

Recognition molecule associated carbohydrate inhibits postsynaptic GABA_B receptors: a mechanism for homeostatic regulation of GABA release in perisomatic synapses

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Abstract

Extracellular matrix molecules are important cues in the shaping of nervous system structure and function. Here, we describe a novel mechanism by which the HNK-1 carbohydrate carried by recognition molecules regulates perisomatic inhibition in the hippocampus. Neutralization of HNK-1 activity by an HNK-1 antibody results in GABA_B receptor-mediated activation of K⁺ currents in CA1 pyramidal cells, which elevates extracellular K⁺ concentration and reduces evoked GABA release in perisomatic inhibitory synapses. This mechanism is supported by pharmacological analysis in hippocampal slices and data showing that the HNK-1 carbohydrate binds to GABA_B receptors and inhibits GABA_B receptor-activated K⁺ currents in a heterologous expression system. We suggest that the HNK-1 carbohydrate is involved in homeostatic regulation of GABA_A receptor-mediated perisomatic inhibition by suppression of postsynaptic GABA_B receptor activity.

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Introduction

Several classes of inhibitory interneurons are involved in feedforward and feedback inhibition of pyramidal neurons in the hippocampus. Perisomatic interneurons represent a subclass of neurons that form inhibitory synaptic contacts on the somata of pyramidal cells (Buhl et al., 1996; Freund and Buzsaki, 1996; Parra et al., 1998). They play important roles in the shaping of neuronal activity by the suppression of repetitive discharges of sodium spikes in pyramidal cells

(Miles et al., 1996) and are responsible for the synchronization of their activity (Cobb et al., 1995; Freund and Buzsaki, 1996; Buzsaki, 1997). In search for the molecular mechanisms underlying the regulation of synaptic activity in the context of adhesion molecules, we were struck by the observation that the somata and synapses of perisomatic interneurons are surrounded by the HNK-1 carbohydrate and the extracellular matrix molecule tenascin-R (Ren et al., 1994; Weber et al., 1999; Yamamoto et al., 1988; Saghatelian et al., 2000; 2001). The HNK-1 carbohydrate, a 3'-sulfated glucuronic acid recognized by a monoclonal antibody has been first described on human natural killer cells (Abo and Balch, 1981). It is carried by many neural recognition molecules, among them tenascin-R (TN-R) and tenascin-C. These molecules and the associated HNK-1 carbohydrate play functionally important roles in neural development and

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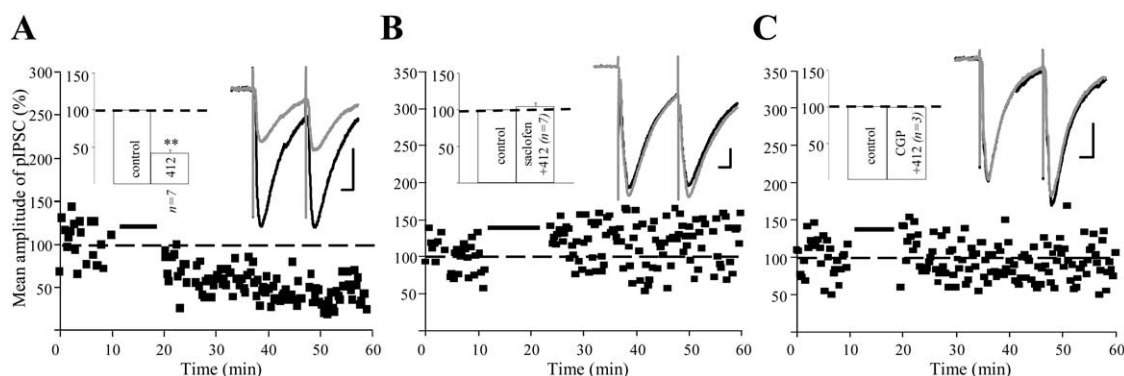


