Involvement of Glutamate Receptors in Strypchnine- and Bicuculline-induced Alloodynia in Conscious Mice

Masahiko Onaka, M.D.,* Toshiaki Minami, M.D., Ph.D.,† Isao Nishihara, M.D.,* Seiji Ito, M.D., Ph.D.‡

Background: Glycine and γ-aminobutyric acid (GABA) are inhibitory neurotransmitters that appear to be important in sensory processing in the spinal dorsal horn. Intrathecal administration of glycine (strypchnine-sensitive glycine receptor antagonist) or bicuculline (GABA_A receptor antagonist) was reported to induce allodynia. Although the strypchnine-induced allodynia was shown to be mediated through the N-methyl-D-aspartate (NMDA)-type glutamate receptor, it is not clear whether the bicuculline-evoked allodynia is mediated through the glutamate receptor system or how different the allodynia induced by strypchnine and bicuculline are.

Methods: Male ddY mice weighing 20 ± 2 g were used in this study. A 27-G stainless-steel needle attached to a microsyringe was inserted between the L5 and L6 vertebrae by a slight modification of the method of Hylden and Wilcox. Drugs in vehicle were injected slowly into the subarachnoid space to conscious mice at 22 ± 2°C. The volume of the intrathecal injection was 5 μl. Studies on allodynia were carried out essentially according to the method of Yaksh and Harty.

Results: The intrathecal administration of strypchnine or bicuculline in conscious mice resulted in allodynia elicited by nonnoxious brushing of the flanks. The maximum allodynia induced by strypchnine was observed 5 min after intrathecal injection, but that induced by bicuculline was observed 5 min after intrathecal injection. Both responses gradually decreased over the experimental period of 50 min. The allodynia induced by strypchnine was dose-dependently relieved by NMDA receptor antagonists (d-AP5, ketamine, and 7-CI-KYN) and non-NMDA receptor antagonists (GAMS and CNQX) but not by metabotropic receptor antagonists (1-AP3 and 1-AP4). On the other hand, allodynia induced by bicuculline was dose-dependently relieved by GAMS, 1-AP3, and 1-AP4, but not by d-AP5, ketamine, 7-CI-KYN, and CNQX. Whereas the strypchnine-evoked allodynia was dose-dependently relieved by the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester (l-NAME) and the soluble guanylate cyclase inhibitor methylene blue, the bicuculline-induced one was dose-dependently relieved by methylene blue but not by l-NAME.

Conclusions: These results demonstrate that both strypchnine- and bicuculline-evoked allodynia were mediated through pathways that include the glutamate receptor and nitric oxide systems but in a different manner. The current study suggests that GABA and glycine may mediate responses to an innocuous tactile stimulus as inhibitory neurotransmitters at presynaptic and postsynaptic sites in the spinal cord, respectively.

(Key words: Antagonists, GABA_A; bicuculline. Antagonists, glycine; strypchnine. Neurotransmitters; glutamate. Pain: Alloodynia. Pharmacology; nitric oxide. Receptors; glutamate.)

