Functional Principles of Posterior Septal Inputs to the Medial Habenula

Authors
Yo Otsu, Salvatore Lecca, Katarzyna Pietrajtis, ..., Caroline Mailhes-Hamon, Manuel Mameli, Marco Alberto Diana

Correspondence
marco.diana@upmc.fr

In Brief
The medial habenula (MHb) contributes to aversive state expression by linking forebrain areas to midbrain monoaminergic centers. Here, Otsu et al. provide a comprehensive characterization, from synaptic physiology to behavioral outcomes, of its glutamatergic afferents from the posterior septum, suggesting that septal stimulation in the MHb promotes locomotion and anxiolysis.

Highlights
- Medial habenular (MHb) neurons receive sparse inputs from the posterior septum (PS)
- PS afferents to the MHb function in a purely glutamatergic mode
- Excitatory ionotropic and inhibitory metabotropic receptors convey PS inputs in the MHb
- PS activation in the MHb increases locomotion and induces anxiolysis

Otsu et al., 2018, Cell Reports 22, 693–705
January 16, 2018 © 2017 The Author(s).
https://doi.org/10.1016/j.celrep.2017.12.064
Functional Principles of Posterior Septal Inputs to the Medial Habenula

Yo Otsu,1,3 Salvatore Lecca,2,4 Katarzyna Pietrajtis,1,7 Charly Vincent Rousseau,1,5 Paı ¨kan Marcaggi,1,6 Guillaume Pierre Dugue,1 Caroline Mailhes-Hamon,1 Manuel Mameli,2,4 and Marco Alberto Diana1,7,8,*

1Institut de Biologie de l’École Normale Supérieure, INSERM U1024, CNRS UMR8197, École Normale Supérieure, PSL Research University, Paris, France
2Institut du Fer à Moulin, INSERM-UPMC UMR-S 839, Paris, France
3Present address: Institut du Fer à Moulin, INSERM-UPMC UMR-S 839, Paris, France
4Present address: Department of Fundamental Neuroscience, University of Lausanne, Lausanne, Switzerland
5Present address: Sainsbury Wellcome Centre for Neural Circuits and Behaviour, University College London, London W1T 4JG, UK
6Present address: INMED/INSERM U901, Parc scientifique de Luminy, 163 route de Luminy, Marseille 13009, France
7Present address: Sorbonne Universités, UPMC Université Paris 06, INSERM, CNRS, Neurosciences Paris Seine-Institut de Biologie Paris Seine (NPS-IBPS), 75005 Paris, France
8Lead Contact
*Correspondence: marco.diana@upmc.fr
https://doi.org/10.1016/j.celrep.2017.12.064

SUMMARY

The medial habenula (MHb) is an epithalamic hub contributing to expression and extinction of aversive states by bridging forebrain areas and midbrain monoaminergic centers. Although contradictory information exists regarding their synaptic properties, the physiology of the excitatory inputs to the MHb from the posterior septum remains elusive. Here, combining optogenetics-based mapping with ex vivo and in vivo physiology, we examine the synaptic properties of posterior septal afferents to the MHb and how they influence behavior. We demonstrate that MHb cells receive sparse inputs producing purely glutamatergic responses via calcium-permeable \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), heterotrimeric GluN2A-GluN2B-GluN1 N-methyl-D-aspartate (NMDA) receptors, and inhibitory group II metabotropic glutamate receptors. We describe the complex integration dynamics of these components by MHb cells. Finally, we combine ex vivo data with realistic afferent firing patterns recorded in vivo to demonstrate that efficient optogenetic septal stimulation in the MHb induces anxiolysis and promotes locomotion, contributing long-awaited evidence in favor of the importance of this septo-habenular pathway.

INTRODUCTION

The habenular complex is a phylogenetically old structure (Stephenson-Jones et al., 2012) involved in the development, maintenance, and extinction of aversive states (Hikosaka, 2010). The habenula links limbic forebrain areas with subcortical nuclei releasing modulatory neurotransmitters (Hikosaka, 2010). In particular, the neurons of its medial subdivision, the habenula (MHb), control the serotoninergic raphe nuclei via its mixed cholinergic/glutamatergic projections (Ren et al., 2011) to the interpeduncular nucleus (IPN) (Gardon et al., 2014; Herkenham and Nauta, 1979). The MHb-IPN pathway has recently been characterized in view of its possible involvement in aversive aspects of nicotine addiction and withdrawal (Hsu et al., 2013) and of fear-related aversive states (Soria-Gómez et al., 2015; Zhang et al., 2016). In contrast with this well identified downstream connectivity, very little is known about the physiology of the only upstream source of glutamatergic excitation to the MHb, the posterior septum (PS) (Herkenham and Nauta, 1977), composed of the septofimbrial (SF) and triangular nuclei (triangular septum [TS]) impinging on the ventral subdivision of the MHb (vMHb), and of the bed nucleus of the anterior commissure contacting the dorsal MHb (dMHb) (Qin and Luo, 2009). Synaptically, early studies identified mixed purinergic and glutamatergic excitatory synaptic inputs to the MHb from the PS in the rat (Edwards et al., 1992), characterized by the complete absence of functional N-methyl-D-aspartate (NMDA) receptors and by the calcium impermeability of the \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors mediating the glutamatergic component (Robertson and Edwards, 1998). These data could not be confirmed (Hamann and Attwell, 1996) and have not been re-examined so far.

Behaviorally, irreversible deletion of these inputs has been found to modify mobility, anxiety, and fear responses in mice (Yamaguchi et al., 2013), indicating their contribution to locomotion and emotional states. However, no information is available concerning the behavioral outcome of real-time modulations of the activity of PS projections.

