Stria medullaris innervation follows the transcriptomic division of the habenula

Iris Juárez-Leal, Estefanía Carretero-Rodríguez, Francisca Almagro-García, Salvador Martínez, Diego Echevarría & Eduardo Puelles

The habenula is a complex neuronal population integrated in a pivotal functional position into the vertebrate limbic system. Its main afference is the stria medullaris and its main efference the fasciculus retroflexus. This neuronal complex is composed by two main components, the medial and lateral habenula. Transcriptomic and single cell RNAseq studies have unveiled the morphological complexity of both components. The aim of our work was to analyze the relation between the origin of the axonal fibers and their final distribution in the habenula. We analyzed 754 tracing experiments from Mouse Brain Connectivity Atlas, Allen Brain Map databases, and selected 12 neuronal populations projecting into the habenular territory. Our analysis demonstrated that the projections into the medial habenula discriminate between the different subnuclei and are generally originated in the septal territory. The innervation of the lateral habenula displayed instead a less restricted distribution from preoptic, terminal hypothalamic and peduncular nuclei. Only the lateral oval subnucleus of the lateral habenula presented a specific innervation from the dorsal entopeduncular nucleus. Our results unveiled the necessity of novel sorts of behavioral experiments to dissect the different functions associated with the habenular complex and their correlation with the distinct neuronal populations that generate them.

The habenula (Hb), an important brain region linking the limbic forebrain to the midbrain and rostral hindbrain, is divided into lateral habenula (LHb) and medial habenula (MHB). It is located in the most dorsal part of the alar plate of prosomere 2 and receives projections from the forebrain via the stria medullaris (sm), and projects to the basal mesencephalon and rostral rhombencephalon through the fasciculus retroflexus.

It was classically divided into medial and lateral portions of the LHb and rostral and caudal portions of the MHB (Herkenham and Nauta; Fig. A). By cytoarchitectural analysis of the habenular complex in the rat brain, the LHb was later divided into 9 subnuclei and the MHB into 5 subnuclei (Andres et al.; Fig. B). This subdivision was confirmed by neurotransmitter distribution. According to topographic, cytochemical, morphological and immunocytochemical criteria, the habenular subdivision described in rat was also shown in the mouse brain. A detailed transcriptomic characterization corroborated the subnuclei previously described in the mouse Hb (Wagner et al.; Fig. C). Accordingly, the LHb was described as displaying a medial division that included central (LHbMC), marginal (LHbMMg), parvocellular (LHbMpc) and superior (LHbMS) subnuclei and a lateral division that included: lateral (LHbL), basal (LHbLB), magnocellular (LHbLMc), marginal (LHbLMg), oval (LHbLO) and parvocellular (LHbLpc) subnuclei. The MHB was subdivided into dorsal (MHBd), superior (MHS), ventral medial (MHBVm), ventral central (MHBVc) and ventral lateral (MHBVl) parts.

In recent years, the development of single cell RNAseq techniques has allowed the study of the expression profile of dissociated habenular neurons. These analyses resulted in the identification by a transcriptomic profile of 12 neuronal clusters that largely coincided with the habenular morphological and transcriptomic subdivisions. Some of them belonged to specific subnuclei and others to subdivisions inside a particular subnucleus. This result unveiled the high complexity displayed by the neuronal subpopulations of the Hb complex.

The sm is the main afferent tract to the Hb complex. This highly fasciculated tract contains fibers originated from different neuronal populations located in the secondary prosencephalon (hypothalamus and telencephalic vesicle; Herkenham and Nauta; Puelles and Rubenstein). The fasciculated sm courses through the most dorsal aspect of the prethalamic prosomere, known as the prethalamic eminence, carrying axons from its multiple origins, caudally into the thalamic prosomere to reach the Hb complex.

Instituto de Neurociencias, Universidad Miguel Hernández de Elche-CSIC, 03550 Sant Joan d’Alacant, Alicante, Spain. *email: epuelles@umh.es
It has been described that sm fibers originate in the hypothalamic, pallidal and septal territories. The MHb reportedly receives inputs from the triangular septal nucleus (TRS), the septofimbrial nucleus (SF), the septal area, the bed nucleus of the anterior commissure (BAC), and from hypothalamic entopeduncular neurons. The LHb, as first described, is innervated by, the entopeduncular nucleus (erroneously identified as basal ganglia and nucleus accumbens), preoptic regions and septum. This description was later confirmed and completed, concluding that the LHb receives inputs from the substantia innominata (SI; Golden et al.; Knowland et al.), dorsal entopeduncular nucleus (EPD), and lateral hypothalamic area (LHA). It must be highlighted that it was recently described that almost all the neuronal populations projecting to the Hb in the chick are colonized by tangentially migrated glutamatergic neurons originated from the prethalamic eminence. There are three heterochronic migratory streams, by which the prethalamic eminence populates hypothalamic, preoptic, pallidal and septal regions. The specific pattern of innervation produced by the different afferent neuronal populations in the LHb or MHb subnuclei has been poorly studied. Only the specific innervation of the LHbLO by the EPD has been described previously.

A selective source of innervation thus possibly underlies different functions associated to the Hb components. In general, the MHb has been associated with the mediation of analgesic, autonomic, reward, anxiety and fear responses. More specifically, the dorsal aspect of the MHb has been related to exercise motivation, regulation of the hedonic state, intrinsic reinforcement circuit and aversive behaviors. The ventral aspect of the MHb has been involved in drug addiction, anxiety, and depression. Therefore, the vertebrate MHb is related to emotional behavior. In contrast, the LHb has been considered as an anti-reward system and appears associated to behavioral and motivational control. In fact, the LHb is involved in regulation of aversion, stress, sleep, mood and maternal behavior. Results obtained in behavioral experiments point out to a LHb relation with learned helplessness response as well as reward, aversion or punishment behavior and depression.

