Natriuretic peptides block synaptic transmission by activating phosphodiesterase 2A and reducing presynaptic PKA activity

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The heart peptide hormone atrial natriuretic peptide (ANP) regulates blood pressure by stimulating guanylyl cyclase-A to produce cyclic guanosine monophosphate (cGMP). ANP and guanylyl cyclase-A are also expressed in many brain areas, but their physiological functions and downstream signaling pathways remain enigmatic. Here we investigated the physiological functions of ANP signaling in the neural pathway from the medial habenula (MHb) to the interpeduncular nucleus (IPN). Biochemical assays indicate that ANP increases cGMP accumulation in the IPN of mouse brain slices. Using optogenetic stimulation and electrophysiological recordings, we show that both ANP and brain natriuretic peptide profoundly block glutamate release from MHb neurons. Pharmacological applications reveal that this blockade is mediated by phosphodiesterase 2A (PDE2A) but not by cGMP-stimulated protein kinase-G or cGMP-sensitive cyclic nucleotide-gated channels. In addition, focal infusion of ANP into the IPN enhances stress-induced analgesia, and the enhancement is prevented by PDE2A inhibitors. PDE2A is richly expressed in the axonal terminals of MHb neurons, and its activation by cGMP depletes cyclic adenosine monophosphates. The inhibitory effect of ANP on glutamate release is reversed by selectively activating protein kinase A. These results demonstrate strong presynaptic inhibition by natriuretic peptides in the brain and suggest important physiological and behavioral roles of PDE2A in modulating neurotransmitter release by negative crosstalk between cGMP-signaling and cyclic adenosine monophosphate-signaling pathways.

One of the most prominent brain areas expressing GC-A is the projection from the medial habenula (MHb) to the interpeduncular nucleus (IPN) in the midbrain (Fig. 1A). This neural pathway links forebrain limbic areas with midbrain modulatory systems and regulates a diverse array of behaviors including pain, anxiety, sleep, and nicotine addiction (14–16). Abundant GC-A mRNA is detected in MHb neurons (17). In addition, strong ANP binding is observed in the IPN (12, 13), which receives dense innervation from the MHb (18, 19). Thus, GC-A is likely expressed in the axonal terminals of MHb neurons and may regulate neurotransmitter release from MHb neurons to IPN neurons.

In this study, we optogenetically activate the MHb-to-IPN pathway and examine whether ANP affects evoked transmitter release and, if so, by which signal transduction cascade (Fig. 1B). We took advantage of ChanT-ChR2-EYFP mice, which allowed us to selectively stimulate the axonal terminals of MHb neurons with light and evoke fast glutamatergic responses in IPN neurons (19). We first demonstrated that natriuretic peptides strongly suppress neurotransmitter release. We then tested whether the presynaptic inhibition is mediated by the activity of PKG, CNG channels, or PDEs. After finding that phosphodiesterase 2A (PDE2A) plays an essential role in the ANP effect on synaptic transmission, we went on to show that PDE2A activity negatively regulates the cyclic adenosine monophosphates (cAMP)-signaling pathway. The results from these experiments reveal strong effects of natriuretic peptides on neurotransmitter release and suggest important roles of presynaptic crosstalk between cGMP and cAMP signals in modulating synaptic transmission.

Results

ANP Application Blocks Glutamate Transmission. We confirmed the functional presence of ANP receptors in the IPN by assaying cGMP accumulation in mouse brain slices (Fig. S1). ELISA revealed that application of PDE inhibitors enhanced cGMP accumulation, and ANP application further increased cGMP levels approximately threefold (Fig. 1C), suggesting that ANP activates guanylyl cyclase and that PDEs are constitutively active in the IPN. We then asked how ANP could influence neurotransmitter release by performing whole-cell patch recordings from IPN neurons of ChanT-ChR2-EYFP transgenic mice (Fig. S1D). In these mice, ChannelRhodopsin 2 (ChR2) is expressed in the so-called “cholinergic” neurons in the MHb, which project their axons to the dorsal and central subnuclei of the IPN (19). Our previous recordings have shown that brief light stimulation of ChR2+ axonal terminals in the IPN produces fast glutamatergic excitatory

Synaptic transmission is dynamically modulated by neuro-peptides, which often act on receptors that belong to the G protein-coupled receptor (GPCR) family (1). In addition to GPCRs, a unique family of receptors known as membrane guanylyl cyclases (GCs) can be activated by neuropeptides such as natriuretic peptides to catalyze the intracellular production of cyclic guanosine monophosphate (cGMP) (2, 3). In animals across taxa, cGMP signals influence cellular physiology by acting on cGMP-stimulated protein kinase G (PKG), cyclic nucleotide-gated (CNG) channels, or cGMP-sensitive phosphodiesterases (PDEs) (3, 4).