By combining a large spectrum of techniques ranging from immunohistochemistry to two-photon calcium imaging, optogenetics-based mapping, and ex vivo and in vivo physiology, we provide here a comprehensive analysis of septo-habenular synaptic properties and identify some of its physiological functions.
Inspired by our recordings of the in vivo firing patterns of MHb-projecting PS neurons, we demonstrate that MHb cells are contacted by sparse fibers that operate exclusively in a glutamatergic mode via calcium-permeable AMPARs, heterotrimeric NMDARs, and inhibitory group II metabotropic glutamate receptors (mGluRIIs). Moreover, we describe the heterogeneity of MHb cell responses to optogenetic stimulation of PS fibers, examining the subtle interactions between ionotropic and metabotropic components in determining the temporal profile of firing changes. Finally, we devise a physiologically relevant optogenetic protocol to demonstrate that activation of the septo-habenular projections increases mouse mobility and produces anxiolysis.

We performed single-unit recordings of the activity of MHb-projecting neurons in the PS of anesthetized mice (Figure 1). Cells were identified using high-frequency collision methods following antidromic stimulation (mean latency from stimulation, 6.2 ± 0.5 ms; n = 12 cells from 3 mice; Figure 1C) via a bipolar electrode placed in the MHb (Figure 1A; Giangetas et al., 2013). Iontophoretic application of pontamine sky blue allowed us to verify post hoc the position of recording sites (Figure 1A).

We examined several distinct parameters of neuronal activity (Figures 1D and 1F). Overall, the firing patterns of PS cells were irregular (Figures 1D and 1E), as illustrated by the coefficient of variation (CV) of the inter-spike interval (ISI; 138.5% ± 7.0%, n = 12). Moreover, most cells showed recurrent bursting.

RESULTS

In Vivo Firing Pattern of MHb-Projecting Neurons in the PS

Our research aimed to provide a comprehensive portfolio of information concerning the synaptic physiology and behavioral role of PS inputs to the MHb. No information was available concerning neuronal activity in the PS before this study. A central initial step of our investigation thus consisted of the identification of in vivo PS firing patterns.
events lasting, on average, 16.2 ± 1.6 ms. In the recorded cells, 54.4% ± 8.9% of the total number of action potentials belonged to a burst, with the number of intra-burst spikes amounting to 2.9 ± 0.1 and occurring at an average intra-burst frequency of 224.8 ± 18.0 Hz (Figure 1F). Bursts per se appeared at an average frequency of 0.6 ± 0.1 Hz. The total firing frequency amounted to 5.0 ± 0.8 Hz.

These data provided relevant information for developing a proper ex vivo analysis of the synaptic properties of the septo-habenular pathway and for achieving consistent in vivo optogenetic activation.

**Individual MHB Neurons Receive Sparse PS Excitatory Input**

We then examined the synaptic properties of the PS afferents in coronal brain slices.

The MHB is located symmetrically on both sides of the third ventricle (Figure 2A). Neurons in the MHB display a typical morphology consisting of short, varicosity-rich dendrites originating from small oval somata (Figure 2A; Kim and Chang, 2005).

We recorded cells exclusively from the vMHB. Following pharmacological block of GABAergic responses, we stimulated incoming excitatory fibers with an extracellular pipette while voltage-clamping MHB neurons (Figure 2B). Plotting the amplitude of the induced currents as a function of stimulation intensity revealed all-or-none responses consistent with stimulation of individual fibers (Figure 2B, right; n = 7). We then complemented these observations with an optogenetic approach. VGlut2-Cre mice were injected in the PS with a double-floxed adeno-associated virus (AAV) virus expressing ChR2 (Supplemental Experimental Procedures). Based on immunohistochemical staining, VGlut2 indeed appeared as a ubiquitous presynaptic marker for MHB glutamatergic afferents (Figure 2A). These injections led to presynaptic expression of ChR2-yellow fluorescent protein (YFP) throughout the vMHB (Figure 2C, a), in the same septal afferents stimulated via the “classical” extracellular electrical method (see below for more details).

PS afferents were optogenetically stimulated. When plotted as a function of light power, the amplitude of the responses showed few step-like increases (3 to 4), probably indicating recruitment of a sparse number of afferent glutamatergic inputs. Both electrical and optogenetic responses were averaged over several repetitions (5 to 20) for each stimulation voltage and light power value, respectively. Error bars represent the SEM of the responses of a single cell to multiple trials at the stimulation intensities indicated on the x axes.

**Excitatory Inputs to MHB Neurons Are Purely Glutamatergic**

Early studies reported that PS inputs showed a mixed glutamatergic/purinergic nature without functional NMDA currents (Edwards et al., 1992; Robertson and Edwards, 1998). We re-examined this issue. We found that inward currents issued from single electrical stimulations (eEPSCs) had a conventional
AMPA/NMDA glutamatergic profile (Figure 3A). The excitatory evoked postsynaptic current (eEPSC) average amplitudes were $369.3 \pm 61.2$ pA and $141.1 \pm 21.0$ pA at recording potentials of $-60$ mV and $+50$ mV ($n = 23$), respectively, with faster decay time constants at $-60$ mV ($1.6 \pm 0.1$ ms) than at $+50$ mV ($21.6 \pm 4.4$ ms, $p < 0.001$, $n = 23$; Figure 3A, a). The $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) ($10 \mu M$) almost completely abolished transmission at the hyperpolarized potential, to $4.4\% \pm 0.9\%$ of the control ($p < 0.001$, $n = 21$), whereas it led to partial reduction of eEPSCs at the positive potential, to $72.4\% \pm 4.4\%$ of the control ($p < 0.001$, $n = 23$). Co-application of the NMDAR antagonist (2R)-amino-5-phosphono pentanoate (APV) ($50–100 \mu M$) completely inhibited the remaining responses at both voltages, to $13.9\% \pm 3.0\%$ of the control ($p < 0.001$, $n = 23$; Figure 3B, b; $p = 0.03$, $n = 5$). The remaining component was completely blocked by co-application of APV, to $1.6\% \pm 1.3\%$ ($p = 0.03$, $n = 5$).

