Coexpression of Multiple Metabotropic Glutamate Receptors in Axon Terminals of Single Suprachiasmatic Nucleus Neurons

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Chen, Gong and Anthony N. van den Pol. Coexpression of multiple metabotropic glutamate receptors in axon terminals of single suprachiasmatic nucleus neurons. J. Neurophysiol. 80: 1932–1938, 1998. Glutamate is the primary excitatory transmitter in axons innervating the hypothalamic suprachiasmatic nucleus (SCN) and is responsible for light-induced phase shifts of circadian rhythms generated by the SCN. By using self-innervating single neuron cultures and patch-clamp electrophysiology, we studied metabotropic glutamate receptors (mGluRs) expressed by SCN neurons. The selective agonists for group I (3,5-dihydroxyphenylglycine), group II ((S)-4-carboxy-3-hydroxyphenylglycine), and group III (l(+)-2-amino-4-phosphonobutyric acid) mGluRs all depressed the evoked IPSC in a subset (33%) of single autaptic neurons, suggesting a coexpression of all three groups of mGluRs in the same axon terminals of a single neuron. Other neurons showed a variety of combinations of mGluRs, including an expression of only one group of mGluR (18%) or coexpression of two groups of mGluRs (27%). Some neurons had no response to any of the three agonists (22%). The three mGluR agonists had no effect on postsynaptic γ-aminobutyric acid (GABA) receptor responses, indicating a presynaptic modulation of GABA release by mGluRs. We conclude that multiple mGluRs that act through different second messenger pathways are coexpressed in single axon terminals of SCN neurons where they modulate the release of GABA presynaptically, usually inhibiting release.

INTRODUCTION

The suprachiasmatic nucleus (SCN) of the hypothalamus is the pacemaker of the mammalian circadian clock and is composed primarily of GABAergic neurons (Chen and van den Pol 1996, 1997; Moore and Speh 1993; van den Pol and Tsujimoto 1985). Glutamate is the major neurotransmitter of the synaptic input from retina to the SCN that mediates light-induced phase shifts of circadian rhythms controlled by the SCN (Cahill and Menaker 1989; Kim and Dudek 1991). Glutamate may activate both ionotropic and metabotropic glutamate receptors in the SCN (for review see van den Pol et al. 1996). Blockade of ionotropic glutamate receptors in the SCN reduces light-induced phase shifting of circadian rhythms (Colwell et al. 1991; Rea et al. 1993). The activation of metabotropic glutamate receptor increases general neuronal activity of SCN neurons in brain slices (Scott and Rusak 1996) and causes a significant alteration in phase shifts of the circadian rhythm (Haak et al. 1997a,b). Metabotropic glutamate receptors (mGluRs) are widely expressed throughout the brain. Although the actions of mGluR agonists were not studied in SCN neurons at the cellular level, in other regions of the brain mGluRs can regulate multiple neuronal functions, such as ion channel modulation, transmitter release, and synaptic plasticity (for review see Nakanishi 1994; Pin and Duvoisin 1995; Schoepp and Conn 1993). In hippocampal neurons, activation of mGluRs inhibits calcium channels (Swartz and Bean 1992), activates nonselective cationic channels (Miller et al. 1995), modulates both excitatory and inhibitory neurotransmission (Fitzsimonds and Dichter 1996; Gereau and Conn 1995), and induces long-term potentiation (Bashir et al. 1993; Bortolotto et al. 1994; O’Connor et al. 1994). mGluRs also modulate ion channels and transmitter release in olfactory bulb (Trombley and Westbrook 1992), cerebellum (Bacher and Garthwaite 1997; Shigemoto et al. 1994), cortex (Choi and Lovinger 1996), and a variety of other brain regions.

There are eight subtypes of mGluRs, categorized into three groups. Activation of group I mGluRs (mGluR 1 and 5) stimulates inositol phosphate hydrolysis, and group II (mGluR 2 and 3) and group III (mGluR 4, 6, 7, and 8) are negatively coupled to adenylate cyclase (Pin and Duvoisin 1995; Schoepp and Conn 1993). Specific agonists for different mGluRs are available. For example, 3,5-dihydroxyphenylglycine (DHPG) specifically activates group I mGluRs, (S)-4-carboxy-3-hydroxyphenylglycine (4C3HPG) activates group II mGluRs, and l(+)-2-amino-4-phosphonobutyric acid (l-AP4) activates group III mGluRs (Hayashi et al. 1994; Schoepp et al. 1994; Thomsen et al. 1994; Tones et al. 1995; Wright and Schoepp 1996).

