Research Report

Evidence for NMDA receptor in the afferent synaptic transmission of the vestibular system.

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Abstract
This study aimed to define the pharmacology and physiological role of the N-methyl-D-aspartate (NMDA) receptor in the synapse between the hair cells and primary afferent neurons in the vestibular system. The spontaneous and mechanically evoked spike discharges of vestibular nerve fibers were extracellularly recorded in isolated inner ear from the axolotl (Ambystoma tigrinum). Pressure ejection of NMDA (10^{-6} to 10^{-3} M) elicited a dose-dependent increase of the basal spike discharge from the vestibular nerve fibers. Extracellular magnesium antagonized the NMDA effect in a dose-dependent manner. D(-)-2-amino-5-phosphonovaleric acid (AP5, 10^{-5} to 10^{-3} M) and 7-chloro-kynurenic acid (7ClKyn, 10^{-6} to 10^{-3} M) inhibited the basal activity of the vestibular nerve fibers. 7ClKyn also diminished the responses elicited by the mechanical stimulation of the preparation. Glycine (10^{-9} to 10^{-6} M) applied by bath substitution enhanced the NMDA response, and the glycine agonist D-serine partially reversed the 7ClKyn inhibitory action. These results suggest that NMDA receptors participate in the generation of the basal spike discharge of vestibular system primary afferent neurons, but its activation is not critical for the response to brief mechanical stimuli.

Key words: glycine, NMDA, kynurenic, neurotransmitter, hair cell, afferent synapse, semicircular canal, inner ear.

1. Introduction

Since Curtis and colleagues [4] demonstrated the excitatory actions of glutamate in the spinal neurons, a great deal of research has shown that glutamate and probably aspartate, homocysteate and quinolinic acid function as excitatory neurotransmitters in the central nervous system (for Reviews see refs. 25 and 13). From a pharmacological standpoint, several types of excitatory aminoacid (EAA) receptors have been defined. Briefly, at least three types of ionotropic receptors i.e., N-methyl-D-aspartic (NMDA), kainic (KA) and α-amino-3-hydroxy-5-methyl-4-isoxasole propionic acid (AMPA) receptors, the last two usually referred to as non-NMDA receptors, have been found [24]. A fourth type of ionotropic receptor which specifically binds 2-amino-4-phosphonobutyric acid (AP4) is still under debate. A metabotropic type EAA receptor linked to a G protein has also been shown to exist [23, 13].

Among the EAA receptors, the NMDA type receptor deserves particular attention since it is modulated by several compounds. Glycine potentiates NMDA-mediated effects [10] acting as a coagonist by binding specific, strychnine insensitive sites on the NMDA receptor. Also the polyamines, spermine and spermidine and Zn^{+2} have been shown to modulate this receptor by binding specific sites [19, 16]. Moreover, the associated ionic channel can be blocked by extracellular Mg^{2+} in a voltage dependent manner [12, 14].

In the vestibular system, the hypothesis that an EAA is the transmitter between the hair cells and the afferent neurons has received wide support. It has been shown that KA, QA and NMDA excited the vestibular afferent neurons [2, 22, 20, 17, 15]. KA receptors have been specifically located by immunocyto-chemistry at the postsynaptic densities of the primary afferent neurons in the frog saccule [5]. In the rainbow trout, glutamate was concentrated in the hair cell layer.

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and was released from hair cells by elevated extracellular potassium [6, 7] and, in the isolated frog semicircular canal glutamate was released under the influence of mechanical stimuli [29].

However, the existence of NMDA receptors on afferent neurons has not been conclusively shown. NMDA produced a dose-dependent increase in the firing rate of the vestibular afferent neurons of the frog [2, 17, 28], the axolotl [22, 20] and the ampulla of Lorentzini of the skate [1]; although NMDA actions were about three orders of magnitude less potent than non-NMDA agonist effects. The specific NMDA antagonist AP5 produced a small inhibitory action on the basal spike discharge of the afferent neurons of the axolotl and the frog [22, 28]. Moreover, there is no evidence confirming the suppressive action of extracellular Mg²⁺ on the NMDA effects. In the cochlear afferents of the guinea pig, it has been found that NMDA also produced an excitatory response, although no significant actions of extracellular glycine or Mg²⁺ were found [18]. In isolated chick cochlear ganglion neurons, ionic currents elicited by glutamate were depressed by Mg²⁺, suggesting an NMDA receptor involvement [27]. However, it is still not clear if these actions are due to the activation of an atypical NMDA receptor, since NMDA actions in hair cell systems show only minimal interaction with Mg²⁺, and no modulatory action of glycine has been reported.

