Group I Metabotropic Glutamate Receptor Activation Produces a Direct Excitation of Identified Septohippocampal Cholinergic Neurons

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Wu, Min, Tibor Hajszan, Changqing Xu, Csaba Leranth, and Meenakshi Alreja. Group I metabotropic glutamate receptor activation produces a direct excitation of identified septohippocampal cholinergic neurons. J Neurophysiol 92: 1216–1225, 2004. First published March 24, 2004; 10.1152/jn.00180.2004. Septohippocampal cholinergic neurons innervate the hippocampus and provide it with almost its entire acetylcholine. Axon collaterals of these neurons also release acetylcholine within the septum and thereby maintain the firing activity of septohippocampal GABAergic neurons. A loss of septohippocampal cholinergic neurons occurs in various neurodegenerative disorders associated with cognitive dysfunctions. Group I metabotropic glutamate receptors have been implicated in septohippocampal-dependent learning and memory tasks. In the present study, we examined the physiological and pharmacological effects of a potent and selective group I metabotropic glutamate receptor (mGluR) agonist 5,3,5-dihydroxyphenylglycine (DHPG) on rat septohippocampal cholinergic neurons that were identified in brain slices using a selective fluorescent marker. In whole cell recordings, DHPG produced a reversible, reproducible and a direct postsynaptic and concentration-dependent excitation in 100% of septohippocampal cholinergic neurons tested with an EC50 of 2.1 μM. Pharmacologically, the effects of DHPG were partially/completely reduced by the mGluR1 antagonists, 7-hydroxyn-myonolycoproban[bichromen-1a-carboxylic acid ethyl ester and (+)-2-methyl-4-carboxyphenylglycine. Addition of the mGluR5 antagonist, 2-methyl-6-(phenylethyl)pyridine hydrochloride, reduced the remaining response to DHPG, suggesting involvement of both receptor subtypes in a subpopulation of septohippocampal cholinergic neurons. In double-immunolabeling studies, 74% of septohippocampal cholinergic neurons co-localized mGluR1α-immunoreactivity and 35% co-localized mGluR5-immunoreactivity. Double-immunolabeling studies at the light and electron-microscopic levels showed that vesicular glutamate transporter 2 terminals make asymmetric synaptic contacts with septohippocampal cholinergic neurons. These findings may be of significance in treatment of cognitive deficits associated with neurodegenerative disorders as a group I mGluR-mediated activation of septohippocampal cholinergic neurons would enhance the release of acetylcholine both in the hippocampus and in the septum.

INTRODUCTION

Septohippocampal cholinergic neurons of the medial septum/diagonal band of Broca (MSDB) control the activity of the hippocampus via direct cholinergic projections and via a muscarinic activation of the septohippocampal GABAergic pathway (Alreja et al. 2000; Wu et al. 2003b). A loss or atrophy of septohippocampal cholinergic neurons accompanies the cognitive deficits in neurodegenerative disorders such as Alzheimer’s disease, alcoholic Korsakoff syndrome (Izquierdo 1989; Kopelman and Corn 1988), Parkinson’s disease, Lewy body dementia, and Down’s syndrome (Arendt et al. 1995; Mulson et al. 1989; Whitehouse et al. 1982). Therapeutic measures aimed at preventing degeneration of cholinergic neurons and/or maximizing the function of the remaining neurons are therefore considered key strategies for treatment of cognitive disorders. Identifying neurotransmitters that enhance septohippocampal cholinergic neuron activity, and thereby increase hippocampal and septal acetylcholine (ACh) release could be important for treatment of cognitive disorders and understanding normal cognition.

L-glutamate is the principal excitatory neurotransmitter in the brain that acts through fast-acting ionotropic as well as through the slower-acting G-protein-coupled metabotropic receptors (mGluRs). The group I metabotropic receptors, which are comprised of the mGluR1 and mGluR5 subtypes are widely distributed in the brain and mediate the slow excitatory postsynaptic effects of glutamate (Gee et al. 2003; Marino et al. 2001; Pisani et al. 2001; Valenti et al. 2002; Zheng and Johnson 2003). Group I mGluRs are required for formation of fear memory and long-term potentiation (LTP) in the amygdala (Rodrigues et al. 2002). mGluR1 may also mediate the excitatory effects of β amyloid 1–42 on human neurons (Blanchard et al. 2002). Mice lacking mGluR1 are severely impaired in a hippocampus-dependent context-specific associative-learning task (Aiba et al. 1994) and blockade of group I mGluRs prevents some types of spatial learning (Balschun and Wetzel 2002; Petersen et al. 2002). Although group I metabotropic glutamate receptors are known to modulate the activity of hippocampal neurons, their effects on the activity of long-projecting cholinergic neurons that are critical for spatial memory is not known.