Multiple subtypes of mGluRs were found in the SCN with immunocytochemical and molecular biological methods (Ghosh et al. 1997; Romano et al. 1995; van den Pol et al. 1994). The unique expression of all eight cloned mGluRs in the SCN allowed us to test at the cellular level whether a single neuron may express functional mGluRs from each of the three groups. This paper, by using whole cell recordings in single-cell cultures of SCN neurons, demonstrates that all three groups of mGluRs are coexpressed in the axon terminals of a single neuron.

METHODS

Microisland culture

Microcultures of central neurons were demonstrated to be a powerful model in studying neurotransmission, neuromodulation, and synaptic plasticity (Bekkers and Stevens 1991; Chen and van den Pol 1996, 1997; Purshpan et al. 1986; Issacson and Hille 1997; Johnson 1994; Tong et al. 1996). Microisland culture of SCN neurons was performed as previously described (Chen and van den Pol 1996, 1997). Briefly, the SCN was dissected from brain slices...
containing the optic chiasm of neonatal Sprague-Dawley rats (P1–P2). The tiny tissue blocks were enzymatically treated for 30 min with a solution containing 10 units/ml papain, 0.5 mM ethylenediaminetetraacetic acid, 1.5 mM CaCl₂, and 0.2 mg/ml l-cysteine. Cells were dissociated by mechanical trituration after washing the enzyme twice with culture medium. The medium contained minimal essential medium (GIBCO) supplemented with 10% fetal calf serum (Hyclone) and serum extender (Collaborative Research), 100 units/ml penicillin/streptomycin, and 6 g/l glucose. A very low density of cells (2,000 cells/cm²) was plated on preconstructed drug application apparatus, we have previously shown that repeated application of glutamate and mside (5 mM) at a holding potential of 0 mV was used to evoke an autaptic response (Fig. 1) at a holding potential of −70 mV. The recording chamber was constantly perfused (2 ml/min) with a bath solution containing (in mM) 155 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose, pH 7.3. Experiments were carried out at room temperature (22°C) between 8:00 A.M. and midnight.

Drugs were applied through a series of glass flow pipes (400 μm id) fed by gravity. By using the same recording system and drug application apparatus, we have previously shown that repeated application of glutamate and γ-aminobutyric acid (GABA) through flow pipes induced reproducible-amplitude ionic currents (Chen et al. 1995). A Picospritzer (General Valve) under computer control was employed to puff GABA locally to recorded neurons through a micropipette with a tip diameter of 2–3 μm. mGlur agonists DHPG, 4ClHPG, and l-AP4 were purchased from Tocris. GABA, (−)bicuculline, Mg-ATP, and Na₂GTP were purchased from Sigma.

**Immunocytochemistry**

Microisland cultures were washed with 0.1 M phosphate buffer (PB) and fixed for 20 min in 3% glutaraldehyde for GABA immunostaining. After fixation, cultures were washed three times with washing buffer (0.4% Triton + 0.1% l-lysine + 1% BSA in PB) and incubated for 20 min in blocking buffer (2% whole goat serum in washing buffer) and then incubated overnight with primary antibody. The primary GABA antibody (from Incstar) was diluted by 1:5000. The primary antibody was washed off with several changes of washing buffer and then incubated with a biotinylated secondary antibody (1:200, Vector) for 1 h. After washing the second antibody, cultures were incubated in an avidin–biotin–peroxidase conjugate (Vector Labs) for 45 min. After washing the reagent, cultures were incubated in diaminobenzidine and hydrogen peroxide for 3–4 min. Controls that received only normal serum without primary antibody showed no specific staining. Specificity of the GABA antiserum (Decavel and van den Pol 1990) was described in detail previously.