In conclusion, under our recording conditions, we could find no evidence for mixed ionotropic neurotransmission at PS-MHb inputs, which appear to operate in a purely glutamatergic mode.

AMPA Currents Are Mediated by Calcium-Permeable Receptors

AMPA transmission has been reported to be mediated by calcium-impermeable receptors in the MHb (Robertson et al., 1999). Consistently, our immunohistochemical data showed expression of both GluA1 and GluA2 AMPAR subunits in the MHb (Figure 4A). The insertion of GluA2 into AMPARs leads to both calcium impermeability and linearity of the IV curves of the currents. Surprisingly, examination of the electrically evoked AMPA currents (Figure 4B) revealed that synaptic currents displayed high rectification indexes ($4.8 \pm 0.3$, $n = 23$), calculated as the ratio between AMPA EPSC amplitudes at $-60$ mV and at $+50$ mV. In comparison, perfect linearity of the current voltage (IV) curves of calcium-impermeable receptors would provide a theoretical rectification index of 1.75. Furthermore, the amplitude of AMPA EPSCs was rapidly and potently reduced, to
the NASPM effect on calcium transients and EPSC amplitudes were similar (data not shown). Calcium increases were observed in several ROIs along dendrites (control, black; following NASPM, blue). However, rise times and amplitudes were largely heterogeneous, suggesting intracellular calcium diffusion from precise entry spots, probably identifying synaptic sites. Pooled data are represented as mean ± SEM.

23.6% ± 2.2% of the control (p = 0.016, n = 7; Figure 4C) by the calcium-permeable AMPAR blocker 1-Naphthyl acetyl spermine (NASPM) (20–50 μM). We then performed two-photon calcium imaging experiments to examine the spatial distribution of the divergent permeability of AMPARs. We scanned the dendritic appendages of MHb cells loaded with Fluo-5F (500 μM), looking for calcium increases triggered by electrical stimulation of the incoming afferents. Evoked calcium transients could indeed be detected (Figure 4D; n = 9). Transients were normally limited to restricted areas of individual neurites and showed varying kinetics and amplitudes in regions of interest (ROIs) distributed along the responding processes. Application of NASPM (20–50 μM) decreased calcium signal amplitudes to 16.3% ± 2.6% of the control (Figure 4D; p = 0.016, n = 7), with a time course closely mirroring the temporal development of eEPSC inhibition. Thus, in contrast to previous studies, our data establish the activation of calcium-permeable AMPARs by PS inputs to the MHb.

**Subunit Composition of NMDARs in the MHb**

We then examined the properties of the NMDA component. Immunohistochemical staining revealed robust expression of GluN1, GluN2A, and GluN2B subunits along the dorso-ventral MHb axis (Figure 5A).

Consistently, with the *in vivo* intra-burst frequencies of PS neurons (Figure 1), we used short (10 pulses), high-frequency (200 Hz) stimulation trains to obtain the IV curves of pharmacologically isolated NMDA currents (Figure 5B). The IV curves of NMDA currents showed the typical Mg²⁺- and voltage-dependent rectification properties of NMDARs (Figure 5B; Paoletti et al., 2013), with their rectification increasing with extracellular Mg²⁺ concentrations. In Mg²⁺-free solution, and at concentrations of 100 μM and 1.5 mM, total charge values recorded at –80 mV amounted to 92.9% ± 13.3% (n = 16), 21.6% ± 5.9% (n = 12), and 4.1% ± 1.1% (n = 10, p = 0.001) of the value obtained at +50 mV (Figure 5B), respectively. The charge transferred by the train-evoked currents was completely blocked by APV, to 4.7% ± 1.3% of the control (n = 12 at +50 mV, p < 0.001).

We then analyzed the properties of individual NMDA currents in GluN2Ako mice (Figure 5C). The NMDA/AMPA current ratio was dramatically reduced in this mouse line with respect to wild-type (WT) littermates, from 17.4% ± 1.5% (n = 15) to 2.7% ± 0.4% (n = 12, p = 0.001; Figure 5C, a). This change was not associated with a significant difference in the AMPA EPSC amplitudes measured at −60 mV, which amounted to 588.6 ± 180.1 pA in the WT (n = 15) and to 756.0 ± 149.5 pA (n = 13, p = 0.3) in mutants. In contrast, the decay time course of NMDA EPSCs was dramatically slower than in the WT (Figure 5C, b), with time constants being 111.7 ± 33.9 ms in mutants (n = 7) and 22.9 ± 2.9 ms in control mice (n = 15, p < 0.001), consistent with the absence of the rapidly deactivate GluN2A subunits.

We finally tested pharmacological agents interfering specifically with GluN2A-, GluN2B-, and GluN2C/GluN2D-containing receptors: Zn²⁺ (300 nM), Ro25-6981 (1 μM), and (3-Chlorophenyl) [3,4-dihydro-6,7-dimethoxy-1-[4-methoxyphenyloxy] methyl]-2(1H)-isoquinolinyl)methane (CIQ) (20 μM), respectively (Paoletti et al., 2013).