The MSDB, which contains the septohippocampal cholinergic neurons and controls the hippocampal theta rhythm and associated spatial learning and memory processes, has a high density of glutamate fibers originating from the frontal cortex (Jaskiw et al. 1991), nucleus reuniens thalami (Bokor et al. 2002), entorhinal cortex (Leranth et al. 1999), and supramammillary nucleus (Leranth and Kiss 1996) or even locally (Wu et al. 2003a). Whether the septohippocampal cholinergic neurons are synaptically innervated by glutamate fibers is not known. It is also not known whether MSDB neurons express group I mGluRs. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
metabotropic glutamate receptors. In light of the importance of the group I metabotropic glutamate receptors in septohippocampal pathway-related learning and memory tasks, the goals of the present study were to study the cellular and pharmacological actions of group I metabotropic glutamate receptor agonists on selectively labeled rat septohippocampal cholinergic neurons in brain slices, determine whether mGlurR1 and/or mGlur5 immunoreactivity is present within the MSDB and if it co-localizes within the septohippocampal cholinergic neurons, and determine whether septohippocampal cholinergic neurons are synaptically innervated by glutamatergic terminals.

METHODOLOGY

All animal protocols used in this study were compliant with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Yale University.

Labeling of septohippocampal cholinergic neurons using Cy3-192IgG

Septohippocampal cholinergic neurons were identified in the living state using the fluorescent marker, Cy3-192IgG, as previously described (Alreja et al. 2000; Wu et al. 2000, 2003b). This technique exploits the fact that septohippocampal cholinergic neurons, but not the GABAergic neurons of the MSDB, exclusively express the low-affinity nerve growth factor receptor, p75. Thus Cy3-192IgG, a conjugate of the fluorochrome, Cy3 and an antibody against the p75 receptor (192IgG), is taken up only by cholinergic neurons and thus selectively labels only the cholinergic subpopulation. The specificity of this marker and its inert nature has been thoroughly confirmed by us (Alreja et al. 2000; Wu et al. 2000, 2003b) and by others in an earlier study (Hartig et al. 1998).

Cy3-192IgG (3–5 μl; 0.4 mg/ml) was stereotaxically injected bilaterally into the lateral ventricle of anesthetized rats with a Hamilton syringe (22-gauge needle) at a rate of 0.5 μl/min. The coordinates used were: 0.8 mm posterior from bregma, 1.2 mm lateral from midline, and 3–4 mm below the dura. Two to 5 days later, slices containing the MSDB were prepared from Cy3-192IgG-injected rats and used for electrophysiological recordings. All recordings were performed on neurons contained within the medial septum and the vertical limb of the diagonal band of Broca as cholinergic cell bodies in these regions project primarily to the hippocampus.

Slice preparation for electrophysiological recordings

Brain slices containing the MSDB were prepared from Cy3-192IgG-injected male Sprague-Dawley albino rats (2–4 wk old) using methods detailed previously (Alreja and Liu 1996). Briefly, rats were anesthetized with chloral hydrate (400 mg/kg ip) and killed by decapitation. The artificial cerebrospinal fluid (ACSF; pH: 7.35–7.38), equilibrated with 95% O2-5% CO2, contained (in mM) 128 NaCl, 3 KCl, 1.25 NaH2PO4, 10 d-glucose, 26 NaHCO3, 2 CaCl2, and 2 MgSO4. After decapitation, brains were removed and placed in a petri dish containing ACSF and trimmed to yield a small block containing the MSDB. From each brain, two coronal slices of 380 μm thickness were obtained with a Vibratome 1500 (Vibratome, St. Louis, MO) and transferred to a Plexiglas recording chamber (1.5-ml volume) on the fixed stage of an Olympus BXX50WI scope. The slices were maintained at 33 ± 0.5°C. One or 2 h later, the slice was used for recording. The chamber was continuously perfused with normal ACSF at a rate of 2–3 ml/min. Visualized whole cell patch-clamp recordings on identified septohippocampal cholinergic neurons were performed using the IR-DIC setup (see following text).

