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Long-Term Potentiation of Glutamatergic Pathways in the Lamprey Brainstem

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Plasticity of synaptic transmission has been investigated in the lamprey brainstem. In this preparation, neurons of the vestibular nuclei make monosynaptic connections to reticulospinal neurons of the posterior (PRRN) and middle (MRRN) rhombencephalic reticular nuclei. This glutamatergic projection shows a long-lasting enhancement in efficacy following a brief high frequency (50 Hz) train of stimuli (tetanus). Enhancement was found on the inputs from the octavomotorius intermediate (nOMI) and octavomotorius posterior (nOMP) nuclei to the reticulospinal neurons of the PRRN and MRRN, and persisted for the entire duration of the recordings. The enhancement was limited to the pathway that received tetanic stimulation for the inputs to any given reticulospinal neuron. It depended upon the activation of postsynaptic processes at least in part; dialysis of the reticulospinal neuron by recording with a whole-cell patch pipette prevented its induction. This dialysis-dependent abolition of enhancement was shown to be dependent on a change in Ca^{2+} concentration in the postsynaptic neuron. The enhancement was not affected by blockade of NMDA receptors with D,L-2-amino-5-phosphonopentanoate (AP5) but was prevented by the metabotropic glutamate receptor (mGluR) antagonist (R,S)-α-methyl-4-carboxyphenylglycine (MCPG). In conclusion, this study demonstrates that vestibular inputs to reticulospinal neurons are capable of undergoing long-term potentiation (LTP) and that this LTP shows synapse specificity. Furthermore, this LTP is activated by synaptic stimulation of a subtype of the mGluR family and its induction is mediated by changing Ca^{2+} concentrations in the postsynaptic neuron.

[Key words: long-term potentiation, glutamate metabotropic receptors, reticulospinal neurons, vestibular inputs, excitatory amino acids, lamprey]

Reticulospinal neurons, which constitute the major source of descending inputs to the spinal cord in lampreys (McClellan, 1984; McClellan and Grillner, 1984; Buchanan et al., 1987; Ohta and Grillner, 1989), receive inputs from several sensory modalities. The vestibular system relays information to the reticulospinal system (Rovainen, 1979; Bussières and Dubuc, 1992; Deliagina et al., 1992a,b; Orlovsky et al., 1992) in large part from the vestibulospinal neurons of two vestibular nuclei, the nuclei octavomotorii intermediate (nOMI) and posterior (nOMP). These neurons make *en passant* synapses with reticulopinal neurons of the posterior rhombencephalic reticular nucleus (PRRN; Rovainen, 1979). The synapses are glutamatergic and comprise an electrical component, a fast α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor-mediated component, and a slower NMDA receptor mediated component (Alford and Dubuc, 1993a). The mechanisms underlying fast excitatory synaptic transmission are similar to that demonstrated in the majority of vertebrate synapses (Collingridge and Lester, 1989).

We have investigated the plasticity of the vestibulo-reticular synapse which relies on the activation of glutamate receptors (Dubuc et al., 1988; Alford and Dubuc, 1993a). Plasticity in the brainstem of the lamprey is of interest for two reasons in particular. The entire CNS of this animal may be maintained *in vitro* for extended periods (Rovainen, 1967), making a study of the properties of plasticity as they relate to the function of the CNS at a systems level a feasible objective. Additionally, the central synapses of the lamprey are uniquely available for investigation at the cellular level, both pre- and the postsynaptically (Ringham, 1975; Alford and Grillner, 1991). Disynaptic excitatory inputs to reticulospinal neurons, from the optic nerve and the VIIIth cranial nerve, have been shown to demonstrate posttetanic potentiation following repetitive (10 Hz) stimulation (Wickelgren, 1977a). Longer-term integrative phenomena have not previously been demonstrated in the lamprey reticulospinal system.

LTP, defined as a long-lasting potentiation of synaptic efficacy following a high frequency burst of synaptic transmission (Bliss and Lømo, 1973), is induced following activation of one or both of two glutamatergic receptor types. In the CA1 region of the hippocampus, and in the cortex, the activation of NMDA receptors on the dendrites of the postsynaptic neuron are known to be necessary for its initiation (Collingridge et al., 1983); however, activation of metabotropic glutamate receptors has also been implicated (Bashir et al., 1993). In the CA1 region of the hippocampus, these receptors may act in tandem with the activation of the NMDA receptor in this process (Bortolotto et al.,

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1994). On mossy fiber inputs to the pyramidal cells of the CA3 region of the hippocampus, induction of LTP may rely on the activation of metabotropic glutamate receptors alone (Sugiyama et al., 1990; Bashir et al., 1993). The action of both receptor subtypes on the postsynaptic neuron in initiating LTP is likely to involve, and require, a rise in the concentration of dendritic Ca^{2+} (Regehr and Tank, 1990), either by entry through the receptor ionophore or by release from intracellular stores (Alford et al., 1993).

In this study we show that vestibular inputs to the reticulospinal neurons of the lamprey MRRN and PRRN are capable of undergoing LTP. The mechanism of activation of this LTP has also been investigated, as have some of its basic properties.

This work has been presented in abstract form (Alford and Dubuc, 1993b).

Materials and Methods

All experiments were performed on the *in vitro*, isolated brainstem of larval and young adult lampreys *Petromyzon marinus*. The brain was dissected free of all tissue except the ventral cranium, which was retained for support. The tissue was superfused with lamprey artificial cerebrospinal fluid (aCSF) of the following composition: NaCl, 104.5 mM; KCl, 2 mM; $CaCl_2$, 2.6 mM; $MgCl_2$, 1.8 mM; glucose, 4 mM; $NaHCO_3$, 20 mM; this was bubbled with 95% O_2, 5% CO_2 (Wickelgren, 1977b) and titrated to a pH of 7.4 with NaOH. The aCSF was continuously superfused over the preparation to enable drug application and was maintained at approximately 9°C.

Microelectrodes or patch pipettes were positioned over the MRRN or PRRN in the 4th ventricle to target a visibly identified reticulospinal neuron. Microelectrode recordings were made with thin-walled glass intracellular microelectrodes filled with 4 M potassium acetate (30–50 MΩ impedance), except when the cells were filled with dye for which electrodes were filled with 1 M potassium acetate plus 0.5% Lucifer yellow dipotassium salt (90–110 MΩ impedance). Recording techniques were conventional and performed in current clamp in bridge mode. Whole-cell patch recordings were made using a technique modified from that of Blanton et al. (1989). Patch pipettes were pulled to a tip resistance of between 5 and 8 MΩ when filled with a solution of the following composition: potassium methane sulfonate, 102.5 mM; $CaCl_2$, 1 mM; $MgCl_2$, 1 mM; NaCl, 1 mM; HEPES, 5 mM; EGTA, 10 mM, titrated to a pH of 7.2 with KOH. In a small number of experiments the EGTA concentration was lowered to 0.05 mM and $CaCl_2$ was not included in the solution. Patch pipette seals were obtained under current clamp. Current pulses of 1 nA for 100 msec were repeatedly delivered and positive pressure applied to the interior of the pipette. The pipette was advanced through the intervening epithelial cell layer; pressure and current pulses were removed on observation of a small increase in pipette resistance. Any junction potential was nulled whereupon suction and DC negative current were applied as the pipette sealed to cell attached configuration, before suction was removed. The interior of the pipette was maintained at −70 mV and whole-cell configuration was achieved by the application of a rapid pulse of suction simultaneously to +10 nA current for 100 μsec. Seal resistances on cell-attached recording were typically between 5 and 10 GΩ (Alford and Dubuc, 1993a). All current-clamp patch recordings were made under current-clamp conditions using an Axoclamp 2A amplifier (Axon Instruments Inc.) in bridge mode. Voltage-clamp patch-clamp recordings were made using an Axopatch 200A amplifier (Axon Instruments). Achieving a whole-cell configuration was similar but performed under voltage clamp. Series resistances errors (20–25 MΩ) were compensated approximately 70% following compensation of electrode and somatic capacitances.