In WT mice, Zn²⁺ and Ro25-6981 had similar inhibitory effects on train-evoked NMDA responses (Figure 5D). Charge transfer was reduced to 62.8% ± 6.7% (n = 11, p = 0.001) and 63.9% ± 6.7% (n = 13, p < 0.001) of the pre-drug period, supporting the presence of both GluN2A and GluN2B subunits (Figure 5D, graph). As expected, in GluN2Ako mice, Zn²⁺ had no effect (100.3% ± 4.2% of the control, n = 5, p = 1.0), whereas Ro25-6981 inhibited the responses to a greater extent than in...
WT mice (to 17.4% ± 3.4% of the control, n = 5, p = 0.03 with respect to pre-drug period; p = 0.003 with respect to the WT), suggesting that, in GluN2Ako mice, NMDARs are predominantly composed of GluN2B-GluN1 heterodimers (Figure 5D; Hatton and Paoletti, 2005). Finally, we examined the effect of CIQ, a drug specifically potentiating GluN2C/GluN2D-containing receptors. In our experiments, CIQ produced a small inhibition of NMDA responses to 87.6% ± 3.6% of the control (n = 9, p = 0.01; Figure 5D, graph), indicating a lack of GluN2C/GluN2D subunits in the MHb.

In conclusion, our experiments support the existence of conventional, heterotrimeric NMDARs containing GluN1-GluN2A-GluN2B in the MHb (Paoletti et al., 2013).

Ionotropic and Metabotropic Glutamate Receptors Differentially Regulate MHb Cell Firing

Both excitatory and inhibitory metabotropic receptors (mGluRs) can be activated by synaptic glutamate release, with effects on firing spanning several hundred milliseconds. We examined the possible presence of functional mGluRs in vMHb neurons by puffing agonists of group I, group II, and group III mGluRs on cells recorded in the loose cell-attached (LCA) configuration (Figure S1). Ventral MHb cells are indeed spontaneously active in slices (Görlich et al., 2013). We found that mGluRIIs are functionally expressed in virtually all cells, and that their activation potently inhibits firing via development of slow outward currents (Figure S1). In contrast, mGluRIIs and mGluRIs are either not expressed or expressed at low levels in the vMHb respectively.

Besides ionotropic excitatory receptors, the glutamate released from PS inputs might thus also activate inhibitory mGluRIIs. We therefore examined the effect on MHb neuronal activity of optogenetically stimulated PS fibers. VGluT2-Cre mice were injected in the PS with a double-floxed ChR2-expressing AAV. The optogenetically stimulated fibers were most likely the same type of afferents activated electrically (Figure 6A). Indeed, liEPCSs were purely glutamatergic. In the whole cell configuration, their amplitude was completely blocked by co-application of NBQX and APV, to 1.3% ± 0.3% (n = 28, p = 0) and 3.1% ± 1.2% (Figure 6A, right graph; n = 11, p = 0.003) of the control at −60 mV and +50 mV. Furthermore, neither the AMPA rectification index (4.8 ± 0.6, n = 22, p = 0.4) nor the NMDA/AMPA ratio (Figure 6A; 36.0% ± 4.3%, n = 18, p = 0.5) were significantly different from the values obtained with electrical stimulation.

In the LCA configuration, we examined the changes in firing frequency upon optogenetic stimulation of PS afferents. We used 5-s-long trains at 20 Hz, a train pattern allowing reliable optogenetic stimulation over the short ex vivo recording times. We pooled MHb cells into three distinct groups according to the response patterns found. The first group comprised MHb neurons in which ChR2 activation produced a dramatic increase in the firing rate during stimulation, to 774.5% ± 125.1% of the control (Figure 6B; n = 48, p < 0.001), followed by action potential inhibition in the 2 s after train end, to 30.0% ± 6.9% of the control (p < 0.001). We found that both phenomena were dependent on activation of ionotropic glutamate receptors. Co-application of NBQX and APV indeed eliminated the late inhibition of the firing rate,
which was 83.6% ± 19.0% of the pre-stimulation period after drug application (n = 11, p = 0.4). The early excitatory phase was instead replaced by a prominent inhibition, to 46.8% ± 8.8% of the control (n = 15, p = 0.01), which was mediated by mGluRII activation because it was canceled by subsequent application of the mGluRII antagonist LY341495 (1 μM; spike rate during light trains, 112.7% ± 12.9%; n = 11; p = 0.6). A subset of this cell group was also recorded in the voltage clamp (VC) configuration. Here, optogenetic stimulation invariably produced AMPA and NMDA liEPSCs, showing the presence of a direct synaptic connection (see Figure 6E and also Figure 6A for average amplitudes and pharmacological profile; n = 28). The firing pattern in response to PS fiber activation was also examined in current clamp (CC) recordings (Figure S2). The results fully confirmed the findings of LCA recordings (Figure S2), further supporting the hypothesis that the inhibition of the firing rate following excitation was due to AMPAR/NMDAR-dependent activation of intrinsic conductances (Figure S2B) and not to activation of local inhibitory interneurons (which are not present in the MHb; Figure S4). These data thus demonstrate that AMPAR and NMDAR activation can entrain MHb cells firing very efficiently, prevailing over inhibitory metabotropic components.

In a second group of neurons, optogenetic stimulation trains decreased spike rates to 49.7% ± 3.9% of the control (n = 53, p < 0.001). We found that this inhibition was due to mGluRII activation because LY341495 prevented the decrease in firing, which amounted to 107.7% ± 7.7% of the control (n = 11, p = 0.003). Interestingly, the glutamate transporter blocker DL-threo-β-Benzyloxyaspartic acid (DL-TBOA) (100 μM) potentiated the spike rate inhibition of this group of cells, with frequencies reduced to 65.6% ± 6.2% in the control
period and to 38.8% ± 8.0% during drug application (n = 11, p = 0.004; Figure S3), suggesting an extrasynaptic localization of mGluRIIs and the possibility of spillover-mediated heterosynaptic inhibition in MHb glomeruli (Kim and Chang, 2005).

