NogoA restricts synaptic plasticity in the adult hippocampus on a fast time scale

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Whereas the role of NogoA in limiting axonal fiber growth and regeneration following an injury of the mammalian central nervous system (CNS) is well known, its physiological functions in the mature uninjured CNS are less well characterized. NogoA is mainly expressed by oligodendrocytes, but also by subpopulations of neurons, in particular in plastic regions of the CNS, e.g., in the hippocampus where it is found at synaptic sites. We analyzed synaptic transmission as well as long-term synaptic plasticity (long-term potentiation, LTP) in the presence of function blocking anti-NogoA or anti-Nogo receptor (NgR) antibodies and in NogoA KO mice. Whereas baseline synaptic transmission, short-term plasticity and long-term depression were not affected by either approach, long-term potentiation was significantly increased following NogoA or NgR1 neutralization. Synaptic potentiation thus seems to be restricted by NogoA. Surprisingly, synaptic weakening was not affected by interfering with NogoA signaling. Mechanistically of interest is the observation that by blockade of the GABAa receptors normal synaptic strengthening reoccurred in the absence of NogoA signaling. The present results show a unique role of NogoA expressed in the adult hippocampus in restricting physiological synaptic plasticity on a very fast time scale. NogoA could thus serve as an important negative regulator of functional and structural plasticity in mature neuronal networks.

Changes in the connectivity of neurons—synaptic plasticity—regulate the fine-tuning of neuronal networks during development and during adult learning. Synaptic plasticity includes functional and structural modifications at neurons and may be the underlying mechanism for learning and memory processes (1). The storage of new information therefore might depend on ever changing neuronal networks. On the other hand, recent data indicate that the large scale organization of neuronal networks is kept remarkably stable to maintain a constant flow of information and to support long-term memory storage (reviewed in ref. 2).

In the CA1 region of the hippocampus, changes in neuronal activity can lead to changes in synaptic weight. Molecular mechanisms include here changes in the number or properties of neurotransmitter receptors, retrograde messengers, structural changes at synapses, and activation of transcription/translation (3). What is less clear is whether molecular mechanisms restricting changes in synaptic weight and thus stabilizing the synapse also play a role as well. In this context it is interesting to note that preventing further potentiation of a given set of synapses in a neuronal network can be induced by a homeostatic shutdown of long-term potentiation (LTP) after intense stimulation (4). In the search for such molecular stabilizers, we investigated the protein NogoA, which has been identified as a negative regulator of structural changes in the CNS (5). NogoA prevents neurite outgrowth in the adult CNS after injury (6) and regulates the progressive restriction of plasticity during development (7–9). In the adult CNS, the bulk of NogoA is found in myelin, but interestingly, neuronal NogoA expression persists in those regions of the CNS that are known to be particularly plastic, e.g., the hippocampus and the olfactory system (10, 11, see also ref. 12). In the mature CNS both known receptors for NogoA, Nogo66 receptor 1 (NgR1) and the paired Ig-like receptor B (PirB), negatively modulate activity-dependent synaptic plasticity. In ngR1 knockdout (9) and in pirB knockout mice (13), ocular
dominance plasticity continues after the end of the critical period, suggesting that NgR1 and PirB signaling stabilizes the neural circuitry and limits experience-dependent plasticity. In addition, NgR1 signaling can influence LTP in concert with FGF2 (14) as well as long-term memory (15).

It is noteworthy that NogoA/NgR1 are expressed in pyramidal cells of the hippocampus (12), that their expression is regulated by neuronal activity (16, 17), and that NgR1 is located at synapses in the adult CNS (18). However, the physiological role of neuronal NogoA in the hippocampus of adult animals has remained largely unexplored (for a review see ref. 5).

Here we report a unique, acute physiological function of NogoA in the mature hippocampus acting on a fast time scale. Our results suggest that NogoA is involved in specifically stabilizing synaptic weight.

