

Multiple NPY Receptors Coexist in Pre- and Postsynaptic Sites: Inhibition of GABA Release in Isolated Self-Innervating SCN Neurons

Gong Chen and Anthony N. van den Pol

Section of Neurosurgery, Yale University School of Medicine, New Haven, Connecticut 06520

Although NPY has been shown to influence the action of many transmitters in the brain, modulation of GABA, the primary inhibitory transmitter, has not been detected with electrophysiology. Using whole-cell patch-clamp recording, we found that NPY has a large modulatory effect on GABAergic neurons of the suprachiasmatic nucleus (SCN) that act as the circadian clock in the mammalian brain. NPY, acting at both Y1- and Y2-like receptors, reduced the frequency of spontaneous miniature inhibitory postsynaptic currents while having little effect on the postsynaptic GABA receptors, suggesting a presynaptic mechanism of NPY action. In single self-innervating neurons, application of either Y1 or Y2 agonists to the same neuron significantly inhibited the evoked autaptic GABA release. The use of

single-neuron microcultures has allowed the demonstration that a single peptide, NPY, has two different receptors coded for by different genes in the same axon terminal. The Y1 and Y2 agonists also inhibited whole-cell calcium currents when applied to the same neuron, indicating a coexistence of Y1- and Y2-like receptors in the postsynaptic cell body. The self-innervating cell model we use here may be applicable generally for discriminating presynaptic versus postsynaptic actions of other neurotransmitters and neuromodulators and locating their subtype receptors.

Key words: NPY; GABA; suprachiasmatic nucleus; NPY receptor; Y1–Y5 receptors; presynaptic; postsynaptic; autapse; microculture

The suprachiasmatic nucleus (SCN) of the hypothalamus controls circadian rhythms in mammals (Moore and Eichler, 1972; Stephan and Zucker, 1972; Inouye and Kawamura, 1979; Ralph et al., 1990; van den Pol and Dudek, 1993). NPY is found in axons in the SCN that originate in the intergeniculate leaflet (Harrington et al., 1985; Card and Moore, 1989; Botchkina and Morin, 1995). The SCN contains one of the highest concentrations of NPY in the mammalian CNS (Allen et al., 1983; Chronwall et al., 1985; Pelletier, 1990). Most SCN neurons are GABAergic (van den Pol and Tsujimoto, 1985; Okamura et al., 1989; Moore and Speh, 1993), and GABA is found in 50% of all presynaptic axons in the SCN (Decavel and van den Pol, 1990). Many of these arise from intrinsic axons of the SCN (van den Pol, 1980). GABA may be colocalized with NPY in some SCN axon terminals (Francois-Bellan et al., 1990). NPY administration into the SCN phase shifts the circadian rhythms (Albers and Ferris, 1984; Shibata and Moore, 1993), and the GABA receptor antagonist bicuculline can block this phase shift (Huhman et al., 1995), suggesting a functional interaction between GABA and NPY in SCN. Although NPY can play an important role in SCN phase shifts (Albers and Ferris, 1984; Moore and Card, 1990; Shibata and Moore, 1993), little is known about the cellular mechanisms of NPY action in the SCN.

NPY may activate different subtypes of receptors. In the past NPY receptors generally have been divided into two cloned types, Y1 and Y2 (Wahlestedt et al., 1990; Herzog et al., 1993; Colmers

and Bleakman, 1994; Gerald et al., 1995; Nakamura et al., 1995; Rose et al., 1995). With the recent cloning of new NPY receptors, including Y4 (Bard et al., 1995) and two different Y5 receptors, one cloned from mouse (Y5m, Weinberg et al., 1996) and one cloned from rat (Y5r, Gerald et al., 1996), the pharmacology of NPY actions has become more complicated. However, in some limited respects, Y4 and Y5 receptors show different sensitivity to Y1 and Y2 agonists and can be considered to demonstrate preferential Y1- or Y2-like properties.

NPY is one of the most abundant peptides in the nervous system (Tatemoto et al., 1982; Adrian et al., 1983). Cytochemically, NPY colocalizes with neurotransmitters such as noradrenaline (Simonneaux et al., 1994), GABA (Hendry et al., 1984) and other peptides (Chronwall et al., 1984) in presynaptic nerve terminals. NPY receptors modulate neurotransmitter release (Wahlestedt et al., 1986; Colmers et al., 1987, 1991; Wiley et al., 1990; Simonneaux et al., 1994), hormone and peptide secretion (McDonald et al., 1985; Walker et al., 1988; Kalra et al., 1992; Larsen et al., 1994; Scheenen et al., 1995), whole-cell calcium currents (Bleakman et al., 1991; Wiley et al., 1993; McQuiston et al., 1996), and gene expression (Li et al., 1994; Garcia de Yébenes et al., 1995) in both peripheral and CNS.

In a previous study based on digital imaging, we found that NPY reduced GABA-mediated calcium rises in synaptically coupled developing neurons (Obrietan and van den Pol, 1996). In the present study, we investigated the mechanism and site of action of NPY modulation in SCN neurons at the single-cell level by using whole-cell patch-clamp recording to study single self-innervating (autaptic) neurons in a microculture model. Autaptic connections have been reported in the SCN on the basis of ultrastructural evidence (Guldner and Wolff, 1978) and in other brain regions such as the cortex (Lubke et al., 1996). Autaptic neuron microcultures have been found to be a useful tool in elucidating syn-

Received June 20, 1996; revised Aug. 29, 1996; accepted Sept. 5, 1996.

This work is supported by National Institutes of Health Grants NS 34887 and NS 10174, the National Science Foundation, and the Air Force Office of Scientific Research. We thank Dr. Y. Yang for technical help.

Correspondence should be addressed to Dr. Anthony van den Pol, Section of Neurosurgery, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520.

Copyright © 1996 Society for Neuroscience 0270-6474/96/167711-14\$05.00/0

aptic functions, especially in establishing the amino acid transmitter identity of living neurons (Furshpan et al., 1986; Bekkers and Stevens, 1991; Johnson, 1994). Furthermore, the same mechanisms and neurotransmitters that exist in cultured neurons also have been detected in homologous regions *in vivo* or in brain slices. Single SCN neurons in multicelled cultures express circadian rhythms of electrical activity similar to the electrical rhythms found *in vivo* (Bos and Mirmiran, 1990; Welsh et al., 1995). Even epileptiform electrical activity and long-term potentiation have been found in single isolated neurons from regions of the brain where the same phenomena are found *in situ* (Segal, 1991, 1994; Tong et al., 1996).

In the present paper we use single self-innervating neurons primarily because they provide a simple model system to study the mechanisms of action of modulators on neurotransmission in the absence of the large number of heterogeneous synaptic interactions that occur in brain slices or in multicelled cultures. This is important in light of the different NPY responses that have been reported in the SCN with extracellular recording in slices that include excitation (Mason et al., 1987), inhibition or excitation (Albers et al., 1990), or excitation followed by inhibition (Liou and Albers, 1991). We demonstrate here with single-neuron microcultures that NPY Y1- and Y2-like receptors coexist in the same SCN neuron and that both receptors mediate the inhibition of presynaptic GABA release and postsynaptic whole-cell calcium currents. The single self-innervating neuron microculture seems to be a useful model to differentiate presynaptic versus postsynaptic effects of transmitters and their modulators and to locate subtype receptors in a single neuron.

MATERIALS AND METHODS

Cell culture

Two types of SCN cultures were used in the present study. One was a multicellular culture with a high density of neurons in the culture dish that formed a substantial number of synaptic connections. The other was a microculture of single isolated neurons in microislands.

Multicellular culture. The multicellular culture of SCN neurons is similar to previously described cultures of hypothalamic neurons (Chen et al., 1995). Briefly, the brain was taken from postnatal 1-d-old Sprague Dawley rats anesthetized with Nembutal and cut coronally in 400 μ m brain slices, and the suprachiasmatic nuclei were punched out with beveled hollow needles (22 G) or dissected out by fine forceps from the brain slice containing the chiasma. The SCN tissues were then incubated for 30–40 min in an enzyme solution containing 10 U/ml papain, 0.5 mM EDTA, 1.5 mM CaCl_2 , and 0.2 mg/ml L-cysteine. After enzyme treatment, the tissues were washed twice with culture medium and mechanically triturated in culture medium to dissociate cells. After centrifuging (1600 rpm) and discarding the supernatant to remove the debris of dead cells or connective tissues, we resuspended the cells and plated them on the center of coverslips coated with poly-D-lysine (1 mg/ml) in 35 mm culture dishes. The culture density was $\sim 50,000$ cells/cm². The culture medium contained minimal essential medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (HyClone, Logan, UT) and Serum Extender (Collaborative Research, Bedford, MA), 100 U/ml penicillin/streptomycin, and 6 gm/l glucose. The cultured cells were maintained in an incubator at 37°C and 5% CO₂ for up to 4 weeks and fed twice weekly. Cytosine arabinoside (5 μ M) was added to cultures after 2 d of plating to inhibit the proliferation of astrocytes.

Microculture. Most steps in the formation of microcultures were similar to those described for the above multicellular cultures. The main differences were that the coverslips were specially treated and the total plating density was very low. The coating of the cover glass is a modification of previous methods (Furshpan et al., 1986; Bekkers and Stevens, 1991; Johnson, 1994). The 35 mm coverslips were covered first with a thin layer of 0.15% agarose solution and allowed to dry. A slide on which a grid of 81 squares (each 1 \times 1 mm) had been scratched was used as a template and placed under culture dishes (containing agarose-coated coverslips) to guide the applying of droplets of poly-D-lysine. A micropipette (tip

broken to 20–80 μ m), connected with a syringe, was filled with poly-D-lysine solution (0.5 mg/ml) and used to apply a microdrop of the solution to the center of each grid square on the cover glasses. The water in each microdrop evaporated within a few minutes, and dots of poly-D-lysine, with a diameter of 100–500 μ m, provided an adhesive substrate for neurons. A very low density of cells (~ 2000 –5000 cells/cm²) was plated on the cover glasses containing the poly-D-lysine dots. Only those neurons sitting on the poly-D-lysine dots survived. There were usually 1–8 neurons falling on each poly-D-lysine dot after plating. Two weeks later, ~ 3 –6 dots (microislands) in each dish contained only a single neuron, usually accompanied by astrocytes confined within the dots. In some microcultures, astrocytes (8000–10,000/cm²) were plated 2 d before plating neurons onto coverslips containing dots of poly-D-lysine to help neuronal growth. There were no perceptible differences between the microcultures with and without preplated astrocytes, probably because all the surviving neurons in the microcultures without preplated astrocytes also were accompanied by astrocytes coming from the dissociated tissues. Cytosine arabinoside (5 μ M) was added to cultures on the fourth day of plating neurons (or astrocytes) to arrest astrocyte proliferation. The microcultures were maintained similarly to the mass cultures and fed once weekly. The electrophysiological recording usually was performed in microcultures older than 19 d *in vitro* (DIV).

Intracellular labeling and staining. A typical SCN neuron microculture is illustrated in Figure 2A. The neuron was filled with 1% biocytin during whole-cell recording. After filling, cells were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde for several hours. Plasma membranes were permeabilized with 0.3% Triton X-100 and labeled with an avidin–horseradish peroxidase complex (Vector Labs, Burlingame, CA). After washing in PBS, cultures were incubated in diaminobenzidine and hydrogen peroxide until the labeled cells were brown. This reaction product was then intensified with 0.1% osmium tetroxide for 1 min. Cells not filled with biocytin showed no color, and labeled cells had a dark brown or black appearance.

