Current and Calcium Responses to Local Activation of Axonal NMDA Receptors in Developing Cerebellar Molecular Layer Interneurons

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Abstract

In developing cerebellar molecular layer interneurons (MLIs), NMDA increases spontaneous GABA release. This effect had been attributed to either direct activation of presynaptic NMDA receptors (preNMDARs) or an indirect pathway involving activation of somato-dendritic NMDARs followed by passive spread of somatic depolarization along the axon and activation of axonal voltage dependent Ca²⁺ channels (VDCCs). Using Ca²⁺ imaging and electrophysiology, we searched for preNMDARs by uncaging NMDAR agonists either broadly throughout the whole field or locally at specific axonal locations. Releasing either NMDA or glutamate in the presence of NBQX using short laser pulses elicited current transients that were highly sensitive to the location of the spot and restricted to a small number of varicosities. The signal was abolished in the presence of high Mg²⁺ or by the addition of APV. Similar paradigms yielded restricted Ca²⁺ transients in interneurons loaded with a Ca²⁺ indicator. We found that the synaptic effects of NMDA were not inhibited by blocking VDCCs but were impaired in the presence of the ryanodine receptor antagonist dantrolene. Furthermore, in voltage clamped cells, bath applied NMDA triggers Ca²⁺ elevations and induces neurotransmitter release in the axonal compartment. Our results suggest the existence of preNMDARs in developing MLIs and propose their involvement in the NMDA-evoked increase in GABA release by triggering a Ca²⁺-induced Ca²⁺ release process mediated by presynaptic Ca²⁺ stores. Such a mechanism is likely to exert a crucial role in various forms of Ca²⁺-mediated synaptic plasticity.

Introduction

In the central nervous system, postsynaptic NMDARs are often seen as canonical coincidence detectors in induction of synaptic plasticity. In addition, several lines of evidence indicate that NMDARs are present on presynaptic elements arguing for their possible involvement in presynaptic plasticity processes [1,2]. Presynaptic NMDARs [preNMDARs] have been anatomically or functionally detected at both GABAergic [3–6] and glutamatergic [7,8] termini (see [9] for review). Moreover, their implication in long-term plasticity has been suggested in various structures including the visual cortex [6], the neocortex [10,11] and the cerebellum [8,12,13]. In the cerebellum, Lorchamp et al. [14] have shown that the preNMDARs expressed in parallel fibers (PFs) could mediate an increase in the frequency of miniature IPSCs recorded in Purkinje cells. On the other hand, several studies have suggested that MLIs express preNMDARs. Application of exogenous NMDA increases the frequency of miniature IPSCs (mIPSCs) [3,12,15] following an elevation of presynaptic Ca²⁺ [16]. This signal is thought to be provided by Ca²⁺ influx through preNMDARs rather than by activation of voltage-dependent Ca²⁺ channels (VDCCs) [4,17], and to be amplified through a Ca²⁺-induced Ca²⁺ release (CICR) process [4]. Two main sources of glutamate have been proposed to activate preNMDARs on MLIs: spillover triggered by an intense stimulation of parallel fibers (PFs) [12,18] or retrograde signaling from Purkinje cell dendrites [4,19]. In cerebellar slices, the tonic activation of preNMDARs [3,15] has been attributed to a spillover mechanism that is likely to be physiologically limited by glial glutamate transporters [15]. Moreover, glutamate spillover was proposed to contribute to long term potentiation of GABAergic synapses through preNMDAR stimulation [12] whereas retrograde activation of preNMDARs has been proposed to potentiate GABA release at the MLI-Purkinje cell synapse [4].

Even though activation of NMDARs clearly affects GABA release, it was recently argued that MLIs may not express axonal NMDARs [20,21]. It was suggested instead that NMDARs are exclusively expressed in the somatodendritic domain, and that the depolarization induced by somatodendritic NMDARs is transmitted to the axon compartment due to the passive properties of the axon cable, leading to presynaptic VDCC activation [22]. A similar mechanism may apply to other neurons as well, so that the very existence of functional presynaptic NMDARs in brain neurons remains controversial [23].
In the present work we show that NMDA superfusion leads to axonal Ca$$^{2+}$$ elevations under somatic voltage clamp and we map the NMDA sensitivity over the entire cell using wide field and local uncaging of NMDAR agonists. These methods reveal discrete spots of high sensitivity in the axon domain indicating that MLI could contain functional preNMDARs. Finally, we reexamine the pharmacological properties of NMDA-induced mIPSC enhancement, finding little sensitivity to VDCC blockers. Altogether the data provide a characterization of the effects of preNMDARs activation on cellular current and Ca$$^{2+}$$ signaling.