Results

Hippocampal Slices Treated with Function Blocking Antibodies Against NogoA. To study possible acute effects of NogoA in regulating synaptic plasticity, we treated wild-type (WT) mouse acute hippocampal slices with the NogoA-specific function blocking antibody (Ab) 11c7 (19) or control Ab (anticyclosporin) for 1 h and induced under these conditions LTP at the CA3-CA1 Schaffer collateral pathway. Theta burst stimulation (TBS) to hippocampal slices of adult mice (P40–P60) was applied 20 min after baseline recording. The 11c7 Ab-treated slices showed already in the induction phase a higher LTP, which resulted in a significant difference 55–60 min after TBS (Fig. 1A; P = 0.02, t test). The average potentiation in 11c7-treated WT slices was 168 ± 7.4% (n = 14 slices per 7 animals), whereas control Ab-treated slices showed a potentiation of 141 ± 8.0% (n = 13 slices per 6 animals) 55–60 min after the TBS.

To investigate the role of NogoA in controlling protein-synthesis dependent L-LTP, we recorded the post-TBS potentiation for 3 h in 11c7-treated WT slices compared with control Ab-treated ones (Fig. 1B). Indeed the difference between both treatments became even more pronounced and was highly significant 3 h after TBS (P = 0.01, t test). The average potentiation in 11c7-treated WT slices was 158 ± 6.7% (n = 4/2) and 126 ± 5.7% (n = 4/2) in control Ab-treated slices, respectively.

We next investigated the effect of 11c7 treatment on the expression of long-term depression (LTD), induced by a 15-min low-frequency (1 Hz) stimulus (LFS) protocol. NogoA neutralization did not induce any differences in LTD induction or maintenance compared with control Ab-treated slices (Fig. 1C). At 55–60 min after LFS, the average depression was 85 ± 2.2% in 11c7 (n = 17/8) and 84 ± 1.9% in control (n = 18/10) Ab-treated slices.

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The authors declare no conflict of interest.

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To investigate whether the strengthening of LTP upon NogoA neutralization is due to changes in presynaptic components necessary to support LTP, we probed whether synaptic fatigue was altered under 11c7 conditions. We therefore analyzed for the first burst of the three TBS trains the ratio of the fourth field excitatory postsynaptic potential (fEPSP) to the first fEPSP in each burst. The results were as follows: first train 11c7, 204 ± 26%; control, 175 ± 14%; P = 0.35; second train 11c7, 128 ± 20%; control, 97 ± 7%; P = 0.15; 11c7, 134 ± 24%; control, 107 ± 6%, P = 0.28; 11c7, n = 10; control, n = 12. Overall, we could not find any significant difference between control Ab- and 11c7-treated slices. In addition, we examined short-term plasticity by measuring paired-pulse facilitation (PPF), characterized by a strong presynaptic component (20). PPF was induced by applying two stimuli separated by different time intervals (10, 20, 40, 80, and 160 ms) and recording the evoked fEPSPs. Fig. 1D shows a small increase in 11c7-treated slices, which is, however, not significantly different at any interstimulus interval (ISI) measured.

Changes in the height of potentiation during the induction and maintenance of LTP could also be due to changes in baseline synaptic transmission. We therefore recorded an input-output curve, using the fiber volley as an indicator of presynaptic stimulation strength and plotted a given fiber volley size against the fEPSP slope (Fig. 1E). With this approach we could not find any significant differences between 11c7 or control Ab-treated slices.

To confirm the penetration of the NogoA blocking Ab and identify its binding sites, the recorded hippocampal slices underwent immunostaining for mouse antibodies. We observed high levels of 11c7 Ab bound to possibly Mosby cells of the polymorphic layer of the dentate gyrus (Fig. S1 A and C, open arrows) and also especially to pyramidal cells of the CA3 region (Fig. S1 A, C, and D, closed arrows) up to 200 μm into the slice. In the latter, both the cell bodies and the dendrites were labeled (Fig. S1C). In the CA1 region, strongly labeled cells were sparser and only the cell bodies were stained (Fig. S1 A and E, arrowheads). No staining could be observed in slices treated with control antibodies (Fig. S1 B and G–I) indicating a specific binding of the 11c7 Ab. This is in line with EM data and biochemical evidence (14, 21, 22). Together, these results clearly show that NogoA is specifically involved in restricting the height of synaptic potentiation on a fast time scale. On the other hand, NogoA signaling is not mediating or preventing mechanisms of synaptic weakening and it does not significantly change properties of baseline synaptic transmission.