Electrophysiological recording

Whole-cell voltage-clamp and current-clamp recordings were made with an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA) or a List EPC-7 amplifier. Patch pipettes were pulled from thin-wall borosilicate glass (World Precision Instruments, Sarasota, FL). The pipette resistance after filling with the electrode solution was 4–6 M Ω . The seal resistance was ~ 10 G Ω after forming a tight seal. The series resistance after breaking through the tip membrane patch was 15–28 M Ω and partially (40–60%) compensated by the amplifier. Data were acquired with AxoData software (Axon Instruments) and processed by Axograph software (Axon Instruments) and Igor Pro software (WaveMetrics) on an Apple Macintosh computer. The data sampling rate was 1–10 kHz, filtered at 1 kHz by an 8-pole Bessel filter (Frequency Devices, Haverhill, MA). The presynaptic release in an autaptic neuron was evoked by a brief positive current injection (2 msec, 10–40 pA) in current-clamp mode or a voltage pulse (2 msec, 60–70 mV) in voltage-clamp mode. In some voltage-clamp experiments, the capacitance current induced by the brief voltage pulse was subtracted by using P/4 protocol (see below). The postsynaptic GABA response was evoked by a brief pressure ejection of GABA (50 μ M, 50 msec, 4–7.5 psi), through a micropipette (2–3 μ m tip diameter) connected to a pneumatic microinjector (Narishige USA, Greenvale, NY) under computer control. The recording chamber was perfused constantly by bath solution at a rate of 1.5–2 ml/min. NPY, its analogs, and bicuculline were applied mainly through a series of glass flow pipes (400 μ m inner diameter) fed by gravity. Alternatively, these drugs occasionally were applied by adding them to the perfusing solution. Experiments were performed at room temperature (22°C), as done in previous studies (Bekkers and Stevens, 1991; Tong et al., 1996). The data are reported as mean \pm SEM, and paired Student's *t* tests were used in comparing two groups of data.

Solutions

The standard bath solution contained (in mM): 162.5 NaCl, 2.5 KCl, 2 CaCl_2 , 10 HEPES, and 10 glucose, pH 7.3. TTX (1 μ M) and AP5 (100 μ M)/CNQX (10 μ M) were added into bath solution when spontaneous GABA activity was recorded. Two types of pipette solutions were used to record GABA-mediated events (spontaneous or evoked). One contained (in mM): 145 KMeSO₄, 1 MgCl₂, 10 HEPES, 2 K₄-BAPTA, 4 Mg-ATP, and 0.5 Na₂-GTP, pH 7.3. When this pipette solution is used, the reversal potential for GABA-gated Cl[−] channels is more negative than the resting membrane potential and resulted in the normal direction of IPSPs or

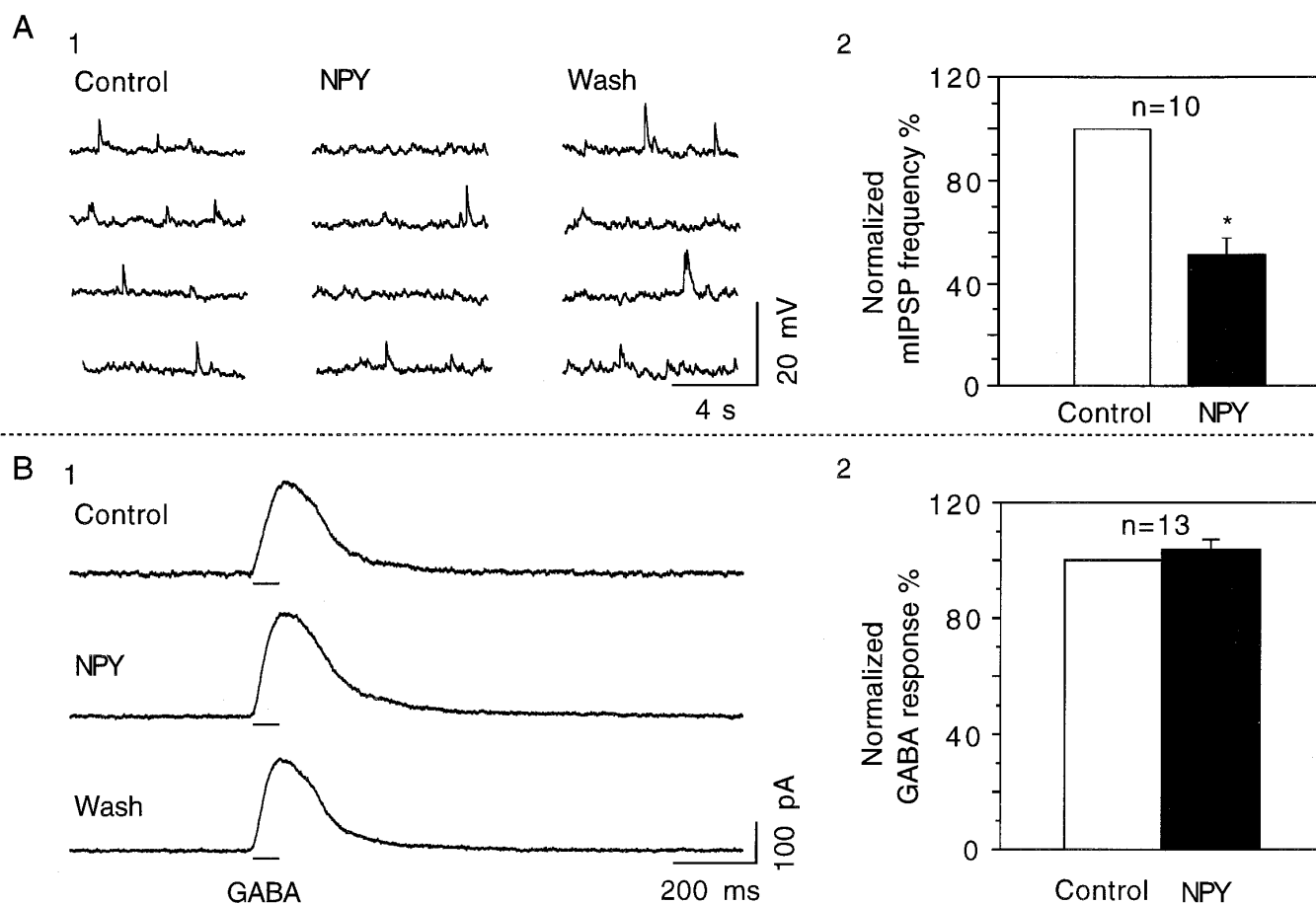


Figure 1. NPY reduces mIPSP frequency but has little effect on postsynaptic GABA responses. **A1**, Consecutive traces showing that NPY (80 nM) reduced the miniature inhibitory postsynaptic potentials (mIPSPs) in the presence of TTX (1 μ M) and glutamate receptor antagonists AP5 (100 μ M) and CNQX (10 μ M). The upward IPSPs are attributable to a KCl-based pipette solution. **A2**, Quantification indicating that NPY depressed the mIPSP frequency to approximately one-half of the control ($p < 0.01$). **B1**, NPY has little effect on postsynaptic GABA responses. **B1**, Pressure-ejected (5 psi, 50 μ M) GABA-induced postsynaptic responses before, during, and after the application of NPY (200 nM). **B2**, Quantitative illustration of the null effect of NPY on postsynaptic GABA responses ($p > 0.1$).

IPSCs. The other pipette solution contained (in mM): 145 KCl, 10 HEPES, 2 K₄-BAPTA, 4 Mg-ATP, and 0.5 Na₂-GTP, pH 7.3. With this pipette solution, the reversal potential for GABA-gated Cl[−] channels is close to 0 mV and resulted in an opposite direction of IPSPs or IPSCs to the normal direction. The advantage of the KCl-based pipette solution is to provide a greater driving force for the GABA-gated Cl[−] channels and increase the amplitude of recorded IPSPs or IPSCs. For the recording of whole-cell barium currents, the bath solution contained (in mM): 114.5 NaCl, 40 TEA-Cl, 2.5 KCl, 5 BaCl₂, 10 HEPES, and 10 glucose plus 1 μ M TTX, pH 7.3; the pipette solution contained 135 CsCl, 2 K₄-BAPTA, 10 HEPES, 4 Mg-ATP, 0.5 Na₂-GTP, and 10 creatine phosphate, plus 50 IU/ml creatine phosphokinase, pH 7.3. The sodium and potassium currents were blocked by TTX and the combination of TEA⁺, Ba²⁺, and Cs⁺. The leak current and the capacitance current were subtracted by a P/4 protocol, i.e., four subpulses with opposite direction and 1/4 amplitude of the main command voltage pulse were applied to induce leak and capacitance currents and then subtracted from the main evoked current.

Pharmacological agents

GABA, (−)-bicuculline methiodide (BIC), D,L-2-amino-5-phosphopentanoic acid (AP5), cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX), tetrodotoxin (TTX), tetraethylammonium hydrochloride (TEA-Cl), Mg-ATP, phosphocreatine, and phosphocreatine kinase were purchased from Sigma (St. Louis, MO). Neuropeptide Y (NPY, porcine), [Leu³¹, Pro³⁴]-NPY, NPY 13-36, and PYY were obtained from Peninsula Labs (Belmont, CA).

RESULTS

NPY inhibits GABAergic spontaneous activity

Although NPY has been found to inhibit noradrenergic, acetylcholinergic, and glutamatergic neurotransmission in some neuronal systems, direct evidence of NPY modulation of GABA, the most important inhibitory transmitter in the CNS, is lacking. In cultured SCN neurons, inhibitory GABAergic events dominated spontaneous activity. NPY (80 nM, 2–4 min) substantially inhibited the GABA-mediated spontaneous activity, as shown in Figure 1A. Four successive traces in each condition of control, NPY application, and after wash are displayed in Figure 1A1. The frequency, but not the amplitude, of spontaneous events was reduced significantly by NPY, and the inhibition was reversible. TTX (1 μ M) was maintained in the bath solution to prevent action potential-dependent release of GABA. Glutamate receptor antagonists AP5 (100 μ M) and CNQX (10 μ M) also were included in the bath solution to abolish excitatory events. The spontaneous GABAergic events are upward in the current-clamp mode because of the KCl-based pipette solution, and all of these events were blocked by bicuculline (30 μ M), a GABA_A receptor antagonist (see Fig. 4). Data from 10 neurons examined are summarized in Figure 1A2. NPY (80 nM) reduced the frequency of

spontaneous activity to $51.5 \pm 6.1\%$ of the control ($p < 0.01$, paired t test) with little effect on the amplitude of the spontaneous events, suggesting a presynaptic mechanism of the NPY action. In 4 of 10 neurons, the reduction of the frequency of spontaneous activity showed continuing recovery, but not full recovery, after 20 min of buffer rinse of NPY. Although long-term experiments were beyond the scope of the current work, these preliminary observations were consistent with our previous finding in immature SCN neurons that NPY may have exerted long-lasting effects on GABA release (Obrietan and van den Pol, 1996).

To address directly the question of whether NPY inhibited the postsynaptic GABA receptors, we used a pneumatic microinjector to pressure-eject GABA ($50 \mu\text{M}$) onto neurons through a micropipette ($2\text{--}3 \mu\text{m}$ tip). A typical example is illustrated in Figure 1*B*. NPY (200 nM) applied for ~ 2 min had little effect on the GABA-evoked postsynaptic responses (Fig. 1*B1*). Data shown in Figure 1*B2* are from all 13 neurons examined. NPY had no consistent effect on postsynaptic GABA receptor responses. In 4 of 13 neurons, we observed an increase of $\sim 20\%$, rather than a decrease, in the GABA response after NPY application, whereas in three neurons there was a 10–13% decrease of the GABA response after NPY application. In the other six neurons, the GABA responses were not affected by NPY. Together, after normalizing the control response as 100%, the relative response to GABA in the presence of NPY was $103.7 \pm 3.5\%$, not significantly different from the control ($p > 0.1$). This indicates that NPY inhibits the GABA neurotransmission of SCN neurons mainly via a presynaptic mechanism.