GLYCINE and γ-aminobutyric acid (GABA) are both inhibitory neurotransmitters that mediate fast synaptic inhibition in the nervous system.1 Their actions are to bind specifically to glycine and GABA_A receptors, respectively. This is followed within milliseconds by the gating or opening of an integral chloride ion channel, which results, in general, in the hyperpolarization of the recipient neuronal cell.2 Pharmacologically, glycine receptors are defined by the antagonism by the convulsive alkaloid strypchnine (strychnine-sensitive glycine receptor antagonist), in contrast to the strypchnine-insensitive glycine-binding site that is associated with N-methyl-D-aspartate (NMDA) subclass of glutamate receptors. On the other hand, the convulsant alkaloid bicuculline blocks the hyperpolarizing actions of GABA and nerve stimulation. Molecular models show that GABA is isosteric with a GABA-like moiety in the bicuculline molecule, suggesting a competitive interaction on the GABA_A receptor.
Previous studies demonstrated that glycine and GABA are important in sensory processing in the spinal dorsal horn as inhibitory neurotransmitters. Intrathecal administration of strychnine or bicuculline to conscious mice was reported to induce allodynia, a state of discomfort and pain evoked by innocuous stimuli; the mice showed squawking, biting, and escaping in response to low-threshold stimuli. A growing body of evidence suggests that the pharmacology of the system activated in the pathologic state "allodynia" may differ from that activated under normal circumstances by high-threshold thermal, chemical, and mechanical stimuli. In fact, it was previously reported that intrathecal administration of the opioid and \(\alpha_2\)-receptor agonists could produce a definitive inhibition of the spinal response to noxious stimuli but had little effect on the strychnine-induced allodynia. On the other hand, adenosine analogs showed the powerful effect on strychnine-induced allodynia at doses that have only a mild analgesic effect on hyperalgesia. Transmission at neural synapses is mediated by a variety of receptors that specify neurotransmitter interactions and transmit information into target cells in the spinal cord. In recent years, much attention has been directed toward the excitatory transmission mediated through the glutamate receptors in the central nervous system and it has been suggested that there are mechanisms whereby interactions between excitatory and inhibitory neurotransmitter systems can modulate signal transmission in the spinal cord. The glutamate receptors are classified in three groups, NMDA, non-NMDA (AMPA/kainate), and metabotropic receptors. It has been reported that the NMDA receptor is a voltage-gated ion channel that, once activated, allows \(Ca^{2+}\) to enter the neuron. This increase in intracellular \(Ca^{2+}\) triggers a cascade of events that include activation of the constitutive form of nitric oxide synthase. Nitric oxide diffuses to its site of action, where it activates soluble guanylate cyclase and increases the intracellular content of cGMP. Although strychnine-induced allodynia was reported to be mediated through the NMDA-type glutamate receptor, it is not clear whether the bicuculline-evoked allodynia is mediated through the glutamate receptor system or how different the allodynia induced by strychnine and bicuculline are. The current study was designed to assign the involvement of glutamate receptors and nitric oxide system in strychnine- and bicuculline-evoked allodynia and seek the difference in the mechanisms of action between them by use of antagonists for glutamate receptors and inhibitors of nitric oxide system.

Materials and Methods

Intrathecal Administration and Studies on Allodynia

Male ddY mice weighing 20 ± 2 g were used in this study. The animals were housed under conditions of a 12-h light-dark cycle and a constant temperature of 22 ± 2°C and 60 ± 10% humidity. A 27-G stainless-steel needle (0.35 mm OD) attached to a microsyringe was inserted between the L5 and L6 vertebrae by a slight modification of the method of Hylden and Wilcox. Drugs in vehicle were injected slowly into the subarachnoid space of conscious mice at 22 ± 2°C. It was previously confirmed by use of Coomassie brilliant blue that the injected solution did not extend to the cervical segments.

Studies on allodynia were carried out according to the method reported previously. Control mice were given physiologic saline (5 µl). Drug-treatment groups were injected with 5 µl of vehicle containing various doses of test agents. After the intrathecal injection, each mouse was placed in an individual 14 × 10 × 12-cm Plexiglas enclosure with wood chips on the floor and observed. Allodynia was assessed once every 5 min over a 50-min period by light stroking of the flank of the mice with a paintbrush. The allodynic response was ranked as follows: 0 = no response; 1 = mild squawking with attempts to move away from the stroking probe; and 2 = vigorous squawking evoked by the stroking probe, biting at the probe, and strong efforts to escape. Each mouse was tested for 50 min following intrathecal injection. To evaluate the effects of various doses of blocking agents on strychnine- and bicuculline-induced allodynia, we assessed the scores at 5 min after intrathecal injection of strychnine for the former and the scores at 10 min after intrathecal injection of bicuculline for the latter.

The animals were used for only one measurement in each experiment. This study was conducted with the approval of the local ethics committee and in concordance with the guidelines of the Ethics Committee of the International Association for the Study of Pain.

Drugs

Strychnine (mw 334.4; a strychnine-sensitive glycine receptor antagonist) and N\(^{\text{6}}\)-nitro-L-arginine methyl ester (L-NAME; an inhibitor of nitric oxide synthase) were obtained from Sigma (St. Louis, MO). Bicuculline (a \(\alpha_2\)-receptor blocker), and NMDA receptor antagonist were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bicuculline (a \(\alpha_2\)-receptor blocker), and \(N^\text{6}\)-nitro-L-arginine methyl ester (L-NAME; an inhibitor of nitric oxide synthase) were obtained from Sigma (St. Louis, MO).

Statistics

The statistical analysis was performed with the aid of IBM SPSS Statistics (version 22.0). The Friedman's test was used to compare multiple groups and the Bonferroni correction was applied for multiple comparisons.