Fluorescence and infrared imaging

Infra-red differential interference contrast imaging (IR-DIC) was performed to visualize neurons for patch-clamp recording using an Olympus Optical BX-50 microscope (Olympus, Tokyo) using methods described in earlier studies (Alreja et al. 2000; Wu et al. 2000). Cy3-192IgG-labeled neurons were visualized using the appropriate fluorescence filter. A neuron viewed with infrared optics was considered same as that viewed with fluorescence optics when the infrared image and the fluorescent image of the neuron had the same position and orientation.

Whole cell recordings from visualized septohippocampal cholinergic neurons

The image of the cells in the slice was displayed on a video monitor, and glass pipettes used for electrophysiological recordings were visually advanced through the slice to the surface of the cell from which recordings were made. Whole cell patch-clamp recordings were performed using previously described methods (Alreja and Liu 1996). In brief, low-resistance (2.5–3.5 MΩ) patch pipettes were filled with a solution containing (in mM) 120 K methylsulfonate, 10 HEPES, 5 BPAT K+, 20 sucrose, 2.38 CaCl2, 1 MgCl2, 1 K2ATP, and 0.1 GTP(pH: 7.32–7.35).

Data were acquired using an Axoclamp-2B and pClamp 9 (Axon Instruments, Foster City, CA). In current-clamp recordings, the output signal was filtered at 10 kHz. Voltage-clamp recordings were performed using the continuous single-electrode voltage-clamp mode. The current and voltage signals were amplified and displayed on storage oscilloscopes and continuously recorded on a chart recorder (Gould 2200).

Agonist and antagonist application

Antagonists were delivered via bath application. Agonists were applied via a Y-tube (Wu et al. 2003a). Briefly, a homemade U-tube (PE 20 tubing) was converted to a Y-tube by gluing in a1-cm MicroFil tubing (100 μm ID, 164 μm OD; CMF34GxL, World Precision Instruments, Sarasota, FL) to a small hole made in the U-tube. This modification allowed the tip to be placed within 100–150 μm from the surface of the recorded neurons. This way, several agonists could be applied to a single neuron and it took only 15 s to switch from one drug to another. The time of application of drugs was determined by an electronically controlled solenoid. A delay between onset of the valve and response of a neuron was <500 ms. Quick removal of the agonist was ensured both by the rapid bath superfusion (2–3 ml/min) and the absence of any leakage of agonist from the Y-tube.

Double-immunolabeling studies

ANIMALS. Male Sprague Dawley rats (n = 10; 280–300 g; Charles River Laboratories, Wilmington, MA) were maintained on a 12/12 h light/dark cycle and provided with unlimited access to water and rat chow.

Tissue processing for light microscopy. Rats were deeply anesthetized using a ketamine-based anesthetic (ketamine (25 mg/ml), xylazine (1.2 mg/ml), and acepromazine (0.03 mg/ml) in saline; 3 ml/kg im) and perfused transcardially with 50 ml phosphate-buffered saline (PBS; pH = 7.60) followed by 400 ml 4% paraformaldehyde diluted in PBS. For electron microscopy, a fixative containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 15% picric acid in PBS was used. Subsequently, the brains were removed and postfixied in 4% paraformaldehyde in PBS at 4°C overnight. Coronal vibratome sections (50 μm thick) were cut into four separate sets throughout the MSDB and collected in PBS. The tissue was immunostained as described in the following text, then the sections were mounted on
gelatin-coated slides, air-dried, cleared in xylene, and coverslipped with Permount. Fluorescent sections were mounted from PBS and wet coverslipped with Vectashield (Vector Laboratories, Burlingame, CA). Immunoreactions were analyzed under either an Olympus BX60 light (Olympus Optical, Tokyo) or a Zeiss Axiophot epifluorescent microscope both equipped with a Zeiss AxioCam digital camera.