The effect of NPY on cell input resistance was examined by injecting a series of current pulses to evoke a series of voltage changes in the current-clamp mode. In eight neurons tested, NPY ($80\text{--}200 \text{ nM}$) had little effect on the cell input resistance. The average cell input resistance before and during NPY application was 1.3 ± 0.2 and $1.2 \pm 0.2 \text{ G}\Omega$, respectively. The effect of NPY on the resting membrane potential was examined also. In 11 neurons tested, NPY ($80\text{--}200 \text{ nM}$) slightly hyperpolarized 8 neurons, depolarized 2 neurons, and had no effect on 1 neuron. The average resting potentials before and after NPY application were -58.0 ± 1.6 and $-59.7 \pm 1.7 \text{ mV}$, respectively.

Presynaptic inhibition of NPY on autaptic GABA release

Previous studies on the mechanism of NPY action were performed in mass cell cultures or brain slices (Wahlestedt et al., 1986; Walker et al., 1988; Colmers et al., 1991; Bleakman et al., 1992; Simonneaux et al., 1994; Obrietan and van den Pol, 1996). Because results were obtained from a net of many neurons, it is difficult to know how NPY acts on a single neuron and whether NPY Y1 and Y2 subtype receptors cofunction in the same neuron. The present study used single-neuron microcultures in which a single neuron in a microisland formed synapses with itself. In our single-neuron microcultures of SCN cells, 47 neurons showing autaptic responses were GABAergic and only 4 neurons were not, confirming that the majority of SCN neurons are GABAergic. Figure 2*A* shows a typical single autaptic SCN neuron in a microisland culture, filled with biocytin during electrophysiological recording and fixed and stained with avidin–HRP after the completion of experiments. Traces shown in Figure 2*B,C* are electrical responses recorded from the illustrated neuron. The neuron was current-clamped, and a brief positive current injection (20 pA , 2 msec) invariably evoked a single action potential, followed by a large evoked IPSP (Fig. 2*B*, control trace). The IPSP

was reversibly blocked by $30 \mu\text{M}$ bicuculline (*BIC*, Fig. 2*B*, middle trace), indicating its GABAergic origin. Figure 2*C* shows that NPY (200 nM) reversibly blocked the GABAergic IPSP. In the presence of NPY, the large IPSP that follows the action potential was totally eliminated. Similarly, NPY (200 nM) totally blocked the evoked GABA response in another two autaptic neurons and, in a fourth neuron, reduced the autaptic GABA response to 28% of the control. In only one neuron tested did NPY have no effect on the evoked autaptic response. Thus, NPY strongly inhibits presynaptic GABA release in single SCN neurons.

A traditional way to identify a presynaptic versus a postsynaptic effect of a chemical is to examine its effect on the frequency of miniature spontaneous activity and postsynaptic responses, as illustrated in Figure 1. In studying the mechanism of NPY action, we found that single-neuron microcultures are ideal for discriminating a presynaptic versus a postsynaptic effect of neurotransmitters or neuromodulators. Figure 3 illustrates such a scenario, in which a presynaptic and/or a postsynaptic effect are evident concurrently after a single application of NPY. Autaptic transmitter release was evoked by a brief voltage pulse (2 msec , 60 mV) under voltage-clamp (holding potential = -60 mV) condition. A postsynaptic response was evoked by a brief pressure ejection (50 msec , 5 psi) of $50 \mu\text{M}$ GABA onto the recording neuron under computer control. Figure 3*A* shows both an evoked autaptic response and a postsynaptic GABA response in the same sweep. In Figure 3*B*, NPY (200 nM) abolished the presynaptic response while leaving the postsynaptic response unaffected. The box in Figure 3*B* demonstrates that the sodium action current was not blocked by NPY. Figure 3*C* illustrates that the NPY inhibition is reversible. In Figure 3*D*, bicuculline ($30 \mu\text{M}$) blocked both the pre- and postsynaptic responses, indicating that both are mediated by GABA_A receptors. Similar results were obtained in two other single autaptic neurons. This is the first time that NPY has been demonstrated to inhibit synaptic neurotransmission by acting only presynaptically, but not on postsynaptic receptors in a single neuron.

Both NPY Y1- and Y2-like receptors coexist in the same SCN neuron

A further question regarding presynaptic NPY inhibition is, through which subtype NPY receptor does NPY act? We pursued this question initially by examining the effect of NPY Y1 and Y2 receptor agonists on the miniature spontaneous activity in multicelled cultures. Although recent evidence has revealed several NPY receptors, they have differential sensitivities to activation by [Leu^{31} , Pro^{34}]-NPY (Y1-like: Y1, Y4, Y5) and NPY13-36 (Y2-like: Y2). Y5m and Y5r show greater sensitivity to [Leu^{31} , Pro^{34}]-NPY but also respond weakly to NPY13-36 (Gerald et al., 1996; Weinberg et al., 1996). As in the experiments shown in Figure 1*A*, TTX and AP5/CNQX were included in the bath solution to prevent action potential-mediated release of transmitters and to block glutamate receptors. Figure 4*A–E* shows a typical example of the effect of Y1 agonist [Leu^{31} , Pro^{34}]-NPY (80 nM) and Y2 agonist NPY 13-36 (80 nM) on the spontaneous activity. Both agonists reduced the frequency, but not the amplitude, of the spontaneous events, and for both the inhibition were reversible. Bicuculline eliminated all the spontaneous events, indicating their GABAergic properties. The spontaneous events were inward in voltage clamp because of the use of a KCl-based pipette solution. The general effect of Y1 and Y2 receptor agonists is summarized in Figure 4*F*. By normalizing the frequency of the spontaneous activity in the control condition to 100%, [Leu^{31} , Pro^{34}]-NPY (80

A

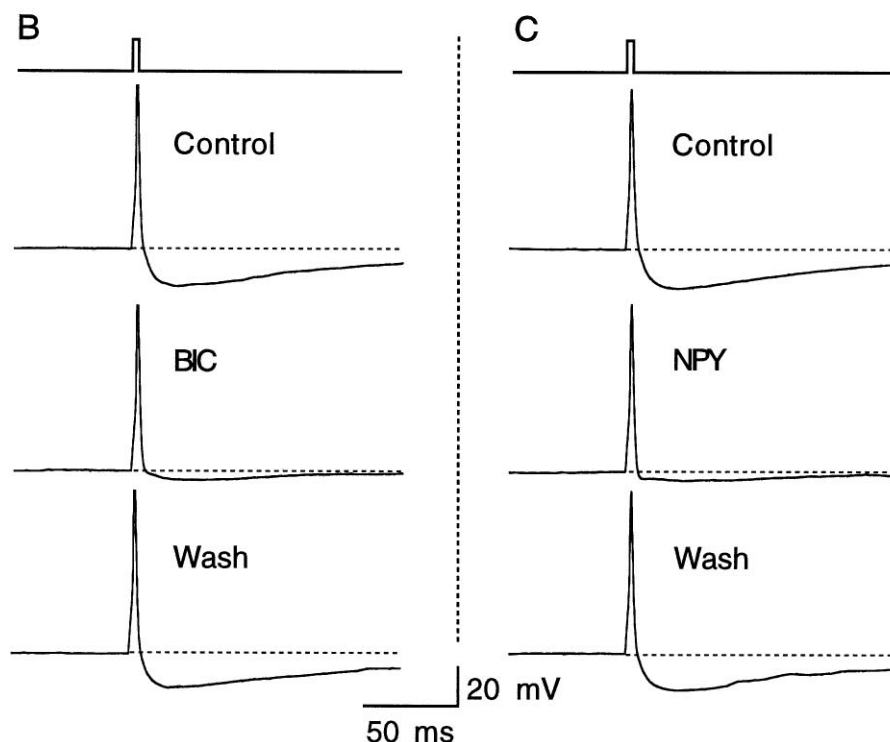
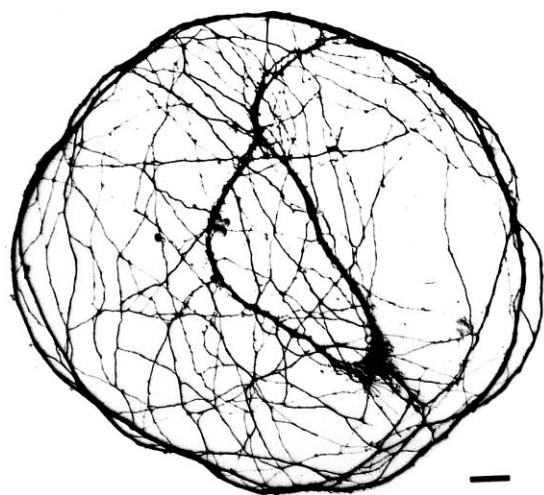


Figure 2. NPY reversibly blocks the evoked autaptic GABA release in single-neuron microcultures. *A*, A single SCN neuron in a microisland culture. The neuron was filled by biocytin during whole-cell recording and stained with avidin-HRP. *B*, *C*, Action potentials and the following autaptic IPSPs initiated by a brief current injection (2 msec, 20 pA) recorded in the single neuron illustrated in *A*. *B*, The evoked autaptic IPSP was reversibly blocked by bicuculline (BIC, 30 μ M). *C*, The evoked autaptic IPSP also was reversibly blocked by NPY (200 nM).

nM) reduced the frequency to $59.3 \pm 5.5\%$ of the control ($p < 0.01$, $n = 11$), and NPY 13-36 (80 nM) reduced the frequency to $57.4 \pm 9\%$ of the control ($p < 0.05$, $n = 10$). For both agonists the inhibition was significant and reversible. These experiments demonstrate that NPY inhibits the GABA neurotransmission in SCN neurons through both Y1- and Y2-like receptors.

The above experiments on multicellular cultures demonstrated that both NPY Y1- and Y2-like receptors are involved in the action of NPY on SCN cells. However, whether the two subtype receptors coexist in the same neuron or are distributed among different neurons was not clear. A single-neuron microculture provides an opportunity to study the distribution of Y1- and Y2-like receptors in single neurons. In total, 30 single autaptic

neurons were examined to study the effect of [Leu³¹, Pro³⁴]-NPY (80 nM) and NPY 13-36 (80 nM) on the evoked autaptic responses. In all, 9 of 30 neurons showed no reduction or $<10\%$ reduction in the evoked responses by both agonists. Different neurons showed different sensitivity to Y1 and Y2 agonists. [Leu³¹, Pro³⁴]-NPY reduced the evoked response by up to 88%, and NPY 13-36 reduced the response by up to 84%. Some neurons were more sensitive to the Y1 agonist and less sensitive to the Y2 agonist, some showed the opposite response, and some were equally sensitive to both agonists. The sequential application of Y1 and Y2 agonists was random among neurons. Some neurons were given the Y1 agonist first and then the Y2 agonist, and in others the opposite procedure was used. In most cases, the first applica-