Graphs

Fig. 1. Time course of the effect of intrathecal injection of strychnine (C), bicuculline (O), and saline (S) on allodynia. Each group consisted of five mice and the percentage of the cumulative score was evaluated every 5 min. The values (mean ± S.E.M.) are shown as a percentage of the possible score over the observation period following each injection (15.5 ng–2.5 µg for strychnine and 0.975 µg for bicuculline).

*P < 0.01 vs. saline.
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Fig. 1. Time courses (A) and dose-dependency (B) for the effect of intrathecal injection of strychnine and bicuculline on allodynia. Studies on allodynia were conducted as described in materials and methods. Mice were injected with 0.25 μg strychnine (●) and 1.25 μg bicuculline (○). Each column in A represents the percentage of the maximum possible cumulative score of six to eight mice evaluated every 5 min (mean ± SE). The values (mean ± SE, n = 6–8) of allodynia shown in B are expressed as a percentage of the maximum possible score over the 50-min observation period following different doses (12.5 ng–2.5 μg).

Time (min)

-\log[10] Dose (μg)

Results

Effect of Intrathecal Strychnine or Bicuculline on Allodynia

Intrathecal administration of strychnine and bicuculline resulted in prominent agitation responses, such as vocalization, biting, and escape from the probe, to tactile stimuli applied to the flank. Brushing of the face or tactile stimulation of the forepaws did not elicit any response, indicating that allodynia appeared limited to the caudal dermatomes of the body.

Figure 1A presents the time courses of allodynia evoked by strychnine (0.25 μg/mouse) and bicuculline (1.25 μg/mouse). Strychnine-induced allodynia showed the maximum effect at 5 min after intrathecal injection, gradually decreasing over the 50-min experimental period. On the other hand, the bicuculline-induced allodynia was evoked by the first stimulus at 5 min after intrathecal injection, but the maximum effect was observed at 10 min. The response was long-lasting and did not disappear by 50 min. Both strychnine- and bicuculline-induced allodynia showed the respective patterns of time courses similar to those shown in figure 1A, over a wide range of doses from 25 ng to 2.5 μg/mouse. When the scores of allodynia obtained for the overall 50 min were cumulated and expressed as a percent of the maximum possible score, both strychnine- and bicuculline-induced allodynia

for multiple comparison. IC₅₀ values were calculated using the computer program of probit test.

Statistics

The statistical analyses were carried out by analysis of variance. Statistical significance (*P < 0.05, **P < 0.01) was further examined with Duncan’s test.
showed a gradually increased pattern (25 ng–2.5 µg; fig. 1B), and mice displayed convulsions at a dose of 25 µg/mouse or more. The intrathecal administration of saline in conscious mice had no effect on allodynia.

Effects of NMDA Receptor Antagonists on Strychnine- and Bicuculline-evoked Allodynia

The effects of various antagonists for the glutamate receptor family on the allodynia were evaluated by the values obtained 5 min after injection of 0.25 µg strychnine or 10 min after injection of 1.25 µg bicuculline. The scores of allodynia induced by strychnine at 5 min and bicuculline at 10 min were 83.3% and 75.0% of the maximum possible score, respectively, and were taken as 100%.

We first investigated the involvement of the NMDA receptor in the strychnine- or bicuculline-induced allodynia by using D-AP5, ketamine, and 7-Cl-KYNA. The allodynia evoked by strychnine was dose-dependently blocked by D-AP5, ketamine, and 7-Cl-KYNA with IC₅₀ values of 589 ng, 147 ng, and 8.03 ng, respectively (fig. 2). On the other hand, the allodynia caused by bicuculline was not blocked by D-AP5, ketamine, or 7-Cl-KYNA (fig. 2).

Effects of Non-NMDA Receptor Antagonists on Strychnine- and Bicuculline-evoked Allodynia

We investigated the involvement of non-NMDA receptors in allodynia caused by strychnine or bicuculline with GAMS and CNQX. The allodynia caused by strychnine was dose-dependently blocked by GAMS and CNQX with IC₅₀ values of 1.17 µg and 8.76 ng, respectively (fig. 3). The allodynia caused by bicuculline was dose-dependently blocked by GAMS with an IC₅₀ value of 214 ng (fig. 3A) but not blocked by CNQX (fig. 3B).

Effects of Metabotropic Glutamate Receptor Antagonists on Strychnine- and Bicuculline-evoked Allodynia

We further investigated the effect of L-AP3 and L-AP4 on allodynia caused by strychnine or bicuculline. The allodynia caused by bicuculline was dose-dependently antagonized by L-AP4 with an IC₅₀ value of 85.6 ng (fig. 4B) but was partially blocked by L-AP3 (fig. 4A). On the other hand, the allodynia caused by strychnine was not antagonized by L-AP3 or L-AP4 (fig. 4). These results demonstrated that NMDA and non-NMDA receptors in the spinal cord were involved in the strychnine- and bicuculline-induced allodynia but that kainate and metabotropic receptors were not.