**IMMUNOHISTOCHEMICAL PROCEDURES.** Vesicular glutamate transporter 2 (VGLUT2)/choline acetyltransferase (ChAT) double immunoperoxidases labeling. To visualize glutamatergic axons and septohippocampal cholinergic neurons, immunostaining for VGLUT2 [VGLUT1 expression is very low in the MSDB (Fremeau et al. 2001)] and ChAT was applied, respectively. For simultaneous immunoperoxidase labeling of VGLUT2- and ChAT-containing structures, sections were incubated overnight in a mixture of polyclonal guinea pig anti-VGLUT2 (Chemicon, Temecula, CA; 1:20,000) and polyclonal sheep anti-ChAT (Chemicon; 1:2000) antisera diluted in PBS containing 2% normal horse serum and 0.5% triton X-100; overnight at room temperature (RT) followed by ABC Elite (Vector Laboratories; 1:500 in PBS; 2 h at RT). After a brief rinse in Tris-buffered saline (TBS; pH = 7.60), VGLUT2-immunoreactive (IR) elements were visualized using a nickel-diaminobenzidine chromogen (0.4 mg/ml 3,3′-diaminobenzidine tetrahydrochloride, 0.4 mg/ml nickel ammonium sulfate, and 0.0006% hydrogen peroxide dissolved in TBS). This reaction resulted in the black staining of VGLUT2-IR elements. The ChAT-IR neuronal structures were visualized by incubating the sections (that were already immunostained for VGLUT2) in biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:500 in PBS containing 2% normal horse serum and 0.5% triton X-100; overnight at room temperature (RT)) followed by ABC Elite (Vector Laboratories; 1:500 in PBS; 2 h at RT). After a brief rinse in Tris-buffered saline (TBS; pH = 7.60), VGLUT2-immunoreactive (IR) elements were visualized using a nickel-diaminobenzidine chromogen (0.4 mg/ml 3,3′-diaminobenzidine tetrahydrochloride and 0.0006% hydrogen peroxide dissolved in TBS) that resulted in light brown staining of ChAT-IR neurons.

**Quantitative analysis and digital imaging.** Quantitative analysis of VGLUT2 input to ChAT-positive neurons in the MSDB. Sections from five animals were double-immunostained for VGLUT2 and ChAT as described in the preceding text. Then by high-magnification light-microscopic screening of both sides of the MSDB at three different rostrocaudal levels [rostral: Bregma +0.80; middle: Bregma +0.40; caudal: at Bregma, ChAT cells were counted and sorted into two groups. ChAT neurons were sorted into the first group if they were contacted by VGLUT2 boutons according to the following criteria: ChAT cells were considered as targets of VGLUT2-IR boutons if at least one VGLUT2 varicosity was found in close apposition either to the cell body or to a dendrite being continuous with the perikaryon. Close appositions were defined as sites suggestive of synaptic input, i.e., side-to-side contacts with both the VGLUT2 bouton and the ChAT profile being strictly in the same focal plane with no discernible gap between the structures. Cholinergic cells that did not fulfill these criteria were sorted into the second group. Finally, the amount of cholinergic neurons contacted by VGLUT2 boutons (1st group) was expressed as the percentage of all ChAT cells in the MSDB (1st group + 2nd group).

**Quantitative analysis of expression of mGlur5 in cholinergic neurons.** All sections from five animals double-labeled either for mGlur1α/ChAT or mGlur5/ChAT were analyzed. Using the ×40 objective lens, pairs of digitized images were taken first with the filter for FITC then for Texas Red. The photos covered the entire area of the MSDB. The mGlur5-IR neuron profiles were counted and the corresponding image pairs were superimposed using the Adobe PhotoShop 6.0 software. Thereafter, ChAT-positive and double-labeled cells were counted in the merged pictures. Group means were calculated and the amount of double-labeled neurons was expressed as percentages of the total number of cholinergic cells. Tables of pictures were assembled and lettering was added using the Adobe PhotoShop software.

**Drugs.**

S-3,5-dihydroxyphenylglycine (DHPG), (+)-2-methyl-4-carboxyphenylglycine (LY367385), 7-hydroxy-6-methyl-2-aminoclopropan[b]chromeno-1a-carboxylic acid ethyl ester (CPCCoEt), and 2-methyl-6-(phenylethyl)pyridine hydrochloride (MPEP) were all obtained from Tocris. Muscarine chloride were obtained from Sigma. TTX was obtained from Alomone Labs. Glutamate receptor antagonists (AP-5, 50 μM; CNQX, 25 μM) and GABA A receptor antagonists (bicuculline or gabazine, 10–30 μM) were also obtained from Sigma. Most drugs were diluted in ACSF from previously prepared stock solutions that were prepared in water and stored at −20 °C. CPCCoEt was mixed in DMSO to obtain a 100 mM stock solution and diluted in ACSF to a final concentration of 100 μM. MPEP was also dissolved in DMSO to
obtain a 30 mM stock solution and diluted in ACSF to a final concentration of 30 µM. CdCl₂ was diluted from a 200 mM stock solution and used at a final concentration of 200 µM.