**Electrophysiology**

Detailed experimental procedures for whole cell voltage-clamp recordings were described in our previous work (Chen and van den Pol 1996, 1997). A List EPC-7 amplifier and an Apple Macintosh computer with AxoData software were used to acquire data. Data were sampled at 2–5 kHz and filtered at 1 kHz by an eight-pole Bessel filter and off-line processed by Axograph and Igor Pro software. Micropipettes (WPI) were pulled from borosilicate glass with a vertical Narishige puller. The pipette resistance was about 3–4 MΩ after filling with pipette solution. The series resistance was about 10–20 MΩ and compensated by 50–70% with the amplifier. The pipette solution contained (mM) 145 KCl, 0.5–2 K4-bis(o-aminophenoxy)-N,N,N’,N’-tetraacetic acid, 10 N-2-hydroxyethylpiperazine-Ν’-2-ethanesulfonic acid (HEPES), 4 Mg-ATP, 0.5 Na₂GTP, pH 7.3. For electrophysiological recording, only a single neuron sitting on an astrocyte microisland (as illustrated in Fig. 1) was chosen for each experiment. After forming a tight-seal for whole cell recording, a brief depolarizing pulse (1–2 ms, 70–80 mV) was used to evoke an autaptic response (Fig. 1) at a holding potential of −70 mV. The recording chamber was constantly perfused (2 ml/min) with a bath solution containing (in mM) 155 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose, pH 7.3. Experiments were carried out at room temperature (22°C) between 8:00 A.M. and midnight.

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RESULTS

Multiple mGluR expression in single SCN neuron

Multiple mGluRs are found throughout the brain. All eight subtype mGluRs were detected in the SCN with immunocytochemistry and complementary deoxyribonucleic acid—polymerase chain reaction (cDNA-PCR) (Ghosh et al. 1997; Romano et al. 1995; van den Pol et al. 1994). Because the cellular function of these mGluRs has not been studied in the SCN, we investigated the mGluR modulation in SCN microcultures with patch-clamp electrophysiology. Figure 1A shows a solitary SCN neuron immunostained with GABA in microculture (23 days in vitro). Figure 1Bi illustrates the autaptic inhibitory postsynaptic current (IPSC) preceded by a sodium action current (INa), evoked by a short depolarization in the single neuron shown in A. Figure 1B2 shows the inhibition of the IPSC by GABA-A receptor antagonist bicuculline (BIC, 30 μM). Consistent with previous findings, our data show that most SCN neurons in microculture are GABAergic and consistent with previous analyses based on cytochemistry and RNA analysis (Ghosh et al. 1997; van den Pol et al. 1994, 1995), express functional mGluRs.

To study the possible function of mGluRs in the SCN, glutamate was applied to SCN neurons while blocking ionotropic glutamate receptors. Figure 2 shows that in the presence of ionotropic glutamate receptor antagonists d,L-2-amino-5-phosphonopentanoic acid (AP5, 100 μM) and cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX, 20 μM), application of glutamate (50 μM) inhibited the evoked autaptic IPSC, indicating that activation of mGluRs can modulate GABAergic neurotransmission of SCN neurons (n = 6). Glutamate application had no effect on IPSCs in three other neurons tested, suggesting a heterogenous expression of mGluRs in individual SCN neurons. Therefore we used more selective agonists to characterize the subtypes of mGluRs expressed in SCN neurons.

Because the eight subtypes of mGluRs are divided into three groups, we tried to determine which subgroup of mGluRs was expressed in single SCN neurons with different specific mGluR agonists. There are a variety of mGluR agonists for each group. In this study, DHPG (20–200 μM), 4C3HPG (20–200 μM), and l-AP4 (50–500 μM) were chosen to activate different groups of mGluRs because of their high selectivity for group I, II, and III mGluRs respectively (Hayashi et al. 1994; Schoepp et al. 1994; Thomsen et al. 1994; Tones et al. 1995; Wright and Schoepp 1996). For each agonist, we used a concentration equivalent to that previously published (Libri et al. 1997; Pisani et al. 1997; Taylor et al. 1995; Yu et al. 1997). Some experiments used a relatively high concentration to maximize the possibility of detecting a mGluR response. Figure 3 illustrates examples of different single SCN neurons expressing different group of mGluRs. Figure 3A illustrates an example showing only a response to group III mGluR agonist l-AP4 (500 μM) but not to DHPG (200 μM) or 4C3HPG (200 μM). B: single SCN neuron showing a response to only the group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG, 200 μM) but no response to the group II agonist (S)-4-carboxy-3-hydroxyphenylglycine (4C3HPG, 200 μM) or group III agonist and L(+)-2-amino-4-phosphonobutyric acid (l-AP4, 500 μM). Each labeled trace corresponds to a labeled point in the line graph (same for B and other figures). The sodium current and capacitance current are not shown (same for B and other figures). Note that DHPG had a long-lasting inhibitory effect on the IPSC. C: single neuron not responding to any of the 3 mGluR agonists. Time of drug application indicated by rectangle.
FIG. 4. Coexpression of group I and group III mGluRs in a single neuron. Application of DHPG (50 μM) and 1-AP4 (100 μM) inhibited the autaptic IPSC, whereas 4C3HPG (200 μM) had no effect. DHPG inhibition of the IPSC only partially recovered after washing off the agonist.