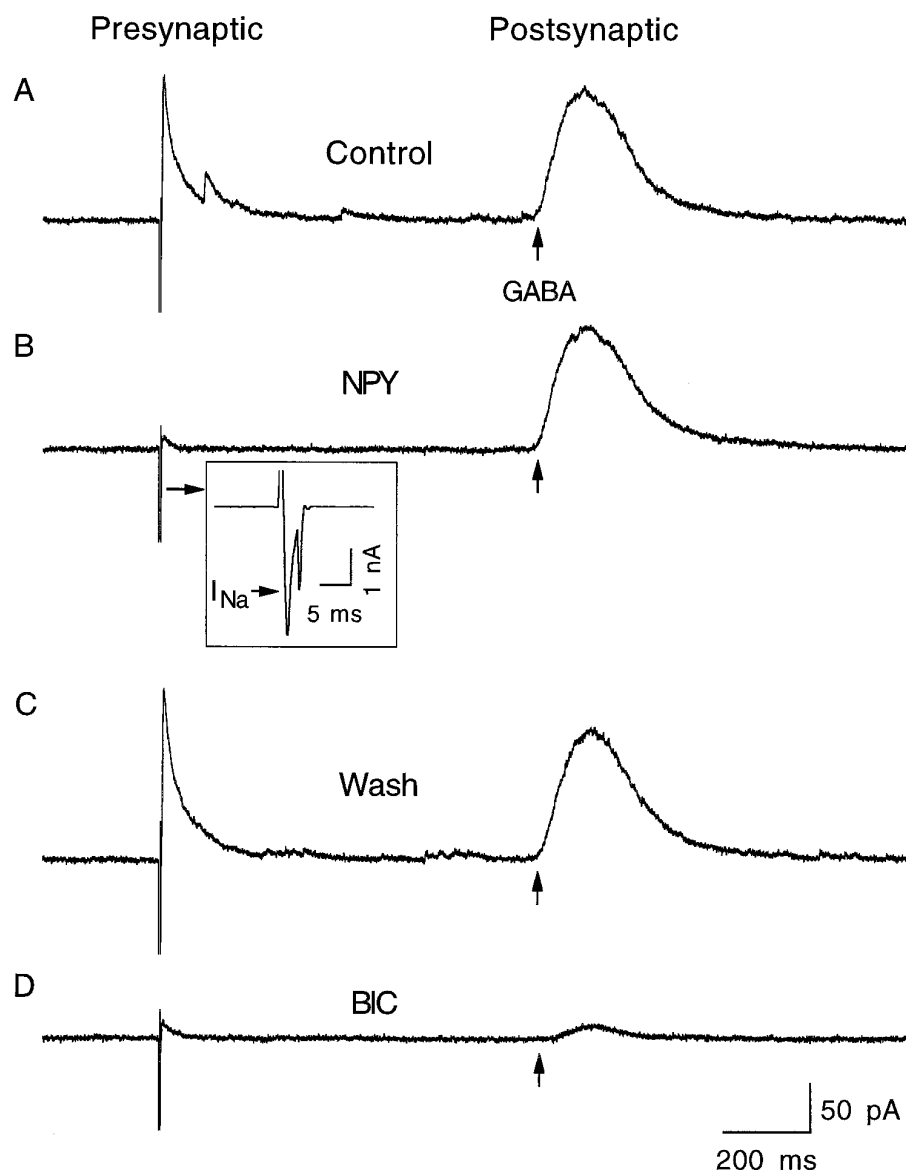


Figure 3. NPY inhibits presynaptic GABA release, but not the postsynaptic GABA response, in the same autaptic SCN neuron. *A*, Presynaptically released GABA (triggered by 2 msec, 60 mV voltage pulse) induced IPSC and pressure-ejected GABA-evoked response in the same recording. *B*, NPY (200 nM) eliminated the presynaptic release but did not affect the postsynaptic response. The box shows the sodium action current in the presence of NPY. *C*, The inhibition of NPY on presynaptic release was reversible. *D*, Bicuculline abolished both the pre- and the postsynaptic GABA responses. Capacitance currents and action currents were truncated.

tion gave the larger inhibition, but in some cases the second application produced larger inhibition. Figure 5*A* illustrates a representative of the action of both [Leu³¹, Pro³⁴]-NPY and NPY 13-36 in the same autaptic neuron. Both agonists were applied for 2 min, and the evoked responses were reduced gradually during the application of the agonists. The recording traces corresponding to the four points in the graph (*i*, *ii*, *iii*, *iv*) are illustrated on the top panel. Both [Leu³¹, Pro³⁴]-NPY and NPY 13-36 substantially inhibited the evoked response in the same neuron, indicating the coexistence of Y1 and Y2 receptors in the presynaptic nerve terminals. In this particular neuron, the inhibition through Y1 receptors was larger than through Y2 receptors. Figure 5*B* summarizes the data of the 21 neurons sensitive to [Leu³¹, Pro³⁴]-NPY and NPY 13-36 and also the data on NPY (80 nM) and PYY (80 nM). [Leu³¹, Pro³⁴]-NPY reduced the evoked response by $48.7 \pm 7.0\%$ ($p < 0.002$; $n = 15$), and NPY 13-36 reduced the evoked response by $30.4 \pm 6.5\%$ ($p < 0.02$; $n = 14$). NPY reduced the response by $41.4 \pm 10.7\%$ ($p < 0.02$; $n = 10$). PYY reduced the evoked response in one neuron by 100% and by 39% in another neuron. The strong inhibitory effect of PYY reduces the possibil-

ity of a significant involvement in these neurons of a putative Y3 subtype of NPY receptor that is insensitive to PYY (Colmers and Bleakman, 1994).

Inhibition of whole-cell barium currents

Previous studies suggest that an inhibition of calcium currents may underlie the inhibition of synaptic transmitter release (Colmers et al., 1988; Walker et al., 1988; Toth et al., 1993). We therefore explored whether the inhibition of NPY on GABA neurotransmission was mediated by an inhibition of calcium currents in SCN neurons and whether Y1- or Y2-like receptors were involved in the inhibition of the calcium currents. The recorded neurons were held at -80 mV, and a command pulse (80 mV, 40 msec) was applied to evoke a barium current (through voltage-activated calcium channels). As in the effect on the autaptic release, both Y1 and Y2 agonists were found to inhibit the whole-cell barium currents (I_{Ba}) in SCN neurons. Also similar to the autaptic release, Y1 and Y2 agonists produced different inhibition of I_{Ba} in different individual neurons. A typical example of the effect of both agonists in the same neuron is shown in Figure

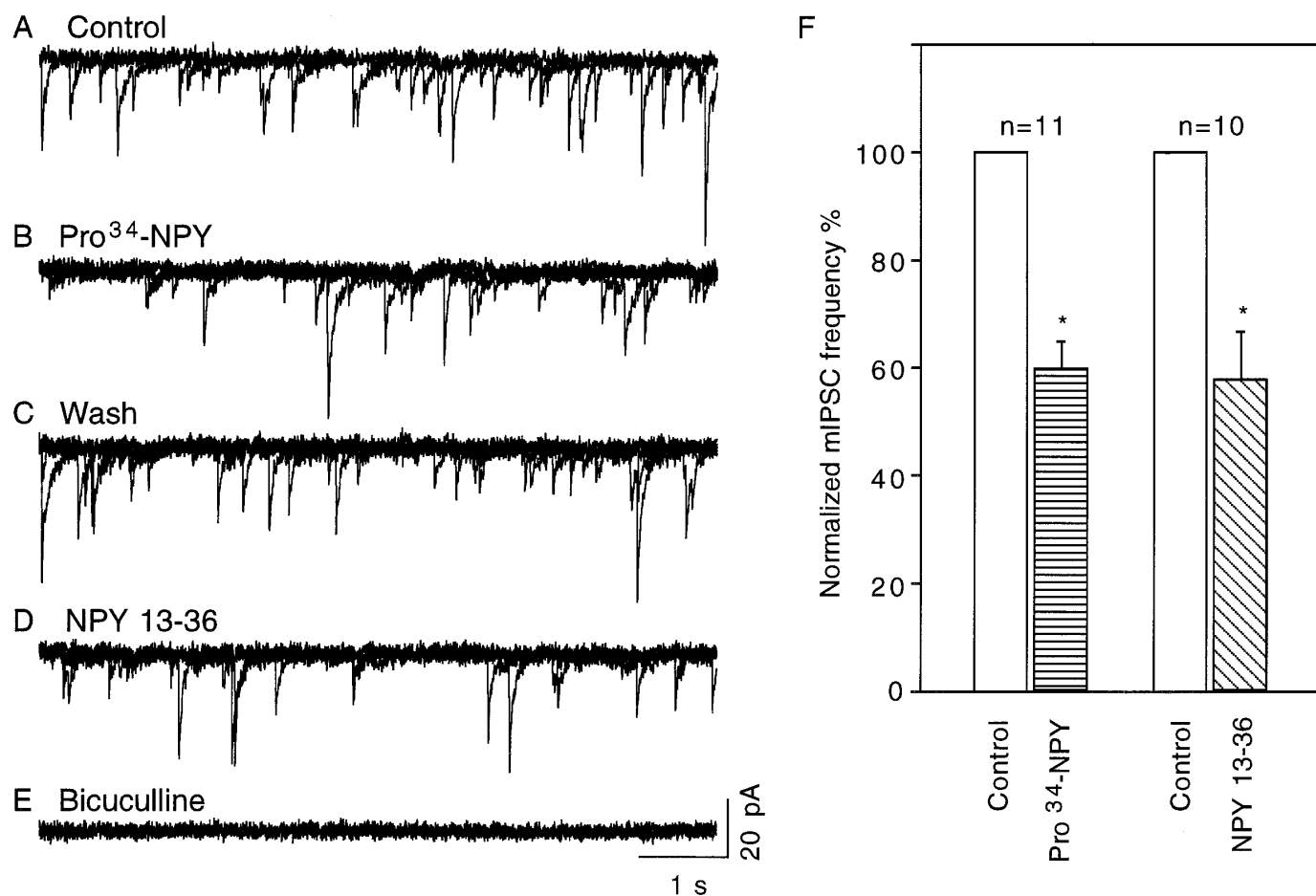


Figure 4. Both NPY Y1- and Y2-like receptors mediate the inhibition of miniature spontaneous GABA activity. *A*, Superimposed four consecutive traces illustrating control mIPSCs in the presence of TTX and AP5/CNQX. *B*, NPY Y1 receptor agonist, $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY (80 nM) reduced the mIPSC frequency. *C*, The inhibition of $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY was reversible. *D*, NPY Y2 receptor agonist NPY 13-36 (80 nM) also reduced the mIPSC frequency recorded from the same neuron. *E*, All of the mIPSCs were abolished by 30 μM bicuculline. *F*, Quantitative analysis showing that both $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY and NPY 13-36 significantly reduced the mIPSC frequency to <60% of the control.

6. *A1* shows that NPY 13-36 substantially suppressed the I_{Ba} , and the suppression was reversible. *B1* shows that in the same neuron $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY also reversibly inhibited the I_{Ba} , although much less than did NPY 13-36. *A2* and *B2* illustrate the I - V curves of I_{Ba} before and after the application of NPY 13-36 and $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY. Except for the reduction of I_{Ba} by both agonists, the shape of the I - V curves was not changed by either NPY 13-36 or $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY. The potential at which the I_{Ba} reached the peak was ~ 0 mV, and the reversal potential for I_{Ba} was approximately +50 mV, not altered by NPY 13-36 and $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY. Figure 7*A* illustrates the typical time course of the action of NPY 13-36 and $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY on the I_{Ba} . The I_{Ba} was reduced within 20 sec of the application of either agonist. In this particular neuron, in contrast to the one shown in Figure 6, $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY produced a greater inhibition than NPY 13-36. The inhibitory effect usually was washed out within 1–2 min, faster than the recovery of the evoked release. CdCl_2 (100 μM) reversibly abolished the I_{Ba} ($n = 4$). The overall reduction of I_{Ba} by NPY 13-36 (80 nM) and $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY (80 nM) and by NPY (80 nM) and PYY (80 nM) is summarized in Figure 7*B*. NPY reduced the I_{Ba} by $32.9 \pm 7.3\%$ ($p < 0.02$; $n = 7$). $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY reduced the I_{Ba} by $42.6 \pm 7.1\%$ ($p < 0.001$; $n = 18$). NPY 13-36 reduced the I_{Ba} by $36.7 \pm 7.2\%$ ($p < 0.001$; $n = 17$).

PYY reduced the I_{Ba} by $23.2 \pm 5.1\%$ ($p < 0.01$; $n = 5$). All 36 neurons (9–30 DIV) examined showed a reduction in the I_{Ba} by NPY or its analogs, confirming a robust expression of NPY receptors, including both Y1- and Y2-like receptors, in SCN neurons. No developmental changes in NPY inhibition of I_{Ba} and GABA release were observed in cultured neurons examined.