Fig. 2. Effects of NMDA receptor antagonists on strychnine- and bicuculline-induced allodynia. Strychnine (○, 0.25 µg) or bicuculline (●, 1.25 µg) was injected simultaneously with various doses of D-AP5 (□), ketamine (△), or 7-Cl-KYNA (■) into the subarachnoid space. Assessment of allodynia was made as described in Materials and Methods. The maximum score of strychnine (0.25 µg/mouse at 5 min) alone or bicuculline (1.25 µg/mouse at 10 min) alone is taken at 100% as the control. Statistical analyses were carried out by Duncan’s test. **P < 0.01, as compared with strychnine- or bicuculline-injected group.
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![Graphs showing the effects of strychnine and bicuculline on alldynia.](image)

**Fig. 3. Effects of non-NMDA receptor antagonists on strychnine and bicuculline-induced alldynia.**

Strychnine (○, 0.25 μg) or bicuculline (●, 1.25 μg) was injected simultaneously with various doses of GAMS (A) or CNQX (B) into the subarachnoid space. Assessment of alldynia was made as described in materials and methods.

The alldynia (data not shown). On the other hand, the alldynia caused by bicuculline was blocked by methylene blue with an IC₅₀ value of 120 pg (fig. 5B) but not altered by l-NAME (fig. 5A). These results demonstrate that the nitric oxide system in the spinal cord is involved in both strychnine- and bicuculline-induced alldynia.

**Discussion**

It was previously reported that intrathecal administration of strychnine or bicuculline to conscious mice receptors were involved in the bicuculline-induced alldynia.

**Involvement of the Nitric Oxide System in Strychnine- or Bicuculline-evoked Alldynia**

To examine whether the nitric oxide system is involved in inducing alldynia, we investigated the effects of l-NAME and methylene blue on alldynia caused by strychnine and bicuculline. The alldynia caused by strychnine was dose-dependently blocked by l-NAME and methylene blue with IC₅₀ values of 68.8 pg and 38.6 μg, respectively (fig. 5). l-NAME, an inactive isomer of l-NAME, did not block the strychnine-induced alldynia.

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induced allodynia and that strychnine-induced alldynia was dose-dependently relieved by NMDA antagonists. In the current study, we first demonstrated that the glutamate receptor system involves the bicuculline- and the strychnine-induced allodynia. However, whereas the latter was inhibited by NMDA receptor antagonist and non-NMDA receptor antagonists, the former was inhibited by the kainate receptor antagonist GAMS and metabotropic receptor antagonists (figs. 2–4), suggesting that the interactions of strychnine and bicuculline with the glutamate receptor system are different. This was supported by the difference in the blockade by 1-NAME and methylene blue of allodynia evoked by strychnine and bicuculline (fig. 5). One of the mechanisms for touch-evoked allodynia was believed to result from removal of tonic or evoked inhibition from pathways relaying information about innocuous tactile stimuli. Yaksh suggested that the blockade of inhibition by spinal strychnine and bicuculline must either be presynaptic on the large primary afferent or postsynaptic on the second-order neuron and activated only by the large afferent input. Glycine binding is found throughout the spinal gray, with that in the dorsal horn being largely found in laminae II, III, and the lateral aspect of V. The glycergic neurons in the laminae II and III receive a major monosynaptic input from myelinated low-threshold cutaneous primary afferents, and glycine is considered to act as a postsynaptic inhibitory transmitter. On the other hand, GABAergic neurons are present in laminae I–III of the rat spinal cord, and many of neurons with somata in lamina I–III are inhibitory interneurons containing GABA. These GABA-containing terminals frequently are present in the presynaptic axons at axoaxonal synapses and in presynaptic dendrites in the dorsal horn. Strychnine inhibits depolarization of excitatory axon terminals by a transmitter released from other axon terminals that form axoaxonal synapses with the excitatory terminals of the primary afferent neuron. Although GABA produces postsynaptic inhibition by hyperpolarizing the postsynaptic cell, it can act as a depolarizing transmitter on the presynaptic terminals of certain primary afferent neurons to produce postsynaptic inhibition. L-Glutamate is a known neurotransmitter of primary afferents and descending projections from the brain and perhaps neurotransmitters of some intrinsic spinal neurons. Among the glutamate receptor family, the NMDA receptor and AMPA receptor were reported to be located mainly at postsynaptic site in the spinal cord, but the kainate receptor was likely to be located at presynaptic site in the spinal cord. On the basis of the selective depressant action of L-AP4 in spinal and certain hippocampal pathways, the metabotropic receptor was proposed to be located at a presynaptic site and possibly function as autoreceptors, controlling the release of neurotransmitters. Nitric oxide has been suggested to act as a retrograde transmitter. That is, activation of the NMDA receptor results in the production of nitric oxide by nitric oxide synthase in a postsynaptic neuron from which it rapidly diffuses to enter the presynaptic neuron. Thus, nitric oxide may modulate excitability and enhance synaptic connection through activation of guanylate cyclase in postsynaptic neuron. Synaptic potentiation, and GABA receptor-mediated inhibition, may be how presynaptic inhibition is relieved in the central nervous system. As a consequence of these observations, it was suggested that nitric oxide may modulate presynaptic transmission by inhibiting transmitter release or by activating postsynaptic neurons.
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Guanylate cyclase in presynaptic terminals and postsynaptic neurons. The concept of a retrograde transmitter recently came into favor in studies of long-term potentiation, and it has been suggested that this may be how presynaptic and postsynaptic connections in the central nervous system are strengthened as a consequence of frequent release. This may explain the difference in the effects of L-NAME and methylene blue on allostomy evoked by strychnine and biccuculline. Taken together, these results suggest that the action of strychnine may be mediated by glutamate receptors on postsynaptic sites, and that the action of bicuculline may be mediated by glutamate receptors on presynaptic site.