FIG. 5. Coexpression of all 3 groups of mGluRs in a single autaptic SCN neuron. Application of DHPG (50 μM), 1-AP4 (100 μM), and 4C3HPG (50 μM) all depressed the evoked IPSC in the same neuron. Note that the inhibition of 1-AP4 and 4C3HPG was mostly reversible, but DHPG produced a longer-lasting depression of the IPSC.
whereas one-fifth of the neurons showed no mGluR responses at all. As for each group of mGluRs, most SCN neurons expressed group III mGluRs (71%), and fewer neurons expressed group I (58%) or group II (42%) mGluRs. The percentages of neurons expressing three groups, two groups, one group, and no expression of mGluRs were 33, 27, 18, and 22%, respectively. The degree of inhibition by DHPG, 4C3HPG, and l-AP4 in responsive cells was $-24.8 \pm 0.7$, $-26.9 \pm 1.3$, and $-30.2 \pm 0.6$ (mean ± SE), respectively.

**Postsynaptic GABA receptors are not modulated by mGluRs**

The modulation of evoked autaptic IPSCs by activation of mGluRs may be due to a change of the presynaptic GABA release or alternatively to a change in the postsynaptic GABA receptor responses. To differentiate a pre- versus postsynaptic action in mGluR modulation of GABA neurotransmission, we compared the effect of mGluR agonists on presynaptic GABA release-induced responses with postsynaptic receptor responses evoked by pressure ejection of GABA from an external pipette recorded in the same cell. Figure 6A illustrates such an experimental arrangement. First, a brief depolarizing pulse in the recorded neuron triggered an autaptic GABA release from axon terminals. Then a short puff of GABA (10 ms, 100 μM) from a micropipette onto the recorded neuron elicited a postsynaptic GABA receptor response. Figure 6, B – D, illustrates the simultaneous recording of presynaptic GABA release-induced responses and pressure ejection of GABA-induced postsynaptic responses in the same sweeps. Whereas the presynaptic GABA release induced-responses were significantly depressed by application of mGluR agonists, the postsynaptic GABA receptor responses showed no change in the same cell (Fig. 6, B – D). Figure 6E shows the grouped data on postsynaptic GABA receptor responses before and after treatment of mGluR agonists. None of the three agonists tested had detectable modulatory effects on postsynaptic GABA receptor responses (paired Student’s t-test, $P > 0.5$). As flow-pipe application of GABA may activate receptors in a wider area of the membrane than synthetically released GABA, it is possible that a subtle postsynaptic action of mGluRs at the synapse may have gone undetected. However, even when the amplitude of the response to flow-pipe application of GABA was smaller than to synthetically released GABA, we still were unable to detect an effect of the mGluR agonists on the postsynaptic cell. These results indicate that the modulation of GABA neurotransmission by mGluRs occurs primarily at presynaptic sites in SCN axon terminals.

**TABLE 1. Multiple expression of mGluRs in single autaptic SCN neurons**

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<th>mGluR Agonist</th>
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<td>4C3HPG</td>
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<td>l-AP4</td>
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Each single neuron was treated with three agonists. An agonist inducing a change of the autaptic inhibitory postsynaptic current >10% of the control was considered effective (+); otherwise no effect was assumed (−). mGluR, metabotropic glutamate receptors; SCN, suprachiasmatic nucleus; DHPG, 3,5-dihydroxyphenylglycine; 4C3HPG, (3)-4-carboxy-3-hydroxyphenylglycine; l-AP4, L(+)-2 amino-4-phosphonobutyric acid.