In Caenorhabditis elegans, a membrane GC acts on the presynaptic terminals of olfactory neurons to induce a behavioral switch (5). Several membrane GCs and their associated peptide ligands are expressed in the mammalian brain. For example, GC-C is activated by the gut peptide hormones guanylin and uroguanylin to amplify postsynaptic responses of midbrain dopamine neurons (6). Another member of the membrane GC family, GC-A (also named NPR-A), is the receptor for atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), and its activation reduces blood pressure and volume in the cardiovascular system (7–9). Both natriuretic peptides and their receptors are expressed in several discrete brain areas (10–13), but it remains unclear how ANP affects behaviors and modulates synaptic transmission in the brain.

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postsynaptic potentials (EPSCs), whereas tetanic stimulation generates slow cholinergic responses (19).

We chose to analyze the effects of ANP application only on fast glutamatergic EPSCs with peak amplitudes over 100 pA for their stability. Because of the difficulty in obtaining stable cholinergic EPSCs with repetitive episodes of tetanic stimulation, the effect of ANP on acetylcholine release was not examined in this study. Axonal terminals were stimulated by 5-ms blue light pulses using an optical fiber with its tip directly above the IPN.

As cGMPs can also be generated by soluble GCs (sGCs) following the stimulation of nitric oxide (NO), we applied the nitric oxide synthase blocker No-Nitro-l-arginine methyl ester hydrochloride (l-NAME) to exclude the potential effects of NO/sGC signaling. The GABA_A-receptor blocker picrotoxin was also added to the bath solution to eliminate GABAergic currents. Consistent with the glutamatergic nature of the fast EPSCs (19), brief light stimulation evoked EPSCs that were resistant to the application of a mixture of two nAChR antagonists (hexamethonium and mecamylamine) (Fig. 1D). Strikingly, bath perfusion of ANP (100 nM) resulted in an immediate reduction in the fast EPSCs, leading to an almost complete abolishment of EPSCs in all cells tested after 10 min of ANP treatment (reduction ratio = 95%; Fig. 1D–F). ANP at a lower concentration (10 nM) produced ∼65% reduction and was partially reversible with washing (Fig. S2 A and B), suggesting a dose-dependent inhibition of EPSCs. Light-evoked EPSCs were similarly blocked by BNP (500 nM; Fig. S2 C and D), which activates GC-A with a lower affinity (20).

We examined whether ANP acted pre- or postsynaptically. To test whether the ANP effect resulted from postsynaptic actions on IPN neurons, we measured excitatory currents evoked by puffing the glutamate receptor agonist AMPA before and after ANP application in the presence of the sodium channel blocker tetrodotoxin (TTX). ANP did not change the AMPA currents (Fig. S2 E and F). Cell-attached recordings from IPN neurons revealed that ANP did not affect action potential firing frequency in response to AMPA application (Fig. S2 G and H). In addition, ANP application did not affect the amplitude or frequency of spontaneous EPSCs recorded in IPN neurons, which likely receive glutamatergic inputs from sources in addition to the MHB (Fig. S2 I–L). In rat pituitary and vasopressin neurons, natriuretic peptides can affect neuronal intrinsic properties by opening potassium channels (21, 22). We directly recorded from MHB neurons to examine whether ANP could target the soma of MHB neurons and affect their intrinsic excitability. Pressure injection of ANP did not elicit any currents, and bath perfusion did not affect the intrinsic properties or synaptic responses of MHB neurons (Fig. S2 M–O). These results indicate that neither inactivation of postsynaptic receptors in the IPN nor inhibition of neuronal firing at the soma of MHB neurons is responsible for the blockade of glutamate neurotransmission, suggesting that the ANP effect results from GC-A activity in presynaptic axonal terminals.

