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# LATERAL HABENULAR NEURONS PROJECTING TO REWARD-PROCESSING MONOAMINERGIC NUCLEI EXPRESS HYPERPOLARIZATION-ACTIVATED CYCLIC NUCLEOTID-GATED CATION CHANNELS

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**Abstract—The lateral habenular complex (LHb) is a key signal integrator between limbic forebrain regions and monoaminergic hindbrain nuclei. Major projections of LHb neurons target the dopaminergic ventral tegmental area (VTA) and the serotonergic dorsal (DR) and median raphe nuclei (MnR). Both monoaminergic neurotransmitter systems play a central role in reward processing and reward-related decision-making. Glutamatergic LHb efferents terminate on GABAergic neurons in the VTA, the rostromedial tegmental nucleus (RMTg), and the raphe nuclei, thereby suppressing monoamine release when required by the present behavioral context. Recent studies suggest that the LHb exerts a strong tonic inhibition on monoamine release when no reward is to be obtained. It is yet unknown whether this inhibition is the result of a continuous external activation by other brain areas, or if it is intrinsically generated by LHb projection neurons. To analyze whether the tonic inhibition may be the result of a hyperpolarization-activated cyclic nucleotid-gated cation channel (HCN)-mediated pacemaker activity of LHb projection neurons, we combined retrograde tracing in rats with *in situ* hybridization of HCN1 to HCN4 mRNAs. In fact, close to all LHb neurons targeting VTA or raphe nuclei are equipped with HCN subunit mRNAs. While HCN1 mRNA is scarce, most neurons display strong expression of HCN2 to HCN4 mRNAs, in line with the potential formation of heteromeric channels. These results are supported by quantitative PCR and immunocytochemical analyses. Thus, our data suggest that the tonic inhibition of monoamine release is intrinsically generated in LHb projection neurons and that their activity may only be modulated by synaptic inputs to the LHb.**


**Key words: lateral habenula, ventral tegmental area, raphe, reward, rat.**

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*Abbreviations:* ABC, avidin-biotin-elite complex solution; AP, action potential; DR, serotonergic dorsal; Hb, habenula; HCN, hyperpolarization-activated cyclic nucleotid-gated cation; HCN-ir, HCN-immunoreactivities; LHb, lateral habenular complex; MnR, median raphe nuclei; PB, phosphate buffer; PBS, phosphate buffered saline; VTA, ventral tegmental area; WGA-apo-HRP-gold, gold-labeled wheat germ agglutinin coupled to horseradish peroxidase.

The lateral habenular complex (LHb) is implicated in a variety of biological functions, such as arousal, feeding, pain processing, and reward transmission (Geisler et al., 2003; Matsumoto and Hikosaka, 2007). Because of its central role in the modulation of monoamine circuitries, the LHb is thought to be involved in the etiology of major depression, bipolar disorder, and schizophrenia (Ellison, 1994; Lecourtier et al., 2004; Shepard et al., 2006; Li et al., 2011). The importance of this idea is supported by the fact that deep brain stimulation of the habenula has been successfully applied to alleviate major depression in an otherwise therapy resistant patient (Sartorius and Henn, 2007; Sartorius et al., 2010).

The LHb relays information from limbic forebrain structures (Herkenham and Nauta, 1977; Sutherland, 1982) to systems, which mainly influence emotion and motivation. These include the dopaminergic ventral tegmental area (VTA), and the serotonergic dorsal (DR) and median raphe nuclei (MnR) (Herkenham and Nauta, 1979; Sutherland, 1982; Geisler, 2003; Omelchenko, 2009; Brinschwitz, 2010). Unsuccessful behaviors activate the LHb, which in turn suppresses the activity of dopaminergic cells in the VTA (Ji and Shepard, 2007; Matsumoto and Hikosaka, 2009), via GABAergic neurons in the rostromedial tegmental nucleus (RMTg) (Omelchenko and Sesack, 2009; Brinschwitz et al., 2010; Li et al., 2011). A similar mechanism has been described for the inhibition of serotonergic cells in the raphe (Wang and Aghajanian, 1977). Thus LHb activation provokes a decreased dopamine and serotonin release in various forebrain regions (Lecourtier et al., 2006, 2008; Matsumoto and Hikosaka, 2007).