The present study was undertaken with the aim of investigating the possible physiological role and the actual characteristics of the NMDA receptor involved in the synaptic transmission between the hair cells and primary afferent neurons in the vestibular system. To this end, the basal and the mechanically evoked discharges of vestibular nerve fibers were extracellularly recorded, before and after administration of NMDA receptor agonists or antagonists.

2. Materials and methods

Experiments were carried out in isolated labyrinth of the axolotl (Ambystoma tigrinum) as reported previously [22, 15]. Briefly, larval axolotls weighing 30 - 60 g were decapitated, and the otic capsule opened ventrally. The nerve fibers of the anterior and lateral canals were dissected up to the brainstem. The cartilaginous otic capsule was cut and isolated from the cranium. The isolated inner ear was transferred to a recording chamber and continuously perfused with Mg²⁺ free Ringer solution of the following composition (in mM): KCl 2.5, NaCl 111, CaCl₂ 1.8, glucose 10, HEPES 5, pH 7.4. In some of the experiments this solution was modified by addition of MgCl₂ (1 or 3 mM). Multiunit extracellular recordings were obtained from the semicircular canal nerve using a suction electrode. Electrical activity amplified by means of a conventional AC amplifier, was filtered at cutoff frequencies of 100 and 2000 Hz and monitored in an oscilloscope. The signal was also led to a magnetic tape recorder and to a window discriminator, the output of which was connected to a microcomputer for on-line analysis of discharge rate [21]. In some of the experiments the preparation was mechanically stimulated. For this, the recording chamber, the manipulators and the amplifier were mounted on a rotating table. Record was led out through a rotating connector, displayed in an oscilloscope and stored on a tape recorder. The spike discharge frequency of semicircular canal nerve fibers was analyzed on line by means of a window discriminator and a computer. The rotating table was driven by a servo-controlled DC motor. The mechanical stimulus control signal was derived from a function generator.
table driven by a DC controlled motor. A function generator output was fed to the DC amplifier to produce sinusoidal accelerations (0.2 Hz) of the rotating table (Fig. 1). Typically, the preparation was stimulated during 30-s periods in control conditions and 30 s, 1.5 min and 5 min after drug administration.

Drugs were either bath or locally applied as indicated in each case. For bath application, the desired drug concentration was added to the perfusion Ringer and the recording chamber bath completely replaced using a rapid exchange suction-perfusion system. For local application, 20 µl of the drug were ejected from a pipette (100 µm tip diameter, flow rate 7-10 µl/s) positioned near (less than 500 µm) the origin of the afferent fibers. The concentrations given herein are those originally in the pipette. Since the bath volume was 2 ml, the drug concentration decays exponentially to about 1% of its original value in a few milliseconds [26]. When more than one drug was applied, sufficient time (at least 10 min) was allowed to wash off the previous drug, and no drug was tested until the basal discharge level was stable. In those trials designed to study the response to mechanical stimulation, the perfusion remains closed during 7 min after drug administration.

N-methyl-D-aspartic acid, kainic acid, 7-chlorokynurenic acid and D(-)-2-amino-5-phosphonovaleric acid were obtained from Cambridge Research Biochemicals. Glycine and D-serine were from Sigma Chemicals Co.

To construct the concentration-response relationship of the various drugs, the spike discharge was normalized as a percent of change with respect to control conditions, and the mean and standard error calculated from these values. Comparisons of the mechanical responses were done by obtaining the mean of the peak response in at least three cycles of the sinusoidal stimulus period (the first and last cycles were eliminated). To determine the statistical significance of drug effects a paired \( t \)-test was performed.

3. Results

Recordings of the vestibular nerve fibers activity were obtained from 93 preparations. Sinusoidal accelerations of the rotating table produced a clear sinusoidal modulation of the spike discharge of the semicircular canal nerve fibers. The preparation remained in adequate condition, as judged by its regular basal discharge and its response to mechanical stimuli, during long periods (more than 8 hr).