Whole-cell VC analysis revealed that light pulses induced liEPSCs only in a small percentage of cells (Figure 6E; average amplitude, 8.2 ± 5.8 pA, with only 3 of the 14 neurons showing detectable liEPSCs), whereas stimulation trains consistently activated small but detectable mGluRII-dependent outward currents at a recording potential of ~45 mV (Figure S2C). Thus, in most cases, AMPARs and NMDARs were either not activated or activated only weakly by glutamate release, the dominant effect being inhibitory mGluRII activation.

We finally found MHb neurons in which optogenetic stimulation produced no effect (Figure 6D; 103.6% ± 3.0% of the control; basal firing rate range, 0.5–6.7 Hz; n = 41; p = 0.9). As expected, VC recordings revealed no detectable current (Figure 6E; liEPSC amplitude, 0.9 ± 0.8 pA; n = 19). In this case, inefficient infection of PS cells probably led to the lack of ChR2 expression in the presynaptic afferent(s).

In conclusion, we demonstrated that optogenetic activation of PS inputs produces complex bi-phasic excitatory/inhibitory responses in MHb cells. In the Discussion, we describe several diverse factors possibly concurring to shape the spatiotemporal profile of the responses.

In Vivo Optogenetic Stimulation of PS Inputs to the MHb
We then investigated the efficacy of distinct stimulation patterns of PS inputs. The fatigue observed ex vivo with continuous stimulations (Figure 2B, a) suggested that the bursting firing patterns of PS neurons recorded in vivo (Figure 1) may be imperative for maintaining synaptic efficacy over long activity periods. We first tested continuous optogenetic trains with a duration (30 s) and frequency (20 Hz) commonly used under in vivo conditions. We examined the reliability of this protocol in modifying neuronal activity. Only neurons showing excitation/inhibition cycles were examined. In both LCA (Figure 7A, a) and VC (Figure 7A, b) recordings, we found that these stimulation trains rapidly lost much of their efficacy in increasing the firing rate (Figure 7A, a, top trace).
Similarly, in VC recordings, the charge transferred in the last seconds of stimulation was 42.8% ± 11.1% of the charge transferred at train onset (n = 22, p = 0.0004; Figure 7A, b, top trace). Importantly, NQOX completely blocked all charge transfer at -60 mV (to 0.3% ± 0.2% of the control trains, n = 9, p < 0.0001; data not shown), further confirming the purely glutamatergic nature of these synapses.

Continuous optogenetic stimulation, thus, does not represent a valuable strategy for proper activation of PS inputs to the MHb. Our in vivo data on PS firing led us to apply brief light-on periods (20 Hz, 1 s) alternated with light-off periods (2 s) over a total duration of 30 s (Figure 7A). Optogenetic stimulation at 20 Hz represents an upper limit for ChR2 stimulation in trains. By using 1-s-long trains, we compensated for the fact that this value is 10-fold lower than the average intra-burst frequency (Figure 1F).

In both LCA and VC recordings, this protocol was very efficient over long stimulation periods. The firing rate at intermittent stimulation onset was smaller than for continuous trains (399.8% ± 68.9%, n = 16, p = 0.006 with respect to continuous trains; Figure 7A, a, bottom trace). Nevertheless, in the last 4 s, periodic stimulation still significantly increased the firing rates (305.2% ± 84.0%, n = 16, p = 0.003 with respect to the pre-stimulation in LCA, p = 0.006 versus the same time window of continuous trains; Figure 7A, a).

In VC recordings, the charge transferred at train end was not different from the onset and, in percentage, greater than for continuous trains (132.3% ± 13.5%, n = 22, p = 0.3 and p = 0.001 versus the first 4 stimulation seconds and versus the corresponding time window for continuous stimulation, respectively; Figure 7A, b). The intermittent protocol allowed us to examine how prolonged in vivo activation of PS inputs to the MHb may affect behavior.

Finally, we tested whether the effect of in vivo stimulation could be attributed either to the activation of ionotropic (the cell group shown in Figure 6B) or of mGluRII glutamate receptors (Figure 6C). The intermittent protocol was applied to mice that were previously injected intraperitoneally with the blood-brain barrier-permeable mGluRII antagonist LY341495 (1–4 mg/kg). We found that optogenetic stimulation still increased locomotion in mice, to 174.9% ± 23.8% of the control (n = 10, p = 0.8 versus intermittent stimulation under control conditions; Figure 7C, green bar in the graph), suggesting that the neurons responsible for this behavioral response are the ones excited by AMPAR and NMDAR activation (Figure 6B).

We then examined the effect of PS inputs on anxiety levels (Yamaguchi et al., 2013). First, no difference was detected in the percentage of time spent by ChR2-expressing mice in the center of the open field during intermittent stimulation patterns (35.7% ± 7.4% before light onset versus 35.4% ± 4.1% during stimulation, p = 0.9; data not shown). In contrast, in the elevated plus maze (EPM) test, ChR2-injected mice significantly spent more time in the open arms during stimulation with respect to preceding control periods (3.3% ± 1.5% versus 0.3% ± 0.1%, p < 0.05, n = 11; Figure 7D, a). The EPM results may be affected by the increased locomotion of the mice. We therefore also performed the marble burying (MB) test. In this test, ChR2-expressing mice buried significantly fewer marbles during light stimulation than YFP-expressing animals (9.4 ± 2.0 with n = 10 versus 16.1 ± 1.8 with n = 8, p = 0.03; Figure 7D, b), confirming the anxiolytic effect of intermittent, physiology-like stimulation of PS inputs to the MHb.

In conclusion, these results illustrate the validity of our intermittent stimulation pattern for eliciting optogenetic responses. Furthermore, they demonstrate that PS activation can control mobility and anxiety states.

**DISCUSSION**

Our work represents a comprehensive characterization, from synaptic components to behavioral outcomes, of the septo-habenular glutamatergic afferent system. These data fill a longstanding information gap. On one side, the role of the PS as a source of glutamatergic inputs to the MHb has indeed been known for decades in morphological terms (Herkenham and Nauta, 1977). In contrast, much less functional information was available before this study, certainly because of the sparse innervation of MHb cells (Figure 2) and of the laboriousness in activating these inputs in slice preparations before the advent of optogenetics.