Our present aim is to analyze the possible differential innervation of the multiple habenular subnuclei considering the differential origin of their afferent fibers. The observed distribution was confronted with the subnuclear organization and location of limbic system functions associated to the habenula.

Results

First, we selected several gene expression patterns, inspired by published single cell RNAseq experiments as examples of Hb subnuclear organization markers (Quina et al.). The images, form rostral to caudal, were color-coded and overlapped by Adobe Software. In the MHb, Asic4 nicely labelled the MHbS and the MHbD subnuclei, while Spon1 was expressed in the MHbVl subnucleus and Myo16 in the remaining MHb ventral components (central and medial; Fig. 2A, 'A', 'A'). The Cubn and Wif1 expression allowed us to discern between the two dorsal MHb components. Being Cubn expressed in the MHbS and Wif1 in the MHbD (Fig. 2B, 'B', 'B'), Kcnmb4 was mainly expressed in the MHbVl and MHbVc allowing us to discern between the positive MHbVc and negative MHbVm (Fig. 2B, 'B', 'B'), both populations where positive for Myo16. Kcnmb4 was also expressed in...
the LHbMS (Fig. 2B, B’, B”). The three ventral components of the MHb shared the expression of Tacr1 (Fig. 2C, C’, C”). Finally, in the LHb, Chrm2 was expressed in the medial region, including the LHbLPc, LHbLMc an LHbLB (Fig. 2C, C’, C”). While Pvalb was expressed in the LHbLO and in the LHbMPc and LHbMC (Fig. 2C, C’, C”). Once we identified all the different subnuclei of the Hb at the three selected section levels, we proceeded with the analysis of the connectivity experiments.
Neuronal populations targeting the medial habenula. We confirmed four neuronal populations that target the MHb. They mainly belong to the septal region and are the triangular septal nucleus (TS), the medial septal nucleus (MS), the septofimbrial nucleus (SF) and the bed nucleus of the stria terminalis, medio-central division (BST). The TS (Fig. 3A) projects to the LHbVM, LHbMS and the MHbD. The GPF + axonal terminals extend along the anteroposterior axis of the habenular complex (Fig. 3B–B”). The proximity of the MS and the median preoptic nucleus (MnPO) did not allow us to find experiments restricted to MS (Fig. 3C). In a first analysis MS/MnPO terminals reached the LHbVC and partially the MHbVM and MHbD (Fig. 3D–D”). The injections in the SF also affected the MnPO (Fig. 3E). The terminal labelling overlap between the MS and SF experiments allowed us to ascribe the MnPO projection area to the LHbMMg (Fig. 3D’ and F”). The SF nucleus would thus project to the MHbVI and the MHbD (Fig. 3F–F”). The contralateral MHbS was partially labelled due to positive fibers crossing through the habenular commissure (Fig. 3F”) SF also projected to the LHb, with terminals found in the LHbMPc and LHbMC (Fig. 3F). Finally, we identified an injection in the BST (Fig. 3G), medio central division, that specifically labelled the LHbS (Fig. 3H–H”).

In summary, the MHb dorsal area is principally innervated by TS, SF and medio central BST while the three ventral MHb subnuclei have differential innervations. The medial part is innervated by the TS, the central part by the MS and the lateral part by the SF. Note that in the case of the MHbD we cannot exclude that some of the positive fibers are not terminals but passing fibers to others nuclei due to the location of the sm.

Neuronal populations targeting the lateral habenula. The LHb receives projections originated from different brain regions. We detected terminals originated from the preoptic area and others parts of the subpal-lium, terminal and peduncular hypothalamus. The GPF + fibers displayed a diffuse distribution in the LHb when compared to the MHb pattern.

We found four populations from the preoptic area, terminal hypothalamus and subpallial area: the lateral preoptic area (LPO), the medial preoptic area (MPO) and the anterior hypothalamic nucleus (AHN) and the substantia innominate (SI). The LPO injection in the preoptic area (Fig. 4A) illustrated the main projection into the LHbLMe and LHbLB (Fig. 4B, B”). We also found scattered axons in the LHbLMg, LHbMPc and LHbMC (Fig. 4B–B”). Some fibers were also detected in the dorsal LHb including LHbMS, LHbLPc and LHbLMg. Therefore, LPO fibers innervate the medio-central and dorsal LHb areas. The MPO injections always included surrounding territories. We selected an injection that affected partially the MPO and also labelled the medial pre-optic nucleus (MPN; Fig. 4C). The database contains specific MPN injections that did not display any habenular projections. Therefore, the projections observed only in the medial aspect of the LHb, concerning the LHbMMg and LHbMCp must be due largely to the MPO (Fig. 4D–D”). In the terminal hypothalamus, the AHN injection (Fig. 4E), that also affected a perifornical nucleus (PeF; peduncular hypothalamic population) displayed projections into the medial area of the LHb, including the LHbMMg, LHbMPc and LHbMC subnuclei (Fig. 4F–F”). Thus, the LHb medial territory is mainly innervated by LPO, MPO and AHN populations. The LPO also targets the LHb central territory (Fig. 4B–B”). The subpallial area contains a neuronal population that targets the LHb, namely SI, intermediate stratum of the diagonal domain. The injection in the SI labelled the magnocellular preop-tic nucleus (MA; Fig. 4G), and the fibers were distributed in a diffuse pattern throughout the LHb (Fig. 4H–H”).

In the latter’s medial part, the axons concentrated in the LHbLB and LHbLMc subnuclei (Fig. 4H”) and in its caudal part, the fibers also occupied the LHbMS and LHbLPc (Fig. 4H”).

