

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

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Edited* by Paul W. Sternberg, California Institute of Technology, Pasadena, CA, and approved September 14, 2012 (received for review June 4, 2012)

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.

presynaptic modulator | neurotransmission | ChannelRhodopsin 2

Synaptic transmission is dynamically modulated by neuropeptides, 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.

One of the most prominent brain areas expressing GC-A is the projection from the medial habenula (MHb) in the epithalamus 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 ChAT-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 ChAT-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

Author contributions: F.H. and M.L. designed research; F.H., J.R., J.-e.Z., and W.Z. performed research; F.H., W.Z., and M.L. analyzed data; and F.H. and M.L. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1209185109/-DCSupplemental.

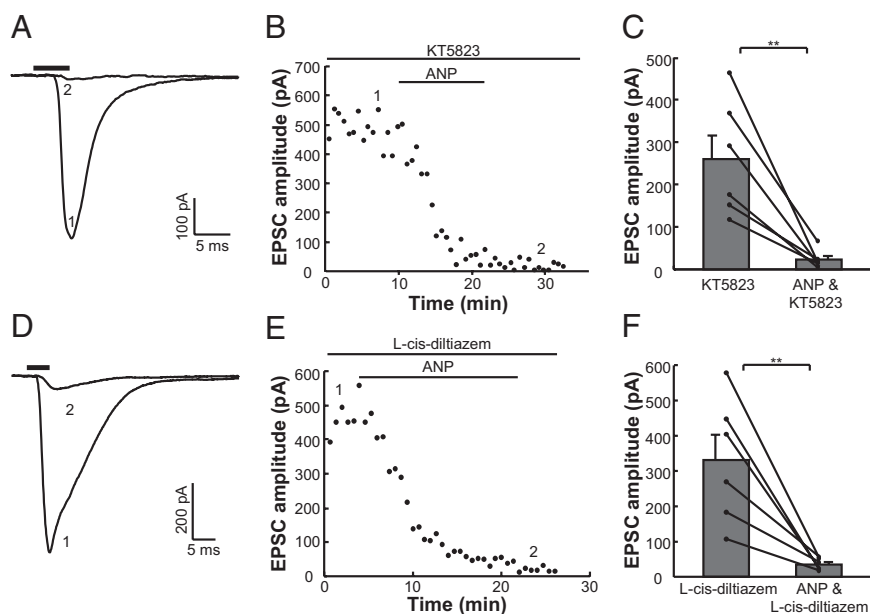


Fig. 2. Blockade of glutamate transmission by ANP does not depend on the activity of PKG or CNG channels. (A and B) ANP remains effective in blocking glutamate transmission following the pretreatment of the PKG-specific inhibitor KT5823 (2 μ M). A fast glutamatergic EPSC was recorded in the presence 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 μ M) 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.

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 several 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 μ M) (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 μ M), 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. S4 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. S4C). 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 response latency. In 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 60-7550 into the IPN before testing (Fig. 3D). These results thus suggest that ANP potentiates SIA by modulating the