Recent studies suggest that the LHb tonically suppresses the activity of brainstem monoaminergic cells and that this inhibition may be modified when required by the ongoing situation (Kim and Chang, 2005; Matsumoto, 2009). This inhibition seems to be near-maximal, as stimulation of the LHb produced only minimal further decreases of extracellular dopamine levels (Lecourtier et al., 2008). Currently it is not known how and where this tonic inhibition is generated.

Two possible models may underlie this phenomenon. LHb neurons may be more or less continuously activated by other brain areas. Alternatively, spontaneous activity may be directly generated within the LHb and only modulated by other areas. Here we hypothesize that the tonic activity responsible for the inhibition of monoaminergic

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cells is intrinsically generated within the LHb projection neurons.

Spontaneously active cells in the LHb are known (Kim and Chang, 2005; Weiss and Veh, 2011). It is not clear, however, whether these cells are responsible for the tonic inhibition of midbrain monoaminergic neurons. In heart and nerve cells spontaneous activity is generated by a family of ion channels (Ludwig et al., 1998), the so called hyperpolarization-activated cyclic nucleotide-gated cation (HCN) channels. This family includes four members (HCN1 to HCN4) with different functional properties (Lüthi and McCormick, 1998).

In the present investigation, therefore, we have analyzed the regional and cellular localization of HCN1 to HCN4 mRNAs and proteins in the habenular complex. Our interest was focused on those LHb neurons, which project to the VTA, the DR, and the MnR. Bearing in mind that the LHb appears to be involved in the pathomechanisms of important neuropsychiatric disorders, the present work may be helpful to identify novel potential targets for pharmacotherapeutical intervention.

## EXPERIMENTAL PROCEDURES

### Animals and chemicals

In the present experiments 25 adult male Wistar rats were used. They were obtained from the department for experimental medicine (FEM) at Charité Berlin. The animals (250–300 g) were kept in group housing under standard conditions (22 °C; 12/12 h light/dark cycle; water and food *ad libitum*). All animal experiments were carried out in compliance with institutional guidelines and were approved by the Regional Berlin Animals Ethics Committee. Six animals were used for *in situ* hybridization and immunocytochemistry, seven animals were used for tracing, and 12 for PCR experiments. If not otherwise specified, all chemicals were obtained from Sigma-Aldrich, Munich, Germany.

### Tracer injection

Rats were deeply anesthetized by placing them into a plexiglas chamber ventilated with 5% isoflurane (in 100% $O_2$). After adjusting them in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA), anesthesia was continued with a 3% isoflurane/oxygen mix (flow rate of 0.6 L/min) through a rat anesthesia adapter (David Kopf Instruments). During surgery, core body temperature was maintained through a heating pad connected to a feedback looped rectal sensor. An amount of approximately 0.5 $\mu$l of the retrograde tracer, gold-labeled wheat germ agglutinin coupled to horseradish peroxidase (WGA-apo-HRP-gold, E-Y laboratories, San Mateo, CA, USA) was manually injected into the right VTA, MnR, or DR according to the atlas of Paxinos and Watson (1998) using 1.0 mm glass pipettes (Science products, Germany) pulled to an outer tip diameter of 30–40 $\mu$m.

For injections into the VTA the following coordinates were used (Paxinos and Watson, 1998): bregma, −6.5 mm; lateral extent, +0.5 mm; ventral (from dura), −7.9 mm; injection angle, 0°. Coordinates for MnR were: bregma, −7.8 mm; lateral extent, +0.8 mm; ventral, −7.8 mm; injection angle, 6°; and for DR: bregma, −7.8 mm; lateral extent, +0.9 mm; ventral, −5.8 mm; injection angle, 6°.

Micropipettes were left *in situ* for 5 min before and 15 min after injection to avoid spread of tracer along the injection tract. After surgery was completed, animals were allowed to recover from anesthesia and placed into their home cages.