Fig. 1. Reduction of perisomatic inhibitory currents (pIPSCs) by the HNK-1 antibody (Ab) requires activity of GABA_B receptors. Individual experiments and statistical summaries (bars) demonstrating the inhibiting effect of the HNK-1 Ab (412; 50 μg/ml) on pIPSCs in normal extracellular solution (A) and a lack of effects in the presence of GABA_BR antagonists, 20 μM 2(OH)-saclofen (B) or 200 nM CGP54626A (C). Data are presented as normalized pIPSC amplitudes. The periods of Ab application are shown by bold black lines. Insets show the corresponding averaged pIPSCs in control recordings (*black curves*) and 10–20 min after the end of Ab injection (*grey curves*), *n* indicates the number of cells. Scale bars for all experiments are 25 pA and 10 ms. Stars show significant differences (***P* < 0.01; paired *t*-test). The antagonists were included into the bath medium 30 min before injection of the HNK-1 Ab.

regeneration (for review, see Schachner and Martini, 1995; Faissner et al., 1997). Our previous study (Saghatelian et al., 2000) revealed that HNK-1 carried by tenascin-R in the CA1 region of the hippocampus is involved in the regulation of perisomatic, but not dendritic, inhibition of pyramidal cells via reduction of presynaptic GABA release, resulting in an increased level of long-term potentiation. The present study gives insights into the mechanisms underlying this regulation. We demonstrate that the HNK-1 antibody neutralizes the constitutive inhibition of postsynaptic GABA_B receptors by endogenous HNK-1, thereby allowing activation of outward K⁺ currents in pyramidal neurons and retrograde modulation of GABA release in perisomatic inhibitory synapses, resulting in reduction of GABA_A receptor-mediated evoked inhibitory postsynaptic currents.

Results

Inhibition of perisomatic inhibitory currents by the HNK-1 antibody is mediated by GABA_B receptors

To investigate the functional role of the HNK-1 carbohydrate in the regulation of inhibitory synaptic transmission, we injected the HNK-1 monoclonal antibody (Ab) into hippocampal slices. Application of the HNK-1 Ab into hippocampal slices started 10–20 min after control recordings of postsynaptic currents and lasted for 10 min. The Ab strongly inhibited pIPSCs evoked by extracellular stimulation of perisomatic interneurons in the pyramidal layer of the CA1 region of the mouse hippocampus. Mean amplitudes of the currents after HNK-1 Ab application were 41.7 ± 4.2% (*n* = 7) relative to the pIPSCs recorded before injection of the Ab (Fig. 1A). The effect evoked by Ab usually reached a stable level 10–20 min after the end of Ab application, a time likely to be required for diffusion of the

Ab to the target area. In agreement with our previous findings (Saghatelian et al., 2000) application of HNK-1 Ab did not affect the paired-pulse ratio (PPR) of pIPSCs (0.8 ± 0.09 and 0.78 ± 0.07 before and after Ab application, respectively).

Since GABA_A receptor (GABA_AR)-mediated currents are often known to be regulated via GABA_BRs, we investigated the effects of the selective GABA_BR antagonists 2(OH)-saclofen and CGP54626A on inhibition of pIPSCs evoked by the HNK-1 Ab. Bath application of these antagonists slightly increased the mean amplitude of pIPSCs (124 ± 11.2%). These observations can be explained by a disinhibitory effect of the antagonists on the presynaptic GABA_BR-mediated inhibition of GABA release (Dutar and Nicoll, 1988; Davies and Collingridge, 1993). Application of the HNK-1 Ab in the presence of GABA_BR antagonists left pIPSCs unchanged. Mean amplitudes of the currents after HNK-1 Ab application were 106.3 ± 5.7% (*n* = 7) and 98.9 ± 2.4% (*n* = 3) relative to the pIPSCs recorded before injection of the Ab in the presence of saclofen and CGP54626A, respectively (Fig. 1B and C). PPR of pIPSCs recorded after HNK-1 Ab application in the presence of saclofen (0.86 ± 0.06) and CGP54626A (0.81 ± 0.07) were not different from those recorded before Ab administration (0.84 ± 0.05 and 0.82 ± 0.08 for saclofen and CGP54626A, respectively). These experiments imply that the activation of GABA_BRs is involved in HNK-1 Ab evoked reduction of pIPSCs.

