Reversed synaptic effects of hypocretin and NPY mediated by excitatory GABA-dependent synaptic activity in developing MCH neurons

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In the mature brain, GABA generally acts as the primary inhibitory neurotransmitter; in contrast, during brain development, this role is reversed, and GABA can exert excitatory actions. Although there are a large number of studies examining neuropeptide modulation of GABA in the mature brain, we know almost nothing about neuropeptide modulation of GABA during development. Here, we examine the actions of hypocretin, an excitatory neuropeptide, and neuropeptide Y (NPY), an inhibitory peptide, on the actions of GABA during the development of green fluorescent protein (GFP)-labeled hypothalamic neurons that synthesize melanin-concentrating hormone (MCH). MCH neurons play a role in energy homeostasis and are active during sleep (Hasani et al. 2009; Qu et al. 1996; Shimada et al. 1998; Verret et al. 2003). In developing MCH neurons, excitatory synaptic input is primarily GABA-mediated due to the positive shift in the Cl⁻ reversal potential, whereas glutamate plays only a minor excitatory role (Li and van den Pol 2009); similar mechanisms of GABA excitation are found in developing neurons in other brain regions (Ben-Ari et al. 1989; Gulledge and Stuart 2003). In developing hypothalamic neurons, GABA synaptic activity is considered excitatory because it produces an inward current, depolarizes the membrane potential, summates with glutamate, raises cytoplasmic calcium, and evokes spikes or increases spike frequency (Chen et al. 1996; Gao and van den Pol 2001; Obrietan and van den Pol 1995).

In the hypothalamus, many or most of the neurons contain a complex array of neuropeptides in addition to the fast amino acid transmitters GABA or glutamate. The hypocretin peptide, synthesized by neurons in the lateral hypothalamus/parafornical area, is involved in enhancing cognitive arousal and its loss causes narcolepsy (Lin et al. 1999; Nishino et al. 2000). Hypocretin neurons are interspersed with MCH neurons in the lateral hypothalamus and send excitatory synaptic input to the MCH neurons (Guan et al. 2002; van den Pol et al. 2001, 2004). Both MCH and hypocretin neurons project widely throughout the brain and spinal cord, innervating >100 different sites (Bittencourt et al. 1992; Peyron et al. 1998; van den Pol 1999). A neuropeptide synthesized in the hypothalamic arcuate nucleus, NPY, plays an important orexigenic role in hypothalamic function (Schwartz et al. 2000). NPY axons terminate on or near MCH neurons (Broberger et al. 1998; Elias et al. 1998). Hypocretin and NPY exert opposing actions on mature MCH cells; hypocretin enhances activity, whereas NPY attenuates activity (van den Pol et al. 2004). Many neuropeptides and their receptors are expressed early in development, suggesting early actions of the peptides. Hypocretin is found embryonically in cell bodies and axons (van den Pol et al. 2001), and neurons express hypocretin receptors (Yamamoto et al. 2000) and respond electrically to hypocretin on the day of birth or earlier (van den Pol et al. 2001, 2002). Similarly, strong expression of NPY is found during embryonic periods of brain development (Beloosesky et al. 2006; Botchkina et al. 1995).

Having established that GABA plays an excitatory role in developing MCH cells, here we ask whether neuropeptides involved in energy homeostasis and cognitive arousal modulate excitatory GABA synaptic transmission or exert a direct effect on MCH neurons during hypothalamic development.

MATERIALS AND METHODS

A transgenic mouse that expressed GFP selectively in MCH neurons was used and is described in detail elsewhere (van den Pol et al. 2001). A transgenic mouse that expressed GFP selectively in MCH neurons was used and is described in detail elsewhere (van den Pol et al. 2001).
Hypothalamic slices of 300- to 350-μm thickness were cut on a vibratome in a cold, high-sucrose buffer that contained (in mM): 220 sucrose, 3 KCl, 6 MgCl₂, 1 CaCl₂, 1.23 NaH₂PO₄, 26 NaHCO₃, and 10 glucose. Slices were then maintained in artificial cerebrospinal fluid (ACSF) buffer for ≥1 h before recording. ACSF buffer contained (in mM): 124 NaCl, 3 KCl, 2 MgCl₂, 2 CaCl₂, 1.23 NaH₂PO₄, 26 NaHCO₃, and 10 glucose (300 mosM). The high-sucrose buffer and ACSF were continuously bubbled with 95% O₂-5% CO₂. Experiments involving mice were approved by the Yale University Institutional Animal Care and Use Committee.