Synaptic Blockade Effect of ANP is Mediated by PDE2A. cGMP molecules can activate the cGMP-sensitive kinase PKG and cGMP channels to regulate a broad set of physiological processes (3,4). We applied membrane-permeable PKG inhibitors to investigate whether PKG activity is required for ANP to reduce glutamatergic synaptic transmission. ANP maintained its ability to block glutamate transmission following preincubation in KT5823 (2 μM), an inhibitor that interferes with the PKG catalytic domain (Fig. 2 A and B). In the presence of KT5823, ANP application reduced EPSC amplitude by ∼90% (n = 6 cells; Fig. 2C), which is comparable to the reduction amplitude without KT5823 (Fig. 1F). Similarly, ANP reduced EPSCs by over 90% following preincubation in Rp-8-pCPT-cGMP (10 μM), a PKG inhibitor acting on the enzyme regulatory domain (n = 7 cells; Fig. S3 A and B). In addition, the amplitude of evoked EPSCs was not reduced by bath perfusion of two membrane-permeable PKG activators (100 μM 8-pCPT-cGMP or 200 μM 8-BrcGMP; Fig. S3 C–F). The application of PKG activators to prevent the neuromodulatory effect blockade by ANP and the inability of two PKG activators to mimic ANP suggest a minimal role of PKG in mediating the presynaptic inhibition of glutamate release following GC-A activation. Furthermore, blocking the cGMP-sensitive CNG channel with l-cis-diltiazem (10 μM) did not affect the presynaptic inhibition of EPSCs by ANP (Fig. 2 D–F), suggesting that CNG channels also do not mediate the ANP effect.
In addition to PKG and CNG channels, some PDEs are stimulated by cGMP signals. For example, PDE2A is stimulated by cGMP to hydrolyze both cGMPs and cAMPs (23). Efforts in studying PDE2A functions have been facilitated by the development of selective PDE2A inhibitors, including 2-(3,4-dimethoxybenzyl)-7-[(1R)-1-{[(1R)-1-hydroxyethyl]-4-phenylbutyl]-5-methylimidazo[5,1-f][1,2,4]triazin-4(3H)-one (labeled as BAY 60–7550 for simplicity) and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (24). The fast EPSCs evoked by light stimulation were potentiated by nearly threefold from basal levels after bath application of BAY 60–7550 (1 μM) (Fig. 3 A–C). Moreover, preincubation in BAY 60–7550 largely eliminated the effect of ANP on reducing fast EPSCs. The potentiated currents were eliminated by the AMPA-type glutamate receptor antagonist CNQX (10 μM), demonstrating that they are glutamatergic in nature (Fig. 3B). Even in the presence of ANP, BAY 60–7550 enabled rapid reversal of EPSCs following the blockade by CNQX (Fig. 3B), further suggesting an antagonism of the ANP effect by BAY 60–7550. Similarly, light-evoked EPSCs were potentiated and their blockade by ANP was largely prevented by EHNA (Fig. 3 A and B), another commonly used PDE2A inhibitor (24). The efficacy of both PDE2A inhibitors on blocking ANP effects strongly suggests that PDE2A serves as the downstream transducer of cGMP signals produced by GC-A activation.

We asked whether ANP influences behaviors and whether PDE2A inhibitors can block the behavioral effects of ANP. Because the MHB is implicated in regulating behaviors related to pain and stress (25), we infused ANP into the IPN to examine its effects on nociceptive responses in behaving mice (Fig. S4 C). In hot-plate tests, ANP infusion alone did not change the latency of behavioral response to heat (Fig. 3D). Challenging mice with forced swimming, which could induce stress-induced analgesia (SIA), indeed increased the latency of the response to heat (Fig. 3D). This stress-related pain behavioral paradigm, the hot-plate latency was further lengthened following ANP injection into the IPN. In addition, this lengthening of the response latency was blocked by infusion of BAY 60–7750 into the IPN before testing (Fig. 3D). These results thus suggest that ANP potentiates SIA by modulating the