The HNK-1 antibody reduces inhibitory synaptic transmission via triggering postsynaptic K⁺ currents

GABA_BRs are coupled to inwardly rectifying Kir3 K⁺ channels and N/P/Q-type Ca²⁺ channels (Mott and Lewis, 1994). When blocking Kir3 channels with 1.5 mM extracellular Ba²⁺, injection of the HNK-1 Ab did not inhibit

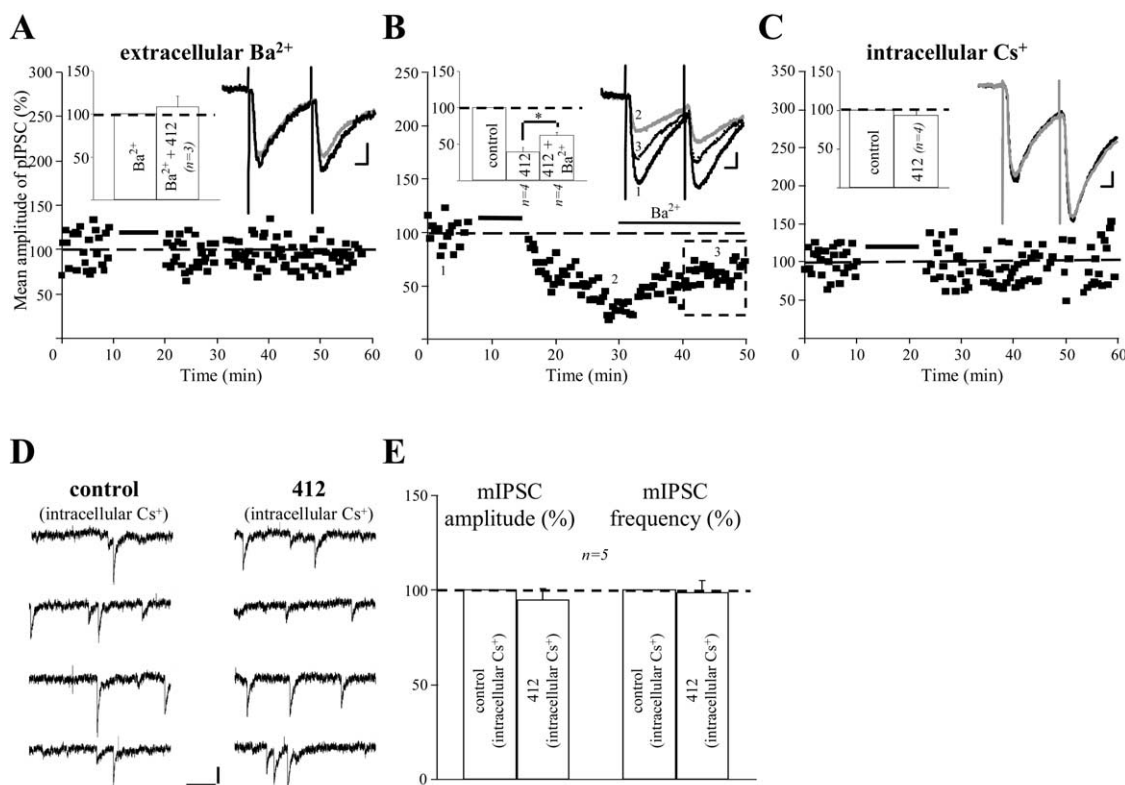


Fig. 2. Block of K^+ channels inhibits the effects of the HNK-1 antibody. A and C, Individual experiments and statistical summaries (bars) demonstrating a lack of effect of the HNK-1 Ab (412; 50 μ g/ml) on pIPSCs recorded in the presence of 1.5 mM extracellular Ba^{2+} (A) or after substitution of intracellular K^+ by Cs^+ (C). Insets show mean pIPSCs before (black curves) and 10–20 min after HNK-1 Ab application (grey curves). In B, application of Ba^{2+} following application of the HNK-1 Ab (50 μ g/ml) rescued the pIPSCs. The time period of addition of Ba^{2+} to the bath solution is shown by a thin black line. Insets show averaged pIPSCs in control recordings (1), 10–20 min (2) and 30–40 min after the end of Ab injection under 20 min exposure to Ba^{2+} (1.5 mM) (3). Mean amplitudes of pIPSCs recorded 30–40 min after the end of Ab injection with and without postapplication of Ba^{2+} revealed a statistically significant difference ($*P < 0.05$, t -test). The dotted square shows the time window for mean pIPSC measurement 30–40 min after the end of Ab injection under 20 min exposure to Ba^{2+} . In A–C, the bold black lines indicate the time period of HNK-1 Ab application. Data are presented as normalized pIPSC amplitudes, n indicates the number of cells. Scale bar, 25 pA and 10 ms. D and E, Lack of effect of the HNK-1 Ab (412; 50 μ g/ml) on spontaneous mIPSCs when K^+ ions in the intracellular solution were substituted by Cs^+ . mIPSCs were isolated by perfusion with 1 μ M TTX, 25 μ M CNQX and 50 μ M AP5. D shows four consecutive sweeps of mIPSC recordings before (control) and 10–20 min after HNK-1 Ab application. Scale bar, 30 pA and 300 ms. E shows mean mIPSC amplitudes and frequencies \pm SEM before and 10–20 min after the end of HNK-1 Ab application. The mean amplitudes of mIPSCs before and after Ab application were 17.8 ± 1.7 pA and 17.1 ± 2.3 pA, and the mean frequencies before and after Ab application were 3.37 ± 0.17 Hz and 3.43 ± 0.08 Hz, $n = 5$, respectively.