**PS Afferents to the MHb Are Purely Glutamatergic**

Early studies dating back more than 20 years suggested that PS inputs co-released glutamate and ATP (Edwards et al., 1992; Robertson and Edwards, 1998). The glutamatergic component was reported to be mediated by calcium-impermeable AMPARs without concomitant NMDAR activation. Our data depict a strikingly contrasting portrait. We indeed provide evidence that these inputs are purely glutamatergic, with responses produced by activation of both calcium-permeable AMPARs and GluN2A-GluN2B-GluN1 heterotrimeric NMDARs (Figures 4 and 5).

**Septo-habenular Glutamatergic Inputs Stimulate Locomotion and Produce Anxiolysis**

VGluT2-Cre mice injected with either a double-floxed ChR2- or a YFP-expressing virus were implanted with an optic fiber cannula just above the third ventricle (Figure 7B).

Mouse locomotion in an open-field arena was examined before, during, and after laser stimulation trains both with the continuous and with the periodic laser-on/laser-off 20-Hz protocol used before (Figure 7C). We examined locomotion because the MHb contributes to rodent mobility (Yamaguchi et al., 2013). We found that continuous stimulation of ChR2-expressing mice did not alter locomotion (110.0% ± 17.4%, n = 11, p = 0.9 versus the pre-light control period). In contrast, intermittent stimulation led to a significant augmentation of the total distance traveled (200.0% ± 37.3%, p = 0.02 and p = 0.04 with respect to the control period and to continuous stimulation, n = 11; Figure 7C). After the stimulation protocol was terminated, mouse mobility returned to control levels (110.6% ± 12.1%, p = 0.6; Figure 7C).

Locomotion remained unaltered in mice injected with a control virus, the distance traveled amounting to 123.6% ± 15.5% (n = 8, p = 0.4; data not shown) of the pre-stimulation period for the continuous pattern and to 106.3% ± 5.5% (n = 8, p = 0.2; data not shown) for the intermittent one.
Differences exist in the experimental conditions, possibly explaining these contradictions, mainly the use of young rats (Edwards et al., 1992) versus adult mice (here) and the ambient versus physiology-like temperatures (here) at which data were collected. Moreover, the purinergic component was generally triggered by higher stimulation intensities than the glutamatergic one (Robertson and Edwards, 1998), with extracellular applied voltages that can lead to transient electroporation of cellular membranes (Hamann and Attwell, 1996).

One physiological implication of such a variety of calcium sources in MHB neurons could obviously be synaptic plasticity. Among other roles, the MHB may be a central region for mediating nicotine and opioid addiction-related aversive states (Fowler et al., 2011; Frahm et al., 2011; Gardon et al., 2014). Synaptic plasticity may thus be central for putting in place the circuitual modifications associated with repeated drug use. It will therefore be important to investigate whether PS inputs to the MHB show forms of long-term synaptic plasticity and their possible behavioral implications.

**MHB Integration Properties of PS Inputs at the Cellular and Circuital Levels**

Our data illustrate a rather complex pattern of integration properties of the sparse PS inputs by MHB cells. We have shown that activation of the ionotropic components of glutamatergic EPSCs, when present, is extremely efficient in driving MHB neurons (Figures 6 and 7). In contrast, if AMPA and NMDA components are absent, then the net stimulation effect often appears to be powerful activity inhibition. The activation of mGluRIs and the consequent decrease in firing may be explained by two configurations: either postsynaptic densities only express metabotropic receptors, or mGluRIs located at the periphery of the active zones sense glutamate diffusing from heterosynaptic sites. We are in favor of the latter hypothesis. First, blocking glutamate transporters potently increased the inhibitory effect of metabotropic activation on firing (Figure S3), suggesting a peripheral position of the receptors with respect to glutamate release sites. Furthermore, PS afferents appear to form glomerular specializations in the MHB (Kim and Chang, 2005), where dendrites from several distinct cells converge, providing an ideally "closed" environment for efficient glutamate spillover.

On a more circuitry-centered point of view, inhibition via mGluRIs may be considered as a lateral inhibition-like synaptic phenomenon. Individual PS fibers might indeed lead to strong activation of their corresponding postsynaptic targets and to inhibition of close-by, not directly contacted MHB cells, increasing the contrast of the incoming information. It is relevant to underline that, inside the classically identified ventral and dorsal MHBs, several molecular markers distinguish further sub-regions (Aizawa et al., 2012; Görlich et al., 2013; Gordon et al., 2014) and that two specific areas in the zebrafish equivalent of the mammal vMHB have been recently found to have opposite roles in social contrast resolution processes (Chou et al., 2016). The equivalence of circuitual organization between zebrafish and mouse still remains uncertain. However, this mGluRII-mediated inhibitory mechanism might contribute to refining the spatial profile of the activated habenular sub-regions, optimizing information transfer via this epithalamic relay.

**General Remarks on MHB Excitatory Inputs: Behavioral Consequences**

In this study, we illustrated an efficient optogenetic protocol for in vivo PS fiber stimulation in the MHB. Our findings suggest that the PS controls locomotion (similar to Yamaguchi et al., 2013) and induces anxiolyis. The latter result is surprising in view of the general assumption that MHB activation is coupled to enhancement of negatively valued states and of the finding that deleting PS inputs decreases anxiety (Yamaguchi et al., 2013). Importantly, though, increased MHB excitability and/or output efficiency can also contribute to relieving negative states, for example, during fear conditioning (Soria-Gómez et al., 2015; Zhang et al., 2016).