We recently reported that intrathecal administration of prostaglandin (PG)E_2 or PGF_2 alpha to conscious mice induced allostomy. The time courses of allostomy evoked by PGF_2 alpha and PGF_2 alpha coincided with those by strychnine and biccuculline, respectively. Although the PGF_2 alpha-induced allostomy was inhibited by NMDA and non-NMDA receptor antagonists similar to the strychnine-induced one, the PGF_2 alpha-induced allostomy was inhibited by kainate and metabotropic receptor antagonists similar to the bicuculline-induced one. Furthermore, whereas the PGF_2 alpha-induced allostomy was inhibited by L-NAME and methylene blue similar to the strychnine-induced one, the PGF_2 alpha-induced allostomy was inhibited by methylene blue, but not by L-NAME, similar to biccuculline-induced one. The modes of inhibition of strychnine- and biccuculline-induced allostomy by glutamate receptor antagonists and nitric oxide synthase inhibitor were the same as those of the agents for PGF_2 alpha and PGF_2 alpha-induced allostomy, respectively. Thus, many neurotransmitters are involved in the modulation of incoming pain information through a number of local receptor systems at different sites, and the disorder of the association may evoke allostomy.

Opioid-insensitive pain evoked by innocuous tactile stimulation is one of the most difficult problems in pain management. The features of strychnine- and biccuculline-induced allostomy apparently resemble those of patients suffering from postherpetic neuralgia or causalgia. The clinical allostomy may involve plastic changes in neural connectivity and synaptic strength in the spinal cord. Hao et al. developed an animal model that produces tonic and chronic states of allostomy in rats lasting several days and 1-3 months, respectively, after spinal cord injury induced photochemically by laser irradiation. They demonstrated that, although the NMDA receptor was involved in the development of allostomy through excitotoxicity, once the allostomy had developed, NMDA receptor antagonists were ineffective in relieving it. Furthermore, they showed that systemic L-NAME induced an analgesic effect on chronic allostomy-like behavior and suggested that production of nitric oxide may be involved in the maintenance of this abnormal pain-related condition in rats with spinal cord injury. Similarly, the established allostomy induced by strychnine was not blocked by the glycine receptor agonist taurine and the NMDA receptor antagonist ketamine. Furthermore, we reported that the established allostomy induced by intrathecal injection of PGE_2 was blocked by L-NAME but not by the PGE receptor and NMDA receptor antagonists. These results imply that the allostomy, once developed, does not require the continued agonist occupancy of receptors in our animal model. The alteration of gene expression associated with allostomy remains to be clarified. The current pharmacologic studies demonstrate that allostomy is induced by at least two different mechanisms and that strychnine- and biccuculline-induced allostomy could be two distinct models for pathologic pain and serve as screening drugs for chronic pain.

References


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