Experiments were performed to investigate the synaptic plasticity of the monosynaptic connection between neurons of either the nOMI or nOMP and reticulospinal neurons of the PRRN or MRRN. Vestibulospinal neurons in these two vestibular nuclei (nOMI and nOMP) are known to make strong excitatory connections with lamprey reticulospinal neurons (Rovainen, 1979; Alford and Dubuc, 1993a). In all experiments, glass-coated tungsten microelectrodes were used to activate the axons of these vestibulospinal neurons as they course in the basal plate before making contact with reticulospinal neurons. To identify a monosynaptic response, the stimulation electrode was moved systematically over the lateral edge of the basal plate. At a constant stimulus intensity, responses were evoked in the reticulospinal neuron for each location of the stimulus electrode, to identify a location which gave the shortest response latency. Figure 1 illustrates the responses elicited by microstimulating different locations in the lateral basal plate. Upon activation of axons from vestibulospinal neurons, a characteristic response was elicited in reticulospinal neurons. It consisted of a large EPSP (see 6, 12, and 13 in Fig. 1) with a short latency and a sharp onset. The positions from which these responses were obtained correspond well to previously defined locations of vestibulospinal pathways investigated anatomically. These responses differ significantly from those elicited by stimulation of other sites in the basal plate which comprised EPSPs of smaller amplitude and of slower rise time. The latter EPSPs result most likely from the activation of axons originating from other alar plate neurons which also project to reticulospinal neurons.

In those cases in which the electrode was loaded with Lucifer yellow, at the end of the experiment, the tissue was removed and fixed in 4% formaldehyde for 1 hr. The brainstem was then removed from the ventral cranium, dehydrated in increasing concentrations of ethanol and cleared in methyl salicylate. The tissue was then imaged on a laser scanning confocal microscope (Bio-Rad MRC 600, Bio-Rad Inc.) using the 488 nm line of an argon ion laser for excitation and an FITC filter set. The volume data was rendered in Voxel View (Vital Images Inc.).

Throughout the text all levels of significance are from Student *t* tests expressed as a probability, *p* that the results are from the same population. All means are expressed with their corresponding standard errors and all recordings of tetanus induced long term increases in synaptic response were made at least 30 min following the tetanus. Drugs were obtained from the following sources: MCPG, Tocris Neuramin, Bristol, UK; AP5, EGTA, Lucifer yellow, Sigma.

Results

A total of 65 reticulospinal neurons were recorded. Resting membrane potentials recorded with microelectrodes following stable impalement ranged from −70 to −80 mV. If, during the recording, the membrane potential deviated by more than 5 mV from the initial value the experiment was terminated. Recorded input impedances of the neurons were highly dependent on the recording technique chosen. For recordings made with microelectrodes, impedances ranged from 17 to 25 MΩ; for recordings made in whole-cell patch mode, impedances ranged from 150 to 310 MΩ. This disparity in measurements made with the two techniques is typical.

To study, in further detail, the morphology of the reticulospinal neurons that were recorded from, the fluorescent dye, Lucifer yellow was included in the recording pipette in a number of experiments. At the termination of the experiment the tissue was fixed, cleared and examined under fluorescence laser scanning confocal microscopy. All neurons visualized in this way showed the characteristic morphology of larger, medially located reticulospinal neurons of the PRRN (Fig. 2*B*). Although the properties of MRRN neurons are somewhat different to those of the PRRN, the neurons are larger with lower input impedances and spike much more readily, the responses to stimulation of the vestibulospinal nuclei were qualitatively similar to those in the PRRN. LTP could be readily evoked in neurons of both reticular nuclei.

Low frequency stimulation (1 shock every 30 sec), over the axons of either the nOMI or nOMP in the rhombencephalon, evoked compound PSPs in reticulospinal neurons of the PRRN and of the MRRN. In the PRRN, these potentials comprise, principally, an EPSP which shows an electrical, an AMPA and an NMDA component. This is followed by an IPSP which is largely mediated by glycine receptors with a small GABA component (Dubuc et al., 1988; Alford and Dubuc, 1993a). The IPSP is, however, not readily visible when recorded at resting membrane potentials of −70 to −80 mV, but is much more marked at more depolarized potentials (not illustrated). Responses evoked in re-

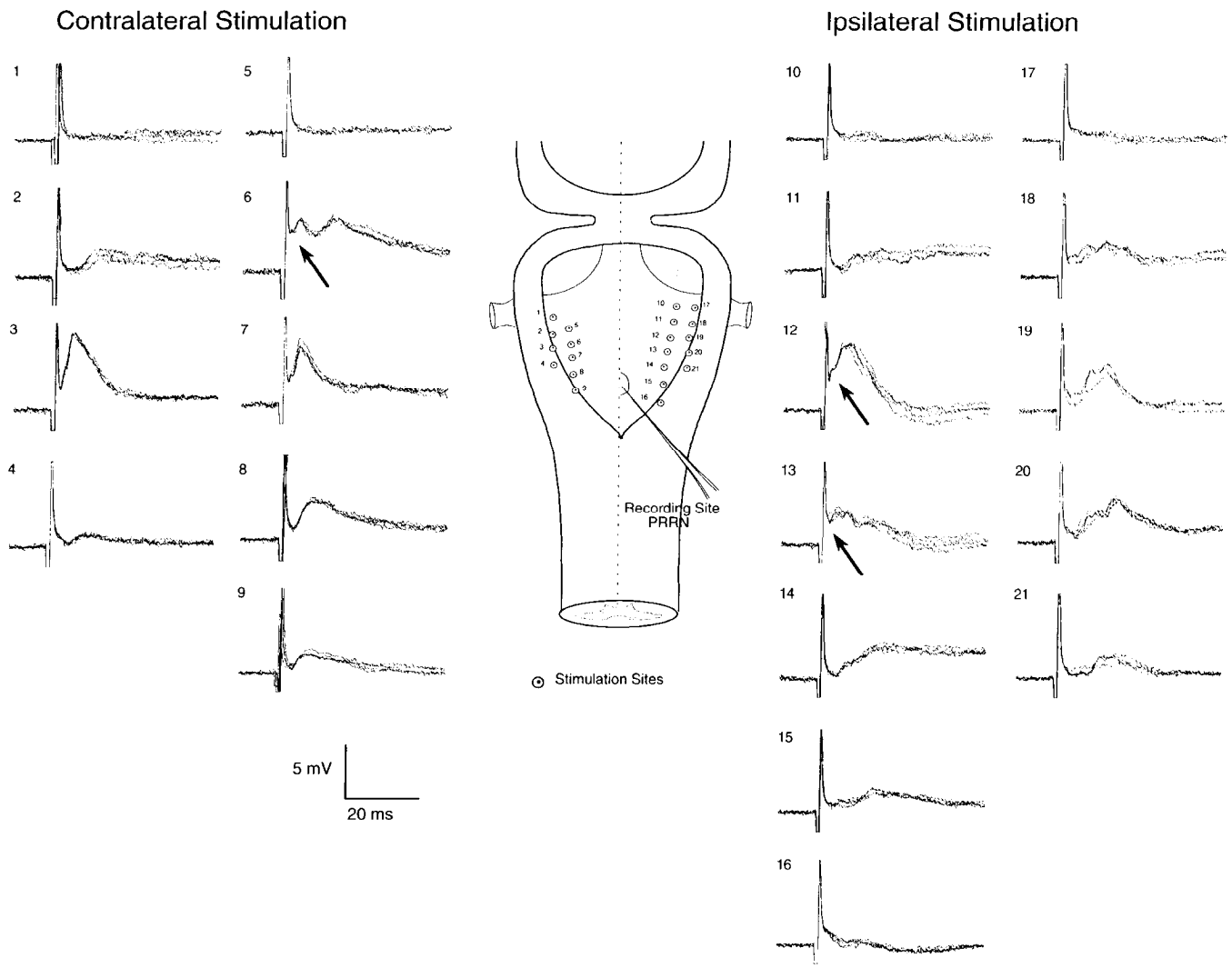

Figure 1. Intracellular responses of a reticulospinal neuron (V_m = −78 mV) in the rostral PRRN, evoked by microstimulation of the basal plate and recorded with a sharp microelectrode. For all stimulation sites (1–21), a glass-coated tungsten microelectrode (1–3 MΩ) was used to deliver single square wave pulses of 1 msec duration, at regular intervals of 2 sec, and at an intensity of 7 μA. Stimulation sites were systematically chosen along the rostro-caudal axis, by tracking the electrode along the lateral border of the alar plate. Specific stimulation sites (e.g., 6, 12, and 13) evoked responses with a very short latency and a sharp onset. These sites correspond to the location of vestibulospinal axons originating from the ipsilateral nucleus octavomotorius intermediate (e.g., sites 12 and 13), and the contralateral nucleus octavomotorius posterior (e.g., site 6; see text).

ticulospinal neurons were defined as vestibular inputs after identification of the pathway under investigation as discussed above in the Materials and Methods section. Typical responses to low frequency stimulation are shown (Fig. 3*A,B* insets) for the dye-filled reticulospinal neuron of the PRRN in Figure 2*B*.