Inhibition of IPSC and I_{Ba} in the same SCN neuron

Because both the presynaptic release and the whole-cell barium currents were inhibited by Y1- and Y2-like receptor agonists, a logical question is whether there is any correlation between these two inhibitory actions. We therefore examined the effect of NPY analogs on both the evoked release and the barium currents in the same self-innervating neuron. Figure 8 shows the results of one such experiment. The effect of $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY (80 nM) and NPY 13-36 (80 nM) was examined first on the evoked IPSCs and then on the I_{Ba} . Figure 8*A* illustrates the recording traces of the evoked IPSCs and the I_{Ba} , corresponding to those points specified in Figure 8*B*. Both the evoked IPSCs and the I_{Ba} were inhibited by $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY and NPY 13-36. The relative amount of reduction in the amplitude of the evoked IPSCs by $[\text{Leu}^{31}, \text{Pro}^{34}]$ -NPY (–83%) and NPY 13-36 (–77%) correlated with the reduction of I_{Ba} (–89% and –52%, respectively). The reduction and

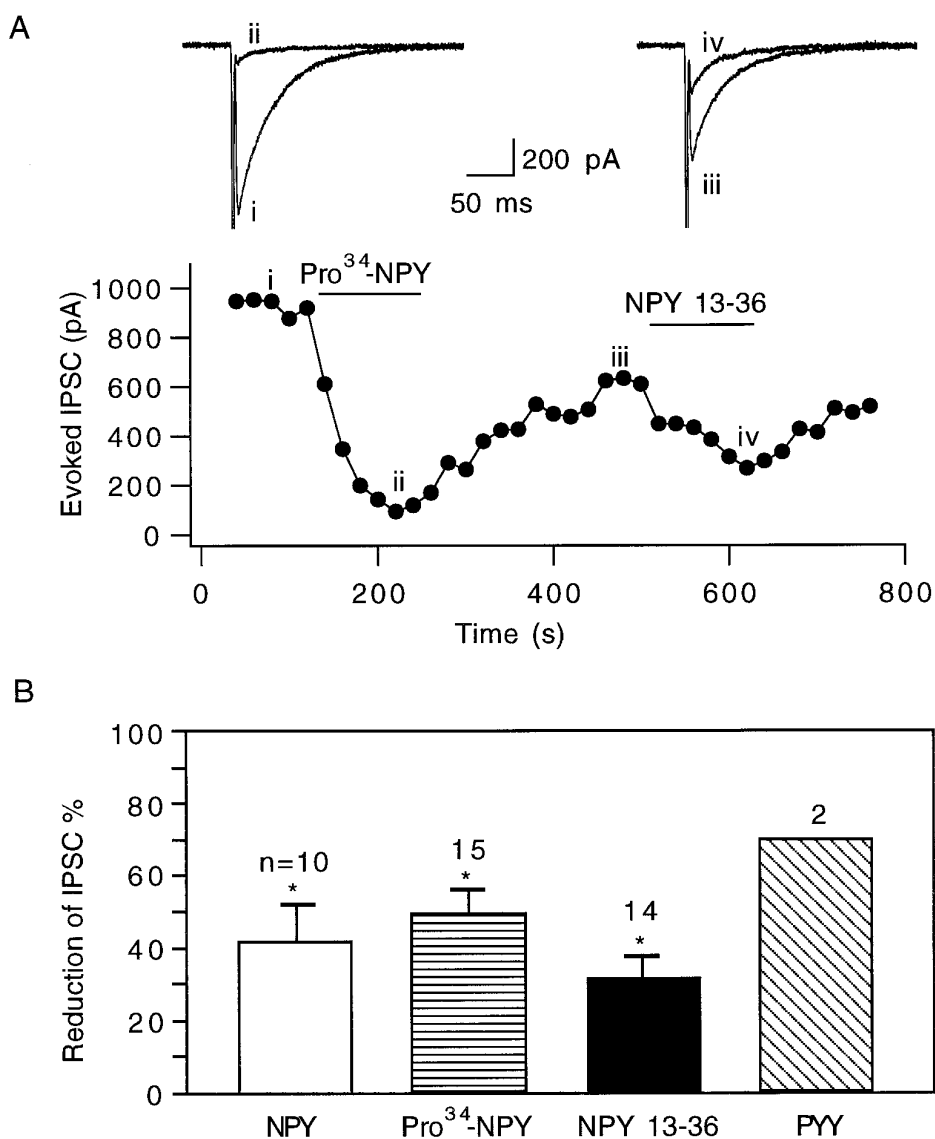


Figure 5. NPY Y1 and Y2 agonists both inhibit the evoked autaptic GABA release in the same single SCN neuron. *A*, $[\text{Leu}^{31}, \text{Pro}^{34}]\text{-NPY}$ (80 nM) and NPY 13-36 (80 nM) both substantially inhibited the evoked autaptic IPSC. The recording traces on the top panel correspond to the four points (*i*, *ii*, *iii*, *iv*) in the graph below. *B*, Group data showing the reduction of the evoked IPSC by NPY, $[\text{Leu}^{31}, \text{Pro}^{34}]\text{-NPY}$, NPY 13-36, and PYY.

the recovery of I_{Ba} seemed to be rapid, whereas those of the evoked IPSCs were relatively gradual. Similar results were observed in four other single autaptic neurons on the inhibition of both the evoked IPSCs and the I_{Ba} by NPY analogs. The overall correlation coefficient between the reduction of IPSCs and I_{Ba} among the five neurons examined was positive ($r = 0.56$). The recorded whole-cell barium currents were evoked mainly through calcium channels at the cell body. Because of the technical difficulties in using patch pipettes to record directly from single boutons, we cannot directly measure calcium currents at the presynaptic terminals. However, on the working assumption that NPY exerts similar actions in the terminal and perikaryon of the same cell, NPY inhibition of presynaptic GABA release may be mediated by inhibition of calcium channels in the presynaptic nerve terminals.

DISCUSSION

The present study with SCN neurons provides for the first time direct electrophysiological evidence that NPY modulates inhibitory GABA neurotransmission in the CNS. Studies in other regions of the brain, for instance in hippocampal slices, found that

NPY selectively inhibits excitatory glutamatergic release without a direct effect on GABA release (Klapstein and Colmers, 1993).

Coexistence of NPY Y1- and Y2-like receptors in the same SCN neuron

The most striking results of the present study are the coexistence of NPY Y1- and Y2-like receptors in both presynaptic axon terminals and postsynaptic neuronal soma of the same GABAergic cell and the demonstration that both Y1- and Y2-like receptors mediate the inhibition of presynaptic GABA release and whole-cell calcium (barium) currents in SCN neurons. These results would not be achieved without the use of single self-innervating neuron microcultures. Our present study indicates two advantages of such single-neuron microcultures in identifying presynaptic versus postsynaptic effects of neurotransmitters and neuromodulators and in locating and discriminating their (sub-type) receptors. Previous studies have indicated that more than one NPY subtype receptor might exist in some preparations. In rat nodose neurons, both Y1 and Y2 receptor agonists modulate calcium currents, although with opposite effects (Wiley et al., 1993). In the rat pineal gland, NPY inhibited presynaptic nor-

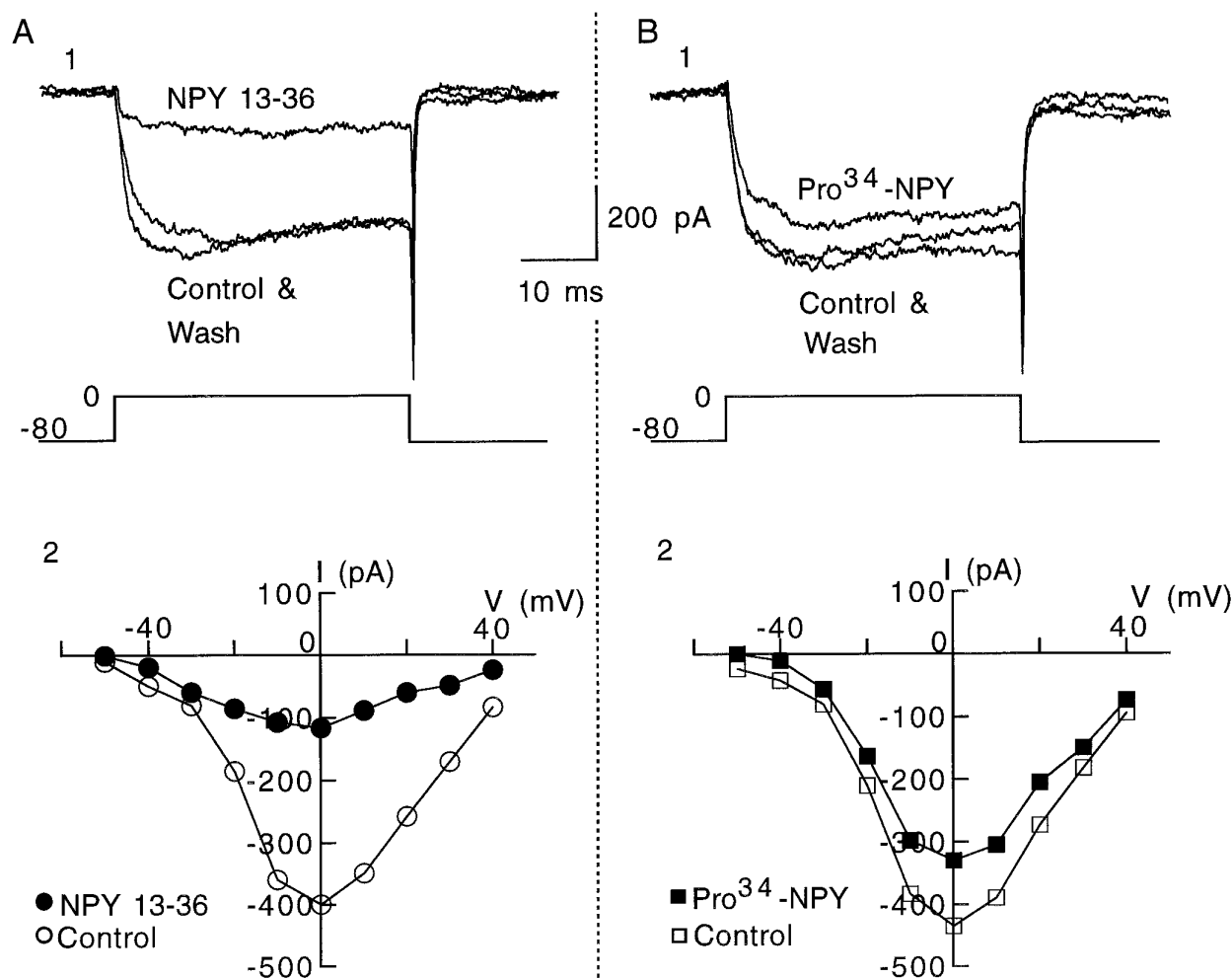


Figure 6. NPY Y1- and Y2-like receptors mediate the inhibition of whole-cell barium currents in the same SCN neuron. *A*, Inhibition by Y2 agonist NPY 13-36 (80 nM). *A1*, Barium currents (I_{Ba}) evoked by test potential from -80 mV to 0 mV before, during, and after the application of NPY 13-36. *A2*, I - V curve of the I_{Ba} before and during NPY 13-36 application. *B*, Inhibition of I_{Ba} by Y1 agonist [Leu³¹, Pro³⁴]-NPY (80 nM) in the same neuron. *B1*, I_{Ba} before, during, and after the application of [Leu³¹, Pro³⁴]-NPY. *B2*, I - V curve I_{Ba} before and during [Leu³¹, Pro³⁴]-NPY application. Notice that NPY 13-36 produced greater inhibition than [Leu³¹, Pro³⁴]-NPY in this neuron. Neither NPY 13-36 nor [Leu³¹, Pro³⁴]-NPY changed the I - V relationship of the barium currents.

adrenaline release via the activation of Y2 receptors, but it decreased cyclic AMP elevation induced by β -adrenaline through a Y1 receptor pathway (Simonneaux et al., 1994), although it is not known whether Y1 and Y2 receptors were in the same cell. In rat dentate granule cells, NPY inhibited calcium currents mainly by the activation of Y1 receptors but also via Y2 receptors in some neurons (McQuiston et al., 1996). In both nodose neurons and dentate granule neurons, only 30% of neurons responded to both Y1 and Y2 receptor agonists.