**Results**

**Activation of Axonal NMDARs Under Somatic Voltage Clamp Conditions**

The original suggestion that MLIs have functional pre-NMDARs has emerged from the observation that bath application of NMDA strongly increases the frequency of mIPSCs recorded in the two cell types that are innervated by MLIs i.e. Purkinje cells and other MLIs [3]. It was however immediately pointed out that, as an alternative or complementary interpretation to the direct activation of preNMDARs, axonal depolarization could result from activation of somatodendritic NMDAR following passive spread of somatic depolarization along the axon cable [3]. The latter component of the response would lead indirectly to GABA release, as the secondary depolarization of synaptic terminals would locally activate VDCCs producing an elevation of GABA release. Such a mechanism was recently described in the case of subthreshold somatic depolarizations [22,24]. In fact, Christie and Jahr (2008, [20]) proposed that the NMDA-evoked depolarization exclusively arises from this indirect mechanism, and does not involve a direct action of NMDA on the axon. In this view, all NMDARs in MLIs would be somatodendritic. According to this hypothesis, somatic depolarization is an obligatory intermediate between activation of somatodendritic NMDARs and the subsequent increase in axonal Ca$$^{2+}$$ concentration. If this is the case, then clamping the soma at a negative holding potential should prevent the effect entirely [20]. We therefore examined local axonal Ca$$^{2+}$$ signalling following bath application of NMDA by performing 2-photon imaging of axon stretches in voltage clamped MLIs. In each experiment the identification of the recorded neurite as the axon was confirmed by analysis of action potential induced Ca$$^{2+}$$ transients, which are markedly larger in the axon than in dendrites [25]. Following this identification, TTX (0.2 μM) and NMDA (50 μM) were sequentially added to the bath. In 7 MLIs, NMDA elicited inward currents with an average peak value of 26±8 pA under somatic voltage clamp (holding potential, −60 mV). Parallel to these currents, the Ca$$^{2+}$$-dependent fluorescence in axonal varicosities increased in all experiments, with ΔF/F0 peak values ranging from 12 to 177% (63±10%, 19 varicosities from 7 cells). The fluorescence changes were transient, and returned to baseline occurring within 2 to 4 minutes after washing the agonist. The two largest Ca$$^{2+}$$ transients in this series of experiments were obtained in basket cell terminals onto Purkinje cell somata. Fig. 1 illustrates one of these experiments, in which a basket cell was challenged with NMDA while imaging boutons on the Purkinje cell layer (Fig. 1A–C). The NMDA-induced current was accompanied by robust Ca$$^{2+}$$ transients in the boutons directly in contact with the Purkinje cell soma. These data indicate that NMDA application is able to elicit large Ca$$^{2+}$$ transients in basket cell terminals in spite of somatic voltage clamp.

In 5 additional experiments, NMDA applications were performed in the presence of 6 mM external Ca$$^{2+}$$. The Ca$$^{2+}$$-dependent fluorescence changes were larger with a peak ΔF/F0 amplitude of 340±89% (n = 5 cells). The effect had a gradual onset: even though a Ca$$^{2+}$$ increase was observed as soon as NMDA was applied, the response typically grew over a time course of several minutes in the continuous presence of NMDA, and it declined rather slowly after washing out the NMDA, with a time for returning to baseline of 2 to 5 min after agonist removal. Such slow kinetics are consistent with a participation of intracellular Ca$$^{2+}$$ stores in the NMDA-induced Ca$$^{2+}$$ response.

In summary, bath application of NMDA elicits significant axonal responses under somatic voltage clamp, both with 2 mM and with 6 mM extracellular Ca$$^{2+}$$. The axonal Ca$$^{2+}$$ response cannot be attributed to voltage escape following activation of somato-dendritic NMDARs since the somatically recorded axonal responses under somatic voltage clamp, both with 2 mM and with 6 mM extracellular Ca$$^{2+}$$. The axonal Ca$$^{2+}$$ response cannot be attributed to voltage escape following activation of somato-dendritic NMDARs since the somatically recorded NMDA-induced currents in these experiments did not exceed 50 pA. Thus, somatic voltage clamp must have prevented depolarization spread from the dendrites to the axon. The axonal Ca$$^{2+}$$ rise cannot be attributed to Ca$$^{2+}$$ diffusion from the somatodendritic compartment since instead of the continuous concentration gradient expected from a diffusion process, a patchy response pattern was observed, with high response locations (which were often found in basket cell terminals) following much lower response locations along the axon course. Therefore these results, like those of similar experiments previously reported on stellate cells [15] are potentially difficult to reconcile with the indirect hypothesis.

**Wide Field Glutamate Photorelease**

The preceding experiments leave the possibility open that NMDA would not bind directly to the MLI axon displaying the Ca$$^{2+}$$ response, but to some other structure (not necessarily in the same cell), which would lead to the axonal response by unknown intermediary steps. Bath NMDA applications are not appropriate in this context since they lead to protracted activation of NMDARs with an imprecise timing. By contrast uncaging experiments provide a much more precise control of NMDAR activation timing. In the next series of experiments, we produced step increases in glutamate concentration by adding the photosensitive precursor of glutamate MNI-glutamate and by applying UV flashes to the entire preparation. Slices were incubated in a low Mg$$^{2+}$$(0.1 mM) HEPES-buffered saline supplemented with MNI-glutamate (final concentration, 0.9 mM) for at least 0.5 h before the beginning of the experiment. MLIs were perfused with Oregon Green BAPTA 1 (OGB-1) through the patch pipette and kept in the presence of NBQX (5 μM) to block AMPARs. Alexa 488 was added to resolve long axonal stretches. Ca$$^{2+}$$ imaging was performed using a CCD camera (see methods).

As depicted in Fig. 2Aa (left panel) the plane of focus typically contained several regions of interest that were selected according to their presumptive axonal or dendritic nature. A train of 4 propagated action potentials led to larger Ca$$^{2+}$$ transients in the axonal compartment and therefore confirmed the identification of axon and dendrites (compare blue and black traces in Fig. 2Ac and Fig. 2Bb).