Stimulation of the pathway at low frequency initiates a PSP of consistent amplitude. Higher frequency stimulation causes a marked adaptation of the response during the course of stimulation. EPSPs were, therefore, evoked at intervals of 30 sec for control periods of from 10 to 30 min. High frequency (tetanic) stimulation was then applied to the same stimulation electrode (50 shocks in 1 sec at test intensity). An immediate and marked potentiation of the low frequency responses was then recorded. The mean increase in response amplitude recorded from the first PSP following the tetanus was 187 ± 19% of the control response. Typically the amplitude of the low frequency responses decayed until stabilizing approximately 15–30 min posttetanus to a significantly greater mean amplitude of 146 ± 9% ($p < 0.05$) of the control response amplitudes (Fig. 3*C*). The potentiation of the response was invariably maintained for the remainder of the experiment. This was for a maximum period of 2.5 hr in this study (Fig. 3*A,B*) before the neurons were lost to recording, either because of a shift in membrane potential as indicated in Materials and Methods, or through loss of the cell impalement. The 10–90% rise time of the low frequency-evoked response was not altered significantly following tetanic stimulation-induced potentiation (6.8 ± 0.9 msec compared to 6.6 ± 0.8 msec prior to the tetanus; all above data are for control neurons in which no other manipulation was applied, n = 10). Further, the latency to onset of the response was unaltered (mean latency to onset of 2.6 ± 0.5 msec). An additional tetanic stimulation of the same pathway after recording a potentiated response, led to little or no further potentiation of the synaptic response; in three of these neurons, a second, identical, tetanus following at least 30 min of low frequency stimulation resulted in an insignificant increase in the mean peak amplitude to 109 ± 9%.

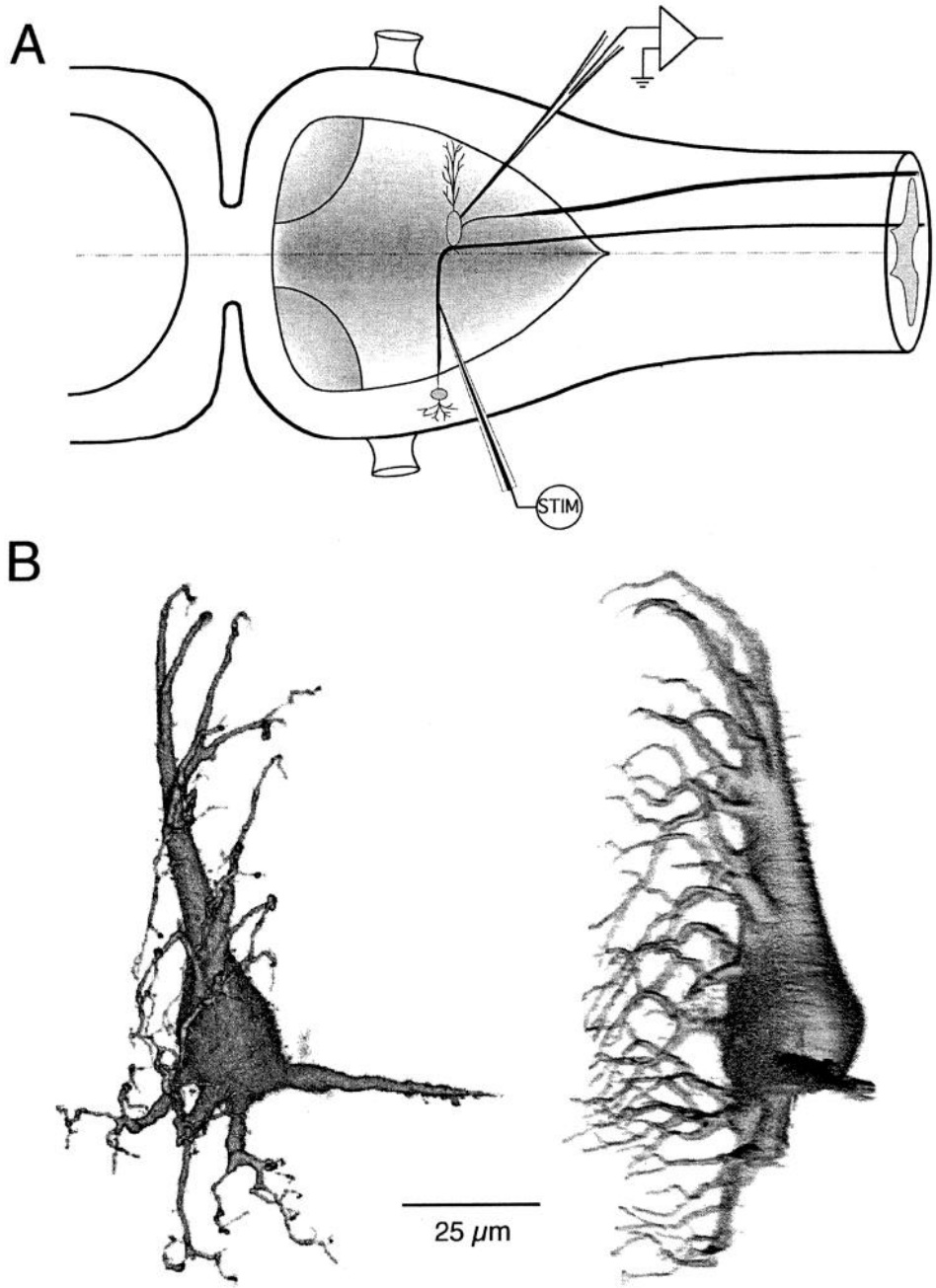

Figure 2. Identification of the neurons recorded in the PRRN. *A*, Schematic representation of the brainstem preparation. Axons of VS neurons are stimulated extracellularly with a glass coated tungsten microelectrode. PSPs are recorded intracellularly using sharp microelectrodes or patch pipettes. *B*, To investigate the morphology of the recorded reticulospinal neurons, the fluorescent dye Lucifer yellow (1%) was included in the electrode in some experiments. At the end of the experiment, cells were loaded with dye by pressure application through the recording pipette. These confocal images were obtained from such a cell. In the *right panel*, the neuron is seen from the ventral aspect of the brainstem; in the *left panel* the neuron is viewed orthogonally from the direction of the spinal cord.

The early component of the response to stimulation represents a pure monosynaptic input to the reticulospinal neurons. To ensure that the potentiation measured represented a change in the amplitude of this monosynaptic component, the early component of the response was measured following each stimulus. This eliminates the possibility that possible late, contaminating polysynaptic components contribute to the measured LTP. To achieve this, the slope of the initial 4 msec of the response was also analyzed. The 4 msec period was chosen because experiments involving single unit inputs to these cells under patch-clamp conditions indicate a time to peak of approximately 4 msec. Single units were recorded by reducing the stimulus intensity to a level that evoked all or none synaptic responses while recording from the reticulospinal neuron under current clamp with whole-cell patch recording (Alford, unpublished observation). A similar profile of potentiation following application of tetanic stimulation was evident when measurements were made of the initial slope of the synaptic response (Fig. 3*B,D*). The mean increase in initial slope of the PSP measured between 30 and 40 min after tetanic stimulation was significant and to 155 ± 14% the control slope (data are pooled from all control preparations; $p < 0.05$). As for the measurements of peak response amplitude, the potentiation of the EPSP slope demonstrated an early phase of higher amplitude falling to a plateau phase after 15–30 min (Fig. 3*B,D*). The time window over which this measurement of slope was recorded is shown in the inset to Figure 3*B*, in which the PSPs recorded before and after LTP induction are superpositioned and shown at a faster timebase.