### Perfusion fixation and brain processing

Three days post surgery, rats were again deeply anesthetized with isoflurane followed by i.p. injection of 45% ketamine hydrochloride (50 mg/ml; Delta Select, Germany), 17.5% xylazine hydrochloride (20 mg/ml, Bayer Vital, Germany), and 37.5% saline, at a dose of 0.32 ml/100 g body weight. Rats were transaortically perfused with Deltadex60 plasma substitute (Delta Select, Pfullingen, Germany) at 37 °C for 10 s, followed by a mixture of 4% paraformaldehyde, 0.05% glutaraldehyde, 0.2% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4) for 25 min, and finally by 0.2 M sucrose in 0.1 M PB (pH 7.4) for another 5 min. Brains were immediately removed, aligned, and cut into predefined coronal blocks. The blocks were soaked in 0.4 M sucrose for 2 h and 0.8 M sucrose overnight for cryoprotection, then shock frozen in hexane at −70 °C and stored at −80 °C until use.

### Silver enhancement and gold toning

Free floating 25 $\mu$m sections were rinsed six times in 0.1 M PB, (pH 7.4). After preincubation in 10% sodium thioglyconat in PB for 30 min and rinsing in 0.15 M sodium nitrate, sections were immersed twice for 20 min in a mixture of solutions A and B of the Intense M Silver Enhancement Kit (Amersham Biosciences, UK). Residual silver ions were removed by fixation for 10 min in 5% sodium thiosulfate in PB. Finally, sections were washed three times in PB. Thereafter sections were *in situ* hybridized with HCN riboprobes.

### Real time PCR

Microdissected habenulas from 12 adult male rats were used for PCR experiments. RNAs were isolated and purified using RNeasy Mini Preparatory Kit (Qiagen, Hilden, Germany) per manufacturer's instructions and reverse transcribed using the Sensiscript RT Kit (Qiagen). TaqMan assays (Applied Biosystems, Darmstadt, Germany) were used for quantitative PCR: HCN1: RN00584498_m1, HCN2: Rn01408575_gH, HCN3: Rn00586666_m1, HCN4: Rn00572232_m1. All assays were used in a duplex PCR reaction with either GAPDH (FG, RAT GAPD MGB) or $\beta$-actin (FG, RAT ACTB MGB) as endogenous controls. Quantitative PCR was set up in triplicates using 10 $\mu$l 2× TaqMan Universal PCR Master Mix, 1 $\mu$l 20× HCN TaqMan assay, 1 $\mu$l 20× endogenous control MGB assay, 3 $\mu$l $H_2O$, and 5 $\mu$l habenula cDNA. A standard thermal profile was used for amplification (one precycle for 10 min at 95 °C followed by 45 cycles with 15 s at 95 °C and 1 min at 60 °C). For each sample Ct values were obtained for HCN assay and endogenous control (EC) GAPDH. As a measure for relative expression ΔCt-values, defined as Ct(HCN)−Ct(EC), were calculated and corrected by a defined efficiency factor for each HCN assay according to manufacturer's instruction. Results were plotted and statistically analyzed with Prism software 4.0 (GraphPad software, La Jolla, CA, USA) using one-way analysis of variance (ANOVA) and post hoc Tukey–Kramer multiple comparison test. The following formula was used to calculate relative fold change: $2^{(Cta-Ctb)}$ in which Cta is cycle threshold average of HCN2, 3, and 4, and Ctb is cycle threshold for HCN1.

### Riboprobe generation

Partial cDNA fragments of each HCN channel (HCN1–4) were PCR amplified using Advantage Taq PCR polymerase mixture (Clontech, Hamburg, Germany), cloned into the pGEM-T vector system (Promega, Karlsruhe, Germany), and sequenced. Primer sequences are denoted in Table 1. For preparation of digoxigenin-labeled riboprobes, appropriately linearized vector DNA (>2 $\mu$g) and the DIG RNA labeling Kit (SP6/T7) (Roche Diagnostics, Mannheim, Germany) were used.