After application of TTX, a UV flash yielded somatic currents (36±4 pA, n = 23; Fig. 2Ad and Fig. 2Bc) accompanied by Ca$$^{2+}$$ transients in dendrites (black traces in Fig. 2Ad and Fig. 2Bc; ΔF/F0:33.7±5.0%, n = 11) as well as in certain regions of the axon (Fig. 2Ad, blue traces 4 and 5; Fig. 2Bc, blue trace 1; ΔF/F0:11.2±1.7%, n = 13). Responsive axonal varicosities were found in 5 out of 6 cells. As discussed above, in the context of the experiments with bath application of NMDA, since the soma was under voltage clamp the only mechanism that could account for a participation of somatodendritic NMDARs to any axonal
response would be Ca\textsuperscript{2+} diffusion along the axon. This can however be ruled out since responsive distal axonal spots follow irresponsible proximal spots as illustrated in Fig. 2Ad. In the case of the diffusion hypothesis, the Ca\textsuperscript{2+} concentration would be expected to gradually decrease from the soma. Importantly, axonal responses rose as fast as dendritic ones, with a delay shorter than the time resolution of imaging (50 ms). This argues against the possibility of slow intermediate steps intervening between NMDAR activation and axonal Ca\textsuperscript{2+} elevation.

**Local Glutamate Photorelease in the Axon**

To further characterize the local sensitivity of MLI axons to NMDA, we restricted photorelease of glutamate by using focused laser illumination [26]. In these experiments, like before, action potentials were blocked by TTX, and the local sensitivity of MLI neurites to glutamate was examined before and after blockage of AMPA receptors, using short and sharply focused laser pulses (405 nm; 0.1 to 1 ms). As shown in Fig. 3Ac, the uncaging spot had a half width of \(-1\) \(\mu\)m. Releasing glutamate in the absence of AMPA receptor blocker elicited somatic current transients that differed markedly according to the location of the laser spot (Fig. 3A,B). Previous work suggest that AMPA receptors are present in both somatodendritic and axonal compartments of MLIs [27,28], so that these experiments were expected to activate a mix of AMPA and NMDA receptors in both cases. When applied to the somato-dendritic compartment, laser pulses evoked robust current responses with rather high amplitude (139\pm 19 pA, \(n = 12\) locations out of 12 cells; Fig. 3A and C) and fast kinetics (20/80% rise time: 1.0\pm 0.1 ms, \(n = 12\); half decay time: 30\pm 4 ms, \(n = 12\); Fig. 3C). Axonal responses obtained under the same conditions displayed a smaller amplitude (28\pm 6 pA, \(n = 67\) varicosities out of 16 cells; Fig. 3A and C) and slower kinetics (20/80% rise time: 2.6\pm 0.5 ms, \(n = 9\) responses from 9 cells; half decay time: 84\pm 16 ms, \(n = 9\) responses from 9 cells; Fig. 3C). The somatodendritic responses were often biphasic and displayed a fast component with a time constant of decay smaller than 5 ms (Fig. 3Ab, black trace), presumably mediated by AMPARs. To isolate the contribution of NMDARs, the same experiments were performed in the presence of NBQX (5 \(\mu\)M). Somatodendritic responses were then found in every cell, however axonal responses were detected in only 12% of the cells (24 out of 202 cells). In responsive cells laser pulses applied along the axons gave rise to detectable currents in 28% of the locations. Uncaging of MNI-glutamate on dendritic spots gave rise to currents with an average amplitude of 42\pm 4.2 pA (\(n = 26\) locations out of 14 cells) with a 20/80% rise time of 8.3\pm 2.8 ms (\(n = 19\) out of 8 cells) and a half decay time of 141\pm 18 ms (\(n = 20\) out of 9 cells). The same experiments performed in the axon elicited currents displaying an average amplitude of 13\pm 2 pA (\(n = 90\) varicosities out of 24 cells), a 20/80% rise time of 17\pm 4 ms (\(n = 53\) responses out of 7 cells) and a half decay time of 172\pm 8 ms (\(n = 53\) responses out of 7 cells).

Axon identification is unambiguous in MLIs; however it could be envisaged that the responses that were attributed to axonal NMDARs were in fact elicited in dendritic structures that happened to be close enough to the targeted axon to be activated by glutamate diffusing away from the release spot. To address this possibility, we assessed the spatial resolution achieved by laser spot uncaging of MNI-glutamate with 1 ms pulse duration in the presence of NBQX (Fig. 3Bd). We found a very abrupt drop in sensitivity when moving the laser spot location away from the neurite, with a length constant of 1.4 \(\mu\)m. In view of these controls sensitive axonal spots that were located within 5 \(\mu\)m from any potential somatodendritic structure were rejected from the above analysis.

These experiments demonstrate that sizable current responses can be elicited from both axonal and dendritic locations following local and short activation of either AMPARs or NMDARs. The results reveal quantitative differences depending on neurite type and pharmacological conditions. Responses are larger and faster for AMPARs than for NMDARs, in accordance with the slower opening kinetics of NMDAR-associated channels than AMPAR-
associated channels. Response onsets are also slower for axonal responses than for dendritic responses, as expected from the filtering effect of the axon cable. Nevertheless axonal responses delays are <10 ms suggesting that axonal responses implicate NMDARs that are located in the axon and not in neighboring cell structures.

Reasons for the relative scarcity of NMDAR-mediated responses to local glutamate uncaging in axonal sites compared to the more reliable axonal responses found with wide field uncaging will be discussed below.

Pharmacological Properties of Axonal NMDARs

While the above experiments strongly suggest the presence of functional NMDARs in the axon they could still be interpreted as reflecting the activation of non NMDA glutamate receptors following displacement of NBQX from the receptor binding site by released glutamate. Therefore it was important to investigate whether the presumptive axonal NMDAR-mediated responses displayed features expected from NMDARs. Three characteristic features of NMDA-gated channels were investigated: Mg^{2+} block, block by APV, activation by NMDA.