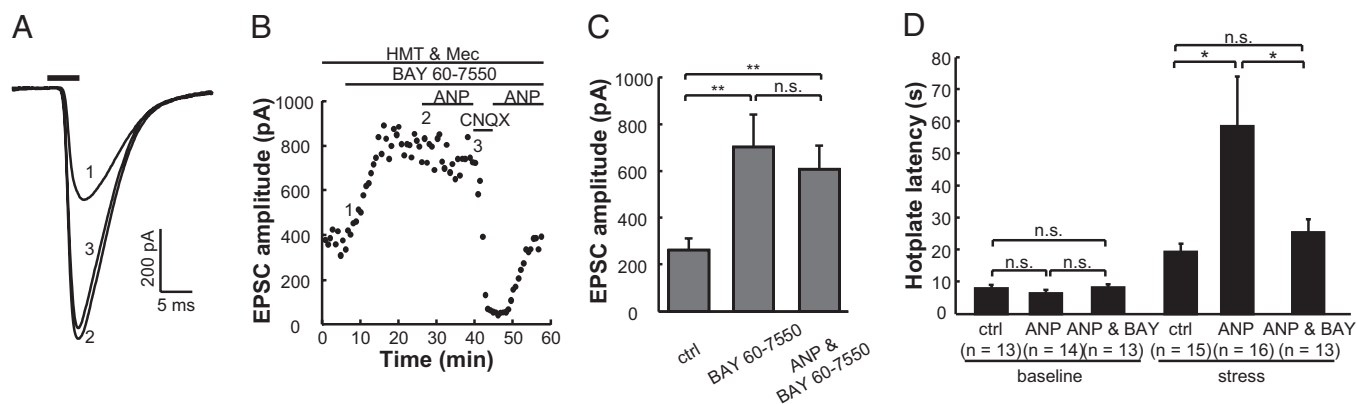


Fig. 3. PDE2A mediates the physiological and behavioral functions of ANP in the MHB-IPN pathway. (A–C) A representative single cell (A and B) and summary data (C) illustrate that BAY 60-7550, a selective PDE2A inhibitor, potentiates fast EPSCs and largely prevents ANP from blocking them (** P < 0.01; 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 \leq 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. S5 *A* and *B*). In the IPN, PDE2A was expressed only in the synaptic terminals (Fig. 4 *A* and *B* and Fig. S5 *C*). PDE2A expression was restricted to ChR2-EYFP⁺ axonal terminals in the dorsal and central IPN subnuclei (Fig. 4 *B* and Fig. S5 *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 60–7750 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*).

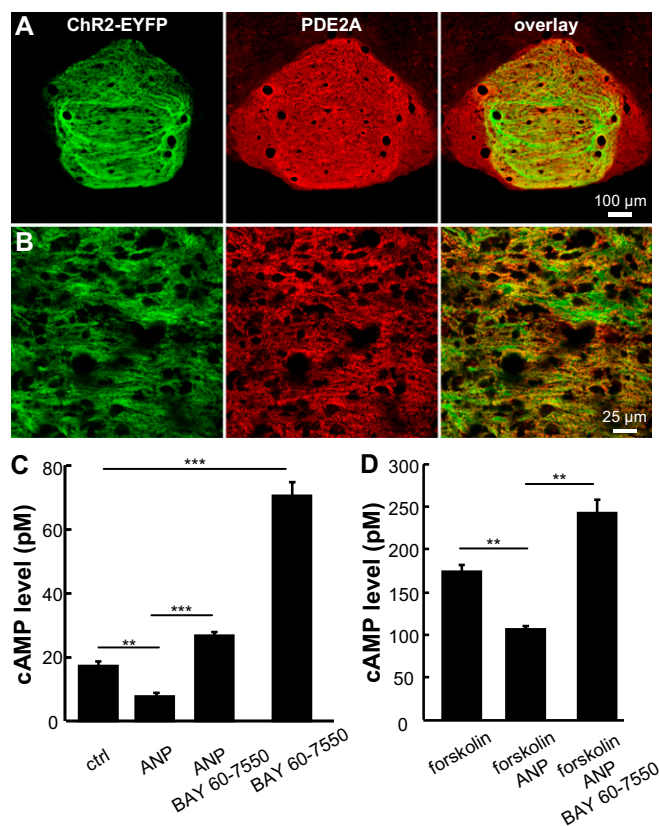


Fig. 4. PDE2A is richly expressed in the axonal terminals of MHb neurons and negatively regulates cAMP levels in the IPN. (*A*) Confocal image of a coronal section from a ChAT-ChR2-EYFP mouse showing the expression of PDE2A (red) in ChR2-EYFP⁺ terminals (green) in the IPN. (*B*) High-magnification views show that essentially all ChR2-EYFP-labeled axonal terminals (green) are immunopositive for PDE2A (red) in the IPN. (*C* and *D*) ANP substantially reduces basal (*C*) and forskolin (25 μ M)-evoked (*D*) cAMP levels in the IPN, and this reduction is reversed by blocking PDE2A with BAY 60–7750 (10 μ M). Application of the PDE2A blocker alone increases cAMP levels (** $P < 0.01$; *** $P < 0.001$; within-group *t* tests; $n = 4$ separate readings for each group). (*C* and *D*) Error bars indicate SEM.

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 protein 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*). 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 $\sim 70\%$ 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–G*), 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. 5 *F*).