**Role of the Nogo Receptor NgR1 on Processes of Synaptic Plasticity.** To analyze whether the role of NogoA on hippocampal LTP involves the known Nogo receptor (NgR) pathway, we tested whether the blockade of the NgR would have an effect on the induction and maintenance of LTP. We applied a function blocking anti-NgR Ab (23) to WT acute slices with the same procedures we used for the anti-NogoA Ab. Fig. 2A shows that the blockade of NgR also leads to higher levels of potentiation following TBS compared with control Ab treatment. At 60 min after LTP induction, we observed a significant difference between anti-NgR Ab-treated slices and controls, with an average potentiation of 175 ± 14.6% (n = 6/2) in the anti-NgR Ab-treated slices and 141 ± 8% in controls (n = 13/6) (P = 0.012).

Again paired-pulse facilitation and baseline synaptic transmission in the anti-NgR Ab experiments revealed no significant differences compared with control Ab-treated slices (Fig. 2 B and C).

**Synaptic Plasticity in NogoA KO Mice.** To have an Ab-independent approach to investigate the role of NogoA in mediating synaptic plasticity, we investigated whether LTP is affected as well in NogoA KO mice. We applied a TBS to hippocampal slices of NogoA KO and WT adult mice (P40–P60). Both genotypes showed an initially equally high posttetanic potentiation (PTP) and at up to 40 min, both curves overlaid (Fig. 3A). Then the fEPSPs from the KO slices became visibly larger than those in the WT slices (Fig. 3A). The average degree of potentiation was 150 ± 7.5% for the NogoA KO mice (n = 14/5) and 136 ± 6.8% for WT animals (n = 14/5) (P = 0.1). Although we could not find a significant difference between the two genotypes, we found a tendency for a higher LTP induction in NogoA KO mice in comparison with WT-derived slices resembling the effect of the acute treatment by the 11c7 Ab.

Next we induced LTD in hippocampal slices of P14–P20 old NogoA KO and WT mice. LTD in the two genotypes showed exactly the same time course and no difference in fEPSP size could be observed (Fig. 3B). At 55–60 min after LFS, the average of both genotypes revealed a depression of 87 ± 1.9% for NogoA KO animals (n = 23/6) and 89 ± 1.8% for WT mice (n = 26/6), respectively.

To examine the presynaptic function, we again analyzed PPF. All intervals tested revealed an overall reduction in PPF in NogoA KO compared with WT mice, which was, however, not significant, resembling the acute treatment with the 11c7 Ab.
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Fig. 2. Nogo-receptor blocking Ab experiments. (A) Hippocampal slices were treated with anti-mNogo receptor (anti-mNgR; filled squares) or control Ab (open squares). LTP was induced by application of TBS (arrow). At 60 min after TBS, a significant difference between anti-mNgR- and control Ab-treated slices could be observed, indicated by the asterisk (P = 0.033, t test).

Inset shows original traces from representative individual experiments; letters correspond to the time point when traces were taken. (B) PPF in 11c7-treated slices is slightly, but not significantly increased. (C) Input-output strength in anti-mNgR-treated slices revealed no significant changes compared with control Ab-treated slices (P > 0.1).

Only at an ISI of 80 ms, a significant difference was found in the comparison between WT and KO mice (P < 0.05, t test; Fig. 3C).