pIPSCs: The mean amplitude of pIPSCs recorded 10–20 min after the Ab injection was $102.1 \pm 7.1\%$ ($n = 3$) of baseline levels (Fig. 2A). PPR of pIPSCs recorded before and after Ab application were 0.76 ± 0.07 and 0.77 ± 0.06 , respectively. In experiments where Ba^{2+} was added into the bath solution after Ab application, the pIPSCs were rescued (Fig. 2B): The mean amplitude of pIPSCs recorded 30–40 min after the end of Ab application, followed by 20 min exposure to Ba^{2+} containing solution was significantly higher than the mean amplitude of pIPSCs measured in this time window without addition of Ba^{2+} ($62.3 \pm 4.0\%$, $n = 4$ versus $39.2 \pm 6.2\%$, $n = 4$, $P < 0.05$, unpaired t -test) (Fig. 2B, inset). The rescue was complete since the mean amplitude of pIPSCs recorded in the presence of Ba^{2+} without injection of the HNK-1 Ab was $57.7 \pm 7.1\%$ ($n = 3$) of baseline levels. PPR of pIPSCs recorded before and after Ab application and following addition of extracellular

Ba^{2+} were 0.81 ± 0.05 , 0.82 ± 0.08 and 0.8 ± 0.05 , respectively. The reduction of pIPSCs by Ba^{2+} could be due to either its presynaptic effect on the docking/fusion machinery of synaptic vesicles (Wiser et al., 2002) or inhibition of Kir channels on glial cells, affecting removal of K^+ from the extracellular space (Walz, 2000).

To investigate whether pre- or postsynaptic Kir3 channels are involved in the HNK-1 Ab effect, we tested the effects of the HNK-1 Ab in the presence of intracellular Cs^+ ions that have been reported to block $GABA_B$ -activated K^+ currents (Gahwiler and Brown, 1985). Replacing K^+ by Cs^+ in the patch pipette solution completely abolished the effect of the HNK-1 Ab. The mean amplitude of pIPSCs after Ab application under these conditions was $93.4 \pm 7.7\%$ ($n = 4$) of baseline levels (Fig. 2C). PPR of pIPSCs recorded before and after HNK-1 Ab application were 0.8 ± 0.12 and 0.78 ± 0.12 , respectively. To insure, that extrusion