RESULTS

The effects of a potent and selective group I mGluR agonist, DHPG (Schoepp et al. 1994), that activates both pre- and postsynaptic group I receptors were tested on septohippocampal cholinergic neurons. Most studies in brain slices have used DHPG at a concentration of 50–100 µM (Kettunen et al. 2003; Lee and Boden 1997; Marino et al. 2001; Merlin and Wong 1997; Pisani et al. 2001; Yamakawa and Hirano 1999). However, clear-cut responses have been reported with 20 µM DHPG (Ireland and Abraham 2002; Raymond et al. 2000) or even 10 µM DHPG (Shahraki and Stone 2003; White et al. 2003). We chose the low concentration of 10 µM DHPG to begin our investigations with this drug.

The effects of 10 µM DHPG were tested on a total of 150 septohippocampal cholinergic neurons that were selectively labeled using the fluorescent marker, Cy3-192IgG (see METHODS) as well as on 19 unlabeled septohippocampal cholinergic-type neurons. All recorded neurons exhibited the electrophysiological signature that is characteristic of septohippocampal cholinergic neurons (Fig. 1A), which includes the presence of inward rectification, but lack of a depolarizing sag in response to hyperpolarizing pulses (Gorelova and Reiner 1996; Griffith and Matthews 1986; Markram and Segal 1990). Also consistent with our published work, none of the recorded neurons responded to muscarine with an excitation. Instead, recorded neurons were either inhibited or not affected by muscarine. This is in contrast to septohippocampal GABAergic neurons which are excited by muscarine (Wu et al. 2000). Because similar responses to DHPG were obtained in both the labeled and unlabeled neurons, data from these cells has been grouped together.

Group I mGluR agonist excites septohippocampal cholinergic neurons via a direct postsynaptic effect

An excitatory response to DHPG was obtained in 100% of neurons tested (n = 169). In current-clamp recordings, rapid applications of DHPG (10 µM, 15 s) depolarized septohippocampal cholinergic neurons tested (Fig. 1A), producing a mean depolarization of 11.8 ± 5.1 mV (n = 4). In voltage-clamp recordings, the excitatory response to DHPG was observed as a 79.5 ± 5 pA inward current (range: 10–300 pA; n = 164) at a holding potential of −65 mV (Fig. 1, B and C).

FIG. 1. Septohippocampal cholinergic neurons are excited by the group I metabotropic glutamate receptor agonist, S-3,5-dihydroxyphenylglycine (DHPG), in a reversible, reproducible, and concentration-dependent manner. A and B: whole cell current-clamp recording from a Cy3-192IgG-labeled septohippocampal cholinergic neuron shows the electrophysiological signature obtained in response to depolarizing and hyperpolarizing steps (step size: 0.1 nA). Note that DHPG reversibly depolarized the cell by 14 mV. C: a voltage-clamp recording from another cell shows that DHPG produced an inward current of 100 pA at a holding potential of −65 mV. Three repeated applications at an interval of 2.5 min show that the DHPG response is reproducible and does not desensitize readily. D: the response to 7 concentrations of DHPG in a single septohippocampal cholinergic neuron voltage-clamped at −65 mV. E: summary concentration-response curve from 3 cells; mean EC₅₀ value: 2.1 µM.
The response to DHPG was reproducible and did not desensitize to repeated applications given at intervals of 2.5 min (Fig. 1B; 1st application: 87.6 ± 10.6 pA; 2nd application: 83.3 ± 9.8 pA; n = 21; P = 0.07). The concentration dependence of the DHPG response was studied by plotting a seven-point concentration-response curve (100 nM to 100 μM) using the Y-tube for drug application. An EC50 value of 2.1 μM was obtained for the three neurons tested with all seven concentrations of DHPG (Fig. 1, C and D).