In addition we recorded an input-output curve. At higher stimulus strength a slight reduction in the size of PPF (Fig. 4B; PTP is plotted in Fig. 4D). Here we saw that the 1-h preincubation led to an increase in PTP in the NogoΔ20 peptide-treated slices compared with controls (Δ20, 161.2 ± 3.5%; Δ20 boiled, 145.1 ± 3.3%; P = 0.0019, t test, Fig. 4D, columns in B). This increase in PTP in NogoΔ20 peptide preincubated slices was stabilized 1 h after TBS at significantly higher LTP values compared with controls (Δ20, 175 ± 7.7% (n = 9/3); Δ20 boiled, 145 ± 7.0% (n = 6/4); P = 0.024). In line with these results is the interpretation that during the 1-h preincubation, the NogoΔ20 peptide with its receptor is already completely internalized from the surface of the cell and mimics therefore the effect of NogoA blockade via antibodies. This is in line with immunostaining data, where we could show that the activation with NogoΔ20 leads to a significant increase in the cytoplasmic immunostaining with NogoA already 10 min after peptide application (Fig. S2 and SI Materials and Methods).

Next we investigated the effect of the NogoΔ20 peptide under similar conditions as used for the 11c7-NogoA Ab treatment. We incubated the slices with NogoΔ20 peptide for 1 h before induction of LTP and until the end of the experiment. Fig. 4C shows the LTP time course and in Fig. 4D the PTP is shown in the third pair of columns (C). This long treatment with NogoΔ20 peptide revealed an even higher increase in PTP than preincubation alone (Δ20, 183.4 ± 2.9%; Δ20 boiled, 136.1 ± 3.3%; P < 0.0001, t test). This again can most likely be explained by the fact that under these conditions, the receptor for NogoΔ20 is removed from the surface. In addition, Fig. 4C shows nicely that during the whole time course of the experiment the NogoΔ20 peptide-treated slices showed a significantly enhanced LTP, which stabilized 60 min after TBS (Δ20, 163 ± 5.4% (n = 17/12); Δ20 boiled, 142 ± 3.6% (n = 12/6); P = 0.008, t test).

PPF as well as the input-output curves revealed no significant differences between the NogoΔ20 and the inactivated peptide, independent of the duration of the treatment (Fig. 4 E and F).

Influence of the GABAergic System on the NogoA-Regulated LTP Enhancement.

We found no evidence that excitatory baseline synaptic transmission is altered under blockade of NogoA. This prompted us to ask whether changes in the GABAergic system might contribute to the observed phenotype. GABAergic transmission has indeed been known for a long time to be implicated in the induction and early maintenance phase of LTP (25). To probe for a GABAergic involvement, we induced LTP in Ab-treated slices by application of picrotoxin (PTX, 50 μM), a GABA_A receptor blocker. PTX was washed for 10 min before the induction of LTP by TBS and applied until the end of the experiments. Within 5 min from the TBS application, the fEPSP size of both Ab treatments (control and 11c7) increased and remained stable until the end of the experiments (Fig. 5). At 55–60 min after TBS, a very high level of LTP could be observed, higher than with NogoA Ab treatment alone and inactivation; however, no difference between the 11c7 treatment and the control Ab was visible [P = 0.61, t test; 11c7, 188 ± 12% (n = 10/5); control, 192 ± 7.4% (n = 13/6)]. To check whether the effect of PTX on LTP induction may mask the Ab treatment, we also tested whether the 11c7 treatment alone would be signifi-

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NogoA KO experiments. WT mice (open squares) and NogoA KO mice (filled squares) were analyzed. No significant difference between NogoA KO and WT mice could be observed 60 min after TBS (P > 0.1, t test). (B) LTD was induced by LFS (900 pulses at 1 Hz) stimulation. After LFS, there is no significant difference between transgenic and WT mice (P > 0.1, t test) (A and B). Insets show original traces from representative individual experiments; letters correspond to the time point when traces were taken. (C) PPf in NogoA KO mice is decreased compared with WT, but only shows a significant difference after an interstimulus interval (ISI) of 80 ms. (D) Input-output strength shows no significant difference between WT and NogoA KO mice.