LTP is specific to the tetanized pathway

LTP of synaptic inputs from vestibulospinal axons to the PRRN is specific to the tetanized pathway. Reticulospinal neurons of

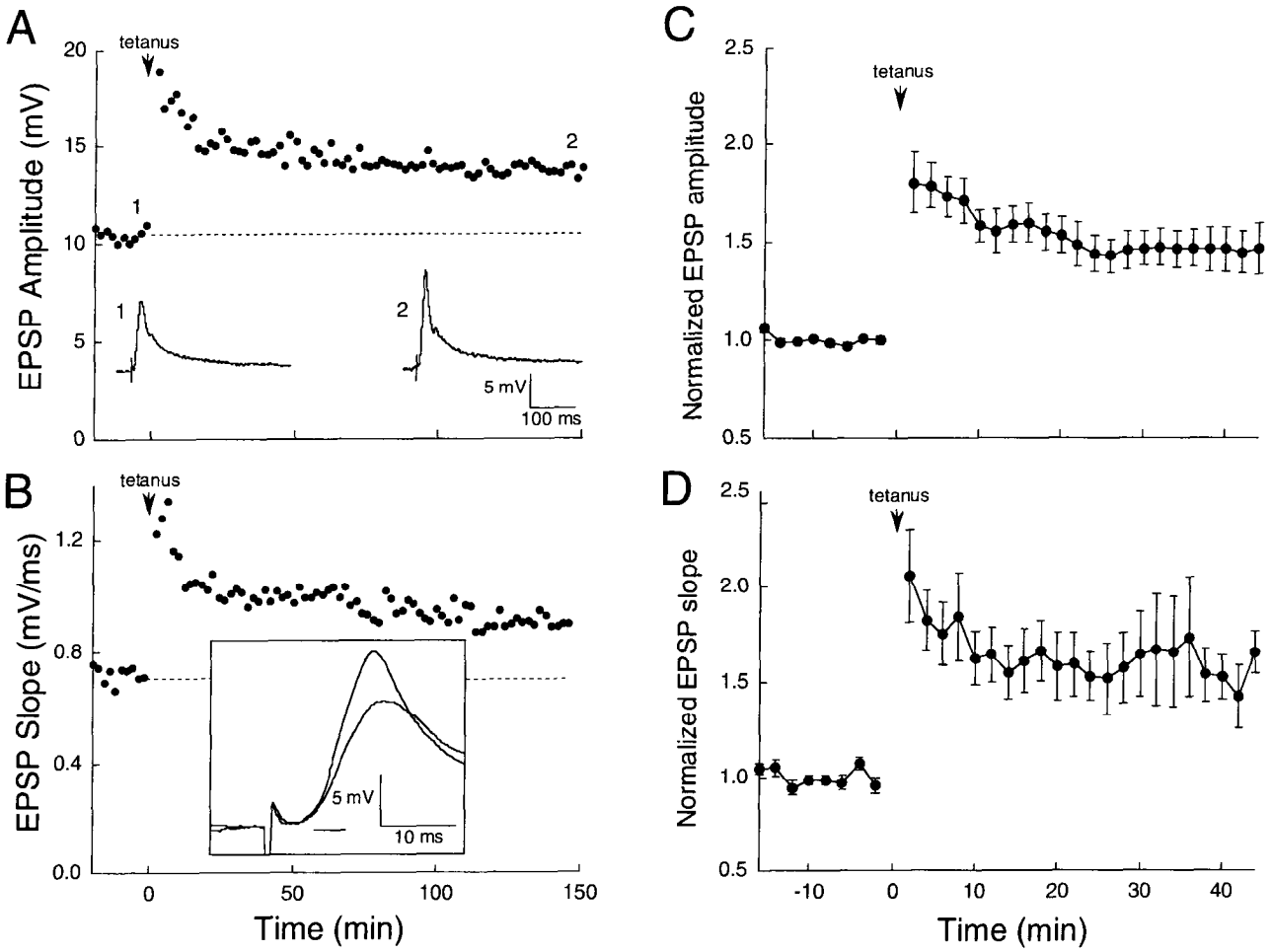

Figure 3. Changes in EPSP amplitude and initial slope induced by tetanic stimulation. *A*, Graph showing a plot of peak EPSP amplitude against time for responses evoked at low frequency (30 sec intervals). The data are from the same cell shown in Figure 2. *Insets* show sweeps taken from the times indicated by the numbers on the graph. A tetanus (50 shocks, 1 sec at test intensity) was evoked at the time marked by the *arrow*. *B*, A similar plot from the same data, but demonstrating the relationship between the slope of the initial 4 msec of the low frequency EPSP. The slope was recorded during the interval marked by the *single horizontal bar* on the *inset* plots. The latter are taken from the same sweeps as the *insets in A* but shown superimposed at a faster time resolution. The EPSP recorded after the tetanus is illustrated in *bold*. *C*, Pooled data showing the time dependency of the peak amplitude of responses and the effect of tetanic stimulation. The graph represents normalized pooled data from 10 reticulospinal neurons of the PRRN in which a tetanus of 50 shocks, in 1 sec at test intensity was given to the vestibular input to these neurons. *D*, Pooled data from the same experiments described in *C* above. This data plots the slope of the first 4 msec of the EPSP with time. In *A* and *B*, error bars denote SEM.

the PRRN were again recorded from with microelectrodes. Stimulus electrodes were placed over axons of the contralateral nOMP and ipsilateral nOMI with respect to a recorded neuron (Fig. 4*A*). Stimuli were then applied alternately to the two electrodes at intervals of 20 sec (interval between repeated stimulation at the same site was 40 sec). After obtaining stable control records for between 10 and 30 min (Fig. 4*C1*), tetanic stimulation was applied to only one of the stimulated pathways. Only the input recorded from the tetanized pathway demonstrated a potentiation (Fig. 4*B,C2*). The nontetanized pathway gave the same response amplitude as had been recorded prior to the application of high frequency stimulation. During a further 30–40 min of stable responses the tetanized pathway remained potentiated in all experiments ($n = 3$) and the nontetanized pathway remained stable at baseline amplitude. Tetanic stimulation applied only to the previously untetanized pathway then evoked a stable potentiation in that pathway leaving the first tetanized pathway inputs stable at its previously potentiated amplitude (Fig. 4*B,C3*).

LTP requires a postsynaptic Ca^{2+} signal

To test if the induction of LTP in reticulospinal neurons of the PRRN involves the activation of a postsynaptic mechanism, neurons were recorded from, under current-clamp conditions, but using whole-cell patch-clamp methods. The internal solution of the patch electrode contained a high concentration (10 mM) of the Ca^{2+} buffer EGTA. In addition, under these conditions, synaptic responses (Fig. 5*A*) were similar to those recorded using microelectrode impalements although input impedances were larger. Stable low frequency responses were obtained as for recordings using microelectrodes. Records were obtained prior to tetanization for up to 15 min under these circumstances. Tetanic stimulation did not initiate LTP. Instead, a brief but relatively small potentiation (mean increase in amplitude of this posttetanic

