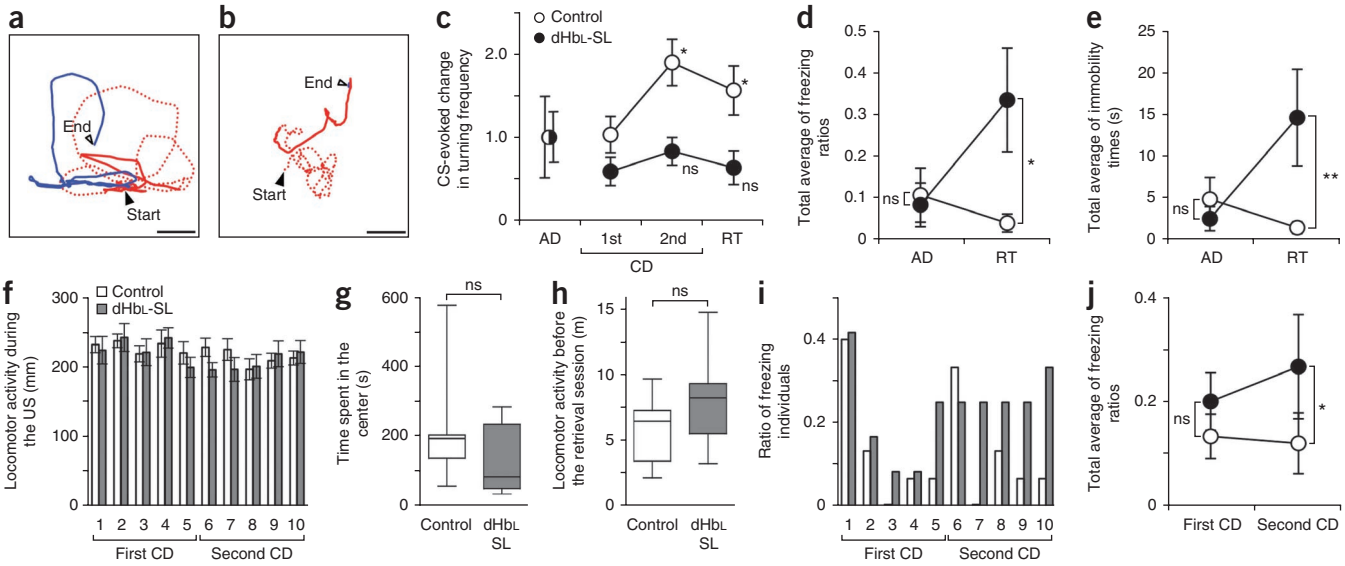

Figure 2 The dHbL-silenced fish showed enhanced freezing responses to the conditioned stimulus instead of flight behaviors. (**a**,**b**) Examples of the control (**a**) and dHbL-silenced (**b**) fish locomotion trajectories during retrieval sessions, before (20 s, red dotted lines), during (8.5 s, red solid lines) and after (20 s, blue lines) the conditioned stimulus (CS) exposure. (**c**) The change in the turning frequency, which was evoked during conditioned stimulus presentation. The values are normalized to the averages of the adaptation sessions. Means ± s.e.m. are plotted. $*P < 0.05$, Wilcoxon signed-rank test (comparison with the first conditioning session). (**d**,**e**) Total average of freezing ratios (**d**) and immobility times (s) (**e**) for 50 s after the offset of the conditioned stimulus. Means ± s.e.m. are plotted. Two-way repeated-measure ANOVA, habenula state (control, silenced) × conditioning (before conditioning, after conditioning) (**d**, $F = 4.66$, $P = 0.04$; **e**, $F = 6.04$, $P = 0.02$). $**P < 0.01$ and $*P < 0.05$, Bonferroni post-tests (control versus dHbL silenced). (**f**) The unconditioned stimulus (US)-triggered locomotor activity at each trial of the first (1–5) and second (6–10) conditioning sessions. Means ± s.e.m. are plotted. Two-way repeated-measure ANOVA, the main effect of Habenula state ($F = 0.27$, $P = 0.61$). (**g**) Exploration time spent in the center of the field (Mann-Whitney *U* test, $P = 0.08$). For **g** and **h**, the middle line represents the median, the box edges indicate quartiles and the vertical bars indicate the range. (**i**) Ratio of freezing individuals in response to the unconditioned stimulus presentations during the conditioning sessions (first, 1–5; second, 6–10). (**j**) Total average of freezing ratios in the first and second conditioning sessions. Means ± s.e.m. are plotted. $*P < 0.05$, χ^2 test with total number of freezing and nonfreezing responses at each session. AD, adaptation session; CD, conditioning session; dHbL-SL, dHbL-silenced fish; RT, retrieval session; ns, nonsignificant ($P > 0.05$).