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**Fig. 2.** Blockade of glutamate transmission by ANP does not depend on the activity of PKG or CNG channels. (A and B) ANP remains effective in blocking glutamate transmission following the pretreatment of the PKG-specific inhibitor KT5823 (2 μM). A fast glutamatergic EPSC was recorded in the presence of the nAChR blockers HMT and Mec and the PKG inhibitor KT5823 (1), and the addition of ANP remained potent in abolishing the EPSC (2). The same conventions were as in Fig. 1 D and E. (C) Summary data show that ANP remains capable of abolishing the fast EPSCs in the presence of KT5823 (**P < 0.01; paired t test; n = 6 cells). (D–F) Representative trace (D) and time course of EPSC amplitude (E) in a single cell as well as population data (F) showing that the application of L-cis-diltiazem (10 μM) does not block the inhibitory effect of ANP on EPSCs (**P < 0.01; paired t test; n = 6 cells). (C and F) Error bars indicate SEM.

**Fig. 3.** PDE2A mediates the physiological and behavioral functions of ANP in the MHB-IPN pathway. (A–C) A representative single cell (A and B) and summary data (C) illustrate that BAY 60–7550, a selective PDE2A inhibitor, potentiates fast EPSCs and largely prevents ANP from blocking them (**P < 0.01; n.s., not significant, P = 0.063; n = 10 cells). (D) Hot-plate response latency for mice injected with aCSF (“ctrl”), ANP, or a mixture of ANP and BAY 60–7550 before and after the stress of forced swimming and hot-plate tests. ANP or the mixture of ANP and BAY 60–7550 did not change hot-plate latency at basal states. Forced swimming increased hot-plate latency, suggesting SIA. ANP potentiated the stress-induced analgesia effect, and this enhancement was blocked by the addition of BAY 60–7550 (P < 0.05; between-group t test; numbers of test mice are shown below the drug name for each group). (C and D) Error bars indicate SEM.
physiological properties of the MHb-IPN pathway. In addition, the behavioral effect of ANP requires the activity of PDE2A.

An earlier study reports PDE2A expression in the MHb and IPN (26), but it was unclear whether PDE2A is specifically expressed in the axonal terminals of MHb neurons. We observed PDE2A immunoreactivity in the somata of MHb neurons (Fig. 5 A and B). In the IPN, PDE2A was expressed only in the synaptic terminals (Fig. 4 A and B and Fig. 5 C). PDE2A expression was restricted to ChR2-EYFP+ axonal terminals in the dorsal and central IPN subnuclei (Fig. 4 B and Fig. 5 C), which are targeted by the axonal projection from MHb cholinergic neurons (18, 19).

Because both cGMPs and cAMPs are the substrates of PDE2A (23), we asked whether ANP application could reduce presynaptic cAMP levels via PDE2A activity. ELISA measurements revealed that ANP significantly reduced the cAMP concentrations in the IPN to less than half of basal levels, and this reduction was prevented by blocking PDE2A activity with BAY 60–7550 pretreatment (Fig. 4 C). BAY 60–7550 alone increased cAMP levels by over fourfold, supporting the role of PDE2A in suppressing constitutive cAMP accumulation and thus transmitter release (Fig. 3). Moreover, ANP substantially reduced cAMP levels following the stimulation of adenylyl cyclase by forskolin, and this reduction was blocked by BAY 60–7550 pretreatment (Fig. 4 D).

These biochemical measurements thus demonstrate that ANP can activate PDE2A to efficiently reduce cAMP levels in the IPN.

Effect of ANP Is Likely Produced by Depleting Protein Kinase A Activity. We investigated how PDE2A shapes downstream signals to block glutamate transmission. Both the cAMP-sensitive kinase A (PKA) and the exchange protein activated by cAMP (Epac) have been implicated in modulating neurotransmitter release in various brain areas (27). By applying the membrane-permeable PKA activator 6-BNZ-cAMP, we tested whether direct PKA activation could rescue fast EPSCs following their blockade by ANP. For all cells tested (n = 6 cells), EPSCs were blocked by ANP and then fully recovered after bath application of 6-BNZ-cAMP (Fig. 5 A–C). In current-clamp mode, 6-BNZ-cAMP reversed the ANP blockade and enabled IPN neurons to be depolarized and fire action potentials in response to light stimulation (Fig. S6 A–C). In contrast to the effectiveness of the PKA activator 6-BNZ-cAMP, application of the selective Epac activator 8-CPT-2Me-cAMP failed to reverse the ANP blockade (Fig. S6 B and C)