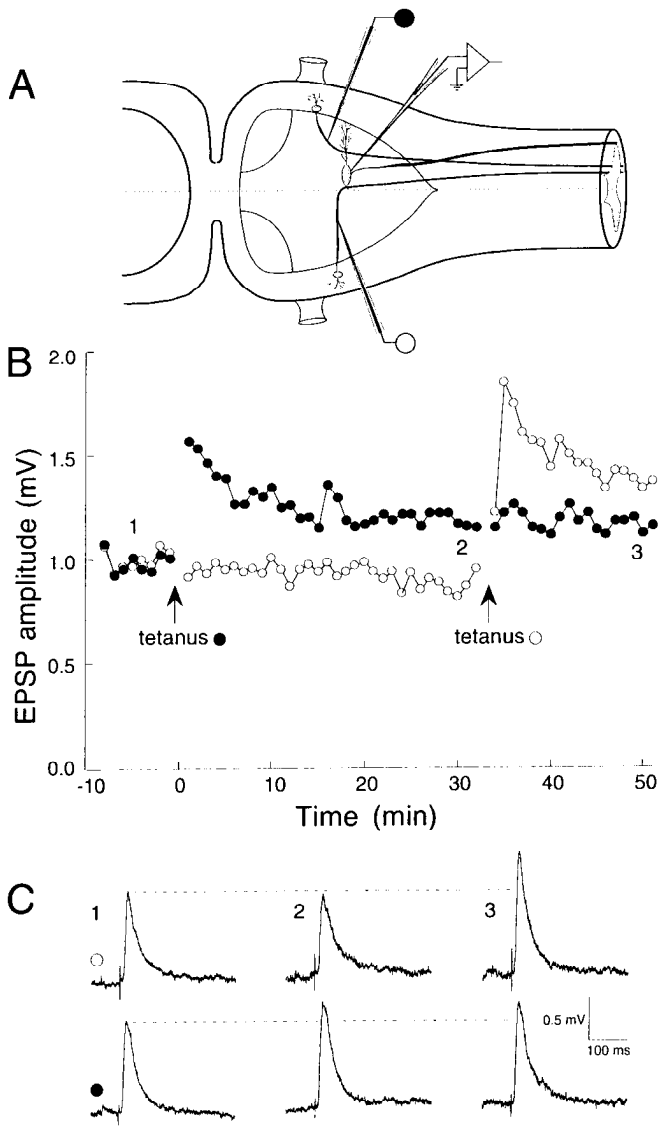

Figure 4. Pathway specificity of LTP. *A*, Diagrammatic representation of the preparation used. Stimulus microelectrodes were positioned over the vestibulospinal fibers from the nOMI ipsilateral to a recorded reticulospinal neuron in the PRRN and the nOMP contralateral to the recorded neuron. Stimuli were applied alternately to each of the electrodes at 20 sec intervals (a 40 sec cycle time). *B*, Graph showing peak amplitudes of the responses following stimulation of axons from the nOMP (*open circles*) and from the nOMI (*solid circles*). Responses were obtained for 10 min prior to tetanic stimulation of the nOMP (50 shocks, 1 sec at test intensity). This stimulation resulted in an increase in amplitude of the low frequency response evoked from the axons of the nOMP but left the response evoked by nOMI stimulation unchanged. Following 33 min of recording the nOMI was tetanised (50 shocks, 1 sec at test intensity). This resulted in a sustained increase in amplitude of the response of low frequency stimulation of axons from the nOMI but left the response of stimulation of axons from the nOMP unchanged. *C*, The individual records of low frequency stimulation before (*C1*) tetanus, and after the two (*C2, C3*) tetani for the stimulated pathways and time periods marked by the numbers. The *dashed lines* indicate the control amplitude. Records shown are averages of three recorded responses at 40 sec intervals.

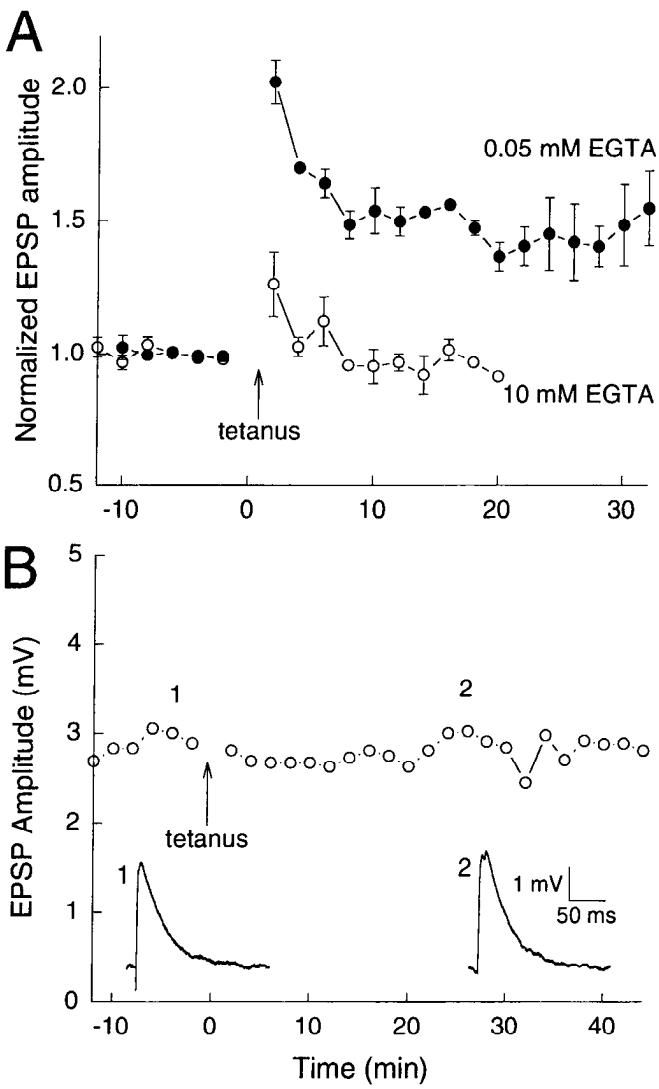

Figure 5. LTP is dependent upon a postsynaptic change in Ca^{2+} concentration. *A*, The graph plots peak amplitude of the mean normalized EPSP amplitude with time. The neurons were recorded under whole-cell patch conditions in current-clamp mode, but under two different conditions. The *open circles* plot the mean response of five cells recorded with a patch electrode containing 10 mM EGTA. Under these circumstances, tetanic stimulation (50 shocks, 1 sec at test intensity) led to a short term potentiation of the low frequency evoked response in some cells but no LTP. The *solid circles* plot the mean response recorded to tetanic stimulation in four cells investigated under current clamp with patch pipettes, but with only 0.05 mM EGTA in the patch pipette. *B*, Plot of peak EPSP amplitudes of a single cell with time before and after the application of a tetanus (50 shock, 1 sec at test intensity) in one neuron recorded with a patch pipette in current-clamp mode when the electrode contained 10 mM EGTA. The *inset* shows evoked PSPs from the time points marked in the graph and show the response before, and after the tetanic stimulation.

potentiation was to 126 ± 12% of control $n = 6$) was observed in some of the cells investigated. Subsequently the response returned to 99 ± 2% of control. No attempt was made to load the neurons with compounds to maintain phosphorylation of postsynaptic ion channels or receptors. This, coupled with the possible dialysis of soluble postsynaptic messengers involved in LTP induction, may have resulted in the failure of these cells to show LTP. Dialysis of the neuron by using whole-cell patch recording did not, however, cause a time-dependent change in response to the low-frequency stimulation recorded (Fig. 5*B*).

In order to rule out the possibility that dialysis of the neurons, in itself, prevented the induction of LTP, further experiments were performed in which the patch pipette solution contained a

much reduced concentration of EGTA (0.05 mM), but was otherwise identical to that used above. Four cells were recorded under current-clamp conditions between 12 and 15 min prior to the application of the tetanus. As for the experiments described above no time-dependent change in the low frequency-evoked EPSP was recorded (Fig. 5*A*; see the control period prior to the application of tetanus). Application of tetanic stimulation caused a sustained and significant increase in the amplitude of the synaptic response similar to that recorded with microelectrodes (mean increase in PSP amplitude was to 148 ± 12% of the control response; $p < 0.05$). These data indicate that, during patch-clamp recording, intracellular chelation of Ca^{2+} in the postsynaptic reticulospinal neuron with EGTA prevents the induction of LTP rather than a dialysis of soluble second messenger molecules between the recording pipette and the cell.