Using an upright Olympus fluorescent microscope with a slice perfusion chamber, we recorded green fluorescent cells with patch pipettes with a resistance of 3–6 MΩ using a HEKA EPC 9 amplifier (HEKA Instruments, Belmore, NY); during recording, slices were maintained at 34°C. Data were analyzed with Axograph 1.3.5 (Berkeley, CA) and IGOR Pro 6.1 software (WaveMetrics, Lake Oswego, OR).

Gramicidin perforated-patch recording (Ebihara et al. 1995), which maintains normal intracellular Cl⁻, was used in some experiments, as described in detail elsewhere (Chen et al. 1996; Li and van den Pol 2009). Gramicidin was dissolved in DMSO at 50 mg/ml and frozen at −80°C. It was diluted into the perforated-patch pipette solution to a final concentration of 50 μg/ml and vortexed. The diluted gramicidin pipette solution was used within 2 h of final preparation. The perforated-patch pipette solution contained (in mM): 145 KMeSO₄, 1 MgCl₂, 10 HEPES, 1.1 EGTA, and 50 μg/ml gramicidin, pH 7.3 with KOH. In other experiments, whole cell recording was used. The pipette solution used contained (in mM): 100–105 KMeSO₄, 25–30 KC1, 1 MgCl₂, 10 HEPES, 1.1 EGTA, 2 Mg-ATP, 0.5 Na₂-GTP, and 10 Na₂-phosphocreatine, pH 7.3 with KOH (with an osmolarity of 285–290 mosM). We have already shown by gramicidin-based recordings that GABA is depolarizing to MCH neurons during the developmental period studied here (Li and van den Pol 2009). Here, we set the Cl− concentration at a level that similarly generates depolarizing actions with a Cl− reversal potential of −41 to −37 mV.

A voltage-ramp experimental protocol consisted of slow (800-ms) voltage ramps from −140 to 0 mV starting from an initial holding potential of −60 mV with K⁺ (for K⁺-channel experiments) or Cs− replacing the K⁺ (for Na⁺-Ca²⁺-exchanger experiments) in the pipette.

Ramps were done in the presence of TTX (1 μM), dl-2-amino-5-phosphonovaleric acid (AP5; 50 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), bicuculline (BIC; 30 μM), and CdCl₂ (200 μM) to block spikes, iotrospic glutamate and GABA synaptic responses, and voltage-dependent calcium channels.

AP5, BIC, CNQX, TTX, and 2-[2-[4-(4-nitrophenyl)methoxy]phenyl]methyl-4-thiazolidinecarboxylic acid ethyl ester (SN-6) were obtained from Tocris Bioscience (Ellisville, MO). NPY and hypocretin-1 were purchased from Phoenix Pharmaceuticals (Burlingame, CA) and muscimol and gramicidin from Sigma (St. Louis, MO). Drugs were applied via a large-diameter (500 μm) flow pipe aimed at the recorded cell. During periods when drugs were not being tested, ACSF was continuously applied by the flow pipe.

**RESULTS**

Developing MCH neurons that expressed GFP were studied with current- and voltage-clamp. The resting membrane potentials (RMPs) of developing MCH neurons from embryonic day 18 to postnatal day 8 (P8) ranged from −40 to −57 mV with a mean RMP of −50.3 ± 1.1 mV based on our previous report with perforated-patch recording, and the GABAₐ reversal potential ranged from −32 to −55 mV (mean −45.1 ± 1.6 mV, n = 24). The GABAₐ reversal potential (EₐGABA) was generally positive to the RMP; EₐGABA ranged from 0 to 13 mV positive to the RMP in the period up to P8 (Li and van den Pol 2009). As development proceeds, in the few days after P8, EₐGABA gradually shifts to become similar to and then negative to the RMP at which point GABA evokes primarily inhibitory actions. Here, we focus on the developmental period P3–P8 during which GABA is primarily depolarizing to MCH cells and little glutamatergic synaptic activity is seen (Li and van den Pol 2009).