We used PKA inhibitors to further examine the role of basal PKA activity in regulating glutamate release. EPSCs were reduced by ∼70% after bath perfusion of a mixture of PKA inhibitors (H89 and Rp-8-Br-cAMP; Fig. 5 D and E). The residual current may be explained by an insufficient ability of PKA inhibitors to fully block intracellular PKA activity after permeating the cell membrane. Additional analyses show that PKA inhibitors did not affect the frequency or amplitude of spontaneous EPSCs in IPN neurons (Fig. S6 D–F), indicating that the substantial suppression of EPSCs by PKA inhibitors results from changes in presynaptic terminals but not in postsynaptic neurons.

Discussion

Since their original discovery in 1980s (7–9, 13), natriuretic peptides and their receptors have been found to be richly expressed in several brain areas (12, 13, 17). However, their physiological functions as well as the underlying signaling mechanisms in the brain remain poorly understood. In this study, we find that ANP application in the IPN abolishes synaptic release of glutamate from habenular axonal terminals. Furthermore, we show that the ANP effects are mediated by PDE2A activity, which in turn depletes cAMP and thus eliminates basal PKA activity. Our data demonstrate a strong effect of presynaptic inhibition by natriuretic peptides and delineate a signaling pathway through which cGMP signals block neurotransmitter release by negatively coupling to cAMP pathways (Fig. S5).

ANP or BNP produces an almost complete blockade of glutamate release by MHb neurons, indicating that they are very strong presynaptic inhibitors. The inhibitory effects of natriuretic peptides appear much weaker in other areas of the nervous system. In the C-fibers from the dorsal root ganglia, BNP reduces glutamatergic EPSCs by ∼30% (28). ANP weakens EPSCs by 46% for the connection between osmoreceptor neurons in the organum vasculosum laminae terminais and the magnocellular neurosecretory cells in the supraoptic nucleus (29). In the retina, BNP functions as a postsynaptic modulator and produces a roughly 40% suppression of GABA<sub>A</sub>-receptor–mediated inhibitory currents in bipolar cells (30).

Our results differ from previous studies that highlight a pivotal role of PKG in mediating the effect of natriuretic peptides in the dorsal root ganglia and the retina (28, 30). For the connection between the MHb and IPN, PKG blockers do not disrupt the shut-off effect of ANP, and PKG activators do not change EPSCs. In contrast, ANP is completely antagonized by PDE2A blockers. Biochemical and imaging studies of culture cells have shown that PDE2A is capable of efficiently depleting cAMPs following cGMP stimulation (23, 31). Our biochemical assays show that ANP reduces cAMP levels in the IPN area of brain slices and that this reduction is fully blocked by applying PDE2A inhibitors. In addition, presynaptic inhibition of glutamate release by ANP is reversed by a PKA activator, and the ANP effect is largely...
mimicked by PKA inhibitors. These results indicate that ANP blocks neurotransmission by depleting presynaptic cAMP concentrations. Interestingly, the signal transduction cascade in the axonal terminals of MHB neurons is reminiscent of that in nonneuronal cells in the periphery. In both adrenal glomerulosa cells and cardiac myocytes, ANP activates its receptors to produce cGMPs, which in turn stimulate PDE2A to reduce intracellular cAMP levels (32–34). Therefore, negative crosstalk between cGMP and cAMP cascades might be one of the conserved mechanisms underlying natriuretic peptide action both inside and outside the nervous system. PDE2A has been thought to serve simply as a cGMP scavenger in olfactory CO2 neurons (35, 36). Our physiological recordings suggest that PDE2A performs the important function of regulating neurotransmitter release by negatively linking cGMP signals to the cAMP pathway.