In contrast, in the present study almost all of the NP4-sensitive SCN neurons responded to both agonists. The whole-cell barium currents were inhibited in most neurons tested for Y1 and Y2 agonists, although different individual neurons showed different sensitivities to each agonist. This suggests a substantial expression of both Y1- and Y2-like receptors in SCN neurons, at least in the neuron soma. In addition to the inhibition of whole-cell barium currents, the evoked presynaptic GABA release assessed in single autaptic neurons in microcultures also was inhibited by both Y1 and Y2 agonists in the majority of SCN neurons ($>70\%$), indicating a coexistence of Y1- and Y2-like receptors at presynaptic nerve terminals of the same SCN neuron. Furthermore, both the

presynaptic release and whole-cell barium currents were inhibited by Y1 and Y2 agonists in the same neuron. These experiments demonstrate that NPY Y1- and Y2-like receptors coexist in both presynaptic nerve terminals and the postsynaptic cell body in SCN neurons, as depicted by the diagram in Figure 9. The recent demonstration that Y4 and Y5 receptors are expressed in the SCN (Bard et al., 1995; Gerald et al., 1996; Weinberg et al., 1996) suggests that they could contribute substantially to the effects detected in the present experiment. NPY Y1 and Y4 receptors show greater activation by the agonist [Leu³¹, Pro³⁴]-NPY and Y2 receptors by NPY13-36, but not [Leu³¹, Pro³⁴]-NPY. Y5r (Gerald et al., 1996) and Y5m (Weinberg et al., 1996) have a greater sensitivity to the Y1 agonist but also have a mild (10–15 times smaller) response to the Y2 agonist. Our previous work showing that the NPY agonist PYY3-36 evoked a large postsynaptic depression of glutamate actions in the SCN (van den Pol et al., 1996) is consistent with the expression of the Y5r receptor. However, a single NPY receptor such as Y5r could not account for responses to both Y1 and Y2 agonists, because the results in the present study demonstrate that some SCN neurons are more sensitive to the Y2 agonist and less to the Y1 agonist, some are the reverse,

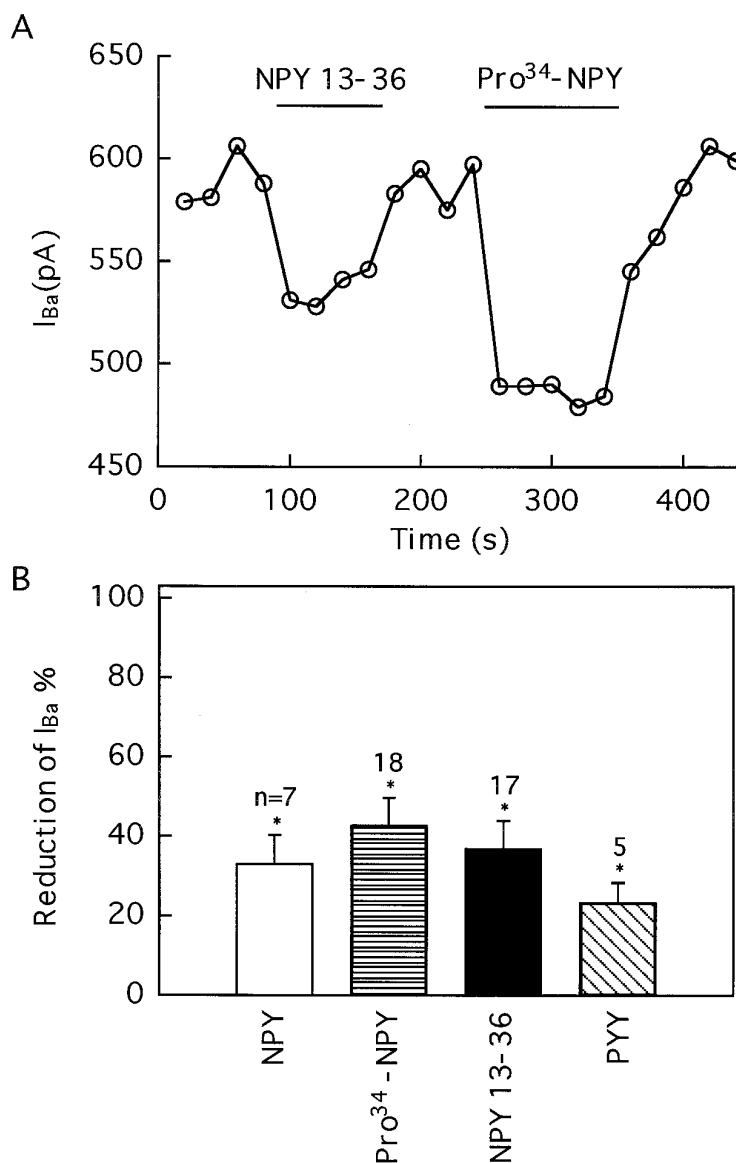


Figure 7. Time course of the action of Y1 and Y2 agonists on barium currents. *A*, Typical example showing the rapid inhibition by and the rapid recovery of I_{Ba} after the application of NPY 13-36 and [Leu³¹, Pro³⁴]-NPY. I_{Ba} was inhibited within 20–30 sec on application of NPY 13-36 or [Leu³¹, Pro³⁴]-NPY and returned to the control level within 2 min after washing out the agonists. In this neuron, [Leu³¹, Pro³⁴]-NPY produced greater inhibition than NPY 13-36. *B*, Summarized data showing the reduction of I_{Ba} by NPY, [Leu³¹, Pro³⁴]-NPY, NPY 13-36, and PYY. The amount of reduction in I_{Ba} was comparable to the reduction in the evoked IPSC (see Fig. 5).

and some are equally sensitive to Y2 and Y1 agonists. These data indicate that the relative expression of multiple NPY receptors differs between SCN neurons. As agonists and antagonists that are more selective for the different NPY receptors become available, their use in the SCN should clarify further the roles of the different NPY receptors found here.

Inhibition of calcium currents

NPY has been demonstrated to inhibit whole-cell calcium (or barium) currents or calcium influx in a number of neurons, mainly peripheral neurons such as rat dorsal root ganglion cells (Walker et al., 1988; Ewald et al., 1989; Bleakman et al., 1991), rat myenteric plexus neurons (Hirning et al., 1990), rat nodose ganglion neurons (Wiley et al., 1993), rat superior cervical ganglion neurons (Foucart et al., 1993; Toth et al., 1993), and central neurons such as rat dentate granule cells (DGC; McQuiston et al., 1996). NPY inhibition seems to be mediated by different NPY receptors in different types of neurons. For example, in DGCs, NPY inhibited calcium currents mainly through Y1-like receptors, and in less than one-third of the cells Y2-like receptors were also involved (McQuiston et al., 1996). In dorsal root ganglion neu-

rons, NPY inhibited barium currents via a Y2-like receptor (Bleakman et al., 1991). In nodose neurons, both Y1- and Y2-like receptors were involved in modulating calcium currents but with opposite actions, i.e., Y2 receptor agonist decreased calcium currents, whereas Y1 receptor agonist increased calcium currents (Wiley et al., 1993). In contrast to previous studies, we demonstrate in the present paper that both Y1- and Y2-like receptors coexist in the same SCN neuron and both inhibit barium currents. Furthermore, SCN neurons seem to be more sensitive to NPY than DGCs are. NPY inhibited only 20–30% of the calcium current (and less with barium currents) at a high NPY concentration of 1 μ M in DGCs (McQuiston et al., 1996), whereas in the present study NPY inhibited 40–50% of barium currents at a low concentration of 80 nM in SCN neurons. The site of NPY action is also different between neurons in the hippocampus and in the hypothalamus. In hippocampal pyramidal neurons, NPY had no effect on barium currents but had an inhibitory effect on presynaptic glutamate release (Bleakman et al., 1992). In DGCs, NPY had an inhibitory effect on calcium currents but no effect on presynaptic release (Klapstein and Colmers, 1993; McQuiston et

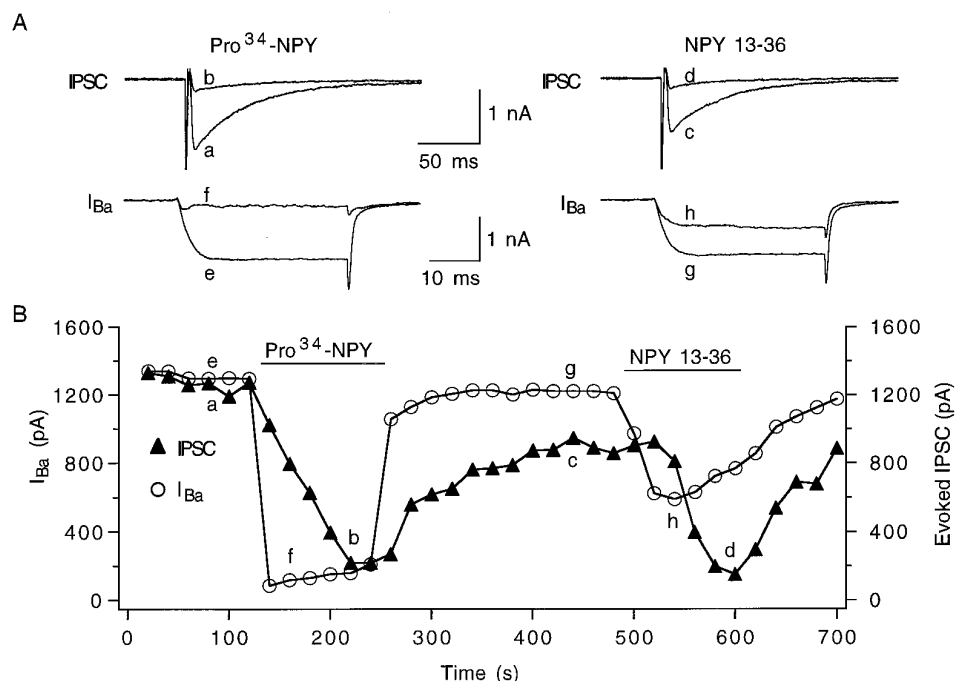


Figure 8. Inhibition of both barium currents and the evoked autaptic GABA release in the same SCN neuron. *A*, Recording traces showing the inhibition of the evoked IPSCs and the I_{Ba} by $[\text{Leu}^{31}, \text{Pro}^{34}]\text{-NPY}$ and NPY 13-36 . Each trace corresponds to a point in *B*. *B*, Comparison between the inhibition of the evoked IPSC and the I_{Ba} by $[\text{Leu}^{31}, \text{Pro}^{34}]\text{-NPY}$ and NPY 13-36 in the same neuron. Note that the relative amount of the inhibition of the evoked IPSC is proportional to the amount of the inhibition of the I_{Ba} . The time course of the inhibition and the recovery of the evoked IPSC is slower than and follows the I_{Ba} .

al., 1996). In contrast, the present study demonstrates that NPY inhibited both the presynaptic GABA release and the whole-cell barium currents in SCN neurons.

With digital calcium imaging, we showed an inhibitory effect of NPY on GABA-mediated calcium rises in developing hypothalamic neurons (Obrietan and van den Pol, 1996). This was based on a mechanism involving a depolarized Cl^- reversal potential (Chen et al., 1996) in which NPY inhibited GABA-mediated depolarizations (Obrietan and van den Pol, 1996). In the present study, we show that NPY can directly modulate voltage-gated calcium channels, independent of GABA actions. In contrast to the presynaptic release, the whole-cell barium currents responded rapidly to NPY and its analogs and also recovered quickly after peptide washout. The inhibition of presynaptic release by NPY was gradual, taking a longer time to reach its minimum amplitude than the inhibition of whole-cell barium currents and usually taking longer to recover. This long-latency recovery of GABA release may serve as a partial mechanistic explanation for the NPY-mediated extended depression of GABA-raised calcium levels in developing SCN neurons (Obrietan and van den Pol, 1996).