**Hypocretin Excites Developing MCH Neurons.** Hypocretin, found in axons terminating on MCH neurons (van den Pol et al. 2004), was tested on developing MCH neurons with perforated-patch recording in mice. Hypocretin (1 μM) evoked a 5.7 ± 0.3 mV (n = 7) peak depolarization. We repeated this experiment with whole cell pipette recording and similarly found that hypocretin evoked a 5.3 ± 0.2 mV (n = 6) depolarization from a RMP of −51.5 ± 2.7 mV (n = 6). In total, 12 of 13 cells responded to hypocretin. When these data were pooled, hypocretin depolarized the membrane potential from the RMP with a mean peak shift of 5.5 ± 0.1 mV (n = 13; P < 0.05, t-test compared with baseline; Fig. 1, A and F). The GABAₐ receptor antagonist BIC (30 μM) reduced the hypocretin-induced depolarization to 3.6 ± 0.2 mV (n = 7; P < 0.05, t-test compared with hypocretin alone; Fig. 1, B and F), suggesting that BIC blocks part of the hypocretin-mediated depolarization. In the absence of hypocretin, BIC alone hyperpolarized the membrane potential by 1–8 mV with a mean hyperpolarization of −3.8 ± 0.2 mV (n = 7; P < 0.05, t-test compared with baseline), indicating that GABA-mediated excitatory synaptic transmission is tonically active and contributes significantly to the membrane potential. This result is consistent with the previous report that in developing MCH neurons, GABA acting at the GABAₐ receptor is depolarizing. As we found relatively little glutamate-mediated synaptic activity in developing MCH cells (Li and van den Pol 2009), it was not studied further here.

To test further whether hypocretin directly modulates developing MCH neurons, TTX (1 μM) was added to the bath to block network spike-dependent hypocretin actions. With whole cell recording from a RMP of −58.0 ± 2.5 mV, TTX reduced the mean hypocretin-induced depolarization to 2.7 ± 0.1 mV (n = 6; P < 0.05, t-test compared with hypocretin alone; Fig. 1, C and F). These data suggest that hypocretin may act by both pre- and postsynaptic mechanisms, as explored in more detail below.

**Postsynaptic Actions of Hypocretin.** To investigate the ionic mechanism underlying the direct effect of hypocretin, we studied the hypocretin-induced membrane depolarization in the presence of ion channel blockers or with ion-substitution studies using whole cell recording. All experiments described in this paragraph were done in the presence of TTX (1 μM), AP5 (50 μM), CNQX (10 μM), and BIC (30 μM). Under these conditions, hypocretin induced a depolarization of 2.5 ± 0.2 mV from a RMP of −60.1 ± 1.8 mV (n = 4; P < 0.05, t-test compared with the depolarization induced by hypocretin in control buffer; Fig. 1G). When 80% of the NaCl was replaced with choline-Cl to investigate Na⁺-dependent ionic mechanisms (Shen and North 1992), the membrane depolarization evoked by hypocretin was reduced to 1.5 ± 0.3 mV (n = 5; P < 0.05, t-test compared with the depolarization induced by hypocretin in a Na⁺ buffer; Fig. 1G). When LiCl was used to replace Na⁺, hypocretin evoked a 1.6 ± 0.2 mV depolarization (n = 5; P < 0.05, t-test compared with hypocretin in a Na⁺ buffer; Fig. 1, D and F); since LiCl can replace Na⁺ in non-selective cation channels but not in the Na-Ca exchanger (NCX; Lee and Boden 1997), these data implicate the NCX in hypocretin postsynaptic action. SN-6 (10 μM), which blocks...
the NCX (Niu et al. 2007), reduced the hypocretin-induced membrane depolarization to 1.2 ± 0.2 mV (n = 9; P < 0.05, t-test compared with the depolarization induced by hypocretin in ACSF with TTX, AP5, CNQX, and BIC; Fig. 1, E and G). These results suggest that the depolarizing action of hypocretin is mediated in part by the opening of NCXs. Voltage-ramp experiments (see MATERIALS AND METHODS) with a Cs\(^+\) -containing pipette solution showed that hypocretin produced an inward current of 18.5 ± 9.8 pA at −120 mV (n = 5) with a reversal potential of −15 ± 2.8 mV (n = 5; Fig. 1H), further supporting the view that activation of the NCX underlies part of the hypocretin-induced direct depolarization.