Four neuronal populations were identified in the peduncular hypothalumus: the paraventricular hypothalamic nucleus (PVH), the dorsomedial hypothalamic nucleus (DMH), the lateral hypothalamic area (LHA) and the dorsal entopeduncular nucleus (EPD). The PVH injection (Fig. 5A) demonstrated a strongly diffuse projection into all the LHb (Fig. 5B–B”). Only in the LHb medial region, the GPF + fibers displayed a more specific pattern within the LHbMMg, LHbMPc and LHbMC (Fig. 5B”). Some fibers were also detected in the dorsal LHb including LHbMS, LHbLPc and LHbLMg. Therefore, LPO fibers innervate the medial-central and dorsal LHb areas. The MPO injections always included surrounding territories. We selected an injection that affected partially the MPO and also labelled the medial pre-optic nucleus (MPN; Fig. 4C). The database contains specific MPN injections that did not display any habenular projections. Therefore, the projections observed only in the medial aspect of the LHb, concerning the LHbMMg and LHbMCp must be due largely to the MPO (Fig. 4D–D”). In the terminal hypothalamus, the AHN injection (Fig. 4E), that also affected a perifornical nucleus (PeF; peduncular hypothalamic population) displayed projections into the medial area of the LHb, including the LHbMMg, LHbMPc and LHbMC subnuclei (Fig. 4F–F”). Thus, the LHb medial territory is mainly innervated by LPO, MPO and AHN populations. The LPO also targets the LHb central territory (Fig. 4B–B”). The subpallial area contains a neuronal population that targets the LHb, namely SI, intermediate stratum of the diagonal domain. The injection in the SI labelled the magnocellular preop-tic nucleus (MA; Fig. 4G), and the fibers were distributed in a diffuse pattern throughout the LHb (Fig. 4H–H”).

In the latter’s medial part, the axons concentrated in the LHbLB and LHbLMc subnuclei (Fig. 4H”) and in its caudal part, the fibers also occupied the LHbMS and LHbLPc (Fig. 4H”).

Four neuronal populations were identified in the peduncular hypothalumus: the paraventricular hypothalamic nucleus (PVH), the dorsomedial hypothalamic nucleus (DMH), the lateral hypothalamic area (LHA) and the dorsal entopeduncular nucleus (EPD). The PVH injection (Fig. 5A) demonstrated a strongly diffuse projection into all the LHb (Fig. 5B–B”). Only in the LHb medial region, the GPF + fibers displayed a more specific pattern within the LHbMMg, LHbMPc and LHbMC (Fig. 5B”). The DMH injection (Fig. 5C) showed a specific terminal pattern that affected mainly the medial LHb territory, including the LHbMMg, LHbMPc and LHbMC (Fig. 5D–D”). The LHA was labelled at the level of the AHN and the resulting projection (Fig. 5E) displayed again a diffuse distribution in the LHb territory (Fig. 5F, F”). Caudally, the terminals concentrated in the LHb central territory particularly in LHbMMg, LHbLB and LHbLPc (Fig. 5F–F”). Accordingly, the DMH targets the LHb medial area while the PVH and LHA distribute in the LHb central territory. The EPD injection (Fig. 5G) labelled fibers that specifically innervated the medial portion of the LHbLO (Fig. 5H”) with a minor projection into neighboring rostral and caudal LHb parts (Fig. 5H”–H”).

Habenular neuronal cell type distribution. The Hb single cell RNAseq experiments demonstrated the presence of various neurotransmitters-related cell types in this neuronal complex. We checked these neuro-transmitter-related patterns testing whether their distribution coincides with habenular subnuclear subdivisions.

Excitatory glutamatergic neurons were predominant in both MHb and LHb. vGlut1 signal was prevalent in all the MHb (Fig. 6A) while vGlut2 expression appeared in both habenular nuclei (Fig. 6B). Choline acetyl-transferase expression labelled cholinergic neurons distributed in the ventral MHb subnuclei (MHbVM, MHbVc and MHbVII; Fig. 6C). Cholecystokinin signal was restricted to the dorsal MHb (MHbS and MHbD; Fig. 6D). The inhibitory marker Gad65 appeared in LHbMPc and partially also in LHbMC as dispersed positive cells (Fig. 6E), while Gad67 transcripts were localized specifically in the MHbS (Fig. 6F). Parvalbumin, specific marker of a subtype of inhibitory gabaergic neurons, was expressed in LHbMPc, LHbMc, LHbLMc and in LHbLO (Fig. 6G). Finally, Somatostatin was located in MHbVM, MHbVc and MHbVI as well as in the LHbMS and LHbLPc (Fig. 6H).