The MHB–IPN pathway is believed to be related to anxiety and nicotine addiction (14, 15). Our behavioral tests indicate that ANP enhances stress-induced analgesia in a PDE2A-dependent manner. This finding is consistent with the facts that the MHB receives input from forebrain septal areas that are involved in stress-related behaviors and that MHB neurons express opiate receptors (18, 25). The exact sources of ANP or BNP in the IPN remain unclear. An early study indicated that ANP may be expressed by MHB neurons (11), suggesting the possibility of presynaptic inhibition by autocrine signaling of ANP. Alternatively, ANP might be transported to the IPN following their production in the heart. Although the exact behavioral context for ANP release into the IPN remains unclear, our results suggest that the ANP signaling can regulate animal behaviors by modulating neurotransmission in the habenulo-interpeduncular pathway. Furthermore, the powerful and selective PDE2A inhibitors have contributed to the elucidation of PDE2A functions but have not yet been put into clinical use (24, 37, 38). We propose that PDE2A inhibitors could be used as pharmacological drugs for treatment of anxiety and nicotine addiction via their actions on MHB axonal terminals.

The efficacy of a PKA activator in reversing ANP-induced presynaptic inhibition supports an essential role of PKA in transmitter release. cAMP can modulate Ca<sup>2+</sup>-dependent exocytosis of secretory cells by acting through PKA- or Epac-dependent signaling pathways (27). In cerebellar and hippocampal synapses, cAMP signals facilitate transmitter release by acting on PKA (39, 40). At the calyx synapse of Held, the potentiotiary effect of cAMP requires the activity of Epac but not PKA (41). In the axonal terminals of MHB neurons, the ANP effect is reversed by a PKA activator but not by an Epac activator, suggesting that certain proteins critical for exocytosis are phosphorylated by endogenous PKA activity in the basal state and that such phosphorylation is required for neurotransmitter release. In addition, the fact that PDE2A inhibitors potentiate glutamatergic EPSCs indicates that increasing cAMP levels may further enhance PKA activity and facilitate vesicle release. In the cortex and many other brain areas, neurotransmitter release is often presynaptically modulated by the activity of GPCRs that influence intracellular cAMP levels (1). The striking inhibitory effect of natriuretic peptides illustrates the power of cGMP signals in shutting off neurotransmitter release by depleting presynaptic cAMP levels through cGMP-stimulated dual substrate PDEs such as PDE2A.

**Materials and Methods**

**Mice.** Experimental protocols were approved by the Animal Care and Use Committee of the National Institute of Biological Sciences, Beijing, and conformed to governmental policies. We used adult CHAT-ChR2-EYFP mice (8–12 wk, 18–25 g) of either physiological recordings of 8-Br-cGMP (500 nM; Sigma), 8-Br-cGMP (50 μM; Sigma), B-NP-CAMP (50 μM; Sigma), 8-Br-cGMP (50 μM; Cayman Chemical), BNP (500 nM; Sigma), 6-BNZ-CAMP (50 μM; Sigma), 8-Br-cGMP (50 μM; Cayman Chemical) were added into the recording solution. Generation of light pulses (5 ms) was digitally controlled with Digidata 1440 (Molecular Devices).
At least 5 min of baseline was collected from each cell.

**Measurement of cGMP and cAMP Levels.** Brain slices (250 μm thick) containing the IPN were prepared from ChAT-ChR2-EYFP mice and recovered in oxy-acetate for 40 min at 34 °C. The tissues were then incubated with the following drugs for 20 min: ANP (100 nM), BAY60-7550 (10 μM), forskolin (25 μM), and 3-isobutyl-1-methylxanthine (IBMX; 1 mM). The IPN area was dissected out using the visual guidance of fluorescent microscopy and lysed with 0.1 M HCl for 5 min. Tissues were further disrupted with an ultrasonic probe for 15 min at 14,000 × g for 10 min. The concentrations of cGMP or cAMP in the supernatant were measured with ELSA kits (NewEast Biosciences Inc., catalog no. 80101 or 80202).

**Histology and Confocal Imaging.** Cells were filled with Neurobiotin (0.25%; Vector Laboratories) in the intrapipette recording solution. Brain slices after recordings were fixed with 4% (wt/vol) parafomaldehyde in 0.1 M PBS, and neurons were stained with Cy3-conjugated streptavidin (1:500; Jackson ImmunoResearch). Fluorescent images were acquired by a Zeiss LSM 710 confocal microscope.