Since the membrane depolarization induced by hypocretin was not completely blocked by the NCX blocker SN-6, we examined the additional involvement of K\(^+\) channels. In the presence of SN-6 to block the NCX, and with a K\(^+\) -containing pipette solution, hypocretin induced a mean inward current of 6 ± 1.5 pA at −60 mV (n = 5) with a mean reversal potential of −90.1 ± 1.6 mV (n = 5; Fig. 1I and J). The hypocretin-mediated current could be blocked by the replacement of intracellular K\(^+\) ions with Cs\(^+\) ions (0.6 ± 0.4 pA at −60 mV; n = 4; Fig. 1J), indicating that K\(^+\) channel closure also contributes to the direct hypocretin-induced membrane depolarization.

Hypocretin enhances GABA excitation in early development by a presynaptic mechanism. To determine the underlying synaptic mechanism of hypocretin depolarization, we examined synaptic activity with whole cell recording and a −60-mV holding potential. In the developing hypothalamus, hypocretin (1 μM) significa-
cantly increased the frequency of spontaneous postsynaptic currents (PSCs) to $251.2 \pm 16.5\%$ ($n = 6; P < 0.05$, t-test) and increased the amplitude to $254.2 \pm 23.6\%$ ($n = 6; P < 0.05$, t-test; Fig. 2, A–C). One-minute application of hypocretin increased the mean PSC frequency from $3.6 \pm 0.7$ to $8.5 \pm 1.4$ Hz and mean PSC amplitude from $3.6 \pm 0.6$ to $9.1 \pm 1.5$ pA ($n = 6; P < 0.05$, t-test). BIC (30 $\mu$M) completely blocked all PSCs (Fig. 2, A–C), indicating that the synaptic activity was mediated by GABA release. Recovery was found after both hypocretin and BIC washout (Fig. 2, A and C). In the mature brain, in the presence of glutamate receptor antagonists AP5 (50 $\mu$M) and CNQX (10 $\mu$M), hypocretin (1 $\mu$M) increased GABA-mediated inhibitory PSC (IPSC) frequency from $4.5 \pm 0.8$ to $8.6 \pm 1.1$ Hz, a $201.2 \pm 21.6\%$ increase over controls ($n = 6; P < 0.05$, paired t-test); the IPSCs were blocked by BIC (30 $\mu$M), indicating that in neonates and adults, hypocretin enhances GABA release but that GABA exerts opposite effects in the neonates and adults.

Hypocretin can act presynaptically to increase transmitter release or postsynaptically (van den Pol et al. 1998). Acting at presynaptic receptors, hypocretin can enhance GABA release in mature neurons (van den Pol et al. 1998), thereby increasing GABA-mediated inhibition. Here, we pursued further the question of whether hypocretin can enhance GABA release presynaptically during a developmental period in which GABA is excitatory (Li and van den Pol 2009).

Hypocretin (1 $\mu$M) increased the amplitude of electrically evoked (50–100 $\mu$A, 0.2- to 0.5-ms duration, 0.1 and 0.2 Hz) evoked postsynaptic potentials from the RMP to $137 \pm 12\%$ of the control in developing MCH neurons in the presence of glutamate receptor antagonists AP5 (50 $\mu$M) and CNQX (10 $\mu$M; $n = 7; P < 0.05$, ANOVA; Fig. 3, A and B). The amplitude of the evoked excitatory postsynaptic potentials (EPSPs) returned to $112 \pm 14\%$ of control levels after hypocretin washout (Fig. 3, A and B). Almost all EPSPs were blockable by BIC (Li and van den Pol 2009). These data suggest that hypocretin does increase GABA actions and possibly release, thereby increasing the excitatory input to MCH neurons. Next, we recorded GABA-dependent, BIC-blockable miniature excitatory PSCs (mEPSCs) with a −60-mV holding potential in the presence of MCH neurons.