The primary excitatory input to the SCN is glutamatergic, both from retina and nonretinal sources (Kim and Dudek, 1991), and glutamate has been shown to raise the cytosolic calcium of SCN neurons (van den Pol et al., 1992). A large proportion of glutamate-evoked calcium rises is attributable to the opening of voltage-gated calcium channels. The direct NPY inhibition of calcium currents demonstrated in the present paper would serve to reduce glutamate-mediated calcium rises in SCN perikarya (van den Pol et al., 1996), potentially serving to reduce the effectiveness of retinal glutamate in activating gene transcription in a circadian time-dependent manner (Rea, 1989; Kornhauser et al., 1990; Rusak et al., 1990).

Inhibition of GABA release and functional role of NPY in SCN

The NPY concentration in the rat SCN displays diurnal fluctuations (Calza et al., 1990). This diurnal change in NPY concentration may be important in modulating the circadian rhythm. NPY

administration into the SCN phase shifts the circadian rhythm (Albers and Ferris, 1984; Medanic and Gillette, 1993; Shibata and Moore, 1993; Huhman and Albers, 1994), and the direction of the phase shift is dependent on circadian time. The NPY-mediated phase shift can be antagonized by bicuculline (Huhman et al., 1995), suggesting that the action of NPY on circadian rhythms may be mediated by modulation of GABA transmission. This is consistent with the present paper that shows a dramatic inhibition of GABA release by NPY in SCN neurons. Morphological evidence provides further support for this interaction in that GABA terminals and NPY terminals are in direct apposition or converge on the same postsynaptic targets in the rat SCN; NPY colocalizes with GABA, probably in axons from the intergeniculate leaflet of the thalamus (Francois-Bellan et al., 1990).

The density of NPY fibers in the SCN is particularly high; in addition, NPY axons are found throughout the medial hypothalamus (Chronwall et al., 1985), including those regions of the hypothalamus that receive SCN innervation. Our finding that NPY inhibits transmitter release from presynaptic SCN axons suggests that NPY may be able to modulate the actions of SCN GABAergic axons not only within the nucleus but also at the terminals outside the nucleus by the same presynaptic mechanism described in the present paper.

Most SCN neurons are GABAergic (van den Pol and Tsujimoto, 1985; Okamura et al., 1989; Decavel and van den Pol, 1990; Moore and Speh, 1993; present study), and many SCN neurons have local axon collaterals that terminate on other SCN cells (van den Pol, 1980). That NPY reduces calcium currents could explain previous observations with extracellular recording that NPY is inhibitory (Albers et al., 1990). That excitatory actions of NPY may exist (Mason et al., 1987) could be explained by a reduction in the presynaptic release of GABA, resulting in disinhibition. We have also found that NPY reduces glutamate release from excitatory axons in the SCN (van den Pol et al., 1996). This observation, together with those reported in the present paper, could account for complex response to NPY, including biphasic responses of excitation and inhibition (Liou and Albers, 1991).

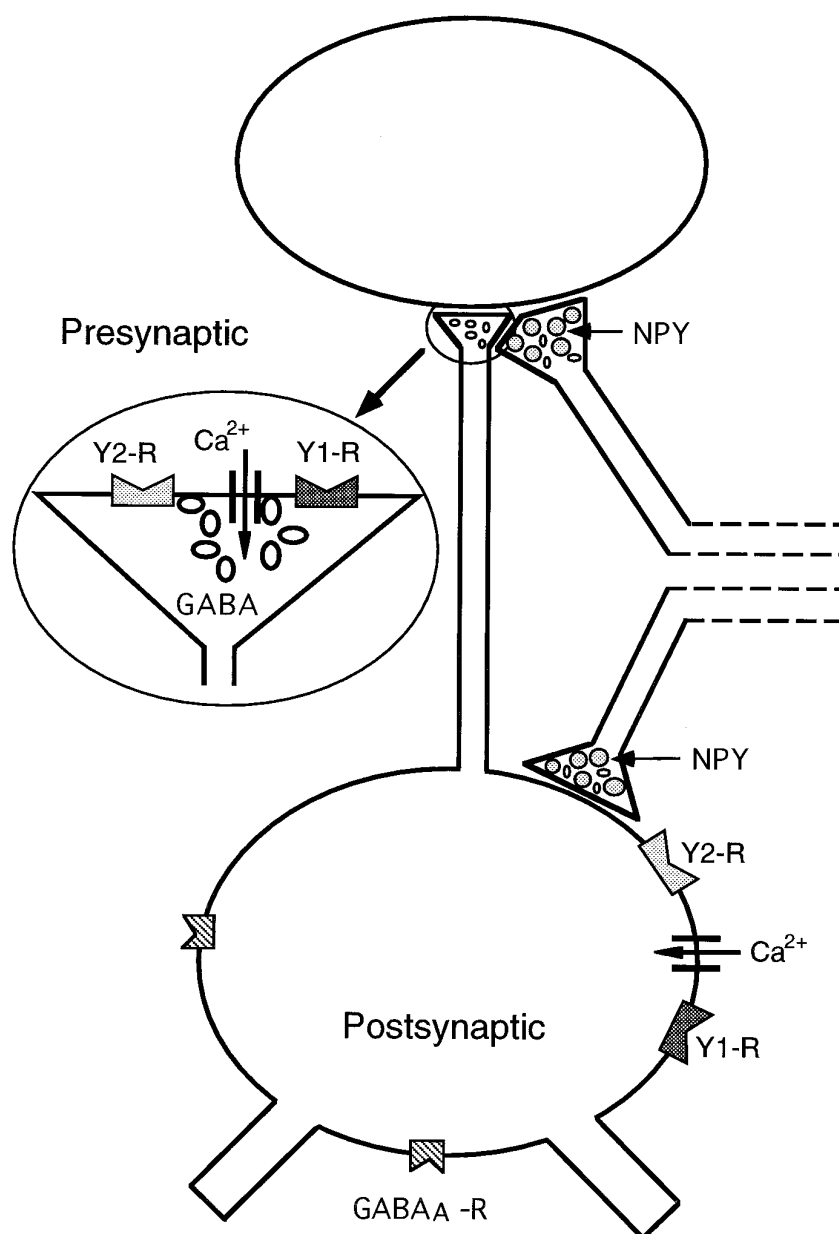


Figure 9. Schematic diagram showing the coexistence of NPY Y1- and Y2-like receptors in both the presynaptic nerve terminals and the postsynaptic cell body.

depending on the time course, mode of application, and circadian state of the SCN. Coupled with the demonstration that single cells express more than one NPY receptor, these data underline the significant level of complexity of NPY actions in the SCN.

REFERENCES

- Adrian TE, Allen JM, Bloom SR, Ghatei MA, Rossor MN, Roberts GW, Crow TJ, Tatemoto K, Polak JM (1983) Neuropeptide Y distribution in human brain. *Nature* 306:584–586.
- Albers HE, Ferris CF (1984) Neuropeptide Y: role in light-dark cycle entrainment of hamster circadian rhythms. *Neurosci Lett* 50:163–168.
- Albers HE, Ottenweller JE, Liou SY, Lumpkin MD, Anderson ER (1990) Neuropeptide Y in the hypothalamus: effect on corticosterone and single-unit activity. *Am J Physiol* 258:R376–R382.
- Allen YS, Adrian TE, Allen JM, Tatemoto K, Crow TJ, Bloom SR, Polak JM (1983) Neuropeptide Y distribution in the rat brain. *Science* 221:877–879.
- Bard JA, Walker MW, Branchek TA, Weinshank RL (1995) Cloning and functional expression of a human Y4 subtype receptor for pancreatic polypeptide, neuropeptide Y, and peptide YY. *J Biol Chem* 270:26762–26765.
- Bekkers JM, Stevens CF (1991) Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell culture. *Proc Natl Acad Sci USA* 88:7834–7838.
- Bleakman D, Colmers WF, Fournier A, Miller RJ (1991) Neuropeptide Y inhibits Ca^{2+} influx into cultured dorsal root ganglion neurones of the rat via a Y2 receptor. *Br J Pharmacol* 103:1781–1789.
- Bleakman D, Harrison NL, Colmers WF, Miller RJ (1992) Investigations into neuropeptide Y-mediated presynaptic inhibition in cultured hippocampal neurones of the rat. *Br J Pharmacol* 107:334–340.
- Boss NPA, Mirmiran M (1990) Circadian rhythms in spontaneous neuronal discharges of the cultured suprachiasmatic nucleus. *Brain Res* 511:158–162.
- Botchkina GI, Morin LP (1995) Organization of permanent and transient neuropeptide Y-immunoreactive neuron groups and fiber systems in the developing hamster diencephalon. *J Comp Neurol* 357:573–602.
- Calza L, Giardino L, Zanni M, Velardo A, Parchi P, Marrama P (1990) Daily changes of neuropeptide Y-like immunoreactivity in the suprachiasmatic nucleus of the rat. *Regul Pept* 27:127–137.
- Card JP, Moore RY (1989) Organization of lateral geniculate-hypothalamic connections in the rat. *J Comp Neurol* 284:135–147.