Pressure ejection of NMDA (10\(^{-6}\) to 10\(^{-3}\) M; \( N = 69 \)) elicited a dose-dependent excitatory effect which began slowly within the first minute of NMDA application, and lasted for more than 5 minutes (Fig. 2). Perfusion of the preparation with a modified Ringer solution containing 1 and 3 mM MgCl\(_2\) (\( N = 16 \) and \( N = 15 \) respectively), diminished the basal discharge rate of the semicircular canal nerve fibers and the response to NMDA (Fig. 2). Maximal effect of NMDA was not recovered in the 3 mM Mg\(^{2+}\) Ringer solution, thus indicating the non-competitive nature of Mg\(^{2+}\) antagonism. It is worth noting that the inhibitory effect of Mg\(^{2+}\) on the NMDA responses is lower than that found in preparations involving isolated central nervous system cells [12, 14], in which, 1 mM Mg\(^{2+}\) is sufficient to block NMDA effects. This could be due

![Figure 2. Semicircular canal nerve fibers response to NMDA. A and B: frequency plots (impulses per second, IPS) of nerve fibers response to NMDA in Mg\(^{2+}\) free and 1 mM Mg\(^{2+}\) Ringer. Drug was applied by pressure ejection from a micropipette (arrow). C: concentration-response relationship of NMDA effects in free, 1 and 3 mM Mg\(^{2+}\) Ringer solution. Control discharge is the mean of spike rate during 2 min basal activity recording. Concentrations are expressed as those originally found in the perfusion pipette. Bars represent the mean ±S.E.M.](image-url)
Figure 3. Semicircular canal nerve fibers response to sinusoidal stimulation was not affected by AP5. A: control recording, B: after AP5 (10^{-3} M) application (arrow). Stim, mechanical stimulus. AP5 diminished the basal discharge from 9.4 ±2.8 ips in control conditions to 3.9 ±1.9 ips after 1 min of its ejection; while leaving the response to mechanical stimuli unaffected.

Figure 4. Concentration-response relationship of AP5 effects on the basal (○) and the mechanically evoked (●) activity of semicircular canal nerve fibers. Drug effects were normalized as percent of change with respect to mean basal and peak mechanical response (control discharge) without drug. Graphs were obtained 1.5 min after drug application. AP5 decreased the basal discharge while the response to mechanical stimuli remained unaltered. Each point represents the mean ± S.E.M. of at least 5 experiments.

Figure 5. 7Cikyn diminished both the basal and the mechanically evoked discharge of the semicircular canal nerve fibers. A: control recording, B: after 10^{-4} M 7Cikyn application (arrow). Stim, mechanical stimulus. 7Cikyn diminished the basal discharge from 16.3 ±3.3 ips in control conditions to 1.4 ±1.0 ips; and the peak response to mechanical stimuli from 60 ips to 32 ips.

to the continuous synaptic input to the vestibular afferents, which may eliminate the Mg^{2+} voltage-dependent blockage of the NMDA receptor.

Local application of the NMDA antagonist D(-)-2-amino-5-phosphonovaleric acid (AP5, 10^{-6} to 0.3*10^{-2} M; N = 28), consistently decreased the basal spike discharge of the semicircular canal nerve fibers in a dose-dependent and reversible manner, but with low potency. After 1 min of its application AP5 1 mM diminished the basal discharge to 57% of the initial level; basal activity slowly recovered during the following 5-10 min. AP5 did not block the response of these fibers to sinusoidal accelerations, even though AP5 was applied at doses at which it produced a significant effect upon the basal spike resting discharge (Fig. 3 and 4). Application of AP5 (10^{-3} M; N = 13) simultaneously with NMDA (10^{-4} and 10^{-3} M) consistently blocked the response to NMDA.

Application of the glycine site antagonist 7-chloro-kynurenic acid (7ClKyn, 10^{-8} to 10^{-3} M, n = 40) consistently diminished both the basal and the evoked spike discharge of the semicircular canal nerve fibers.
Although its action upon the mechanical response was of lower amplitude and duration (Fig. 6), 7ClKyn effect lasted for more than 10 minutes, time after which the discharge of the afferent neurons slowly recovered.