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**Supporting Information**

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**Fig. S1.** Methods of slice preparation for biochemical assays and physiological recordings. (A) Habenulo-interpeduncular projection in a sagittal plane. Dashed lines illustrate the angle of sectioning brain slices (thickness: 250 µm). (B) In one brain slice from a ChAT-ChR2-EYFP transgenic mouse, strong green fluorescence of ChR2-EYFP clearly delineates projection from the medial habenula (MHb) to the interpeduncular nucleus (IPN) via the fiber tract of the fasciculus retroflexus (fr). (C) For biochemical measurement of cGMP or cAMP levels, brain slices were first recovered in artificial cerebrospinal fluid (aCSF) and then incubated with various drugs for 20 min. The IPN area was dissected, lysed in HCl, disrupted with ultrasound, and centrifuged. The concentrations of cGMP or cAMP in supernatant were measured with respective ELISA kits. The green fluorescent image shows the IPN dissected from the brain slice shown in B. (D) For physiological analysis, IPN neurons in brain slices were recorded using the whole-cell patch-clamp approach (red). MHb axonal terminals (green) were stimulated with blue laser light.
Fig. S2. Natriuretic peptides block glutamate neurotransmission by acting on presynaptic axonal terminals but not on postsynaptic AMPA-type glutamate receptors. (A and B) Representative traces and plot of excitatory postsynaptic potential (EPSC) amplitudes across time (A) as well as population data (B) show the effect of reversible inhibition of EPSCs by atrial natriuretic peptide (ANP) at 10 nM (**P < 0.01; ***P < 0.001; paired t test; n = 6 cells). (C and D) Data from a representative cell (C) and summary data (D) show that BNP (500 nM) abolishes EPSCs (**P < 0.01; paired t test; n = 6 cells). (E and F) Current traces of a single cell (E) and summary data (F) show that ANP does not modulate the currents elicited by direct AMPA puff. (n.s., not significant; P = 0.57; paired t test; n = 7 cells). (G and H) Traces of cell-attached recordings from a single cell (G) and population data (H) show that ANP does not reduce the intensities of AMPA-evoked action potential firing (P = 0.58; paired t test; n = 7 cells). (I–L) ANP application does not affect the amplitude and frequency of spontaneous EPSCs.

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Current traces obtained from an interpeduncular nucleus (IPN) neuron in control solution [artificial cerebrospinal fluid (aCSF)] and in the presence of ANP show that ANP did not change the intrinsic membrane property of the cell. In the presence of ANP, spontaneous EPSCs of IPN neurons show a trend of reduction in mean frequency, but the difference is not statistically significant \( (P = 0.27; \text{paired } t \text{ test; before and during ANP application; } n = 7 \text{ cells}) \). Plots of the cumulative probability distributions of spontaneous EPSC amplitudes measured from seven IPN neurons during 80-s recording segments obtained in aCSF and in the presence of 100 nM ANP. Dashed lines indicate mean + SD. In the presence of ANP, spontaneous EPSCs of IPN neurons show a trend of reduction in mean frequency, but the difference is not statistically significant \( (P = 0.27; \text{paired } t \text{ test; before and during ANP application; } n = 7 \text{ cells}) \).

Plots of the cumulative probability distributions of spontaneous EPSC amplitudes measured from seven IPN neurons during 80-s recording segments obtained in aCSF and in the presence of 100 nM ANP. Dashed lines indicate mean + SD.

Bar plot of relative median amplitude (normalized amplitude at 0.5 probability) of spontaneous EPSPs observed in aCSF and in the presence of 100 nM ANP. **\( P < 0.01; \text{paired } t \text{ test; } n = 7 \text{ cells} \).”

Recordings from the somata of MHb neurons show that ANP application does not affect the intrinsic properties or postsynaptic responses of medial habenula (MHb) neurons.

Whole-cell voltage-clamp recordings from the soma of an MHb neuron illustrating that bath perfusion of ANP does not change the intrinsic property of the cell.

Brief ANP puff does not evoke any obvious current in an MHb neuron.

ANP perfusion does not change the acetylcholine-evoked responses of an MHb neuron.