Therefore, specialized neurons with specific neurotransmitters are grouped in the different subnuclei described by transcriptomic methodology, and they also are innervated by different neuronal populations.
Figure 3. Septal projections to the MHb. (A) Injection site in the triangular nucleus of septum (TS; Experiment nº: 125,830,911, identified as Lateral septum rostral part, LSr). (B–B”) Adult mouse habenula coronal sections, from rostral to caudal, showing the fiber distribution originated from the TS nucleus. (C) Injection site in the medial septal nucleus (MS; Experiment nº: 147,162,736). (D–D”) Adult mouse brain coronal sections of the habenula rostral to caudal, displaying the fibers distribution coming from the MS nucleus. (E) Injection site in the septofimbrial nucleus (SF; Experiment nº: 554,021,622). (F–F”) Adult mouse brain coronal sections of the habenula rostral to caudal, showing the fibers distribution originated from the SF nucleus. (G) Injection site in the bed nuclei of the stria terminalis (BST; Experiment nº 159,433,187. (H–H”) Adult mouse brain coronal sections of the habenula rostral to caudal, displaying the fibers distribution coming from the BST nucleus, being the only nucleus that is not from septal territory. Abbreviations: BST: bed nuclei of the stria terminalis; LSc: lateral septal nucleus, caudal part; LSr: lateral septal nucleus, rostral part; MS: medial septal nucleus; LHbLMc: LHbL magnocellular subnucleus; LHbMMg: LHbM marginal subnucleus; LHbMPC: LHbM parvocellular subnucleus; MHbD: MHb dorsal subnucleus; MHbS: MHb superior subnucleus; MHbVc: MHb ventral central subnucleus; MHbVI: MHb ventral lateral subnucleus; MHbVM: MHb ventral medial subnucleus; MnPO: median preoptic nucleus; SF: septofimbrial nucleus; TS: triangular nucleus of septum. Scale bar: 200 μm. Image credit: Allen Institute for Brain Science. [https://connectivity.brain-map.org/].
Figure 4. Preoptic area, terminal hypothalamic and pallidal projections to the LHb. (A) Injection site in the lateral preoptic area (LPO; Experiment nº: 293,942,188). (B–B”) Adult mouse habenula coronal sections, from rostral to caudal, showing the fibers distribution originated from the LPO nucleus. (C) Injection site in the medial preoptic area (MPO; Experiment nº: 299,247,009). (D–D”) Adult mouse habenula coronal sections, from rostral to caudal, displaying the fibers distribution coming from the MPO nucleus. (E) Injection site in the anterior hypothalamic nucleus (AHN; Experiment nº: 292,035,484). (F–F”) Adult mouse habenula coronal sections, from rostral to caudal, showing the fibers distribution originated from the AHN nucleus. (G) Injection site in the substantia innominata (SI; Experiment nº: 302,739,608). (H–H”) Adult mouse habenula coronal sections, from rostral to caudal, showing the fibers distribution originated from the SI nucleus. Abbreviations: AHN: anterior hypothalamic nucleus; AVPV: anteroventral periventricular nucleus; AVP: Anteroventral preoptic nucleus; LHb, lateral habenula; LHbLB: LHbL basal subnucleus; LHbLMc: LHbL magnocellular subnucleus; LHbLMg: LHbL marginal subnucleus; LHbLO: LHbL oval subnucleus; LHbLPc: LHbL parvocellular subnucleus; LHbM: LHb medial territory; LHbMC: LHbM central subnucleus; LHbMMg: LHbM marginal subnucleus; LHbMPc: LHbM parvocellular subnucleus; LHbMS: LHbM superior subnucleus; LPO: lateral preoptic area; MA: magnocellular nucleus; MHB: medial habenula; MHBd: MHB dorsal subnucleus; MPO medial preoptic area; MPN: medial preoptic nucleus; NDB: diagonal band nucleus; PeF: perifornical nucleus; SBPV: subparaventricular zone; SI: substantia innominata; VLPO: ventrolateral preoptic nucleus. Scale bar: 200 μm. Image credit: Allen Institute for Brain Science. [https://connectivity.brain-map.org/].
Figure 5. Peduncular hypothalamic projections to the LHb. (A) Injection site in the paraventricular hypothalamic nucleus (PVH; Experiment nº: 581,641,279). (B–B’) Adult mouse habenula coronal sections, from rostral to caudal, showing the fiber distribution originated from the PVH nucleus. (C) Injection site in the dorsomedial nucleus of the hypothalamus (DMH; Experiment nº: 178,283,239). (D–D’) Adult mouse habenula coronal sections, rostral to caudal, displaying the fibers distribution coming from the DMH nucleus. (E) Injection site in the lateral hypothalamic area (LHA; Experiment nº: 485,239,207). (F–F’) Adult mouse habenula coronal sections, rostral to caudal, showing the fibers distribution originated from the LHA nucleus. (G) Injection site in the dorsal entopeduncular nucleus (EDP; Experiment nº: 539,498,984, identified as internal segment of globus palidus; GPi). (H–H’) Adult mouse habenula coronal sections, rostral to caudal, displaying the fibers distribution coming from the EDP nucleus. Abbreviations: AHN: anterior hypothalamic nucleus; DMH: dorsomedial nucleus of the hypothalamus; EDP, dorsal entopeduncular nucleus; Fx: fornix; LHA: lateral hypothalamic area; LHb, lateral habenula; LHbL: LHbL basal subnucleus; LHbLO: LHbL oval subnucleus; LHBMC: LHbM central subnucleus; LHbMMg: LHbM marginal subnucleus; PH: posterior hypothalamic nucleus; PVH: paraventricular hypothalamic nucleus; RCH: retrochiasmatic area; RE: nucleus of reuniens. Scale bar: 200 μm. Image credit: Allen Institute for Brain Science. [https://connectivity.brain-map.org/].
Discussion

The single cell transcriptomic RNAseq studies of Hashikawa et al. and Wallace et al. have corroborated different subnuclear components of the habenular complex. The MHb is divided into a dorsal part that includes the MHbD and MHbS subnuclei and a ventral part composed by the MHbVm, MHbVc and MHbVl units. On the other hand, the LHb is divided into a dorsal region that involves the LHbMS, LHbLPc and LHbLMg, a central part that includes medial LHbMPc and LHbMC components, a central LHbLMc component and a lateral LHbLO portion, and finally a ventral portion with LHbMMg and LHbLB units. These studies identified the MHb subnuclei by the expression of a specific gene but the LHb components were recognized by a combination of several gene expression patterns. Each distinct MHb component displays internal homogeneity, while the LHb subnuclei show substantial internal heterogeneity. The diverse nature of their neurons indicates an intricate mode of development. It may be hypothesized that the LHb subnuclei are composed of different subsets of neurons that occupy diverse destinations by differential migration processes.