Discussion

NogoA is an inhibitor of axonal regeneration and sprouting following CNS injury (6, 26) and acts as a growth suppressor in the adult CNS (6, 8, 9, 26–28). These effects are thought to be mainly due to oligodendrocytes and myelin NogoA, whereas the functions of NogoA in subpopulations of neurons are largely unknown. To address this question, we have investigated whether interfering with NogoA expression and signaling restricts synaptic plasticity in the adult intact hippocampus where NogoA is expressed by pyramidal as well as interneurons (11, 29). We found a unique physiological role of NogoA in negatively regulating LTP in the hippocampus on a fast time scale. Our findings are overall in line with the observations of Lee et al. (14), who could show that in NgR1 KO mice FGF2-dependent hippocampal LTP was enhanced and LTD was decreased. The present results, acute Ab-mediated neutralization of NogoA or NgR1 showed a more pronounced, FGF2-independent effect on LTP, but no effect on LTD. Similar, but less pronounced, effects were seen in the NogoA KO tissue. This is a phenomenon that is frequently observed when comparing acute interventions with constitutive KOs where compensatory mechanisms are often activated. These results are also in line with the proposed role of NogoA and NgR1 in regulating the stabilization of neuronal circuits and in inhibiting structural rearrangements and thereby limiting, e.g., spinal cord, or ocular dominance (OD) plasticity (9, 27, 30, 31), or regulating the formation of long-lasting memories (15).

NogoA20 Experiments. To complement the Ab and KO loss-of-function experiments, we applied the active NogoA-specific NogoA20 fragment to the hippocampal slices. NogoA20 induces growth arrest of neurites and growth cone collapse via activation of RhoA (32, 33). NogoA20 is rapidly internalized following receptor binding, and its internalization is followed first by intracellular signaling (24). We then studied the short-term application and effects separately from the long-term preincubations and synaptic effects. A 5-min preincubation of the tissue with NogoA20 significantly decreased PTP. This effect is in line with a synaptic weakening effect of NogoA. Sixty minutes after TBS, however, LTD was significantly enhanced, similar to what was seen after NogoA neutralization. Preincubation of hippocampal slices with the NogoA active fragment for 1 h always led to LTD enhancement. These results can be best explained by a diphasic effect: first fast signaling upon internalization occurred leading to synaptic weakening, and then NogoA20 induced down-regulation of the cell surface NogoA receptor complex (24), rendering the neurons temporarily insensitive to the NogoA present in the tissue, thereby causing the observed enhancement of LTD. This is supported by the observation that upon NogoA20 application, an increased cytoplasmic staining for NogoA20 in CA3 and CA1 pyramidal neurons can be observed in comparison to the use of boiled NogoA20 (SI Materials and Methods and Fig. S2).

Role of GABAergic Neurons. Our analysis of hippocampal synaptic plasticity shows that blockade of NogoA signaling causes significant changes in LTD. This cannot be attributed to changes in baseline excitatory synaptic transmission, because our fEPSP analysis of fiber volley size and paired-pulse facilitation were not significantly different under conditions of NogoA or NgR suppression. Although blockade of GABAergic activity can facilitate NMDA receptor-dependent LTD (34), in its natural form it is a key feature of LTD induction. During regular synaptic transmission, a delicate balance between excitation and inhibition is crucial, which needs to be transiently shifted toward less inhibitory activity to allow induction of plastic rearrangements of connectivity. Therefore, it is intriguing to speculate that NogoA does not act only on excitatory pyramidal neurons, but also on inhibitory neurons, as it is suggested by our picrotoxin experiments.