**Fig. S3.** Protein kinase G (PKG) activity does not contribute to the effect of presynaptic inhibition by atrial natriuretic peptide (ANP). (A) ANP remains effective in blocking glutamatergic excitatory postsynaptic potentials (EPSPs) following the pretreatment of Rp-8-pCPT-cGMP (10 μM), a membrane-permeable PKG inhibitor acting on the PKG regulatory domain. \( \text{Right} \) The plot of EPSC amplitudes versus time. The numbers (1, 2) indicate the time points for the averaged traces shown in the \text{Left} panel. \( \text{B} \) Summary data show that ANP remains capable of blocking fast EPSCs in the presence of Rp-8-pCPT-cGMP. **\( P < 0.01; \text{paired } t \text{ test; } n = 7 \text{ cells} \).”

Data from a single cell \( \text{(C and D)} \) and summary data \( \text{(D)} \) show that application of 8-pCPT-cGMP (100 μM), a membrane-permeable PKG activator, does not affect light-evoked EPSCs (n.s., statistically not significant; \( P = 0.78; \text{paired } t \text{ test; } n = 6 \text{ cells} \)).

Application of 8-Br-cGMP (200 μM), another membrane-permeable PKG activator, does not affect EPSCs (n.s., statistically not significant; \( P = 0.73; \text{paired } t \text{ test; } n = 5 \text{ cells} \)).
Fig. S4. Phosphodiesterase 2A (PDE2A) activity is required for the presynaptic inhibition by atrial natriuretic peptide (ANP). (A) Representative traces and time plot of excitatory postsynaptic potential (EPSC) amplitudes show that EHNA, a specific but less potent PDE2A inhibitor, potentiates EPSCs and largely prevents their blockade by ANP. (B) The summary data for the erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) effect. The potentiation of EPSCs by EHNA is much weaker than that by the BAY 60-7550, likely because of the low affinity of EHNA (*P < 0.05; paired t test; n = 7 cells). (C) A representative injection site for drug delivery into the interpeduncular nucleus. Following drug infusion and behavioral tests, Texas Red dextran amines (5%, 0.1 µL) were pressure-injected to visualize the injection site.
Fig. S5. Phosphodiesterase 2A (PDE2A) is richly expressed in the somata and axons of the medial habenula (MHB) neurons that project to the interpeduncular nucleus (IPN). (A) Confocal images of a coronal section show especially rich PDE2A expression (red) in the ventral MHB where ChR2-EYFP+ neurons (green) are located. (B) High-power view illustrates coexpression of PDE2A (red) and ChR2-EYFP (green) in the MHB. Blue, DAPI counterstaining of cell nuclei. (C) Confocal images of a coronal section through the central IPN show PDE2A expression in the axonal terminals of MHB neurons.
Fig. 56. The activity of PKA but not Epac is essential for glutamate transmission. (A) Voltage traces from an interpeduncular nucleus (IPN) neuron show that atrial natriuretic peptide (ANP) abolished light-evoked excitatory postsynaptic potentials (EPSPs) and the application of the selective PKA activator 6-BNZ-cAMP (50 μM) reversed the blockade by ANP. (B) Traces (Left) and time-series plot (Right) of a single cell show that the capability of ANP to block fast EPSCs remained intact following the application of the specific Epac activator 8-CPT-2Me-cAMP (50 μM). (C) Summary data show that ANP completely abolishes fast EPSCs and that 8-CPT-2Me-cAMP fails to reverse it (**P < 0.01; paired t-test; n = 6 cells). (D) Traces show spontaneous EPSCs of an interpeduncular nucleus (IPN) neuron before and during the application of PKA inhibitors. (E) Summary data show that the frequencies of spontaneous EPSCs were not significantly different before and after the application of PKA inhibitors (30 μM H89 and 170 μM Rp-8-Br-cAMP; P = 0.61; paired t test; before and during the application of PKA inhibitors; n = 6 cells). (F) Plots of the cumulative probability distributions of spontaneous EPSC amplitudes measured from six IPN neurons during 80-s recording segments obtained in artificial cerebrospinal fluid (aCSF) and in the presence of PKA inhibitors. Dashed lines indicate mean ± SD. (G) Bar plot of the relative median amplitude of spontaneous EPSCs observed in aCSF and in the presence of PKA inhibitors (P = 0.78; paired t test; n = 6 cells).