To confirm that the potentiation represented an increase in the amplitude of the EPSP, experiments were performed to monitor the effect of tetanic stimulation on IPSP amplitude. Three reticulospinal neurons were recorded under whole-cell patch clamp and held at −70 mV. The vestibular afferents were stimulated to evoke a PSC (Fig. 6*A1*). This response was largely inward current as expected after stimulation of the vestibular excitatory inputs at this holding potential. The neurons were then held at 0 mV, close to the AMPA receptor reversal potential. Stimulation of the vestibular afferents then led to an early inward component, most likely due to the activation of the electrical component of the EPSC. This was followed by an outward conductance, the IPSC. This protocol enabled the visualization of the IPSC without significant contamination by the EPSC. It was not possible to wash in an antagonist to the excitatory component for these experiments because this would have also abolished the polysynaptic IPSC. Tetanic stimulation was then applied and the amplitude of the IPSC measured before and after tetanus. The IPSC amplitude transiently increased then returned to the control amplitude within 5 min of application of the tetanus (Fig. 6*A2*).

Experiments were also performed in the presence of the glycine receptor antagonist strychnine (5 μM). Most of the inhibitory component of synaptic transmission is blocked using this protocol. However, application of strychnine to the brainstem led to extended periods of uncontrolled excitation and depolarization of the reticulospinal neurons. To overcome this problem, the alar plates containing the vestibular nuclei were removed from both sides of the brain as was the spinal cord and all tissue rostral to the medial rhombencephalic reticular nucleus (approx 1 mm rostral to the recording site) in four preparations. Stimulation of the vestibular pathway then led to a similar input to the reticulospinal neurons as without strychnine application (Fig. 6*B*, insets). Reticulospinal neurons of the PRRN were then impaled with microelectrodes and LTP experiments were performed as described above. Tetanic stimulation again led to a maintained increase in EPSP amplitude (Fig. 6*B,C*; $n = 4$; increase was to 137 ± 9% of control after 30 min) that was not significantly different from the potentiation seen in control preparations. This was followed for a minimum of 34 min after application of the tetanus.

LTP does not require the activation of NMDA receptors

A number of forms of LTP have been shown to require the activation of NMDA receptors (Collingridge et al., 1983). To test if this were the case for vestibular inputs to lamprey reticulospinal neurons, experiments were performed in the presence

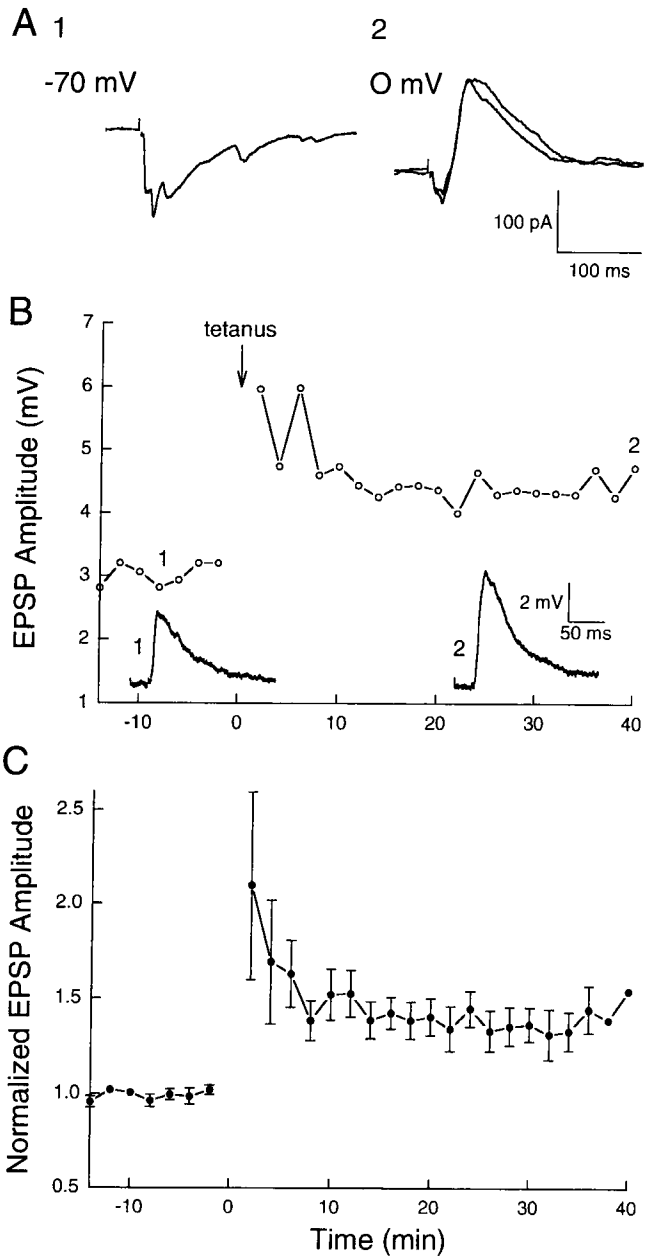

Figure 6. Potentiation is not a result of decreased inhibition. *A1*, Patch-clamp recording of a whole cell current following stimulation of vestibular fibers in the brainstem. The neuron was held at −70 mV, revealing an inward current. *2*, The response in the same cell to the same stimulus but with the cell held at 0 mV. Following an early inward current an IPSC is observed. Superimposed are two traces, the average of four stimuli prior to tetanic stimulation and the average of four stimuli 5 min after tetanus. No significant difference between the traces is seen. *B*, Plot of peak EPSP amplitudes of a single cell with time before and after the application of a tetanus (50 shock, 1 sec at test intensity) in 1 neuron recorded with a microelectrode. The insets shows evoked PSPs from the time points marked in the graph before, and after, the tetanic stimulation. The tissue was perfused in 5 μM strychnine throughout the experiment. To prevent large spontaneous depolarizations the alar plates of the brainstems were removed. Stimuli were applied as for controls (Fig. 3). *C*, Plot of the normalized mean amplitude of EPSPs recorded from four cells in four preparations after the application of 5 μM strychnine to the preparation. Following tetanic stimulation a maintained potentiation of the EPSP amplitude was observed.

of the NMDA receptor antagonist, AP5. Stimuli were applied to the axons of the nOMI and reticulospinal neurons recorded from the ipsilateral PRRN with microelectrodes again containing 4 M potassium acetate. Control responses were obtained from low frequency stimuli. Responses were similar to responses obtained above. All resting membrane potentials were between −70 and −80 mV. In 10 neurons, AP5 (100 μM) was added to the perfusate and responses were recorded under these conditions for a further 15 min. The application of AP5 had no significant effect on the PSP shape or amplitude. AP5 has been shown to block the NMDA component of synaptic transmission at this dose and after this period of washin (Alford and Dubuc, 1993a; and see Fig. 8*D* below). Observation of PSPs before and after the application of AP5 stimulated at low frequency demonstrated no significant change in PSP amplitude when recorded at resting membrane potential. Tetanic stimulation was then applied to the stimulated pathway. Stimulation was then reverted to record low frequency PSPs once more. In all cases, potentiation of the low frequency PSP amplitude was observed (Fig. 7*A,B*). The mean increase was to 146 ± 9% ($p < 0.05$; see Fig. 6*B* for pooled data). This potentiation lasted for the period of recording in all cases (minimum 30 min, maximum 60 min). In three of these neurons, after at least 30 min of recording and following washout of the AP5, a second period of tetanic stimulation was applied which resulted in no significant further potentiation of the low frequency PSPs, as observed in cases where no prior application of AP5 had occurred. The mean increase in peak amplitude of the synaptic response on application of this second tetanus was to 104 ± 4% of the control amplitude and was not significant.