Regeneration and Increase of Plasticity. It is well established that severed adult mammalian CNS axons show very little, if any, regenerative axonal growth. Nevertheless, considerable spontaneous sensory-motor recovery can be accomplished in particular after smaller lesions (35, 36). Recovery is use dependent, and exercise substantially improves sensory and motor recovery after incomplete transection of the spinal cord (37). Cortical representations, for example, undergo well-adaptive changes (36, 38). Elimination of NogoA or its receptor NgR1 in KO mice or blockade of their function with specific antibodies enhances anatomical regeneration, compensatory fiber growth, and functional recovery following lesions in the spinal cord or brain; for reviews see refs. 6, 26, 39. In addition to the decrease of inhibitory signals in the environment of the growing fibers and the hypothesized
Mechanism of Action of NogoA in Modulating Synaptic Plasticity. A crucial question is how the function of NogoA can mechanistically be explained in the context of our current knowledge about processes of synaptic plasticity. One conceptual view would be that NogoA counteracts growth factor-mediated promoting effects on synaptic plasticity. In line with this are the results of Lee et al. (14), which indicate that NogoA acts via the synaptic cytoskeleton to stabilize synaptic function and structure. Another alternative view would be that NogoA acts via the synaptic cytoskeleton to stabilize synaptic function and structure and puts a break on growth-promoting processes. NogoA acts solely via p75NTR, an Nogo1-associated component of the Nogo receptor complex, which has been postulated as a mediator of negative synaptic plasticity (42–44). Because we do not see an effect on LTD after NogoA neutralization, however, it is unlikely that NogoA acts solely via p75NTR. It is intriguing to speculate that NogoA acts via the synaptic cytoskeleton to stabilize synaptic function and structure and puts a break on growth-promoting processes. NogoA is known to mediate neurite repulsion by negatively modulating actin assembly via small GTPases of the Rho family (28, 32, 45, 46). The synaptic actin cytoskeleton is important for the regulation of neurotransmitter release in the presynaptic compartment as well as spine shape changes and anchorage of postsynaptic density (PSD)-associated molecules in the postsynaptic compartment (reviewed in ref. 47). Downstream of RhoA and the RhoA kinase ROCK, NogoA may regulate effectors such as LimK and coflin (28, 32), which are important regulators of F-actin dynamics (28). Indeed phosphocofilin+ PSDs were shown to be increased after LTP induction (48), and limk ko mice show impaired spatial learning capabilities, abnormal spine morphology, and an increase in LTD (49). This supports the concept that NogoA signaling negatively modulates synaptic plasticity and structures underlying NogoA actions could represent a mediator of its effects on LTD.
plasticity via Rho, LimK, and coflin and subsequent changes of the pre- and post synaptic cytoskeleton.

In summary, our results strengthen the idea that neuronal NogoA and its receptors are important for upholding synaptic circuitry and regulating synaptic plasticity. They increase our understanding of how NogoA could mediate its effects in stabilizing neuronal networks, and how suppression of NogoA–Nogo receptor activity could enhance the formation of compensatory circuits and functional recovery after CNS injury. NogoA in the intact, adult hippocampus restricts physiological synaptic plasticity on a fast time scale. We therefore suggest that NogoA plays a crucial role in modulating the balance between plasticity and stability of the mature circuitry in the intact hippocampus thus ensuring the necessary level of stability in an ever changing neuronal network.

Materials and Methods

Slice Preparation and Electrophysiological Recordings. Acute hippocampal slices were prepared from wild-type C57BL/6j mice and from NogoA KO mice according to standard procedures. For LTP experiments, 40- to 60-d-old mice (P40–P60) and for LTD experiments, 14- to 21-d-old mice (P14–P21) were used; for details see SI Materials and Methods. All procedures were approved by the guidelines from Animal Committee on Ethics in the Care and Use of Laboratory animals of TU Braunschweig.

Antibody Treatment. For the Ab treatment, mouse and goat monoclonal Ab were used: A NogoA-specific blocking Ab (Ab 11c7, mg1G1), raised against an 18-aa peptide in the most active region of NogoA (NogoΔ20 region), see ref. 19; a control Ab (anticyclosporin, mg1G1, a gift from Novartis Pharma, Basel, Switzerland), and an Ab against the Nogo receptor, NgR (mNogo receptor affinity-purified goat IgG, R&D Systems). The Ab 11c7 was shown to block the NogoA-mediated neurite outgrowth inhibition in vitro and in vivo (6, 19, 37, 50); for details see SI Materials and Methods.

Pharmacology. The GABA<sub>A</sub>-receptor antagonist PTX was added in a 50-μM concentration 10 min before LTP induction until the end of the experiments.

Statistical Analysis. The average values of the slope function of the field EPSP (mV/ms) per time point were analyzed using a t test.

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