Our results may also be explained by the emerging complexity of afferent synaptic excitation to the MHB. Defying expectations, several groups have indeed reported that GABAergic afferences increase, rather than decrease, neuronal firing in the MHB (Kim and Chung, 2007; Choi et al., 2016; Koppensteiner et al., 2016). Similarly to glutamatergic fibers, GABAergic axons impinging onto MHB cells originate in septal nuclei (Qin and Luo, 2009). Morphologically, GABAergic fibers form classical small terminals on postsynaptic dendrites, externally with respect to glutamatergic glomeruli (M.A.D., unpublished data). Thus, GABAergic terminals produce clearly distinct structures in comparison with glutamatergic ones, and they may therefore efficiently convey different activity patterns onto MHB cells. It is tempting to speculate that, downstream in the raphe nuclei via the IPN relay, this heterogeneity of incoming excitation to MHB cells may produce divergent spatiotemporal patterns of serotonin release throughout the brain and, thus, distinct behavioral outcomes. It will be important in the future to understand how the excitatory role of GABAergic synapses can be reconciled with that of the glutamatergic inputs described here.

In conclusion, by establishing the physiological contour of septo-habenular glutamatergic function, we provide here long-awaited foundations for further exploration of the role of the MHB in brain physiology.

**EXPERIMENTAL PROCEDURES**

All procedures involving experimental animals were performed in accordance with Directive 2010/63/EU, the guidelines of the French Agriculture and Forestry Ministry for handling animals, and local ethics committee guidelines.

**Ex Vivo Electrophysiology**

Adult mice (≥ 3 months old) were used for this study. Coronal slices (300 μm) were prepared, and recordings were performed at 30°C–34°C as described previously (Giber et al., 2015) from both C57/B6 mice, GluN2KO mice and their control littersmates, VGluT2-Cre mice, and, finally, from Thy1-ChR2 mice (line 18; Wang et al., 2007).

All recordings were performed in a bicarbonate buffered solution (BBS) containing the following (mM): 116 NaCl, 2.5 KCl, 1.25 NaH2PO4, 26 NaHCO3, 30 glucose, 2 CaCl2, 1.5 MgCl2, and 5 x 10⁻⁵ minocycline (bubbled with 95% O2, 5% CO2). We recorded neurons located exclusively in the vMHB. For whole-cell (WC) recordings, patch pipettes (resistance, 2.5–4 megaohms [MΩ]) were filled with an intracellular solution containing 150 mM CsMeSO4, 10 mM tetraethylammonium chloride (TEA-Cl), 1 mM CaCl2, 4.6 mM MgCl2, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM K2-creatine phosphate, 0.5 mM EGTA, 4 mM Na2-ATP, 0.4 mM Na2-GTP.
Data analysis was performed with pClamp 10 (Molecular Devices), Origin 6.1 (OriginLab, Northampton, USA), with custom routines written in Igor.
Statistical Analysis

The results are presented as mean ± SEM throughout the manuscript and in all figures. For statistical analyses, Mann-Whitney U test and Wilcoxon signed-rank test were used as appropriate. Statistical significance was set at 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.12.064.