In relation to habenular affereces, the MHb is innervated by four neuronal populations (BST, SF, TS and MS). It is remarkable that almost all the neuronal populations projecting to the MHb belong to the septal territory. This innervation is strongly compartmentalized and the four different sets of axons target specific MHb subnuclei. The BST (medio central division) specifically innervates the MHbS, the SF targets the MHbVI and the MHbD, the TS reaches the MHbD and MHbVm and finally, the MS innervates the MHbVc. It has been described that the TS innervation of the MHb is accompanied by fibers from the bed nucleus of the anterior commissure (BAC; Yamaguchi et al.; Watanabe et al.), but no specific BAC injection was found in the Allen database. Therefore, the dorsal MHb is under the influence of BST, TS/BAC and SF innervation. The fact that both subnuclei are innervated by different neuronal populations suggests that the functions related to them may be separated between both subnuclei. New and more selective behavioral experiments are needed to dissect the specific function of each dorsal MHb subnucleus. The ventral MHb region is related to anxiety, depression and drug addiction learning. This MHb territory is subdivided in three subnuclei from medial to lateral (MHbVm, Vc and VI). These subnuclei are each innervated by selective neuronal

---

Figure 6. Habenular neuronal cell type distribution. Adult mouse habenula coronal sections displaying the location of specific cell type markers by in situ hybridization. Excitatory neurotransmitters (A–D). (A) vGlut1 gene, displayed in ventral and dorsal MHb; (B) vGlut2 gene, displayed principally in the MHb and in a the LHb as a scattered pattern. (C) ChAT gene, displayed in the ventral MHb. (D) Cck gene, expressed in the dorsal MHb. Inhibitory neurotransmitters (E–H). (E) Gad65 gene, displayed in LHb with a specific expression in LHbMpc and LHbMc subnuclei. (D) Gad67 gene, displayed in a specific pattern in the MHbS subnuclei. (E) Pvalb gene, expressed in specific pattern in the LHbMpc, LHbMc and LHbLO subnuclei, also some scattered positive cells were detected in LHbLmc. (D) Sst gene, displayed in the ventral MHb part. Abbreviations: LHb, lateral habenula; LHbLMc: LHbL magnocellular subnucleus; LHbLO: LHbL oval subnucleus; LHbM: LHb medial territory; LHbMC: LHbM central subnucleus; LHbMpc: LHbM parvocellular subnucleus; LHbMS: LHbM superior subnucleus; LHb: medial habenula; MHbD: MHb dorsal subnucleus; MHb: MHb superior subnucleus; MHbVc: MHb ventral central subnucleus; MHbVI: MHb ventral lateral subnucleus; MHbVm; MHb ventral medial subnucleus. Scale bar: 200 μm. Image credit: Allen Institute for Brain Science. [https://mouse.brain-map.org/].

---
populations. The ventromedial, central and lateral subnuclei are projected upon by the TS/BAC, MS and SF, respectively. This selective innervation also indicates, as in the dorsal MHb, that each ventral subnucleus may be involved in a different function or that collaboration among them is needed for the cited behavioral phenomena (Fig. 7A, B). As stated before, more specific behavioral experiments must be done to properly understand the role of each ventral MHb subnucleus.

The LHb afferences displayed a wide range of origins. The axonal distributions were less specific for the different LHb subnuclei and usually displayed a diffuse pattern. Nevertheless, we noted that preoptic and AH projections cover the medial aspect of the LHb, whereas diverse afferents from peduncular hypothalamic nuclei reach preferentially the central territory, and SI, EPD and reticular nucleus projections target the lateral components of the LHb. This relatively diffuse pattern of innervation coincides with the complex internal subnuclear organization described by the transcriptomic RNAseq experiments. In contrast with the MHb neuronal clusters, the LHb subnuclei required a combination of markers for characterization. We mentioned above the possibility of intermixed neuronal tangential migrations within the LHb components as a plausible explanation of their peculiar partly shared characteristics.

We identified the LPO and MPO in the preoptic area, the AH nucleus in the alar terminal hypothalamus and SI in the subpallial area as territories that project into the LHb. Their axons converged within the medial LHb territory. The LPO projection into the LHb reportedly includes gabaergic and glutamatergic neurons. The balance between these two neurotransmitters modulates the reward/aversion equilibrium in the learning process. The MPO and AH projections into the LHb were previously described but without reference to specific subnuclei or habenular function (Fig. 6A, B). We located PVH, DMH and LHA fibers from the peduncular hypothalamus in the central area of the LHb. The LHA projection into the LHb was previously described and was related with feeding regulation and reward/aversion equilibrium in the learning process. No specific information about the PVH and DMH projections into the LHb was found (Fig. 7A,B). Finally, we identified EPD and SI input to the LHb. These nuclei distribute their projections preferentially from medial to lateral areas of the LHb. It is remarkable that the LHbLO is distinguished among all the LHb nuclei due to a strongly specific innervation by the EPD. The LHbLO neurons, together with the LHbMS, display unique electrophysiological properties when compared to the rest of LHb neurons. They respond to Dopamine with an increment of their firing rate in contrast with the rest of LHb neurons. Therefore, this unit constitutes not only a differentiated morphological structure of the LHb but a distinctive functional entity by itself. The EPD excitatory projections into LHbLO are related with reward prediction errors modulated by neurotransmitters (Shabel et al.; Wallace et al.; Fig. 7A,B). The high quality of Allen Brain Atlas images allowed a high level of magnification. In most of the experiments analyzed we were able to detect varicosities in the positive fibers that could point to axonal boutons. Nevertheless, in order to confirm this fact, double labeling with synaptic proteins should be performed to confirm the presence of axonal boutons in the different territories.