LTP induction requires the activation of a metabotropic glutamate receptor

Activation of metabotropic glutamate receptors (mGluRs) is known to occur in the vestibulo-reticulospinal pathway investigated in this study (Alford and Dubuc, 1993a). Additionally, mGluRs are believed to play a role in LTP induction in mammalian systems. In the absence of a role for NMDA receptor activation in the induction of LTP in this glutamatergic synapse, it was of interest to test for a possible role of mGluRs. MCPG has been shown to antagonize some mGluRs selectively, with little or no effect on other amino acid receptor subtypes (Birse et al., 1993). Consequently, this compound was tested for efficacy at the vestibulospinal/reticulospinal synapse. Low frequency responses were observed with microelectrodes in reticulospinal neurons of the PRRN by stimulation of vestibulospinal axons of the nOMI. After obtaining a stable response, 500 μM MCPG was applied to the superfusate. No discernible effect was recorded upon the evoked synaptic response (Fig. 8*A*). After recording stable responses for a further 15 min, a tetanic stimulation was applied. In all 15 cells tested, a small initial potentiation was obtained following the tetanus. A small, but statistically insignificant, sustained increase was seen in most of the cells investigated in this study such that pooled data demonstrate a mean increase in peak amplitude of the EPSP to 107 ± 13% of the control data (Fig. 8*A*). Subsequent washout of the MCPG had no effect upon the amplitude of the evoked response. To ensure that the application of MCPG did not alter the amplitude of the NMDA component of the synaptic response, in three cells this component was isolated by the application of the AMPA receptor antagonist CNQX (5 μM; Fig. 8*D*). After 5 min of washing a stable synaptic response was recorded of smaller amplitude and slower time to peak (Fig. 8*D*, left panel). MCPG (500 μM) was then added to the perfusate but had no effect on this smaller component (Fig. 8*D*, right panel). Subsequent application of AP5 (100 μM) much reduced this remaining component.

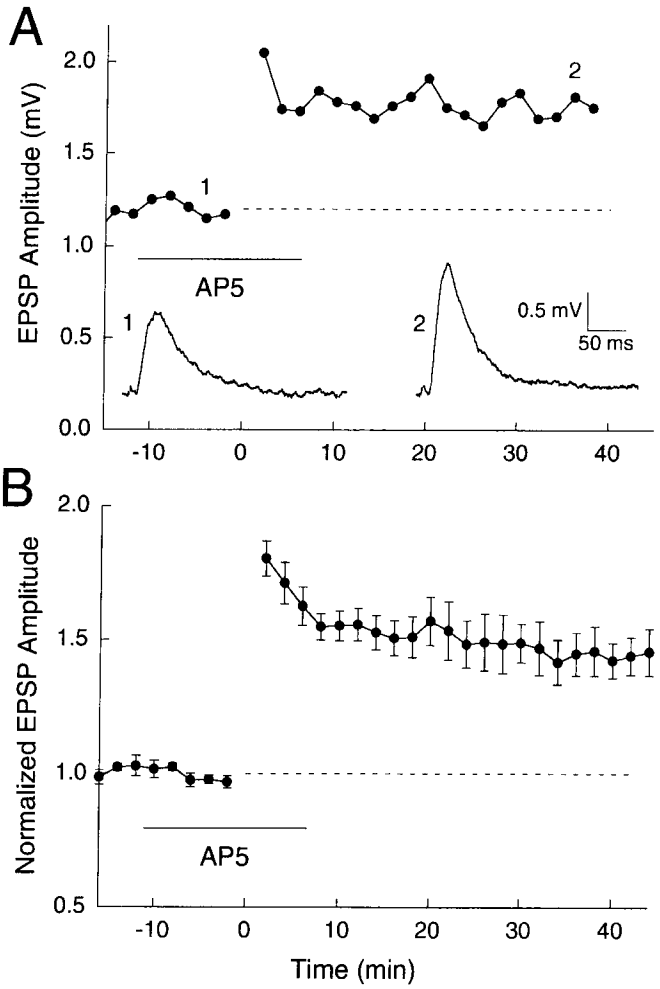

Figure 7. Lack of effect of the NMDA receptor antagonist AP5 on the induction of LTP. *A*, Graph plotting the peak amplitude of low frequency evoked PSPs with time. Stimuli were applied to the nOMI pathway at 30 sec intervals. AP5 (100 μM) was applied for 10 min prior to the tetanus and then washed after tetanic stimulation as indicated by the bar. Representative EPSPs are illustrated below. EPSPs were evoked by low frequency stimulation taken from the time points marked by the corresponding numbers in the graph. *B*, Pooled normalized data from 10 neurons in which AP5 (100 μM) was applied to the preparation during the tetanus, and from 10 min before. The graph is a plot of normalized mean EPSP amplitude versus time.

In six of these neurons, MCPG was washed from the superfusate and recording of responses to low frequency stimulation was continued for 60 min following tetanic stimulation. At the end of this recording period, the pathway was tetanized once more. In two of the neurons tested in this way, LTP was subsequently recorded and lasted until termination of the experiment in both cases (at least 30 min; Fig. 8*B,C*). The mean sustained increase in amplitude was 130 ± 10%. The remaining four neurons did not show any further increase in EPSP amplitude after this second tetanus. It seems unlikely that this failure of the remaining neurons to demonstrate potentiation represents a chance effect of recording from cells which were initially in-