Contrary to the idea of a selective antagonism of 7ClKyn on the glycine site of the NMDA receptor [11], microperfusion of 7ClKyn also diminished the effects induced by kainic acid (not shown). In fact the inhibitory effect of 7ClKyn was stronger than that produced by AP5, thus indicating that at the concentrations used in our experiments 7ClKyn acts as a non-selective EAA antagonist.

To determine whether NMDA responses in the vestibular afferents are also modulated by glycine, we studied the effect of glycine and its interaction with NMDA application. Glycine applied either by bath perfusion ($10^{-9}$ to $10^{-6}$ M; $N = 16$) or by pressure ejection from a micropipette ($10^{-7}$ to $10^{-3}$ M; $N = 31$), did not produce a significant effect on the basal discharge of the semicircular canal nerve fibers. Glycine and NMDA interactions were studied with glycine being applied by bath perfusion, and NMDA
locally by pressure ejection. In these conditions, glycine (10^{-9} to 10^{-6} M, N = 65) potentiated the responses to NMDA, although with low potency and no clear correlation with the concentration of glycine employed (Fig. 7). Glycine also modified the time course of NMDA responses, lengthening their duration by about 30% (not shown).

Since the extracellular level of glycine seems difficult to control in an isolated organ preparation like ours, and 7ClKyn seems not to be acting selectively upon the glycine binding site, the interactions of 7ClKyn and the glycine site agonist, D-serine were studied. Bath application of D-serine (10^{-5} M; N = 5) did not produce a significant change in the basal discharge of the semicircular canal nerve fibers. Bath application of 7ClKyn (10^{-5} M, N = 6) consistently reduced the basal discharge of these fibers to 59% of the control value within 4 min, and blocked their response to the NMDA (10^{-3} M) ejection (Fig. 8). Bath perfusion of 10^{-5} and 10^{-6} M D-serine partially reverted the effect of 7ClKyn (10^{-5} M, N = 4), and restored the response to NMDA (Fig. 8). This suggests that activation of the glycine site is also a requirement for NMDA receptor activation in the axolotl labyrinth nerve fibers.

4. Conclusions

These results show that NMDA produced distinct excitatory actions in the semicircular canal nerve fibers in a typical concentration-dependent manner. AP5 diminished the basal discharge rate of these fibers and blocked the NMDA excitatory effects, but it did not modify the response to mechanical stimuli. The inhibitory action of AP5 upon the basal spike discharge of the semicircular canal nerve fibers suggests that NMDA receptors are normally active in our experimental conditions. NMDA effects were negatively modulated by the extracellular magnesium concentration, and glycine site activation potentiated the NMDA effects. These results suggest that tonically activated NMDA receptors contribute to the basal spike discharge of the vestibular afferents, although they do not mediate the response to brief mechanical stimuli. The NMDA receptors in the axolotl vestibule seems to share the pharmacological properties of those described in the central nervous system [12, 14, 25, 10, 13].

As described in the methods section, in our experiments the drug was applied in volumes of 20 µl. Since bath volume is of 2 ml, drugs diluted quickly to a final concentration of less than 1% of that originally applied. Although the drug initially arrives at a high concentration near the synaptic region it dilutes very quickly to its final concentration. Continuous perfusion of the bath and the existence of diffusional barriers diminish the chance for the drug to reach the basal pole of the sensory epithelia. Hence, the concentration at the synaptic cleft should be very low. These factors should be taken into account for the evaluation of relative drug potencies.

It has been shown that 1 mM Mg^{2+} effectively blocks the response elicited by NMDA in the central nervous system neurons [12, 14]. In our experiments increasing extracellular Mg^{2+} in the bath solution, diminished the basal spike discharge of the semicircular canal nerve fibers and its response to NMDA. However, the effects of Mg^{2+} on the NMDA responses were of lower magnitude than those which have been reported in isolated central nervous system neurons [12, 14]. This can be due to the continuous depolarizing synaptic input to the vestibular afferents, which sustains the basal spike discharge of these neurons and which, may decrease the voltage-dependent block of NMDA receptors by Mg^{2+}. However, the possibility of NMDA receptor in the Ambystoma tigrinum labyrinth being less sensitive to extracellular Mg^{2+}, as has been shown to occur in the retinal ganglion cells of this same animal, could also explain these results [8].