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 $\sim 30\%$ (28). ANP weakens EPSCs by 46% for the connection between osmoreceptor neurons in the organum vasculosum laminae terminalis 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_A-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

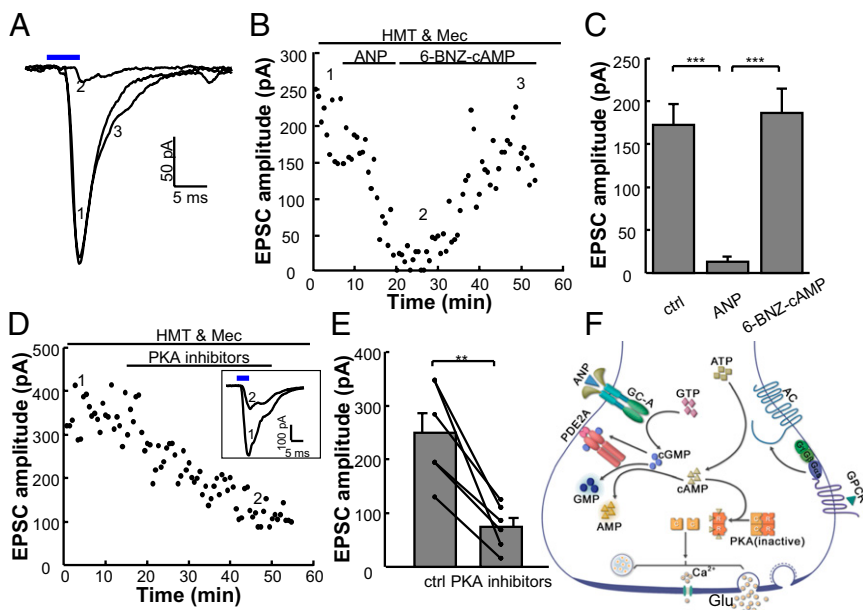


Fig. 5. The ANP/GC-A/PDE2A–signaling pathway is mediated by PKA activity. (A–C) Data from a representative cell (A and B) and group data (C) show that the ANP-induced inhibition is reversed by 6-BNZ-cAMP (50 μ M), a specific PKA activator ($***P < 0.001$; paired t test; $n = 6$ cells). (D and E) PKA inhibitors substantially reduces the amplitudes of fast EPSCs ($**P < 0.01$; $n = 6$ cells). *Inset in D* shows representative traces before and after the application of PKA inhibitors. (C and E) Error bars indicate SEM. (F) Diagram illustrating the potential signal transduction pathway mediating presynaptic inhibition by ANP. At the basal level, cAMP is constitutively produced by GPCR/adenyl cyclase (AC) pathways and activates PKA to phosphorylate certain components essential for glutamate (Glu) release. Upon ANP binding, GC-A produces cGMP and in turn activates PDE2A to hydrolyze both cGMP and cAMP. Hydrolysis of cAMP depletes PKA activity and blocks glutamate release. PDE2A metabolizes cGMP to compartmentalize and terminate the cGMP signals.

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 Ca²⁺-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 potentiatory 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 a 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 (6–12 wk, 18–25 g) of either sex for physiological recordings and C57BL/6 mice (8–12 wk, males) for behavioral tests. The ChAT-ChR2-EYFP mice were a gift of G. Feng (Massachusetts Institute of Technology, Cambridge, MA) and have been characterized in detail (19).

Slice Preparation, Electrophysiology, Photostimulation, and Drug Application.

The methods of slice preparation and whole-cell patch recordings were identical to what was described in our previous study (19). For cell-attached recordings, the pipettes were filled with 150 mM NaCl. Physiological traces were low-pass-filtered at 2.6 kHz, digitized at 10 kHz, and analyzed using a commercial recording system (MultiClamp 700B and Clampfit 10, Molecular Devices). For recording fast EPSCs, neurons were held at -60 or -65 mV. We averaged the amplitudes of five to eight sweeps for each data point. EPSCs amplitudes were measured by subtracting the mean of a 1- to 2-ms window around the peak with the mean of a 10-ms window immediately before light stimulation. Summary data are presented as mean \pm SEM. A level of $P < 0.05$ was used to designate a difference as statistically significant.

For light stimulation, blue light pulses (473 nm) were generated by a diode-pumped solid-state 473-nm laser and delivered by an optical fiber (200- μ m core diameter, N.A. = 0.22). The tip of the optical fiber was submerged in artificial cerebrospinal fluid (aCSF) and placed ~ 1.5 mm above the recording site, resulting in a light intensity of 0.2–20 mW/mm². Generation of light pulses (5 ms) was digitally controlled with Digidata 1440 (Molecular Devices).