that our findings in zebrafish could be extended to mammals. As different parts of the PAG differentially regulate coping strategies for stress in mammals[8], it is worth examining whether the efferents from the dIPN could influence the PAG either directly or indirectly in the zebrafish brain, although a direct connection of the IPN and the PAG has not been proved in mammals. Investigating the interactions among these parts of the brainstem regions in the zebrafish by taking advantage of its relative simplicity and amenability to genetic manipulation will lead to a deeper understanding of the mechanisms by which humans cope with fear and stress in both normal states of mind and psychiatric disorders. Zebrafish show behavioral laterality in recognition of novelty and conspecifics[2]. An intriguing question is whether this could be related to a possible difference in the properties of the left and right habenula-IPN pathways in modulating fear response strategies.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

M.A., H.A. and H.O. designed the experiments and wrote the manuscript. H.O. supervised the research project. M.A. performed most of the experiments using transgenic fish with T.A., R.N., M.T., T. Sassa, T. Shiraki, K.K., T.H. and S.H. H.A. performed the neural tracing study with M.G., M.T. and R.A.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Hikosaka, O. *Nat. Rev. Neurosci.* **11**, 503–513 (2010).
2. Bianco, I.H. & Wilson, S. *Phil. Trans. R. Soc. Lond. B* **364**, 1005–1020 (2009).
3. Herkenham, M. & Nauta, W. *J. Comp. Neurol.* **187**, 19–47 (1979).
4. Ressler, K.J., Paschall, G., Zhou, X. & Davis, M. *J. Neurosci.* **22**, 7892–7902 (2002).
5. Amo, R. *et al. J. Neurosci.* **30**, 1566–1574 (2010).
6. Aizawa, H. *et al. Curr. Biol.* **15**, 238–243 (2005).
7. Wullimann, M.F., Rupp, B. & Reichert, H. *Neuroanatomy of the Zebrafish Brain: A Topological Atlas.* (Birkhäuser, Boston, 1996).
8. Bandler, R., Keay, K., Floyd, N. & Price, J. *Brain Res. Bull.* **53**, 95–104 (2000).
9. Goto, M., Swanson, L. & Canteras, N. *J. Comp. Neurol.* **438**, 86–122 (2001).
10. Reti, I.M., Reddy, R., Worley, P. & Baraban, J. *J. Neurochem.* **82**, 935–944 (2002).
11. Sweeney, S.T., Broadie, K., Keane, J., Niemann, H. & O'Kane, C. *Neuron* **14**, 341–351 (1995).
12. Eisenberg, M., Kobilo, T., Berman, D. & Dudai, Y. *Science* **301**, 1102–1104 (2003).
13. Curado, S. *et al. Dev. Dyn.* **236**, 1025–1035 (2007).
14. Mongeau, R., Miller, G., Chiang, E. & Anderson, D. *J. Neurosci.* **23**, 3855–3868 (2003).
15. Maier, S.F. & Watkins, L. *Neurosci. Biobehav. Rev.* **29**, 829–841 (2005).