Figure 2. Wide field uncaging of MNI-glutamate reveals presynaptic NMDARs. A: MNI-glutamate was uncaged in a wide area of the recording chamber by a UV-flash through the microscope objective (see Methods) in 0.1 mM [Mg^{2+}] and in the presence of both TTX (0.5 μM) and NBQX (5 μM). Fluorescence levels are shown before (a, left) and after (a, right) the flash. b: Reconstruction of the MLI (somatodendritic compartment in black and axon in blue). c: Ca^{2+} transients recorded before TTX application in response to 4 propagated action potentials in an axonal region (blue, region 5 in a) and in a dendritic region (black, region indicated «den» in a). d: MNI-glutamate uncaging in the presence of TTX and NBQX evoked a NMDAR-mediated current (left bottom trace) and Ca^{2+} transients in dendrites (left upper trace) as well as in axonal areas 4 and 5. B: Another experiment following the same experimental paradigm. a: Cell morphology. b: Responses to 4 propagated action potentials obtained before TTX application in axonal area 1 and in a dendritic location. c: Global uncaging of MNI-glutamate in TTX elicited Ca^{2+} transients both in the axonal and in the dendritic compartment (upper blue and black traces respectively), together with an inward current (bottom).

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Because of the well-known blocking action of external Mg$^{2+}$ ions on NMDA-sensitive channels [29], we performed experiments in a low external Mg$^{2+}$ saline ([Mg$^{2+}]_o$, 0.1 mM). Under these conditions, the currents observed in response to axonal MNI-glutamate uncaging in the absence of NBQX were weakly dependent on the holding voltage (Fig. 4Aa,C). However, when Mg$^{2+}$ was added to the bath, the currents became clearly voltage dependent, and started to display the characteristic outward rectification of NMDAR-coupled channels (Fig. 4Ab,C). Intermediate voltage dependence was observed for 200 μM Mg$^{2+}$ in the bath (Fig. 4B,C). Intermediate voltage dependence was observed for 200 μM Mg$^{2+}$ in the bath (Fig. 4B,C).

Direct identification of the responses as arising from NMDARs activation was provided by the fact that when the specific NMDAR antagonist AP-V (50 μM) was added, the uncaging response was blocked (n = 3; Fig. 4D). Finally, we investigated the sensitivity of the axonal channels to NMDA by using a novel NMDA cage instead of MNI-glutamate. Fig. 4E shows current traces obtained by local uncaging of MNI-NMDA (1 mM) in response to 1 ms laser pulses at various locations indicated by numbers on the cell reconstruction. Axonal current responses displaying an average amplitude of 11.5 ± 3.5 pA were recorded in 6 cells out of a total of 29. Collectively, the results indicate that the axonal responses obtained by local MNI-glutamate uncaging in the presence of NBQX reflect the activation of NMDARs.

Axonal Ca$^{2+}$ Responses to Local NMDAR Activation

Next we tested whether local axonal glutamate uncaging (Fig. 5A, blue arrowhead) could produce Ca$^{2+}$ transients that would be associated with the current responses. Local MNI-glutamate uncaging in TTX gave rise to Ca$^{2+}$ transients and to...
current responses both in the axonal (Fig. 5B, left) and in the dendritic domain (Fig. 5B, right). These transients rose with a time course of several hundreds of ms and broadened during this time over a distance of ~10 μm along the axon. Importantly, axonal spots that failed to deliver a current response also failed to exhibit a Ca²⁺ transient. In responsive spots, the amplitude of the axonal laser pulse-elicited Ca²⁺ transient (mean ΔF/F₀, 25.6±0.8%, n = 21 out of 6 cells) was correlated to that of the current (mean, 13.8±0.4 pA, n = 21 out of 6 cells; Fig. 5C, left panel). As a rule, the Ca²⁺ transients elicited by uncaging had a similar amplitude as those obtained using 4 propagated action potentials before TTX application (respectively ΔF/F₀ = 25.6±0.8%, n = 21 and ΔF/F₀ = 34.7±1.3%, n = 15; Fig. 5C, right panel). Therefore the results indicate that a local and short activation of NMDARs in an axon spot is able to induce a large enough Ca²⁺ transient to increase transmitter release in the corresponding axonal area.

Pharmacological Profile of NMDA Effect on Miniature Current Frequency

The previous sections have examined the ability of glutamate to produce NMDAR-mediated current and Ca²⁺ responses in MLIs. An alternative way to characterize the mechanism of action of NMDA is to examine the pharmacological profile of NMDA effect on miniature current frequency. In keeping with the pioneer study by Glitsch and Marty [3], we found that bath application of NMDA (30 μM) in the presence of TTX increased the frequency of mIPSCs with a frequency ratio over control of 9.97±1.19 (n = 8; Fig. 6 A and D). This increase was totally prevented by the presence of MK801 (50 μM) in the bath (ratio over control: 0.86±0.09, n = 4; Fig. 6D). Note that in MLIs, mEPSCs have a much lower frequency than mIPSCs, and that the two types of currents can be unambiguously distinguished on the basis of their decay kinetics [30]; therefore mEPSCs could easily be eliminated from the analysis and pharmacological block of AMPARs was not necessary in these experiments.