The DHPG-induced inward current persisted in 2 μM TTX (Fig. 2, A and D; control: 55.7 ± 12.5 pA; TTX: 54.3 ± 11.9 pA; P = 0.6; n = 7) and in Cd2⁺-containing ACSF (Fig. 2, B and D; control: 97.5 ± 21.7 pA; 200 μM Cd2⁺: 90 ± 24.8 pA; P = 0.08; n = 4). It also persisted in ACSF containing ionotropic glutamate and GABA receptor antagonists (CNQX: 25 μM, AP-5: 50 μM, bicuculline/gabazine, 10–30 μM; Fig. 2, C and D; control: 67.5 ± 19.3 pA; antagonists: 65 ± 19.4 pA; P = 0.4; n = 4), suggesting presence of a direct postsynaptic effect.

**Pharmacological dissection of the group I mGluR-mediated excitation in septohippocampal cholinergic neurons**

As mentioned in the introduction, the group I metabotropic receptors are comprised of the mGluR1 as well as mGluR5 subtypes, both of which are activated by DHPG, the highly selective group I agonist used in this study. In the CNS mGluR1 and mGluR5 subtypes frequently colocalize in the same neurons (Pisani et al. 2001), and interesting interactions between the two subtypes have been reported in some CNS neurons (Poisik et al. 2003). We, therefore used receptor subtype selective antagonists to examine the contribution and possible interactions between mGluR1 and mGluR5 subtypes in septohippocampal cholinergic neurons. To observe possible interactions between mGluR1 and mGluR5 subtypes, the mGluR1 antagonist was applied first in some cells and mGluR5 antagonist in others.

Involvement of mGluR1 was tested using the noncompetitive antagonist, CPCCOEt (n = 12) and the competitive antagonist LY367385 (n = 9). Both CPCCOEt and LY367385 had similar effects, and because the two antagonists have comparable pKb (4.76 and 4.92, respectively) and IC50 values (6.5 and 8.8 μM) (Schoepp et al. 1999), the data from the two antagonists have been pooled. The mGluR1-selective antagonists, CPCCOEt and LY367385 (100–200 μM, 10 min), completely blocked the response to DHPG in 23.8% (5/21) neurons tested and reduced it by 64 ± 4.6% in the remaining 16 neurons. Addition of the mGluR5-selective antagonist, MPEP, reduced the response further in the four cells tested (total...
FIG. 3. Both mGluR1 and mGluR5 mediate the response to DHPG in septohippocampal cholinergic neurons. A: the DHPG response in a septohippocampal cholinergic neuron is reduced by 68% by the noncompetitive mGluR1 antagonist, 7-hydrox-iminocyclopropan-[b]chromen-1α-carboxylic acid ethyl ester (CPCCOEt). Addition of the mGluR5 antagonist, 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), produced a further reduction in the response to DHPG, suggesting involvement of both receptor subtypes. Also note the recovery in the response after washout of antagonists. B: the DHPG response in a septohippocampal cholinergic neuron is only very slightly reduced (10%) by the mGluR5 antagonist, MPEP. Addition of the mGluR1 antagonist, CPCCOEt, reduces the response by 73% suggesting a primary involvement of the mGluR1 subtype. C: another example of a septohippocampal cholinergic neuron in which the mGluR5 antagonist, MPEP, reduces the response to DHPG by 24%. Addition of the mGluR1 antagonist, CPCCOEt, further reduces the DHPG current suggesting involvement of both mGluR1 and mGluR5 subtypes. D and E: similar to CPCCOEt, (+)-2-methyl-4-carboxyphenylglycine (LY367385), a competitive mGluR1 antagonist, also reduces the response to DHPG. In contrast, MPEP produced a smaller reduction. The effect of the antagonists on 3 μM DHPG is also shown. F: bar chart shows that while 70% of neurons exhibited a DHPG response that was mediated via both mGluR1 and mGluR5, in 30% neurons, the response was mediated via mGluR1 alone. An exclusively mGluR5-mediated response was not recorded in any of the neurons tested. G: the relative contribution of mGluR1 and mGluR5 in mediating the DHPG response in cells that showed a mixed pharmacology.
Septohippocampal cholinergic neurons colocalize both mGluR1α and mGluR5 immunoreactivities