Glycine induced a slight potentiation of NMDA-evoked responses on the semicircular canal nerve fibers, the maximal effect of glycine was elicited with a bath concentration of 1 µM, which has been shown to be the saturating concentration for the glycine site in the NMDA receptors [10]. Furthermore, the antagonistic action of 7ClKyn on the basal activity and on the responses elicited by NMDA, and its reversion by D-serine, suggest that NMDA receptor activation in the vestibular nerve fibers also requires the binding of an agonist to the glycine site.

In our experiments 7ClKyn acts as a non-specific EAA receptor antagonist. We did not find previous works suggesting a non-specific action of 7ClKyn. Kemp and colleagues [11] reported that, in crude postsynaptic densities from rat cerebral cortex, the affinity of 7ClKyn for the glycine site is 273-, 302- and >1000-fold higher than its affinity for QA, NMDA and KA receptor sites respectively. They found that 7ClKyn has an affinity ~ 0.3 µM for the glycine site. Thus, concentrations of 7ClKyn in the range we have used could determine its apparent non-
selective antagonistic action. However, the finding that D-serine overcomes the inhibitory action of 7ClKyn upon the NMDA-evoked responses, demonstrates a competitive action between these compounds indicative of glycine site binding as a requirement for NMDA receptor activation.

Inhibitory action of both 7ClKyn and AP5 was greater on the basal discharge than on the peak mechanical response of semicircular canal nerve fibers. We think that different sensitivities of basal and mechanically evoked discharges to the inhibitory action of these drugs, is due to the fact that basal activity is sustained by an excitatory input which is very close to the threshold level of these neurons; thus, little changes in the threshold or in the synaptic input suffice to induce notorious effects upon the basal activity. In contrast, the mechanical stimulation induced a massive release of neurotransmitter from the hair cells, determining an excitatory input to the afferent neurons which is maximal and less sensitive to a reduction in its level. Thus, differences in the synaptic input level determine the apparent pharmacological dissociation between the basal and the evoked activity here reported. In fact, the 7ClKyn concentration- response relationship for the evoked activity shows a displacement to the right with respect to the curve for basal discharge, indicating just a linear decrease of potency. For AP5 other mechanisms can be involved, but they are difficult to evaluate due to the low inhibitory potency of this drug, which was three orders of magnitude less potent than 7ClKyn. Thus, despite the apparent dissociation between the basal and the mechanical activities in the vestibular afferent neurons, our results do not lend support to the hypothesis that evoked and basal release of transmitter are mediated by different mechanisms [9].

Although NMDA excited the semicircular canal nerve fibers, its low potency, and the lack of effect of AP5 on the mechanically evoked responses, suggest that NMDA receptor did not participate in the response of these neurons to brief mechanical stimuli of the type we have used in this work. However, the blocking action of extracellular Mg\(^{2+}\) on the NMDA responses and the D-serine reversion of 7ClKyn actions [25], support the idea of NMDA receptor being involved in the synaptic processing of vestibular system afferent information. Prigioni and colleagues have found that EAA agonists increase the number of excitatory postsynaptic potentials recorded in vestibular afferent fibers, thus suggesting that EAA receptors are located presynaptically [17]. Our results did not conflict with the possibility that EAA receptors can be located both pre- and postsynaptically. However, it seems important to define the role of NMDA receptors in the synaptic coding of vestibular sensory information. NMDA produced a slow and long-lasting response; in contrast, our previous work has shown that KA generated a rapid and very large response of short duration, and QA administration produced long-lasting responses [22, 20, 15]. It is possible that the protracted excitatory action of NMDA be related with the generation of plastic phenomena [3]. We hypothesize that the relative density of each EAA receptor type in the synapse participates in the generation of the dynamical properties of the afferent neurons. Those neurons with a larger proportion of NMDA or QA receptors could codify for sustained stimuli, and those in which KA receptors are predominant, could have a phasic-type response and codify for very rapid and short-lived stimuli, such as a rapid head displacement. It is also possible that the sequential activation of these receptors generates the different phases of the response to mechanical stimuli [22, 20]. Even though the precise interaction between NMDA and non-NMDA receptors remains to be solved; our results suggest that NMDA receptors participate in the generation of a background firing of the afferent neurons, which is essential for bi-directional coding of vestibular information.

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References