A simple approach that can be envisaged to distinguish between direct and indirect mechanisms of NMDA action is a pharmacological block of VDCCs. In the direct mechanism, NMDAR activation in the axon is likely to provide a Ca²⁺ rise even after total VDCC block, thus leading to mIPSC enhancement; in the indirect mechanism however, the response should be abolished by VDCC blockers. Therefore, we assessed the effects of NMDA on mIPSC frequency in the presence of 100 μM CdCl₂ to block high threshold VDCCs. Under these conditions, the mIPSCs frequency ratio over control reached 7.93±1.10 (n = 7) indicating that high threshold VDCCs are not required for the response (Fig. 6 B and D). The increase in mIPSCs frequency was not significantly different in Cd⁰ and in control (P > 0.05; unpaired t-test) although Cd²⁺ ions have been reported to partially block VDCCs [31]. Low threshold VDCCs are not affected by Cd²⁺ ions and are expressed in MLIs [32]. The T-type channel blocker mibefradil (10 μM; [33]) did not alter the potentiation effect of NMDA on mIPSCs frequency (ratio over control : 11.8±2.1, n = 4; P > 0.05; unpaired t-test; Fig. 6D). Taken together, these results concur with those previously obtained on Purkinje neurons [3,17] to indicate that neither high threshold nor low threshold VDCCs are required for the response. They indicate that, contrary to the indirect depolarization theory, VDCC opening is not an obligatory step leading to the increase in GABA release during NMDA application.

Ryanodine-sensitive Ca²⁺ stores have been proposed to participate in the presynaptic Ca²⁺ elevation triggered by preNMDARs activation [4]. In line with this proposal, addition of dantrolene (10 μM), a muscle relaxing agent acting on ryanodine receptors, significantly reduced the potentiating effect of NMDA on mIPSC frequency (ratio over control : 5.9±0.15, n = 4; P < 0.05; unpaired t-test; Fig. 6D). An even stronger effect was obtained by combining dantrolene and Cd²⁺ together (ratio over control : 2.1±0.32, n = 4; P < 0.05; unpaired t-test; Fig. 6D). These results indicate that even though VDCCs are not required for the NMDA response, they contribute together with ryanodine-sensitive Ca²⁺ stores to enhance the response. Such an effect could reflect a functional coupling between VDCCs and the ryanodine receptor. Note that dantrolene alone neither affects the frequency (0.30±0.13 Hz vs. 0.27±0.12 Hz, n = 5; P > 0.05, paired t-test; Fig. 6E, upper panel) nor the amplitude of mIPSCs (138±31 vs. 112±26 Hz, n = 5; P > 0.05, paired t-test; Fig. 6E, lower panel). In summary, intracellular Ca²⁺ stores do not participate in the regulation of the resting Ca²⁺ concentration but that they amplify the response to NMDA.

MLI axons contain neuronal NO synthase (nNOS; [34]), and MLI axon terminals have been suggested to engage an NMDAR-driven NO cascade during cerebellar LTD [16]. However NMDARs and nNOS have also been suggested to be located in PFs [8] so that local activation of NMDARs in PFs could lead to NO release and possibly to an indirect effect on MLI terminals that would be mediated by NO rather than by direct activation of NMDARs. In the presence of the nNOS antagonist L-NNa (10 μM). Under these conditions, NMDA still increased mIPSC frequency in a way that was not significantly different from that obtained in control conditions (frequency ratio over control of 8.74±1.25, n = 4; P > 0.05; unpaired t-test; Fig. 4D). These results indicate that the NMDA-induced enhancement of GABA release in MLIs is due to the activation of NMDARs located in MLI axons rather than to an indirect mechanism involving NO release from neighboring structures.

The effects of NMDA on synaptic activity were readily observed in normal TTX- containing BBS. This indicates that the preNMDARs activation occurs even in the presence of the normal Mg²⁺ concentration (1 mM) without any pre-depolarization to relieve the Mg²⁺ block. To explain this result, it has been postulated that preNMDARs could incorporate NR2C and D subunits in their structure which would render them less sensitive to Mg²⁺ inhibition than their NR1 and NR2A/B counterparts [3,4]. Alternatively, axonal depolarization could amplify pre-NMDAR activation. Zn²⁺ ions are widely used to distinguish NR2A subunits as they appear to be far more selective for NR1/ NR2A receptors than for any other NR2 subunit-containing...
receptors [35]. In the presence of 300 nM Zn\(^{2+}\), NMDA (30 mM) did not significantly increase the frequency of mIPSC (frequency ratio over control of 2.12 ± 2.00, n = 4; Fig. 6D). Additionally ifenprodil, an NR2B selective antagonist, was used to examine the possible involvement of this subunit in the effect of NMDA application. When slices were treated with 10 μM ifenprodil, application of NMDA (30 mM) still increased the frequency of mIPSCs (frequency ratio over control of 3.53 ± 1.57; Fig. 6 C and D), albeit to a smaller extent than in control conditions. These results indicate that both NR2A and NR2B participate in the effects elicited by NMDA on mIPSCs. Because of the high input resistance of MLIs and the rather small volume of their presynaptic terminals, even a small glutamate-evoked current might be sufficient to alleviate Mg\(^{2+}\) block. It is important to

**Figure 5. Axonal Ca\(^{2+}\) signals elicited by local activation of pre-NMDARs.** A: Representative experiment. Top: Fluorescence image (left) and reconstruction (right) of a MLI filled with Alexa 488 (20 μM) and OGB-1 (50 μM). Bottom: Local Ca\(^{2+}\) transients and associated somatic currents obtained in response to axonal (left) and dendritic (right) glutamate release (1 ms laser pulses; 0.9 mM MNI-glutamate; 5 μM NBQX; 0.5 μM TTX). B: Summary data. Left: In responsive axonal spots, the amplitude of Ca\(^{2+}\) transients are correlated to the corresponding somatic current (correlation coefficient R = 0.74). Right: Ca\(^{2+}\) transients elicited in TTX by local glutamate uncaging (Uncaging) had peak amplitudes similar to those obtained in the same axonal spots before TTX application using 4 propagated action potentials (Action potentials). Experiments were carried out in normal extracellular [Mg\(^{2+}\)] (1 mM).