It must be highlighted that it was recently described in chick that the prethalamic eminence, the dorsal subregion of prethalamus found just rostral to the habenular thalamic region, contributes excitatory neurons by tangential migration during embryonic development to almost all the populations described as projecting into the Hb complex. A migratory origin in the prethalamic evidence was demonstrated for the mouse habenulopetal BAC nucleus. The described migration into the peduncular hypothalamic EPD formation nicely explains that this mixed excitatory/inhibitory neuronal population has usually been wrongly assigned to the pallidal territory.
as part of the rodent GPi\textsuperscript{9,17,26,27}. Therefore, the functions assigned to the pallidal GPi\textsuperscript{20,22} seem to correspond to the hypothalamic EPD.

It must be noted that our analysis presents certain limitations due to the fact that we have only used the data obtained from Allen Brain database. In some of the cases it would have been needed more specific and accurate injections or the use of specific viral tracers to label specific neuronal types. Nevertheless, we do not foresee that these specific experiments would strongly modify the conclusions of our work.

The molecular neuronal heterogeneity among the MHb and LHb subnuclei correlates with the distribution of different neurotransmitters. In general, the LHb is divided in two areas (medial and lateral) attending to its different psychobiological functions. The lateral area is related to avoidance behavior to aversive stimuli while the medial part has been involved in despair, helplessness, anhedonia responses and in sleep and circadian rhythms\textsuperscript{54}. Our hodological results open the possibility to develop research lines that uncover the specific roles of the different subnuclei of both LHb and MHb.

**Methods**

**Allen brain atlas.** The Allen Mouse Brain Atlas (© 2021 Allen Institute for Brain Science. Mouse Brain Connectivity. Available at: https://connectivity.brain-map.org) offers Adult Mouse Connectivity Atlas as an image database of axonal projections labeled by viral (rAAV) tracers and visualized using serial two-photon tomography from 2994 experiments.

This resource contains several tools to search through its experiments. The Source Search tool, allows the search of experiments by injection site (Filter source Structures) filtered by mouse line, tracer type and the presence of Intrinsic Signal Images. The Target Search tool allows a “virtual retrograde” search that localizes experiments based on projections located in the structures of interest. Finally, the Spatial Search allows the user to choose either a target signal or injection site based on a voxel selection that retrieves all the experiments with positive signal. The injection summary includes primary and secondary injection structures, the stereotaxic injection Bregma coordinates, the mouse strain, tracer type and the calculated injection summary (%) for the rAAV. The Image Viewer allows to browse the experiment in 2-D and panning through the 140 coronal slices of each experiment. The histogram shows the quantified signal in each structure either by projection volume (mm\textsuperscript{3}) or by projection density, which means the fraction of the area occupied by signal compared to the whole structure. The mouse strains used included wild type and transgenic cre lines. Nevertheless, the rAAV used did not include specific sequences to interact with the cre endonuclease.

**Adult mouse connectivity atlas.** At the identification stage, 754 experiments (Supplementary Table S1) were revisited, using the injection site search, from Septal (30), Hypothalamic (258), Pallidal (67), Striatal (131), and Thalamic (268) areas, according with the habenula-afferent nuclei identified previously. These nuclei were screened by checking both the section images and the projection density window (3D viewer) of each experiment, in order to corroborate the labelled terminations in the habenula. The coronal slices from each confirmed case were reexamined to check the labelled fiber pathway and the terminations in both MHb and LHb.

For the selection of experiments, the following criteria were used: virus volume injected < 0.2 mm\textsuperscript{3} and injection coordinates within anatomical boundaries of the core of interest. Experiments with a massive virus volume injected, or labelling of 4 or more structures to the area of interest, were excluded. In order to systematize the image selection through the Hb between the experiments, we selected three coronal section levels taken 3, 6 and 9 sections rostrally to the habenular commissure. The entire process was carried out through peer analysis, both the selection and screening of the experiments was carried out by two researchers, according to the inclusion and exclusion criteria. For this reason, once all the experiments were collected, each researcher selected one or two experiments from each set that met the criteria. After the first screening, both researchers pooled the results to reach a consensus list of experiments that were suitable in relation to the selection criteria. The experiment number, the amount of virus injected and the Bregma coordinates of the injection were noted (Supplementary Table S1).

All the gene expression images were downloaded from Allen Institute for Brain Science. [https://mouse.brain-map.org/], Mouse Brain (ISH Data). Adobe Photoshop (version 22.1.1) was used for the photo editing program and Adobe Illustrator (version 25.1) was used to generate the figures.

**Data availability**

The datasets generated and/or analyzed during the current study are available in The Allen Mouse Brain Atlas (© 2021 Allen Institute for Brain Science. Mouse Brain Connectivity and Mouse Brain Map (Available at: https://connectivity.brain-map.org and https://mouse.brain-map.org) repository. The accession number to each experiment are contained in Supplementary Table S1.