![Fig. 2. Hypocretin enhances GABA-mediated postsynaptic currents (PSCs) in developing MCH neurons. A: a representative trace shows how hypocretin (1 $\mu$M) increased the amplitude and frequency of PSCs with whole cell recording. BIC (30 $\mu$M) completely blocked all PSCs. The PSC block recovers with BIC washout. B: similar data as in A with expanded time and current scale showing 10 s of control baseline, hypocretin, and hypocretin + BIC. C: bar graph shows the mean effect on the frequency and amplitude of PSCs under the same conditions as A. Error bars indicate SE, and the asterisk indicates that the test group was significantly different from control ($P < 0.05; n = 6$).](http://jn.physiology.org/doi/10.1152/jn.00522.2012/fig2)

![Fig. 3. Presynaptic enhancement of GABA excitation by hypocretin in developing MCH neurons. A: a representative trace shows how hypocretin (1 $\mu$M) increased the amplitude of the electrically evoked (50–100 $\mu$A, 0.2–0.5 ms, 0.1–0.2 Hz) excitatory postsynaptic potential (eEPSP; RMP, $-49$ mV) using perforated-patch recording. The increased amplitude of eEPSP recovered after peptide washout. B: bar graph shows the mean effect on eEPSP amplitude before (Ctrl), during, and after application of hypocretin. C: traces of miniature excitatory PSCs (mEPSC; $-60$-mV holding potential) before, during, and after application of hypocretin (1 $\mu$M) in the presence of TTX (1 $\mu$M), AP5 (50 $\mu$M), and CNQX (10 $\mu$M) and with whole cell recording. Hypocretin increased mEPSC frequency. D: bar graph shows the mean effect of hypocretin on the frequency of mEPSC, as in C. E: cumulative probability of mEPSC amplitude distribution from a single MCH neuron before and during hypocretin application. F: traces show the muscimol (30 $\mu$M)-induced current before (left) and after (right) treatment with hypocretin using perforated-patch recording. Hypocretin was added 60 s before application of muscimol and hypocretin. G: bar graph shows the mean effect of hypocretin on the muscimol (30 $\mu$M)-induced current. *$P < 0.05$.](http://jn.physiology.org/doi/10.1152/jn.00522.2012/fig3)
TTX (0.5 μM) to block action potentials and AP5 (50 μM) and CNQX (10 μM) to block ionotropic glutamate receptors in neonatal mice. Hypocretin (1 μM) significantly increased the frequency of mEPSCs from 100% (control) to 178 ± 16% (n = 8; P < 0.01, ANOVA; Fig. 3, C and D). The frequency of mEPSCs recovered to 111 ± 8.7% of the control after peptide washout. The mean mEPSC amplitude was 7.2 ± 0.2 pA in control, and this increased by 22 ± 6% in the presence of hypocretin and returned to control levels after peptide washout (Fig. 3, C and E). No change in the cumulative probability distribution of mEPSC amplitude was found with a Kolmogorov-Smirnov test (P > 0.05). To investigate further whether hypocretin acts presynaptically during MCH neuron development, a paired-pulse stimulation protocol was used. A change in the paired-pulse ratio (PPR; the ratio of the 2nd EPSC amplitude over the 1st, P2/P1) can be attributable to a presynaptic change in release probability (Manabe et al. 1993; Zucker 1989). Paired-pulse EPSCs were evoked in voltage-clamp (−60-mV holding potential) by two successive stimuli of identical strength at an interval of 30 ms between stimuli (1-ms duration, 5 V, and 5-s interpulse interval). AP5 (50 μM) and CNQX (10 μM) were added to the bath to block glutamatergic currents. The EPSC PPR was significantly decreased by hypocretin (1 μM) from 1.3 ± 0.04 to 1.0 ± 0.05 (n = 6; P < 0.05, ANOVA). Together, the analysis of miniature PSCs and the PPR suggest that hypocretin modulates GABA excitation by activating hypocretin receptors in presynaptic axon terminals, ultimately enhancing GABA release onto MCH neurons.