Neuronal cell bodies in the MSDB displayed immunopositivity for both mGluR1α and mGluR5. The fine red fluorescent dots were mostly associated to the cell membrane (Fig. 4A’). In the majority of cases, however, the cytoplasm was only lightly fluorescent and just occasionally strongly labeled (Fig. 4B’). Besides cell bodies, both mGluR antibodies labeled a large amount of fibers and boutons (0.5–1.5 μm in diameter) resulting in strong immunofluorescence in the neuropil of the MSDB. The mGluR-IR fibers and boutons were noncholinergic, and other markers were not tested. As demonstrated in sections double-labeled for mGluR/ChAT, a large population of cholinergic neurons in the MSDB (73.5%) showed immunoreactivity for mGluR1α (Fig. 4, A and A’), while a considerably smaller proportion (34.5%) displayed immunoreactivity for mGluR5 (Fig. 4, B, B’, C, and C’). These numbers suggested that a population of ChAT cells express both receptors. We next performed double-immunolabeling studies to determine whether glutamatergic terminals innervate septohippocampal cholinergic neurons.

VGLUT2-IR boutons synapse with cholinergic neurons in the MSDB

Antibodies against VGLUT2 were used to visualize glutamatergic terminals within the MSDB because it contains mainly VGLUT2 boutons and VGLUT1 varicosities are sparse (Hajszan et al. 2004). The appearance and distribution pattern of VGLUT2-IR structures were in line with our previous findings (Hajszan et al. 2004; Wu et al. 2003a). Briefly, the VGLUT2-containing neuronal elements appeared as varicosities ranging from 0.5 to 1.2 μm in diameter. The intervaricosic segments of axons were only occasionally stained and very short in length. The overall distribution of VGLUT2-positive varicosities was not homogeneous in the MSDB. They gathered mainly in the medial septal/lateral septum border zone and along the boundaries of the vertical limb of the diagonal band. Along the midline, relatively fewer boutons were observed. The distribution pattern of cholinergic neurons seemed to be similar to that of the VGLUT2 axons, and several close associations were observed between VGLUT2 boutons and cholinergic cell bodies and dendrites (Fig. 5, A and B). In some cases, ChAT-positive cell bodies were completely surrounded by VGLUT2-containing varicosities (Fig. 5B) that resembled pericellular baskets. The quantitative analysis of VGLUT2 input to ChAT-positive neurons in the MSDB showed that ~93% of ChAT neurons were contacted by at least one VGLUT2-containing bouton on their perikarya and/or proximal dendrites.

The notion of this very strong glutamatergic influence on septohippocampal cholinergic neurons was further strengthened by our electron microscopic observations. A total number of 15 VGLUT2/ChAT close associations were analyzed. Seven of the eight VGLUT2-IR boutons closely associated to cholinergic cell bodies (Fig. 5, C and D) were revealed to establish synaptic contacts. Furthermore, all of the seven VGLUT2/ChAT axo-dendritic close associations analyzed were found to form synapses (Fig. 5, E and F). All synapses were of the asymmetric type with prominent postsynaptic densities suggesting that the VGLUT2 input exerts an excitatory influence on cholinergic neurons.
DISCUSSION

The main findings of this study are septohippocampal cholinergic neurons are activated by a group I metabotropic glutamate receptor agonist via a direct postsynaptic mechanism, the excitatory effect is mediated via mGluR1 and mGluR5 subtypes, mGluR1 and mGluR5 receptor immunoreactivity is present within the MSDB and colocalizes with septohippocampal cholinergic neurons, and septohippocampal cholinergic neurons are synaptically innervated by VGLUT2-IR glutamatergic boutons.

Activation of group I mGluRs excites septohippocampal cholinergic neurons via the mGluR1 and mGluR5 subtypes

One hundred percent of identified septohippocampal cholinergic neurons were excited by the selective and potent group I mGluR agonist, DHPG. The EC_{50} value of 2.1 μM, obtained in this study, is comparable to the EC_{50} values of 2 and 30 μM obtained in phosphoinositide hydrolysis studies (Valenti et al. 2002). On the other hand, it was lower than the EC_{50} values of 18 μM (Neale et al. 2001) and 37 μM (Mannaioni et al. 2001) obtained for mGluR1-mediated responses in brain slice studies.

The excitatory effects of DHPG observed in septohippocampal cholinergic neurons were concluded to be of a direct postsynaptic nature as they persisted in the presence of TTX, Cd^{2+} as well as ionotropic glutamate and GABA receptor antagonists. Our findings are supported by the double-immunolabeling studies that demonstrate the presence of mGluR1 and mGluR5 immunoreactivity in 74 and 35% of septohippocampal cholinergic neurons, respectively. The presence of mGluR1 and mGluR5 immunoreactivity has not been previously reported in the MSDB and confirms the primarily...
postsynaptic localization of mGluR1 and mGluR5 reported in brain neurons. Presynaptic mGluR-mediated effects have been reported by a few studies (Merlin and Wong 1997), and mGluR5 is also present postsynaptically in glia (Schoepp 2001).