**DISCUSSION**

This study demonstrates for the first time that the activation of all three groups of mGluRs presynaptically modulates GABA neurotransmission in a single neuron, indicating a coexpression of all three groups of mGluRs in single axon terminals. Microisland culture of single autaptic neurons was used as a powerful model in demonstrating co-release of serotonin.
and glutamate in rat mesopontine neurons (Johnson 1994) and in elucidating mechanisms of long-term synaptic plasticity (Goda and Stevens 1996; Tong et al. 1996). We took advantage of this model to show that multiple mGluRs are coexpressed in axon terminals of a single SCN neuron. Some SCN neurons responded to only a single agonist, some to none of the agonists, and others to various combinations of the three agonists, indicating a degree of variability in mGluR expression among SCN neurons. In addition to the differences in mGluR expression reported here, differences in the expression of neuroactive peptides used by different SCN neurons were previously found (van den Pol and Tsujimoto 1985), and differences in afferent innervation occur within the nucleus. SCN cells can show nonsynchronized circadian rhythms of electrical activity in vitro (Welsh et al. 1995); levels of activity may influence expression of mGluRs (Bessho et al. 1993). It is possible therefore that mGluR receptors or their second messenger systems may show a circadian variation, and this may account for some of the differences we found between cells.

In agreement with our present findings with electrophysiology, previous work in our laboratory with reverse Northern blots and cDNA-PCR detected all eight subtypes of mGluR mRNA in the SCN in vivo (Ghosh et al. 1997). Postsynaptic GABA receptors were not affected by activation of mGluRs in our experiments, suggesting a presynaptic mechanism of mGluR modulation on GABA neurotransmission in SCN neurons. Previous studies with immunocytochemistry did not detect a presynaptic location of mGluR1 but did for mGluR5 in the hypothalamus (Romano et al. 1995; van den Pol et al. 1994, 1995). In light of the cytochemistry results, the presynaptic modulation of GABA transmission by DHPG may be due to an activation of mGluR5. The ultrastructural distribution of group II III mGluRs in the hypothalamus was not explored yet. However, a presynaptic localization of both group II and III mGluRs has been found in other brain areas (Hayashi et al. 1993; Shigemoto et al. 1997), in accordance with our present findings in the SCN.

The presynaptic modulation of evoked GABA release by all three groups of mGluRs in our experiments does not argue against the expression of these mGluRs in postsynaptic membranes. Rather, it suggests that the postsynaptic GABA receptors do not appear to be affected by mGluRs, although the mGluRs reported in postsynaptic cells and dendrites immunocytochemically (van den Pol et al. 1994) may modulate the actions of other receptors, for instance, ionotropic glutamate receptors.

The SCN, composed primarily of GABAergic neurons (Moore and Speth 1993; van den Pol and Tsujimoto 1985), functions as a circadian clock in the mammalian brain. The clock is reset, or phase shifted, by afferent glutamate projections from the retina that synapse directly in the SCN (Cahill and Menaker 1989; Ding et al. 1994; Kim and Dudek 1991). A single brief exposure to light can evoke glutamate secretion in the SCN and phase shift the clock either by 1–2 h, or not at all, depending on the circadian time of the clock, suggesting a differential response to glutamate. Preliminary data indicate that injections of the mGluR agonist t-ACPD into the SCN alters the phase shift in response to light stimulation (Haak et al. 1997). Although we did not address the question of mGluR modulation in a circadian context, our data show a substantial mGluR-mediated presynaptic modulation of GABA release from SCN neurons, and this could play a role in light-induced phase shifting of the circadian clock. In parallel, the long depression of GABA release mediated by group I mGluRs could also serve as a potential cellular substrate for phase shifts in response to light, consistent with the behavioral data (Haak et al. 1997). As we find little evidence for a direct enhancement of activity by mGluR agonists, the reported increase in SCN activity evoked by mGluR agonists (Scott and Rusak 1996) could be due to a decrease in GABA inhibition, rather than a simple direct increase in excitation.

That the mGluRs appear to act primarily on presynaptic SCN axons suggests that glutamate could not only enhance excitation in SCN cells directly but also by reducing GABA release from presynaptic terminals either within the SCN or in its efferent projections. As the different groups of mGluRs we find in SCN neurons can act on several different second messenger pathways (Nakanishi 1994), mGluRs could thus modulate calcium, IP3, and cAMP-related signaling in the presynaptic axon.

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REFERENCES


CHOI, S. AND LOVINGER, D. M. Metabotropic glutamate receptor modulation...