Received: 30 March 2022; Accepted: 6 June 2022
Published online: 16 June 2022

**References**

1. Seigneur, E., Polepalli, J. S. & Südhof, T. C. Cbln2 and Cbln4 are expressed in distinct medial habenula-interpeduncular projections and contribute to different behavioral outputs. *Proc. Natl. Acad. Sci. U. S. A.* 115, E10235–E10244 (2018).
2. Sutherland, R. J. The dorsal diencephalic conduction system: a review of the anatomy and functions of the habenular complex. *Neurosci. Biobehav. Rev.* 6, 1–13 (1982).
3. Herkenham, M. & Nauta, W. J. H. Afferent connections of the habenular nuclei in the rat. A horseradish peroxidase study, with a note on the fiber-of-passage problem. *J. Comp. Neurol.* 173, 123–145 (1977).
4. Herkenham, M. & Nauta, W. J. H. Efferent connections of the habenular nuclei in the rat. *J. Comp. Neurol.* 187, 19–47 (1979).
5. Andres, K., Von Düring, M. & Veh, R. Subnuclear organization of the rathbunenbular complexes. J. Comp. Neurol. 407, 130–150 (1999).
6. Aizawa, H., Kobayashi, M., Tanaka, S., Fukai, T. & Okamoto, H. Molecular characterization of the subnuclei in rat habenula. J. Comp. Neurol. 520, 4051–4066 (2012).
7. Wagner, F., Stroh, T. & Veh, R. W. Correlating habenular subnuclei in rat and mouse by using topographic, morphological, and cytochemical criteria. J. Comp. Neurol. 522, 2630–2662 (2014).
8. Wagner, F., French, L. & Veh, R. W. Transcriptional-anatomic analysis of the mouse habenula uncovers a high molecular heterogeneity among neurons in the lateral complex, while gene expression in the medial complex largely obeys subnuclear boundaries. Brain Struct. Funct. 221, 39–58 (2016).
9. Wallace, M. L. et al. Anatomical and single-cell transcriptional profiling of the murine habenular complex. Elife 9, 1–22 (2020).
10. Hashikawa, Y. et al. Transcriptional and spatial resolution of cell types in the mammalian habenula. Neuron 106, 743–758.e5 (2020).
11. Puelles, L. & Rubenstein, J. L. R. Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuroemeric organization. Trends Neurosci. 16, 472–479 (1993).
12. Puelles, L. & Rubenstein, J. L. R. Forebrain gene expression domains and the evolving prosomeric model. Trends Neurosci. 26, 469–476 (2003).
13. Puelles, L. & Rubenstein, J. L. R. A new scenario of hypothalamic organization: rationale of new hypotheses introduced in the updated prosomeric model. Front. Neuroanat. 9, 27 (2015).
14. Viswanath, H., Carter, A. Q., Baldwin, P. R., Molfese, D. L. & Salas, R. The medial habenula: still neglected. Front. Hum. Neurosci. 7, 1–6 (2014).
15. Hsu, Y. W. A. et al. Medial habenula output circuit mediated by a5 nicotinic receptor-expressing GABAergic neurons in the interpeduncular nucleus. J. Neurosci. 33, 18022–18035 (2013).
16. Watanabe, K., Irie, K., Hanashima, C., Takebayashi, H. & Sato, N. Diencephalic progenitors contribute to the posterior septum through rostral migration along the hippocampal axonal pathway. Sci. Rep. 8, 1–13 (2018).
17. Wallace, M. L. et al. Genetically distinct parallel pathways in the entopeduncular nucleus for limbic and sensorimotor output of the basal ganglia. Neuron 94, 138-152.e5 (2017).
18. Golden, S. A. et al. Basal forebrain projections to the lateral habenula modulate aggression reward. Nature 534, 688–692 (2016).
19. Knowland, D. et al. Distinct ventral pallidal neural populations mediate separate symptoms of depression. Cell 170, 284-297.e18 (2017).
20. Hong, S. & Hikosaka, O. The globus pallidus sends reward-related signals to the lateral habenula. Neuron 60, 720–729 (2008).
21. Li, H., Pullmann, D. & Hou, T. C. Nucleus-encoding in the lateral habenula arises from the entopeduncular region. Elife https://doi.org/10.7554/eLife.41223 (2019).
22. Shabel, S. J., Proulx, C. D., Trias, A., Murphy, R. T. & Malinow, R. Input to the lateral habenula from the basal ganglia. Neuron 79, 1–13 (2021).
23. Lazaridis, I. et al. A hypothalamus-habenula circuit controls aversion. Mol. Psychiatry 24, 1351–1368 (2019).
24. Lecca, S. et al. Aversive stimuli drive hypothalamus-to-habenula excitation to promote escape behavior. Elife https://doi.org/10.7554/eLife.30697 (2017).
25. Stamatakis, A. M. et al. Lateral hypothalamic area glutamatergic neurons and their projections to the lateral habenula regulate feeding and reward. J. Neurosci. 36, 302–311 (2016).
26. Alonso, A., Trujillo, C. M. & Puelles, L. Longitudinal developmental analysis of prethalamic eminence derivatives in the chick by mapping of Tbr1 in situ expression. Brain Struct. Funct. 225, 481–510 (2020).
27. Alonso, A., Trujillo, C. M. & Puelles, L. Quail-chick grafting experiments corroborate that Tbr1-positive eminential prethalamic neurons migrate along three streams into hypothalamus, subpallium and septocommissural areas. Brain Struct. Funct. 226, 759–785 (2021).
28. Kowski, A. B., Geisler, S., Krauss, M. & Veh, R. W. Differential projections from subfields in the lateral preoptic area to the lateral habenular complex of the rat. J. Comp. Neurol. 507, 1465–1478 (2008).
29. Gardner, O. et al. Expression of mu opioid receptor in dorsal diencephalic conduction system: new insights for the medial habenula. Neuroscience 277, 595–609 (2014).