The following drugs were added to the superfusion medium by dilution of stock solutions: ANP (10 or 100 nM; Sigma), BAY 60–7550 (1 μ M; Cayman Chemical), BNP (500 nM; Sigma), 6-BNZ-cAMP (50 μ M; Sigma), 8-Br-cGMP

(200 μM ; Sigma), 8-CPT-2Me-cAMP (50 μM ; Tocris), 8-pCPT-cGMP (100 μM ; Sigma), CNQX or DNQX (10 μM ; Sigma), *l*-*cis*-Diltiazem (10 μM ; Biomol), EHNA (90 μM ; Sigma), H89 (30 μM ; Sigma) and Rp-8-Br-cAMP (170 μM ; Biolog), KT5823 (2 μM ; Biomol), *l*-NAME (100 μM ; Sigma), picrotoxin (50 μM ; Sigma), Rp-8-pCPT-cGMP (10 μM ; Biomol), TTX (1 μM ; Sigma), and a mixture of hexamethonium-Cl (50 μM ; Sigma) and mecamlamine (5 μM ; Sigma). The effectiveness of Rp-8-pCPT-cGMP, KT5823, 8-Br-cGMP, and *l*-*cis*-Diltiazem has been confirmed by recent studies in our group (6, 35). AMPA (17.5 μM ; Sigma) and acetylcholine (1 mM; Sigma) were pressure-ejected using an eight-channel drug delivery system (MPS-1, Inbio Life Science Instrument), with the tip of the drug delivery pipette located \sim 500 μm away from the recording site. Picrotoxin and *l*-NAME was added to the recording solution to block GABA_A-receptor-mediated transmission and the effects mediated by sGCs. 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 oxygenated aCSF for 40 min at 34 $^{\circ}\text{C}$. 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 under the visual guidance of fluorescent microscopy and lysed with 0.1 mM HCl for 5 min. Tissues were further disrupted with an ultrasound probe for 10 min and centrifuged (14,000 \times g) at 4 $^{\circ}\text{C}$ for 10 min. The concentrations of cGMP or cAMP in the supernatant were measured with ELISA 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) paraformaldehyde in 0.1 M PBS, and neurons were stained with Cy3-conjugated streptavidin (1:500; 2 h) in 0.1 M PBS with 0.3% Triton-X. For PDE2A immunostaining, adult mouse

brain was fixed and cryoprotected in 30% (wt/vol) sucrose. Frozen tissue was sectioned coronally at 40 μm thickness with a microtome (Leica CM 1900). After rinsing with 0.3% Triton-X in 0.1 M PBS and blocking with 2% (wt/vol) normal bovine serum for 1 h, sections were then incubated with a rabbit anti-PDE2A antibody in the blocking solutions (1:200, 12 h in 4 $^{\circ}\text{C}$; FabGennix Inc., PD2A-101AP). After rinsing, sections were then incubated with Cy3-conjugated goat anti-rabbit antibody for 2 h at room temperature (1:500; Jackson ImmunoResearch). Fluorescent images were acquired by a Carl Zeiss 510 confocal microscope.

Behavioral Assays. Mice were anesthetized with pentobarbital (80 mg/kg i.p.), and a guide cannula was implanted with its tip 0.5 mm above the IPN. One week later, mice were first exposed to forced swimming in a glass cylinder partially filled with 22–25 $^{\circ}\text{C}$ water for 8 min. After a 2-d recovery, drug or aCSF control (1 μL) was infused into the IPN through an internal cannula for 10 min (RWVD202 Syringe Pump, RWD Life Science). Seven minutes later, mice were placed on a hot plate (Taimeng Technology), the temperature of which was set at 54.5 \pm 0.5 $^{\circ}\text{C}$. We assessed the baseline nociception response by measuring the latency to first hindpaw licking, tapping, or four-paw jumping. To test the effect of SIA the next day, all mice were injected with aCSF or drugs into the IPN and immediately immersed in cold water (4 $^{\circ}\text{C}$) for 2 min. To measure the effect of BAY 60-7550 on ANP (10 μM)-induced SIA enhancement, we first injected 0.5 μL of BAY 60-7550 (25 μM) and then a 0.5- μL mixture of ANP (20 μM) and BAY 60-7550 (25 μM). Mice were then dried with paper towels and rested for 3 min before the hot-plate test.

ACKNOWLEDGMENTS. We thank G. Feng (Massachusetts Institute of Technology) for ChAT-ChR2-EYFP mice and Z. Liu and C. Qin (National Institute of Biological Sciences) for help with behavioral assays. M.L. is supported by China Ministry of Science and Technology 973 Grant 2010CB833902.

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