To test further whether hypocretin modulates GABA activity postsynaptically, the GABA_A agonist muscimol (30 μM) was applied before, during, and after application of hypocretin for 5 min, respectively. TTX (0.5 μM), AP5 (50 μM), and CNQX (10 μM) were added to the bath to block spike-mediated synaptic currents and synaptic glutamatergic currents. With a −60-mV holding potential, the GABA_A agonist muscimol induced an inward current with mean amplitude of 240 ± 35 pA (n = 8) in perforated-patch recordings. The presence of hypocretin did not alter the amplitude of muscimol-evoked currents (5.2 ± 4.7%; P > 0.05; n = 8), arguing against postsynaptic modulation of GABA action (Fig. 3, F and G).

**NPY inhibits developing MCH neurons.** NPY is one of the most common neuroactive peptides in the brain and plays a number of functional roles. In the hypothalamus, NPY acts as an orexigenic peptide that increases food intake and body weight (Saper et al. 2002; Schwartz et al. 2000). NPY-immunoreactive axons are found in contact with MCH neurons (Broberger et al. 1998; Elias et al. 1998), and mature MCH cells respond to NPY (van den Pol et al. 2004).

With perforated-patch recording, NPY (1 μM) substantially hyperpolarized the membrane potential of developing MCH neurons by 7.5 ± 0.5 mV (n = 7) in normal ACSF (Fig. 4, A and D). We repeated this experiment with whole cell recording and found a similar NPY-mediated hyperpolarization of 7.8 ± 0.1 mV (n = 7) from a RMP of −49.8 ± 0.5 mV (n = 7). Combining the two experiments gave a mean hyperpolarization of 7.7 ± 0.1 mV. All neurons tested (n = 14) responded to NPY. After addition of BIC (30 μM), the NPY-induced hyperpolarization was reduced by 46% to 4.2 ± 0.3 mV from a RMP of −54.4 ± 0.5 mV (n = 7; P < 0.05, t-test; Fig. 4, B and D), indicating a contribution of synaptic GABA activity to the NPY-mediated hyperpolarization. In the presence of TTX (1 μM), the NPY-induced hyperpolarization was reduced to 5.3 ± 0.3 mV (n = 7; P < 0.05, ANOVA; Fig. 4, C and D), suggesting both a direct postsynaptic and indirect presynaptic hyperpolarizing action of NPY.

**NPY direct inhibition.** To determine the direct mechanism of NPY actions, we used whole cell recording to test the possibility that K^+ channels were involved. To explore this in immature MCH neurons, voltage-ramps were used, and the current response to NPY was examined. NPY (1 μM) caused a reversible increase in the current response to the voltage-ramp protocols (Fig. 4E). When the NPY and control components were subtracted, net NPY-induced currents were obtained (Fig. 4F). In normal extracellular K^+ (3 mM), the NPY-sensitive current reversed at −97.5 ± 4.8 mV (n = 6), consistent with an increase in K^+ conductance. When the extracellular K^+ concentration was increased from 3 to 15 mM, the NPY (1 μM)-induced current showed an inward rectification and reversed at −57.0 ± 1.2 mV (n = 5), a 40.5-mV positive shift compared with 3 mM K^+ (Fig. 4F).

We next tested the effect of NPY on input resistance by injecting a series of square-wave negative current steps and measuring the voltage response. NPY (1 μM) reduced the input resistance by 12.3 ± 2%, from 828 ± 65 to 726 ± 48 MΩ, a significant decrease (P < 0.05; n = 7, paired t-test), suggesting that NPY opened ion channels (Fig. 4, G and H). The NPY-induced current was blocked by barium (1 mM; Fig. 4F), suggesting an inwardly rectifying K^+ current as has been described in other cells responding to NPY (Fu et al. 2004; Roseberry et al. 2004; Sun et al. 2001). These results suggest that the postsynaptic effect of NPY is mediated by the opening of inwardly rectifying K^+ channels.