30. Hsu, Y. W. A., Morton, G., Guy, E. G., Wang, S. D. & Turner, E. E. Dorsal medial habenula regulation of mood-related behaviors and primary reinforcement by tachykinin-expressing habenula neurons. eNeuro 3, 118–126 (2016).
31. Lee, H. W., Yang, S. H., Kim, J. Y. & Kim, H. The role of the medial habenula cholinergic system in addiction and emotion-associated behaviors. Front. Psychiatry 10, 1–8 (2019).
32. Koppensteiner, P., Galvin, C. & Ninan, I. Development- and experience-dependent plasticity in the dorsomedial habenula. Mol. Cell. Neurosci. 77, 103–112 (2016).
33. Hsu, Y. W. A. et al. Role of the dorsal medial habenula in the regulation of voluntary activity, motor function, hedonic state, and primary reinforcement. J. Neurosci. 34, 11366–11384 (2014).
34. Stamatakis, A. & Stuber, G. Activation of lateral habenula inputs to the ventral midbrain promotes behavioral avoidance. Nat. Neurosci. 15, 1105–1107 (2012).
35. Gold, P. W. & Kadriu, B. A major role for the lateral habenula in depressive illness: physiologic and molecular mechanisms. Front. Psychiatry 10, 1–7 (2019).
36. Li, B. et al. Synaptic potentiation onto habenula neurons in the learned helplessness model of depression. Nature 470, 535–541 (2011).
37. Li, K. et al. GABAergic circuits in the lateral habenula mediate core symptoms of depression. Science 341, 1016–1020 (2013).
38. Matsumoto, M. & Hikosaka, O. Representation of negative motivational value in the primate lateral habenula. Nat. Neurosci. 12, 77–84 (2009).
39. Hikosaka, O. The habenula: from stress evasion to value-based decision-making. Nat. Rev. Neurosci. 11, 503–513 (2010).
40. Proulx, C. D., Hikosaka, O. & Malinow, R. Reward processing by the lateral habenula in normal and depressive behaviors. Nat. Neurosci. 17, 1146–1152 (2014).
41. Zheng, Z. et al. Hypothalamus-habenula potentiation encodes chronic stress experience and drives depression onset. Neuron 110, 1400–1415.e6 (2022).
42. Yang, Y., Wang, H., Hu, J. & Hu, L. Lateral habenula in the pathophysiology of depression. Curr. Opin. Neurobiol. 48, 90–96 (2018).
43. Cui, Y., Yang, Y., Dong, Y. & Hu, H. Decoding depression: insights from glial and ketamine regulation of neuronal burst firing in lateral habenula. Cold Spring Harb. Symp. Quant. Biol. 83, 141–150 (2018).
44. Hu, H. Advances in molecular and circuitry mechanisms of depressive disorder—a focus on lateral habenula. In Advances in Experimental Medicine and Biology Vol. 1180 (ed. Fang, Y.) 135–146 (Springer, 2019).
45. Hu, H., Cui, Y. & Yang, Y. Circuits and functions of the lateral habenula in health and in disease. Nat. Rev. Neurosci. 21, 277–295 (2020).
46. Qiu, L. A., Wang, S., Ng, L. & Turner, E. E. Brn3a and Nurr1 mediate a gene regulatory pathway for habenula development. J. Neurosci. 39, 14309–14322 (2019).
47. Yamaguchi, T., Danjo, T., Pastan, I., Hikida, T. & Nakanishi, S. Distinct roles of segregated transmission of the septo-habenular pathway in anxiety and fear. Neuron 78, 537–544 (2013).
48. Barker, D. J. et al. Lateral preoptic control of the lateral habenula through convergent glutamate and GABA transmission. Cell Rep. 21, 1757–1769 (2017).
49. Risold, P. Y., Canteras, N. S. & Swanson, L. W. Organization of projections from the anterior hypothalamic nucleus: a Phaseolus vulgaris-leucoagglutinin study in the rat. J. Comp. Neurool. 348, 1–40 (1994).
50. Anderson, C. H. & Shen, C. L. Efferents of the medial preoptic area in the guinea pig: an autoradiographic study. Brain Res. Bull. 5, 257–263 (1980).
51. Rossi, M. A. et al. Transcriptional and functional divergence in lateral hypothalamic glutamate neurons projecting to the lateral habenula and ventral tegmental area. Neuron 109, 3823-3837.e6 (2021).
52. Poller, W. C., Madai, V. I., Bernard, R., Laube, G. & Veh, R. W. A glutamatergic projection from the lateral hypothalamus targets VTA-projecting neurons in the lateral habenula of the rat. Brain Res. 1507, 45–60 (2013).
53. Kowski, A. B., Veh, R. W. & Weiss, T. Dopaminergic activation excites rat lateral habenular neurons in vivo. Neuroscience 161, 1154–1165 (2009).
54. Aizawa, H. & Zhu, M. Toward an understanding of the habenula's various roles in human depression. Psychiatry Clin. Neurosci. 73, 607–612 (2019).

Acknowledgements
We thank Pablo Ferao Egea for photography and figure design advice as well as Luis Puelles for critical reading of the manuscript.

Author contributions
All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Conceived and designed the experiments: I.J.L., S.M., D.E. and E.P.; Performed the experiments: I.J.L., E.C.R. and F.A.G.; Analyzed the data: I.J.L., E.C.R., D.E. and E.P.; Wrote the article: I.J.L. and E.P.; Obtained funding: S.M., E.P. and D.E.

Funding
Work supported by MINECO/AEI/FEDER (BFU2013-48230) to E. Puelles and D. Echevarría; MINECO/AEI/ FEDER (SAF2017-83702-R; PID2020-118171RB-I00), GVA (PROMETEO/2018/041), ISCIII ("RD16/001/0010"), co-funded by ERDF/ESF, "Investing in your future", and FTPGB (FTPGB18/SM) to S. Martinez. The Institute of Neurosciences is a "Centre of Excellence Severo Ochoa (SEV-2017-0723)".

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-14328-1.

Correspondence and requests for materials should be addressed to E.P.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022