- Chen G, Trombley PQ, van den Pol AN (1995) GABA receptors precede glutamate receptors in hypothalamic development: differential regulation by astrocytes. *J Neurophysiol* 74:1473–1484.
- Chen G, Trombley PQ, van den Pol AN (1996) Excitatory actions of GABA in rat developing hypothalamic neurones. *J Physiol (Lond)* 494:451–464.
- Chronwall BM, Chase TN, O'Donohue TL (1984) Coexistence of neuropeptide Y and somatostatin in rat and human cortical and rat hypothalamic neurons. *Neurosci Lett* 52:213–217.
- Chronwall BM, DiMaggio DA, Massari VJ, Pickel VM, Ruggiero DA, O'Donohue TL (1985) The anatomy of neuropeptide-Y-containing neurons in rat brain. *Neuroscience* 15:1159–1181.
- Colmers WF, Bleakman D (1994) Effects of neuropeptide Y on the electrical properties of neurons. *Trends Neurosci* 17:373–379.
- Colmers WF, Lukowiak K, Pittman OJ (1987) Presynaptic action of neuropeptide Y in area CA1 of the rat hippocampal slice. *J Physiol (Lond)* 383:285–299.
- Colmers WF, Lukowiak K, Pittman OJ (1988) Neuropeptide Y action in the rat hippocampal slice: site and mechanism of presynaptic inhibition. *J Neurosci* 8:3827–3837.
- Colmers WF, Klapstein GJ, Fournier A, St-Pierre S, Treherne KA (1991) Presynaptic inhibition by neuropeptide Y in rat hippocampal slice *in vitro* is mediated by a Y2 receptor. *Br J Pharmacol* 102:41–44.
- Decavel C, van den Pol AN (1990) GABA: a dominant neurotransmitter in the hypothalamus. *J Comp Neurol* 302:1019–1037.
- Ewald DA, Pang IH, Sternweis PC, Miller RJ (1989) Differential G-protein-mediated coupling of neurotransmitter receptors to Ca^{2+} channels in rat dorsal root ganglion neurons *in vitro*. *Neuron* 2:1185–1193.
- Foucort S, Bleakman D, Bindokas VP, Miller RJ (1993) Neuropeptide Y and pancreatic polypeptide reduce calcium currents in acutely dissociated neurons from adult rat superior cervical ganglia. *J Pharmacol Exp Ther* 265:903–909.
- Francois-Bellan A, Kachidian P, Dosticier G, Tonon MC, Vaudry H, Bosler O (1990) GABA neurons in the rat suprachiasmatic nucleus: involvement in chemospecific synaptic circuitry and evidence for GAD-peptide colocalization. *J Neurocytol* 19:937–947.
- Furshpan EJ, Landis SC, Matsumoto SG, Potter DD (1986) Synaptic functions in rat sympathetic neurons in microcultures. I. Secretion of norepinephrine and acetylcholine. *J Neurosci* 6:1061–1079.
- Garcia de Yebenes E, Li S, Fournier A, St-Pierre S, Pelletier G (1995) Involvement of the Y2 receptor subtype in the regulation of prolactin gene expression by neuropeptide Y in the male rat. *Neurosci Lett* 190:77–80.
- Gerald C, Walker MW, Vaysse PJ, He C, Branchek TA, Weinshank RL (1995) Expression cloning and pharmacological characterization of a human hippocampal neuropeptide Y/peptide YY Y2 receptor subtype. *J Biol Chem* 270:26758–26761.
- Gerald C, Walker MW, Criscione L, Gustafson EL, Batzl-Hartmann C, Smith KE, Vaysse PJ, Durkin MM, Laz TM, Linemeyer DL, Schaffhauser AO, Whitebread S, Hofbauer KG, Taber RI, Branchek TA, Weinshank RL (1996) A receptor subtype involved in neuropeptide-Y-induced food intake. *Nature* 382:168–171.
- Guldner FH, Wolff JR (1978) Self-innervation of dendrites in the rat suprachiasmatic nucleus. *Exp Brain Res* 32:77–82.
- Harrington ME, Nance DM, Rusak B (1985) Neuropeptide Y immunoreactivity in the hamster geniculo-suprachiasmatic tract. *Brain Res Bull* 15:465–472.
- Hendry SH, Jones EG, DeFelipe J, Schmechel D, Brandon C, Emson PC (1984) Neuropeptide-containing neurons of the cerebral cortex are also GABAergic. *Proc Natl Acad Sci USA* 81:6526–6530.
- Herzog H, Baumgartner M, Vivero C, Selbie LA, Auer B, Shine J (1993) Genomic organization, localization, and allelic differences in the gene for the human neuropeptide Y Y1 receptor. *J Biol Chem* 268:6703–6707.
- Hirning LD, Fox AP, Miller RJ (1990) Inhibition of calcium currents in cultured myenteric neurons by neuropeptide Y: evidence for direct receptor/channel coupling. *Brain Res* 532:120–130.
- Huhman KL, Albers HE (1994) Neuropeptide Y microinjected into the suprachiasmatic region phase shifts circadian rhythms in constant darkness. *Peptides* 15:1475–1478.
- Huhman KL, Babagbemi TO, Albers HE (1995) Bicuculline blocks neuropeptide Y-induced phase advances when microinjected in the suprachiasmatic nucleus of Syrian hamsters. *Brain Res* 675:333–336.
- Inouye ST, Kawamura H (1979) Persistence of circadian rhythmicity in a mammalian hypothalamic “island” containing the suprachiasmatic nucleus. *Proc Natl Acad Sci USA* 76:5962–5966.
- Johnson MD (1994) Synaptic glutamate release by postnatal rat serotonergic neurons in microculture. *Neuron* 12:433–442.
- Kalra SP, Fuentes M, Fournier A, Parker SL, Crowley WR (1992) Involvement of the Y-1 receptor subtype in the regulation of luteinizing hormone secretion by neuropeptide Y in rats. *Endocrinology* 130:3323–3330.
- Kim YI, Dudek FE (1991) Intracellular electrophysiological study of suprachiasmatic nucleus neurons in rodents: excitatory synaptic mechanisms. *J Physiol (Lond)* 444:269–287.
- Klapstein GJ, Colmers WF (1993) On the sites of presynaptic inhibition by neuropeptide Y in rat hippocampus *in vitro*. *Hippocampus* 3:103–111.
- Kornhauser JM, Nelson DE, Mayo KE, Takahashi JS (1990) Photic and circadian regulation of c-fos gene expression in the hamster suprachiasmatic nucleus. *Neuron* 5:127–134.
- Larsen PJ, Jukes KE, Chowdrey HS, Lightman SL, Jessop DS (1994) Neuropeptide-Y potentiates the secretion of vasopressin from the neurointermediate lobe of the rat pituitary gland. *Endocrinology* 134:1635–1639.
- Li S, Hong M, Fournier A, St-Pierre S, Pelletier G (1994) Role of neuropeptide Y in the regulation of gonadotropin-releasing hormone gene expression in the rat preoptic area. *Mol Brain Res* 26:69–73.
- Liou SY, Albers HE (1991) Single unit response of neurons within the hamster suprachiasmatic nucleus to neuropeptide Y. *Brain Res Bull* 27:825–828.
- Lubke J, Markram H, Frotscher M, Sakmann B (1996) Frequency and dendritic distribution of autapses established by layer 5 pyramidal neurons in the developing rat neocortex: comparison with synaptic innervation of adjacent neurons of the same class. *J Neurosci* 16:3209–3218.
- Mason R, Harrington ME, Rusak B (1987) Electrophysiological responses of hamster suprachiasmatic neurones to neuropeptide Y in the hypothalamic slice preparation. *Neurosci Lett* 80:173–179.
- McDonald JK, Lumpkin MD, Samson WK, McCann SM (1985) Neuropeptide Y affects secretion of luteinizing hormone and growth hormone in ovariectomized rats. *Proc Natl Acad Sci USA* 82:561–564.
- McQuiston AR, Petrozzino JJ, Connor JA, Colmers WF (1996) Neuropeptide Y1 receptors inhibit N-type calcium currents and reduce transient calcium increases in rat dentate granule cells. *J Neurosci* 16:1422–1429.
- Medanic M, Gillette MU (1993) Suprachiasmatic circadian pacemaker of rat shows two windows of sensitivity to neuropeptide Y *in vitro*. *Brain Res* 620:281–286.
- Moore RY, Card JP (1990) Neuropeptide Y in the circadian timing system. *Ann NY Acad Sci* 611:247–257.
- Moore RY, Eichler VB (1972) Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res* 42:201–206.
- Moore RY, Speh JC (1993) GABA is the principal neurotransmitter of the circadian system. *Neurosci Lett* 150:112–116.
- Nakamura M, Sakanaka C, Aoki Y, Ogasawara H, Tsuji T, Kodama H, Matsumoto T, Shimizu T, Noma M (1995) Identification of two isoforms of mouse neuropeptide Y-Y1 receptor generated by alternative splicing. Isolation, genomic structure, and functional expression of the receptors. *J Biol Chem* 270:30102–30110.
- Obrietan K, van den Pol AN (1996) Neuropeptide Y depresses GABA-mediated calcium transients in developing suprachiasmatic nucleus neurons: a novel form of calcium long-term depression. *J Neurosci* 16:3521–3533.
- Okamura H, Berod A, Julien JF, Geffard M, Kitahama K, Mallet J, Bobillier P (1989) Demonstration of GABAergic cell bodies in the suprachiasmatic nucleus: *in situ* hybridization of glutamic acid decarboxylase (GAD) mRNA and immunocytochemistry of GAD and GABA. *Neurosci Lett* 102:131–136.
- Pelletier G (1990) Ultrastructural localization of neuropeptide Y in the hypothalamus. *Ann NY Acad Sci* 611:232–246.
- Ralph MR, Foster RG, Davis FC, Menaker M (1990) Transplanted suprachiasmatic nucleus determines circadian period. *Science* 247:975–978.
- Rea M (1989) Light increases fos-related protein immunoreactivity in the rat suprachiasmatic nucleus. *Brain Res Bull* 23:577–580.

- Rose PM, Fernandes P, Lynch JS, Frazier ST, Fisher SM, Kodukula K, Kienzle B, Seethala R (1995) Cloning and functional expression of a cDNA encoding a human type 2 neuropeptide Y receptor. *J Biol Chem* 270:22661–22664.
- Rusak B, Robertson HA, Wisden W, Hunt SP (1990) Light pulses that shift rhythms induce gene expression in the suprachiasmatic nucleus. *Science* 248:1237–1240.
- Scheenen WJ, Yntema HG, Willems PH, Roubos EW, Lieste JR, Jenks BG (1995) Neuropeptide Y inhibits Ca^{2+} oscillations, cyclic AMP, and secretion in melanotrope cells of *Xenopus laevis* via a Y1 receptor. *Peptides* 16:889–895.
- Segal MM (1991) Epileptiform activity in microcultures containing one excitatory hippocampal neuron. *J Neurophysiol* 65:761–770.
- Segal MM (1994) Endogenous bursts underlie seizure-like activity in solitary excitatory hippocampal neurons in microcultures. *J Neurophysiol* 72:1874–1884.
- Shibata S, Moore RY (1993) Neuropeptide Y and optic chiasm stimulation affect suprachiasmatic nucleus circadian function *in vitro*. *Brain Res* 615:95–100.
- Simonneaux V, Ouichou A, Craft C, Pevet P (1994) Presynaptic and postsynaptic effects of neuropeptide Y in the rat pineal gland. *J Neurochem* 62:2464–2471.
- Stephan FK, Zucker I (1972) Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc Natl Acad Sci USA* 69:1583–1586.
- Tatemoto K, Carlquist M, Mutt V (1982) Neuropeptide Y—a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* 296:659–660.
- Tong G, Malenka RC, Nicoll RA (1996) Long-term potentiation in cultures of single hippocampal granule cells—a presynaptic form of plasticity. *Neuron* 16:1147–1157.
- Toth PT, Bindokas VP, Bleakman D, Colmers WF, Miller RJ (1993) Mechanism of presynaptic inhibition by neuropeptide Y at sympathetic nerve terminals. *Nature* 364:635–639.
- van den Pol AN (1980) The hypothalamic suprachiasmatic nucleus of rat: intrinsic anatomy. *J Comp Neurol* 191:661–702.
- van den Pol AN, Dudek FE (1993) Cellular communication in the circadian clock, the suprachiasmatic nucleus. *Neuroscience* 56:793–811.
- van den Pol AN, Tsujimoto KL (1985) Neurotransmitters of the hypothalamic suprachiasmatic nucleus: immunocytochemical analysis of 25 neuronal antigens. *Neuroscience* 15:1049–1086.
- van den Pol AN, Finkbeiner SM, Cornell BA (1992) Calcium excitability and oscillations in suprachiasmatic nucleus neurons and glia *in vitro*. *J Neurosci* 12:2648–2664.
- van den Pol AN, Obrietan K, Chen G, Belousov A (1996) Neuropeptide Y-mediated long-term depression of excitatory activity in suprachiasmatic nucleus neurons. *J Neurosci* 16:5883–5895.
- Wahlestedt C, Yanaihara N, Hakanson R (1986) Evidence for different pre- and post-junctional receptors for neuropeptide Y and related peptides. *Regul Pept* 13:307–318.
- Wahlestedt C, Grundemar L, Hakanson R, Heilig M, Shen GH, Zukowska GZ, Reis DJ (1990) Neuropeptide Y receptor subtypes, Y1 and Y2. *Ann NY Acad Sci* 611:7–26.
- Walker MW, Ewald DA, Perney TM, Miller RJ (1988) Neuropeptide Y modulates neurotransmitter release and Ca^{2+} currents in rat sensory neurons. *J Neurosci* 8:2438–2446.
- Weinberg DH, Sirinathsinghji DJS, Tan CP, Shiao LL, Morin N, Rigby MR, Heavens RH, Rapoport DR, Bayne ML, Cascieri MA, Strader CD, Linemeyer DL, MacNeil DJ (1996) Cloning and expression of a novel neuropeptide Y receptor. *J Biol Chem* 271:16435–16438.
- Welsh DK, Logothetis DE, Meister M, Reppert SM (1995) Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron* 14:697–706.
- Wiley JW, Gross RA, Lu YX, MacDonald RL (1990) Neuropeptide Y reduces calcium current and inhibits acetylcholine release in nodose neurons via a pertussis toxin-sensitive mechanism. *J Neurophysiol* 63:1499–1507.
- Wiley JW, Gross RA, MacDonald RL (1993) Agonists for neuropeptide Y receptor subtypes NPY-1 and NPY-2 have opposite actions on rat nodose neuron calcium currents. *J Neurophysiol* 70:324–330.