**NPY attenuates GABA excitation presynaptically.** NPY (1 μM) reduced the frequency and amplitude of spontaneous PSCs by 49 ± 10.8% (n = 8; P < 0.05, t-test) and 57.6 ± 6.8% (n = 8; P < 0.05, t-test), respectively. NPY reduced the mean frequency of PSCs from 3.7 ± 0.6 to 1.7 ± 0.3 Hz and the mean amplitude of PSC from 10.5 ± 2.0 to 5.7 ± 0.8 pA (n = 8; P < 0.05, t-test). BIC (30 μM) completely blocked all PSCs (Fig. 5, A–C), indicating that NPY inhibits GABA-mediated synaptic transmission in developing MCH neurons.

In experiments similar to those using hypocretin above, GABA-mediated synaptic potentials were electrically evoked. NPY (1 μM) decreased the amplitude of the PSP evoked from RMPs to 65 ± 7.1% of the control (Fig. 6, A and B; n = 6; P < 0.05, ANOVA). After washout, the amplitude of the ePSP returned to 95 ± 6.5% of the control.

Further experiments were undertaken to investigate the effect of NPY on mPSCs. NPY (1 μM) decreased the frequency of GABA-mediated mEPSCs by 50 ± 7.2% (Fig. 6, C and D; n = 6; P < 0.01, ANOVA), and the frequency of mEPSCs recovered to 87 ± 7.1% of the control after NPY washout. A modest reduction of 27 ± 5% in the mEPSC amplitude was noted, which recovered to control levels after NPY washout. No significant difference in amplitude distribution was found with a Kolmogorov-Smirnov test (P > 0.05). Next, we used PPRs to corroborate presynaptic effects similar to the hypocretin PPR experiments described above in the presence of glutamate receptor antagonists AP5 (50 μM) and CNQX (10 μM). The EPSC PPR was significantly increased by NPY (1 μM) from 1.25 ± 0.1 in normal ACSF to 1.6 ± 0.05 in the presence of NPY (n = 6; P < 0.05, ANOVA); such a PPR...
increase suggests presynaptic inhibition (Manabe et al. 1993; Zucker 1989). Together, the analysis of miniature PSCs and the PPR is consistent with a presynaptic mechanism whereby NPY reduced the excitatory actions of GABA by presynaptic attenuation of GABA release.

In experiments similar to those described above for hypocretin, muscimol (30 μM)-evoked currents were recorded. NPY (1 μM) had little effect on the amplitude of the muscimol-evoked current (Fig. 6, E and F; 112 ± 8.7% of control; P > 0.05, n = 8), suggesting NPY did not attenuate GABA actions postsynaptically.

**DISCUSSION**

Neuropeptides play a key role in hypothalamic function, and a large number of hypothalamic neurons release neuropeptides. Here, we found that the receptors for two key hypothalamic peptides, hypocretin and NPY, are functional during early stages of postnatal hypothalamic development and are expressed both pre- and postsynaptically. The postsynaptic actions of the peptides were similar in the neonate (present data) and adult (van den Pol et al. 2004). We show that neuropeptides can modulate GABA excitation presynaptically: hypocretin enhanced the release of GABA in developing MCH neurons, thereby depolarizing the cells. In contrast, NPY reduced GABA release onto developing MCH cells and thereby contributed to inhibition by attenuating the excitatory actions of GABA.

Based on electrically evoked transmitter release, analysis of mEPSCs blockable by BIC, and PPR data, hypocretin can enhance GABA release by a presynaptic mechanism during the developmental period when GABA is excitatory. NPY reduced GABA release during the same period by presynaptic mechanisms. Thus in contrast to the actions of the two peptides in the mature brain, the modulatory effect of both peptides on GABA release in the developing brain evokes the opposite resultant GABA actions. These data are consistent with the view that the two sets of neuropeptide receptors are synthesized, transported to axon terminals, and functionally coupled to second messenger pathways during the 1st wk of hypothalamic development. The role of the peptides during development could be related to energy homeostasis and cognitive arousal functions parallel to...
that in the adult (Saper et al. 2002; Schwartz et al. 2000) or could be involved in stabilization or destabilization of synaptic connections that may be use-dependent.