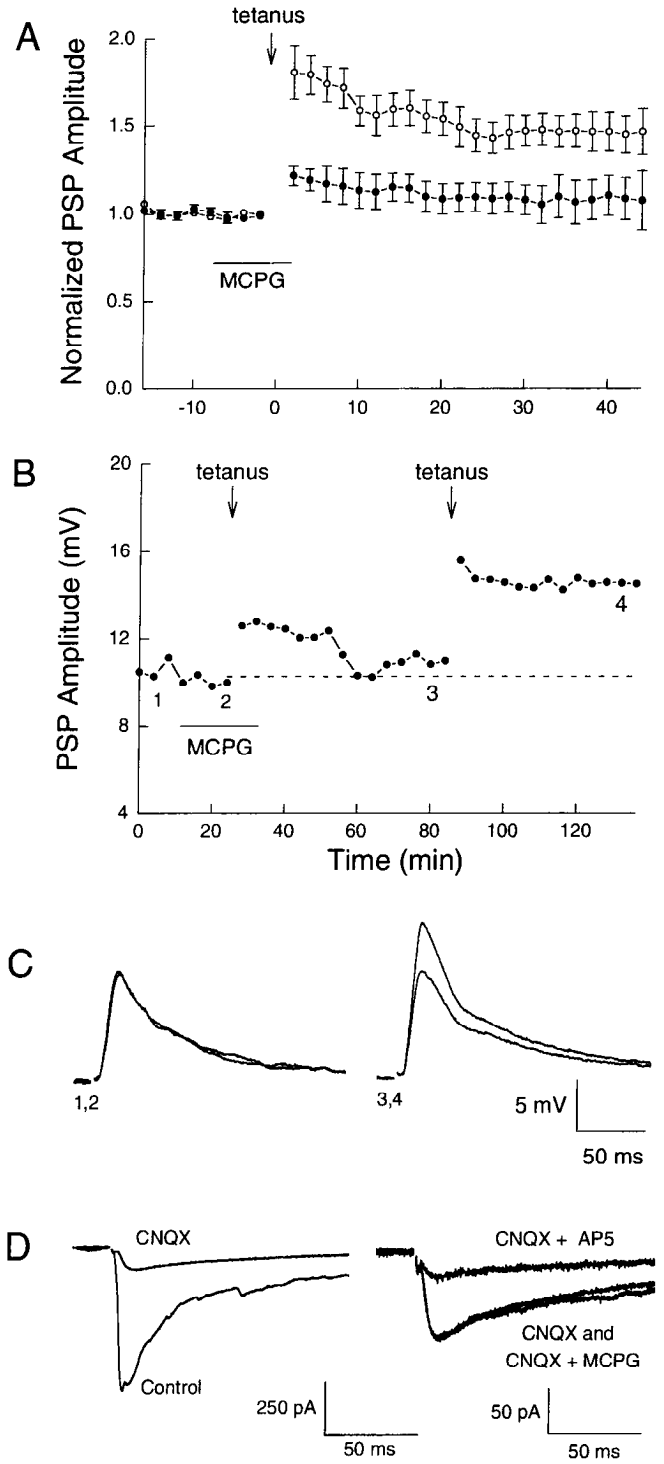

Figure 8. Effects of the mGluR antagonist MCPG on LTP induction. *A*, The *solid circles* plot the normalized mean EPSP amplitudes from 15 cells for which MCPG was added to the preparation during the tetanic stimulation. Application of MCPG (as indicated by the bar) during, and for 10 min prior to, tetanic stimulation prevented the induction of LTP. Control data in which no drug was added to the bath are shown in *open circles*. Note that these control data were taken from Figure 4*A*. *B*, Graph plots the peak amplitude of low frequency evoked PSPs recorded in one neuron following stimulation of axons from the nOMI with time. The mGluR antagonist MCPG (500 μM) was applied for 10 min prior to, and during, tetanic stimulation as indicated by the *horizontal bar*. Tetani were applied when marked by the *arrows*. The second tetanic stimulation, applied after washout of the MCPG, caused the induction of LTP. *C*, Records of evoked PSPs taken from the time points marked by the corresponding numbers in the graph (*B*). *D*, Records of stimulated responses in voltage clamp following whole-cell patch recordings in the presence of 5 μM strychnine. The *right panel* shows the effect of the application of CNQX on the control response. Responses shown are the averages of four evoked EPSCs before and after application of 10 μM CNQX. The *left panel* shows the same response in CNQX but with an expanded vertical axis. Superimposed on this is the average of four evoked responses after the addition of 500 μM MCPG. The smaller response demonstrates the effect of the addition of 50 μM AP5.

capable of showing LTP. In all 41 other cells tested, in which a mGluR antagonist was not applied to the preparation, LTP occurred after the application of tetanic stimulation. It is possible that either MCPG may evoke a long lasting change in the preparation or that washout of the drug was difficult and required extended periods in many of the preparations. Alternatively because the washout period for MCPG appears to be over 1 hr, the extended recording with microelectrodes may have prevented the induction of LTP in many cells.

Discussion

Results from this study reveal that vestibular inputs to brainstem reticulospinal neurons undergo a robust and sustained potentiation following high frequency transmission from the vestibulospinal system. The lower frequency range over which it was possible to evoke this response (20 Hz) implies a physiological role for this form of LTP. This coupled with the accessibility of the *in vitro* lamprey preparation, in which the CNS may be maintained intact, suggests this model may provide an excellent preparation for the study of synaptic plasticity within the sensorimotor systems.

The accessibility of lamprey synapses, both the presynaptic terminal and the postsynaptic element, for electrophysiological investigation also stresses the advantages of this preparation in the elucidation of some of the basic mechanisms of cellular plasticity of vertebrate glutamatergic synapses. Consequently, we have attempted, in this study, to demonstrate some of the basic mechanisms by which this synapse undergoes a long-term plastic change. It is clear that the initiation of LTP in the vestibuloreticulospinal synapse requires a form of postsynaptic activity, although data from this study do not demonstrate whether the maintenance of LTP is either pre- or postsynaptic in nature. The dialysis of reticulospinal neurons by using whole-cell patch-clamp recording with pipette solutions containing high concentrations of EGTA abolished long term changes in synaptic efficacy. This manipulation, similar to results observed in mammalian hippocampus (Lynch et al., 1983; Malinow and Tsien, 1990), will have had its effect solely on the postsynaptic reticulospinal neuron from which the recording was made. This result also indicates that the LTP seen in these experiments must have resulted from a potentiation of synaptic transmission onto the reticulospinal neuron. Dialysis of this neuron could not have altered the properties of polysynaptic pathways later impinging upon this neuron because dialysis would not have affected neurons that were not recorded from directly. Recording from the reticulospinal neurons with a patch pipette solution with a reduced concentration of the Ca^{2+} chelator, EGTA (to 0.05 mM), allowed the induction of LTP of neurons recorded in this manner. This result indicates that a postsynaptic Ca^{2+} signal is required to induce or maintain LTP during or after tetanic stimulation. This situation may be very similar to that seen in the mammalian hippocampus where tetanic stimulation leads to a rise in den-

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dritic Ca^{2+} following activation of the NMDA receptor (Regehr and Tank, 1990; Alford et al., 1993), the metabotropic receptor (Frenguelli et al., 1993) and release of Ca^{2+} from intracellular stores (Alford et al., 1993). This potentiation was also not a result of the decreased activation of inhibitory inputs onto reticulospinal neurons. No change in the amplitude of the inhibitory component was recorded after tetanic stimulation, while the excitatory component recorded in the presence of strychnine was also potentiated.

The components of the EPSP (AMPA, NMDA, or electrical) has not been investigated in this study. It is likely that at the least the AMPA component is potentiated. The early response is potentiated significantly, a response that is unlikely to be mediated by NMDA receptors because of their slow time course. It was not however, possible to conclude whether the electrical component underwent potentiation. We have found this component to be very variable (from 50 to 5% of the intact EPSP amplitude when it was measured. This has also been described for synapses between descending reticulospinal axons and spinal neurons in lampreys (Ohta and Grillner, 1989). It is not without precedent that this component might undergo LTP, however. Electrical inputs to goldfish Mauthner cells have been shown to undergo such a phenomenon (Yang et al., 1990).

Potentiation of the inputs to reticulospinal neurons is homosynaptic, that is, only the pathway that undergoes a period of high frequency stimulation (tetanus) demonstrates LTP. In this respect, this phenomenon appears similar to data obtained in other vertebrate preparations. This result also implies that the reticulospinal neurons in the lamprey are able to contribute to forms of motor learning, specifically with regard to their input from the vestibulospinal system. Physiological rates of synaptic input from the vestibular nuclei to the reticulospinal system have not yet been elucidated. Rates as high as 50 Hz have been recorded in primary afferents of the VIII nerve (Deliagina et al., 1992a) but recordings have not been made from the vestibulospinal neurons in response to natural stimulation in the lamprey. Presumably if such rates are common then a mechanism for depression of the synaptic response must also exist at these synapses. Such specificity of learned responses may be considered critical to the phenomenon of motor learning, if the reticulospinal neurons, as integrators of sensory input to the motor system, are capable of relating past experience to particular sensory modalities. The role of reticulospinal neurons as a component of a motor learning system may be critical in the lamprey which lacks a developed cerebellum.

The lack of susceptibility of this form of LTP to antagonism of NMDA receptors is perhaps surprising since these receptors are known to contribute to synaptic transmission in this pathway (Alford and Dubuc, 1993a). However, in the light of the limited effect of NMDA receptor antagonism on the high frequency mediated response in this preparation, when evoked from resting membrane potential and in the presence of physiological concentrations of extracellular Mg^{2+}, this result may imply that either NMDA receptor activation initiates limited plastic changes, or that these receptors play a different role in synaptic transmission at this pathway.

Roles for mGluRs in various forms of long and short term changes at glutamatergic synapses now appear widespread. There is now strong evidence that these receptor subtypes are involved both in the initiation of LTP in CA3 and CA1 (Anwyl, 1991; Bashir et al., 1993) regions of the mammalian hippocampus in addition to acting as autoreceptors in numerous regions of the CNS, including the brainstem of the lamprey (Alford and Dubuc, 1993a). The efficacy of the mGluR antagonist, MCPG, in preventing the induction of LTP in the brainstem of the lamprey provides strong evidence that this plastic change is mediated by mGluRs. The lack of effect of mGluR blockade on the amplitude or time course of the low frequency-evoked PSP indicates that mGluRs are necessary for the induction phase of LTP in this preparation.

Concluding remarks

This investigation has clearly demonstrated that a long lasting enhancement of synaptic efficacy may occur in the brainstem of the lamprey. This phenomenon demonstrates the basic properties of LTP. (1) It lasts for extended periods. (2) It is not evoked by weak inputs. Ten hertz stimulation of the vestibular nerve leads only to posttetanic potentiation in this preparation, but higher frequency inputs are capable of activating this enhancement (Wickelgren, 1977a). (3) The enhancement is synapse specific; only those inputs that undergo tetanic stimulation show enhancement. Taken together, these findings suggest that these properties are similar to those of LTP in the mammalian brain. Finally, this LTP is evoked not by the activation of NMDA receptors but rather by the activation of mGluRs.

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