ACKNOWLEDGMENTS

The authors would like to thank P. Paoletti for providing GluN2AKO mice and Ro25-6981 and M. Galante, R. Lambert, N. Leresche, and E. Schwartz for critical and extensive commenting on the original manuscript, which was edited by all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Aizawa, H., Kobayashi, M., Tanaka, S., Fukai, T., and Okamoto, H. (2012). Molecular characterization of the subnuclei in rat habenula. J. Comp. Neurol. 520, 4051–4066.
Amaral, D.G., Scharfman, H.E., and Lavenex, P. (2007). The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). Prog. Brain Res. 163, 19–47.
Borgius, L., Restrepo, C.E., Leao, R.N., Saleh, N., and Kiehn, O. (2010). A transgenic mouse line for molecular genetic analysis of excitatory glutamatergic neurons. Mol. Cell. Neurosci. 45, 249–257.
Choi, K., Lee, Y., Lee, C., Hong, S., Lee, S., Kang, S.J., and Shin, K.S. (2016). Optogenetic activation of septal GABAergic afferents entrains neuronal firing in the medial habenula. Sci. Rep. 6, 34800.
Chou, M.Y., Aron, R., Kinoshita, M., Cheng, B.W., Shimazaki, H., Agetsuma, M., Shiraki, T., Aoki, T., Takahoko, M., Yamazaki, M., et al. (2016). Social conflict resolution regulated by two dorsal habenular subregions in zebrafish. Science 352, 87–90.
Edwards, F.A., Gibb, A.J., and Colquhoun, D. (1992). ATP receptor-mediated synaptic currents in the central nervous system. Nature 359, 144–147.
Fowler, C.D., Lu, Q., Johnson, P.M., Marks, M.J., and Kenny, P.J. (2011). Habenular x5 nicotinic receptor subunit signalling controls nicotine intake. Nature 471, 597–601.
Frahm, S., Slimak, M.A., Ferrarese, L., Santos-Torres, J., Antolin-Fontes, B., Auer, S., Filkin, S., Pons, S., Fontaine, J.-F., Tetslin, V., et al. (2011). Aversion to nicotine is regulated by the balanced activity of x4 and x5 nicotinic receptor subunits in the medial habenula. Neuron 70, 522–535.
Gardon, O., Faget, L., Chu Sin Chung, P., Matfas, A., Massotte, D., and Kieffer, B.L. (2014). Expression of mu opioid receptor in dorsal diencephalic conduction system: new insights for the medial habenula. Neuroscience 277, 595–609.
Gibber, K., Diana, M.A., Plattner, V., Duguë, G.P., Bokor, H., Rousseau, C.V., Maglóczyzky, Z., Havas, L., Hangya, B., Wildner, H., et al. (2015). A subcortical inhibitory signal for behavioral arrest in the thalamus. Nat. Neurosci. 18, 562–568.
Glangeat, C., Girard, D., Groc, L., Marsicano, G., Chaouloff, F., and Georges, F. (2013). Stress switches cannabinoid type-1 (CB1) receptor-dependent plasticity from LTD to LTP in the bed nucleus of the stria terminals. J. Neurosci. 33, 19857–19863.
Görlisch, A., Antolin-Fontes, B., Ables, J.L., Frahm, S., Slimak, M.A., Dougherty, J.D., and Ibarz-Talón, I. (2013). Reexposure to nicotine during withdrawal increases the pacemaking activity of cholinergic habenular neurons. Proc. Natl. Acad. Sci. USA 110, 17077–17082.
Hamann, M., and Attwell, D. (1996). Non-synaptic release of ATP by electrical stimulation in slices of rat hippocampus, cerebellum and habenula. Eur. J. Neurosci. 8, 1510–1515.
Hatton, C.J., and Paoletti, P. (2005). Modulation of triheteromeric NMDA receptors by N-terminal domain ligands. Neuron 46, 261–274.
Herkenham, M., and Nauta, W.J. (1977). 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, 123–146.
Herkenham, M., and Nauta, W.J. (1979). Efferent connections of the habenular nuclei in the rat. J. Comp. Neurol. 187, 19–47.
Hikosaka, O. (2010). The habenula: from stress evasion to value-based decision-making. Nat. Rev. Neurosci. 11, 503–513.
Hsu, Y.W., Tempest, L., Quina, L.A., Wei, A.D., Zeng, H., and Turner, E.E. (2013). Medial habenula output circuit mediated by x5 nicotinic receptor-expressing GABAergic neurons in the interpeduncular nucleus. J. Neurosci. 33, 18022–18035.
Kim, U., and Chang, S.Y. (2005). Dendritic morphology, local circuitry, and intrinsic electrophysiology of neurons in the rat medial and lateral habenular nuclei of the epithalamus. J. Comp. Neurol. 483, 236–250.
Kim, U., and Chung, L.Y. (2007). Dual GABAergic synaptic response of fast excitation and slow inhibition in the medial habenula of rat epithalamus. J. Neurophysiol. 98, 1323–1332.
Koppensteiner, P., Galvin, C., and Ninan, I. (2016). Development- and experience-dependent plasticity in the dorsomedial habenula. Mol. Cell Neurosci. 77, 105–112.
Otsu, Y., Marcaggi, P., Feltz, A., Isole, P., Kollo, M., Nusser, Z., Mathieu, B., Kanö, M., Tsujita, M., Sakimura, K., and Diedonné, S. (2014). Activity-dependent gating of calcium spikes by A-type K+ channels controls climbing fiber signaling in Purkinje cell dendrites. Neuron 84, 157–171.
Paoletti, P., Bellone, C., and Zhou, O. (2013). NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. Nat. Rev. Neurosci. 14, 383–400.
Qin, C., and Luo, M. (2009). Neurochemical phenotypes of the afferent and efferent projections of the mouse medial habenula. Neuroscience 167, 827–837.
Ren, J., Qin, C., Hu, F., Tan, J., Qiu, L., Zhao, S., Feng, G., and Luo, M. (2011). Habenula “cholinergic” neurons co-release glutamate and acetylcholine and activate postsynaptic neurons via distinct transmission modes. Neuron 69, 445–452.
Robertson, S.J., and Edwards, F.A. (1998). ATP and glutamate are released from separate neurones in the rat medial habenula nucleus: frequency dependence and adenosine-mediated inhibition of release. J. Physiol. 508, 691–701.

Robertson, S.J., Burnashev, N., and Edwards, F.A. (1999). Ca2+ permeability and kinetics of glutamate receptors in rat medial habenula neurones: implications for purinergic transmission in this nucleus. J. Physiol. 518, 539–549.

Soria-Gómez, E., Busquets-Garcia, A., Hu, F., Mehidi, A., Cannich, A., Roux, L., Louti, L., Alonso, L., Wiesner, T., Georges, F., et al. (2015). Habenular CB1 Receptors Control the Expression of Aversive Memories. Neuron 88, 306–313.

Stephenson-Jones, M., Floros, O., Robertson, B., and Grillner, S. (2012). Evolutionary conservation of the habenular nuclei and their circuitry controlling the dopamine and 5-hydroxytryptophan (5-HT) systems. Proc. Natl. Acad. Sci. USA 109, E164–E173.

Vergnano, A.M., Rebola, N., Savtchenko, L.P., Pinheiro, P.S., Casado, M., Kieffer, B.L., Rusakov, D.A., Mulle, C., and Paoletti, P. (2014). Zinc dynamics and action at excitatory synapses. Neuron 82, 1101–1114.

Wang, H., Peca, J., Matsužaki, M., Matsužaki, K., Noguchi, J., Qiu, L., Wang, D., Zhang, F., Boyden, E., Deisseroth, K., et al. (2007). High-speed mapping of synaptic connectivity using photostimulation in Channelrhodopsin-2 transgenic mice. Proc. Natl. Acad. Sci. USA 104, 8143–8148.

Yamaguchi, T., Danjo, T., Pastan, I., Hikida, T., and Nakanishi, S. (2013). Distinct roles of segregated transmission of the septo-habenular pathway in anxiety and fear. Neuron 78, 537–544.

Zhang, J., Tan, L., Ren, Y., Liang, J., Lin, R., Feng, Q., Zhou, J., Hu, F., Ren, J., Wei, C., et al. (2016). Presynaptic Excitation via GABAB Receptors in Habenula Cholinergic Neurons Regulates Fear Memory Expression. Cell 166, 716–728.