During synaptogenesis, an overabundance of synapses is generated (Rakic et al. 1986). During development, some synapses are maintained, and others are lost. The current view is that more active synapses are maintained, whereas less active or silent synapses may be lost as part of the normal developmental process (Hua and Smith 2004). Most of the work done in this area has focused on excitatory synapses; one function of excitation mediated by GABA during development may be to enhance synaptic stabilization (Owens and Kriegstein 2002). From this perspective, the modulatory action of an excitatory peptide such as hypocretin that enhances GABA release may thereby increase the probability of synapse retention by virtue of increasing postsynaptic excitation. In contrast, inhibitory peptides such as NPY may reduce the probability of synapse stabilization by reducing GABA excitation. Unlike GABA, which is released to a large degree at the synaptic specialization, the dense core granules that contain neuropeptides are generally found in perisynaptic sites at some distance from the actual synapse; peptides are probably not released directly at the synapse and may therefore act on not only the axon that releases them, but also the nearby axons containing the relevant receptors (van den Pol 2012). Neuropeptide modulation of the GABA-mediated rise in intracellular calcium may affect gene expression, cell survival, and neuronal differentiation (Collins et al. 1991).

Although the effect of hypocretin and NPY on GABA actions in developing MCH neurons generates the opposite effect relative to that in the adult MCH neuron, it is noteworthy that each peptide also evoked a direct effect on the developing MCH cell body. In each case, the presynaptic effect of the peptide served to enhance the postsynaptic effect of that peptide: hypocretin direct depolarizing action mediated by activation of an NCX current and attenuation of a potassium channel was enhanced by the increased GABA-mediated synaptic excitation. Similarly, NPY direct hyperpolarizing effect based on an increased conductance of an inwardly rectifying potassium current was enhanced by the reduction in GABA-mediated excitatory synaptic effects. Based on our results, it seems probable that other neuropeptides that modulate GABA release may similarly generate opposite actions in the developing and adult brain.

Together, these data indicate that neuropeptides can influence the excitatory actions of GABA and thereby are in a position to modulate use-dependent pre- and postsynaptic neuronal development.

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Fig. 5. NPY reduces GABA-mediated PSCs in developing MCH neurons. A: a trace was recorded (−60-mV holding potential) from a typical MCH cell showing that NPY reduces synaptic activity, which recovers after washout, using whole cell recording. BIC (30 μM) completely blocked synaptic activity. B: similar traces to those in A with expanded time and current scale showing 10 s of control, NPY, peptide washout, and BIC traces. C: bar graph shows the mean effect on the frequency and amplitude of PSCs as shown in the example in A. Error bars indicate SE, and the asterisk indicates that the test group was significantly different from control (P < 0.05; n = 8).

Fig. 6. Presynaptic inhibition of GABA excitation by NPY in developing MCH neurons. A: traces show that NPY (1 μM) decreased the amplitude of the evoked (50–100 μA, 0.2–0.5 ms, and 0.1–0.2 Hz) eEPSP (RMP, −50 mV) using perforated-patch recording. The decreased amplitude of the eEPSP recovered after peptide washout. B: bar graph shows the mean effect on the eEPSP amplitude before, during, and after application of NPY. C: traces of mEPSCs (−60-mV holding potential; whole cell recording) before, during, and after washout of NPY (1 μM) in the presence of TTX (1 μM). NPY decreased mEPSC frequency. D: bar graph shows the mean effect of NPY on the frequency of mEPSCs as in C. E: traces show muscimol (30 μM)-induced current before (left) and after (right) treatment with NPY using perforated-patch recording. NPY was applied continuously for 30 s before coapplication of muscimol and NPY. F: bar graph shows the mean effect of NPY on muscimol (30 μM)-induced currents. *P < 0.05.
DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Y.L., Y.X., and A.N.v.d.P. conception and design of research; Y.L. and Y.X. performed experiments; Y.L. and Y.X. analyzed data; Y.L. and Y.X. interpreted results of experiments; Y.L. and Y.X. prepared figures; Y.L., Y.X., and A.N.v.d.P. drafted manuscript; A.N.v.d.P. edited and revised manuscript; A.N.v.d.P. approved final version of manuscript.

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