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They exhibit specific amplitude and kinetics that set them apart from conventional miniature events.

A representative experiment is illustrated in Fig. 7. Under control conditions (TTX, 0.5 μM) preminis had a peak amplitude smaller than 30 pA and risetime values ranging from 0.5 to 6 ms (Fig. 7A, B). By contrast conventional miniature events had amplitudes of 30–300 pA and homogeneous risetime values of about 0.4 ms (Fig. 7A, B). In the presence of NMADA, the recording noise increased, but it was still possible to identify the two separate classes of events, and the frequencies of both preminis and conventional miniatures increased (respectively from 0.43 to 0.93 Hz, and from 0.15 to 0.84 Hz; Fig. 7 A–C). Summary results from 7 cells gave a mean increase of 2.5-fold for preminis, and of 4.1-fold for conventional miniature currents (Fig. 7D).

Like the previous results of Fig. 6, the premini frequency increase must be due to activation of preNMDARs because the soma was under voltage clamp. The results of Fig. 7 indicate that the elevation of the axonal Ca2+ concentration elicited by preNMDAR activation is able to induce transmitter release in spite of the somatic voltage clamp. To confirm that premini activation was not due to an indirect mechanism involving neighboring MLIs, mIPSCs and preminis were recorded in the presence of NBQX (20 μM) and Cd2+ (100 μM) to block the indirect pathway. In the presence of NMADA, the frequency of preminis increased from 0.12 to 0.46 Hz. Averaged results from 4 cells gave a mean increase of 3.3-fold for preminis and 5.2 for miniatures (Fig. 7D; open square symbols). These values are not significantly different from those obtained without blockers, thus confirming that the increase in premini frequency is due to activation of axonal NMDARs in the recorded cell.

It is worth noting that the augmentation of premini frequency is of the same order of magnitude as that of conventional miniatures (which originate from unclamped MLIs). The increase measured for preminis is actually smaller than that of conventional miniatures. However, the first value is an underestimate because NMADA decreases the length constant of the axon cable by increasing the membrane permeability of this cable, thus decreasing the effective surface area from which preminis are collected. Since this effect could easily explain the difference between the 2.5-fold increase in premini rate and the 4.1-fold increase in mini frequency, the results suggest that the entire scope of the NMADA-induced frequency increase can be accounted for by the direct activation of axonal NMDARs.

**Discussion**

Altogether our results fail to substantiate an indirect mechanism for the responsiveness of MLI axons to NMADA, and suggest instead a direct mechanism reflecting the activation of NMDARs in the axonal domain of these cells. Here we briefly review the key new findings that favor the direct mechanism, and we proceed with a discussion of the specific features of axonal NMDAR-mediated signaling that emerge from the present work.

**NMDA-induced Axonal Ca2+ Rise and Subsequent GABA Release under Somatic Voltage Clamp**

Clamping the somatic potential at a negative value did not prevent NMADA-induced axonal responses. Large axonal Ca2+ elevations were still elicited by NMADA application and furthermore, these Ca2+ elevations were able to elicit GABA release as demonstrated by the fact that the rate of preminis was significantly enhanced. Thus, contrary to the predictions of the indirect mechanism hypothesis, preventing somatodendritic NMDARs...
from depolarizing the axon compartment does not abolish the NMDA response.

Uncaging Experiments

The results obtained with wide field uncaging confirm that most axons are responsive to NMDA, but they also indicate that this sensitivity is restricted to a small number of varicosities. In addition, they rule out Ca\(^{2+}\) diffusion from the somatodendritic compartment as an explanation for NMDA-induced axonal Ca\(^{2+}\) elevations under voltage clamp because no Ca\(^{2+}\) gradient from soma/dendrite to axon terminals was observed.

Local uncaging either of NMDA or of glutamate in conditions where both AMPA-selective and kainate-selective glutamate receptors are blocked, leads to the generation of an axonal current that can be collected in whole-cell recording. These experiments allow for a much more precise and reproducible agonist application than the previous approach based on iontophoresis [20,21], but they nevertheless suffered from some technical limitations. First, axonal structures were not always available, since the axon was sometimes located under the recording pipette. In other cases the axon was visible but plunged into the slice tissue. Because the loss of 405 nm laser intensity due to scattering is approximately two-fold per 18 μm [26], the uncaging efficiency of local laser stimulation declines quickly with depth. This explains why responsive axonal stretches were immediately near the slice surface. Second, experience proved that only a limited number of laser flashes could be applied before photodamage was evident, so that a relatively small number of locations (typically not more than 5) were tested in a given experiment. These limitations explain that only a minority of uncaging experiments revealed any axonal sensitivity to NMDAR, without implying the existence of non-responsive axons.

In responding axons, only 28% of the probed locations gave rise to a detectable response. This result is in keeping with the fact that in cultured MLIs, 39% of the patches excised from axon terminals did not display functional NMDARs [5] indicating that a significant proportion of varicosities does not contain NMDARs. The sparse nature of axonal NMDA signaling may account in part for the previous failure to detect axonal NMDARs using iontophoretic NMDA applications [20,21,37] as well as a two-photon uncaging of MNi-glutamate [21]. The exact nature of the responsive spots remains to be determined but they presumably include basket terminals onto Purkinje cells since these structures display presynaptic NMDAR immunostaining [38] and since they appeared especially responsive to NMDA in the present work.