Our pharmacological studies, using selective group I metabotropic antagonists in slices, also support the involvement of the mGluR1 and mGluR5 receptor subtypes. In 30% of neurons tested, the DHPG response was mediated solely via mGluR1 and in the remaining cells, ~27% of the response was mediated via mGluR5. Thus the DHPG-induced inward current in septohippocampal cholinergic neurons is mediated primarily via mGluR1 but also by mGluR5 in septohippocampal cholinergic neurons. As compared with pharmacological findings, receptor immunoreactivity was detected in a slightly lower percentage of cells, this may, however, be a limitation of the detection technique.

Group I mGluRs have frequently been shown to colocalize in the same neurons in various CNS regions such as the globus pallidus (Poisik et al. 2003), substantia nigra (Marino et al. 2001) and the striatum (Pisani et al. 2001) and interesting interactions have been noted between the two receptor subtypes in some but not all neurons. Thus in the type II neurons of the rat globus pallidus, blockade of mGluR5 enhances the mGluR1 response and prevents its desensitization. In the present study, no such interactions were observed. The mGluR response in septohippocampal cholinergic neurons did not desensitize on repeated applications of the agonist at intervals of 2.5 min. Applications of the mGluR5 antagonist, MPEP, did not enhance the amplitude of the mGluR1 response. In this regard, septohippocampal cholinergic neurons are similar to the type I globus pallidus, nigral, and hippocampal neurons.

Our results are further strengthened by our double-immunolabeling studies that for the first time investigated glutamatergic innervation of septohippocampal cholinergic neurons at the light and electron-microscopic levels. In these anatomical studies, we have demonstrated the presence of asymmetric synaptic connections with prominent postsynaptic densities between VGLUT2-containing fibers and septohippocampal cholinergic neurons. As mentioned in the introduction, origin of glutamate fibers in the septum is both extrinsic and intrinsic as in recent years several studies have reported the presence of glutamate neurons in the MSDB (Csaki et al. 2000, 2002; Fremeau et al. 2001; Hajszan et al. 2004; Kiss et al. 2002; Lin et al. 2003; Manns et al. 2001). Thus released glutamate from extrinsic or intrinsic glutamatergic fibers is likely to activate the group I metabotropic receptors in septohippocampal cholinergic neurons. In a recent study, we demonstrated that locally released glutamate activates group I mGluRs in septohippocampal GABAergic neurons (Wu et al. 2003a).

Implications of the findings

The discovery that group I mGluRs are expressed by septohippocampal cholinergic neurons is significant for septohippocampal functions because manipulations aimed at reducing mGluR effects have been reported to diminish performance in some learning and memory tasks (see introduction). On the other hand, contradictory effects of mGluR1/5 agonists also have been reported in in vitro models of neuronal cell death. Activation of group I mGluRs is known to either enhance or attenuate excitotoxic neuronal death depending on the experimental conditions (Bruno et al. 2001).

Using knock-out mice and receptor antagonists, the facilitatory effects of group I metabotropic glutamate receptors on cognitive performance have been attributed to the hippocampal mGluRs, thus far, because the hippocampus itself expresses high levels of these receptors. However, considering the dense glutamatergic innervation of the septohippocampal cholinergic neurons and the established role of septohippocampal inputs in maintaining hippocampal theta rhythm and associated mnemonic functions, a group I mGluR-mediated activation of septohippocampal cholinergic neurons would also facilitate hippocampal functions. Thus theoretically, a group I mGluR activation would activate both the septohippocampal GABAergic and cholinergic pathways to the hippocampus and facilitate hippocampal functioning by increasing hippocampal and septal ACh release. It is hoped that the results of the present study will lead to future investigations in which the contribution of septal mGluRs to septohippocampal cognitive functions will be assessed possibly by direct infusions of group I mGluR agonists and antagonists into the nucleus. Group I mGluR effects on the long-projecting septohippocampal cholinergic neurons may especially be useful in enhancing cholinergic function in neurodegenerative disorders that are associated with a reduction in the number of septohippocampal cholinergic neurons.

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