PreNMDAR Activation Leads to Ca\(^{2+}\) Transients

In the presence of OGB-1 in the intracellular recording solution, we were able to simultaneously record Ca\(^{2+}\) transients and NMDA-evoked currents. The amplitudes of the transients obtained with 1 ms laser pulses were comparable to those of summed transients evoked by four action potentials in close succession. It seems therefore plausible that a short-lived activation of preNMDARs leads to a robust release of GABA. These transients were found to spread along the axon over a distance of ~10 μm, possibly reflecting diffusion as well as the recruitment of ryanodine-sensitive intracellular Ca\(^{2+}\) stores [4]. Local amplification by intracellular Ca\(^{2+}\) stores may also account for the negative results obtained with aspartate iontophoresis by [20], since the constant use of the SERCA pump inhibitor cyclosporin acid in these experiments may have emptied Ca\(^{2+}\) stores. Taken together, our data indicate that this axonal sensitivity to NMDA is strong enough to fully account for the effects observed on miniature currents.

Pharmacological Profile of the mIPSC Response to NMDA

According to the indirect mechanism the mIPSC frequency increase elicited by NMDA should be highly sensitive to VDCC blockers. However we found that neither the general VDCC blocker Cd\(^{2+}\), or the T-type VDCC blocker mibefradil, were able to inhibit this effect significantly. These results together with those of a previous study [17], demonstrate that activation of VDCC is dispensable for the mIPSC response. The strength of the inhibitions observed with NR2 subunit antagonists (i.e. Zn\(^{2+}\) and ifenprodil) indicate that the NR3A subunit is not present presynaptically unlike in the neocortex where NR3A-containing preNMDAR promote neurotransmitter release and spike timing-dependent plasticity [39]. Indeed, the expression of NR3A in cerebellum has been shown to be barely detectable after 10 days PN [40]. The half-decay time of preNMDARs current is consistent with mixed involvement of NR2A and -B subunits since it matches the deactivation kinetics of the currents recorded in HEK cells expressing recombinant NMDARs containing either NR2A or NR2B subunit [13].

Functional Role of preNMDARs

In MLIs, activation of preNMDARs i) increases spontaneous and miniature IPSC frequency in Purkinje cells and MLIs; ii) triggers the onset of depolarization-induced potentiation of inhibition (DPI) in immature Purkinje cells [4] and iii) leads to L-LTP in MLIs [12]. Application of D-APV has been shown to slightly decrease the frequency of mIPSCs recorded in Purkinje cells [3,15] suggesting a tonic activation of NMDARs. These results suggest that the regulation of IPSC frequency occurs as soon as the ambient glutamate concentration exceeds normal levels, whether as a result of some local unbalance or because of strong or sustained parallel fibre activity; because of the NMDAR-mediated increase in spontaneous inhibition on postsynaptic MLIs and Purkinje cells, the effects of ambient glutamate on the mean level of cell firing in the molecular layer are then kept within acceptable limits. On a somewhat longer time scale (minutes) the onset of DPI triggers a regulatory loop by which increased MLI inhibition corrects excessive firing of Purkinje cells. Finally I-LTP fulfills a similar role on a still longer time scale (hours). Therefore

Figure 7. NMDA application increases premini frequency in voltage-clamped MLIs. A: Spontaneous synaptic currents in the presence of TTX (preminis are identified as events having a peak amplitude of <30 pA; there is 1 such event in the upper trace, plus one somatodendritic miniature) and after further bath application of 20 μM NMDA (lower trace: 5 preminis plus 2 minis). The soma is voltage clamped at ~60 mV. 2 of the miniature events, one in control and one in NMDA, have their peaks clipped off. B: Plots of 20–80% risetime as a function of peak amplitude in control (2 min duration) and in NMDA (also 2 min), from the same experiment as in A. Conventional miniature currents (minis) appear as a cluster of events with peak amplitudes >30 pA and risetimes <0.7 ms, and presynaptic miniature currents (preminis) appear as a non overlapping cluster of events with peak amplitudes <30 pA and risetimes >0.5 ms. Note that the frequencies of both preminis and minis increase in response to NMDA application. C: Peak amplitude histograms from the data in B (gray: control; open bars: NMDA). D: Dots: summary of 8 experiments (5 with 20 μM NMDA, and 3 with 50 μM NMDA), showing ratios of both premini and mini frequencies in NMDA over control periods. Only 1/8 experiment fails to show an increase in the premini frequency in response to NMDA. Open squares: summary of 4 experiments performed in the presence of NBQX (20 μM), Cd\(^{2+}\) (100 μM) and NMDA (50 μM). Experiments have been carried out in normal extracellular [Mg\(^{2+}\)] (1 mM).

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all three effects may be seen as contributing to reestablishing near-normal conditions in case of excessive neuronal activity.

The strong inhibition of miniature current frequency observed with a combination of VDCG and ryanodine receptor antagonists suggest that the activation of preNMDARs triggers a signaling module involving both VDCCs and Ca²⁺ stores in the amplification of the initial rise in presynaptic Ca²⁺. Moreover, the detection of nNOS [34] and PSD-95 [41] immunoreactivity in MLIs terminals led to propose the existence of NMDAR-containing signaling complexes [16]. Such units are likely to contribute to the NO-dependent form of LTP that is produced at the PF-MLI synapse [18], although a similar cascade has been shown to directly carry surface pores [13]. The recruitment of the NMDAR-NOS Ca²⁺ signaling pathway in MLIs could be distinguished based on the decay kinetics of the two currents which differ by an order of magnitude as reported [30]. For specific experiments, Zn²⁺ was buffered with tricine [N-tris(hydroxymethyl)methylglycine], as described [43].

Wide Field Uncaging of MNI-glutamate

Whole field photolysis of MNI-glutamate was achieved using a pulsed xenon arc lamp (Till Photonics, Germany) as described [44]. Briefly, a high intensity (0.5 ms duration; 80 J) discharge of UV light (360 ± 7.5 nm) was reflected onto the plane of focus using a dichroic mirror and the 60X wavelength immersion objective. For uncaging experiments, the perfusion was turned off to minimize consumption of the cages. A HEPES-buffered solution supplemented with NaHCO₃ to control internal pH was used (composition in mM: 135 NaCl, 4 KCl, 2 NaHCO₃, 25 glucose, 2 CaCl₂, 0.1 MgCl₂ and 10 HEPES, pH 7.4 with NaOH).

Local Uncaging of MNI-glutamate and MNI-NMDA

Laser photolysis was carried out by implementing a 405 nm diode laser (Point Source Illex 2000) coupled with a single mode optical fibre into the epifluorescence condenser of a commercial microscope (Zeiss Axioskop FS1) as described previously [26]. In this implementation the fibre output of the laser was expanded with a 40 mm focal length positive lens in a dual LED lamphouse (OptoLED, Cairn Research, UK) and reflected with a 45° dichroic mirror (425 DCXR, Chroma) into the epifluorescence condenser of the microscope. The epifluorescence condenser was previously adapted to accommodate output of a TILL monochromator (Polychrome 2, TILL Photonics, Germany) and the final dichroic reflector located to an extended UV type (470LP, Chroma). After initial alignment, the laser fibre and first lens were adjusted on three axes to position and focus the uncaging spot in the microscope focus. A spot of approximately 1 μm diameter was formed at the focal plane in a 100 μm pyramidal solution using a CCD camera (TILL Photonics, Germany). The straight-through path of the LED lamphouse received the fibre output of the monochromator for epifluorescence excitation at 488 nm (6.6 μw at 10 nm).

Calcium Imaging

Two different setups were used for Ca²⁺ imaging experiments, either a custom two-photon laser-scanning microscopy (2PLSM) system [45] and references therein) or a single-photon system. The 2PLSM setup was used to assess the axonal Ca²⁺ rise induced by bath application of NMDA. Other experiments were performed on a single photon setup from TILL Photonics (Germany) based on a Polychrome 2 monochromator and a Peltier-cooled CCD camera (IMAGO QE; 1376 × 1040 pixels; pixel size: 244 nm after 53X magnification and 2 μm diameter) with DCMX (Chroma). The rationale behind the selection of the CCD camera is to determine the axonal calcium influx induced by bath application of NMDA. Other experiments were performed on a single photon setup from TILL Photonics (Germany) based on a Polychrome 2 monochromator and a Peltier-cooled CCD camera (IMAGO QE; 1376 × 1040 pixels; pixel size: 244 nm after 53X magnification and 2 μm diameter). The rationale behind the selection of the CCD camera is to determine the axonal calcium influx induced by bath application of NMDA.
potentials (20 ms intervals) were produced by depolarizing the cell for 3 ms to 0 mV from a holding value of ~70 mV thus inducing a propagated action potential [46].

Whole-cell recording pipettes were filled with (in mM) 140 K-gluconate, 5.4 KCl, 4.1 MgCl₂, 9.9 HEPES-K, 0.36 Na-GTP, 4 Na-ATP, pH was adjusted to 7.3 with KOH. For 2-photon laser-scanning imaging experiments, this solution was supplemented with 400 μM Fluo 4 (Invitrogen). For single photon excitation, the solution contained 50 μM Oregon Green 488 BAPTA-1 (OGB-1; Invitrogen) and Alexa 488 fluor (20 μM; Invitrogen) was added for local uncaging to facilitate axon identification early in the experiments. However, ΔF/F₀ values reported here for trains of action potentials (ΔF/F₀ = 34.7 ± 1.3%; n = 15; Fig. 5C) are lower than those previously reported for the axons using OGB-1 alone.

For all Ca²⁺ imaging experiments, neurites identification was confirmed by analysis of action potential induced Ca²⁺ transients, which are markedly larger in the axon than in dendrites as reported [25]. Analysis was performed by calculating the average fluorescence in regions of interest (ROIs) as a function of time as detailed before [45].

The following drugs applied in the bath were used when appropriate: N-methyl-D-aspartate (NMDA), tetrodotoxin (TTX), Mibefradil, calcium chloride (CaCl₂), zinc chloride (ZnCl₂), Henpopri, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), DL-2-Amino-5-phosphonopentanoic acid (AP-V), N0-nitro-L-arginine (L-NNA), dantrolene. All drugs were purchased at Ascent. Other chemicals were purchased at Sigma. 4-Methoxy-7-nitroindolyl-caged-L-glutamate (MNI-Glutamate) and 4-Methoxy-7-nitroindolyl-caged-NMDA (MNI-NMDA) were kindly provided by Tocris Bioscience.

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Author Contributions
Conceived and designed the experiments: AM IL DO TC. Performed the experiments: BR IL TC. Analyzed the data: IL BR AM TC. Contributed reagents/materials/analysis tools: DO YT. Wrote the paper: TC AM.

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