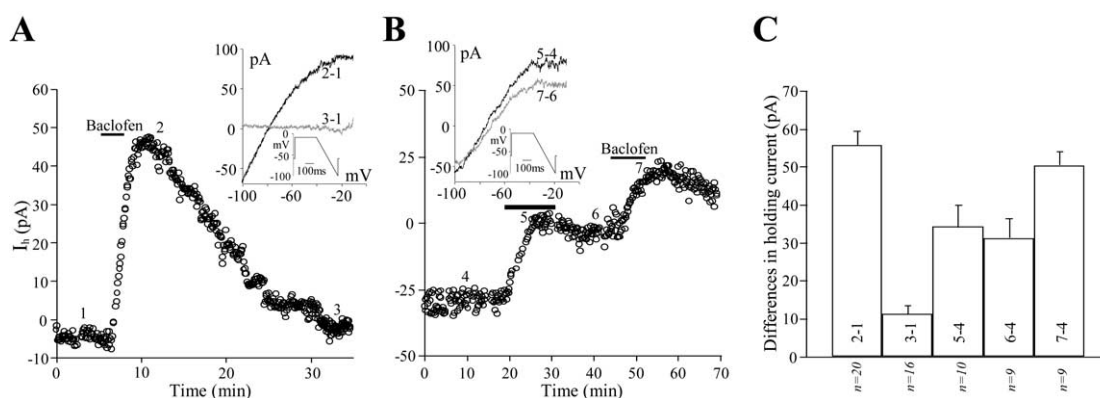


Fig. 3. Occlusion of outward currents evoked by the HNK-1 antibody and GABA_BR agonist baclofen. A and B: Baclofen-induced current in control conditions (A) and after HNK-1 Ab injection (B). The time period of baclofen (40 μM) application is shown by a thin black line. The bold line indicates the time window for the HNK-1 Ab (50 μg/ml) injection. The numbers indicate the time points for holding current measurements. Insets show K⁺ currents activated either by baclofen (A, 2-1; B, 7-6) or by HNK-1 Ab injection (B, 5-4). Curve 3-1 shows the lack of current after washout of baclofen. The K⁺ current was calculated as the difference between currents activated by the ramp protocol (shown below in the insets) before and after the application of either baclofen or the HNK-1 Ab. C, Changes in the holding current induced by application of baclofen (2-1), the HNK-1 Ab (50 μg/ml) (5-4) and application of baclofen following HNK-1 Ab injection (7-4). Bar 6-4 shows the holding current difference after washout of baclofen and the HNK-1 Ab. TTX (1 μM) was present in the bath solution throughout the experiments, *n* indicates the number of cells.

of Cs⁺ from the patch pipette during formation of the gigaseal, did not cause any presynaptic alteration that might account for the absence of an effect of the HNK-1 Ab under these conditions, we recorded from a CA1 pyramidal cell with K⁺-based intracellular solution while approaching this cell with a Cs⁺-containing pipette. Unaltered holding current and frequency of sPSCs demonstrated that extrusion of Cs⁺ from the patch pipette during gigaseal formation does not affect synaptic transmission and, thus, cannot account for the lack of an effect of HNK-1 Ab on pIPSCs with Cs⁺-based intracellular solution (data not shown). Since we previously observed an increase in frequency of miniature inhibitory postsynaptic currents (mIPSCs) with K⁺-based intracellular solution after injection of the Ab (Saghatelian et al., 2000), this effect was also examined in the presence of intracellular Cs⁺. Under this condition, the HNK-1 Ab affected neither the amplitude nor the frequency of mIPSCs (Fig. 2D and E). Expressed as percentage of baseline levels, the mean amplitude and frequency of mIPSCs after the Ab application were $94.95 \pm 5.3\%$ and $98.8 \pm 6.2\%$, respectively ($n = 5$, $P > 0.1$).

Since we could neutralize the effects of the HNK-1 Ab by pharmacological block of GABA_BR and K⁺ channels, we attempted to demonstrate a direct effect of the HNK-1 Ab on GABA_BR-activated K⁺ currents. These currents were evoked by bath application of the GABA_BR agonist baclofen. It induced outward currents at a holding potential of -60 mV, on average 55.7 ± 3.8 pA ($n = 20$; Fig. 3A). These currents are mediated by Kir3 channels, which is shown by the inward rectification of activated currents (Luscher et al., 1997) [Fig. 3A, curve (2-1) in the inset] and their block by intracellular Cs⁺ (1.3 ± 0.9 pA, $n = 3$). Similarly, administration of the HNK-1 Ab activated inwardly rectifying K⁺ current of 34.2 ± 5.8 pA [$n = 10$; Figs. 3B and C, curve (5-4) in the inset]. This current was, most likely,

mediated by the activation of GABA_BR by spontaneously released GABA after removal of the inhibitory action of the HNK-1 carbohydrate on GABA_BR. Administration of baclofen before Ab application completely blocked this current (0.9 ± 1.5 pA, $n = 7$). Bath application of baclofen following the HNK-1 Ab administration further increased the holding current by 19.2 ± 3.7 pA ($n = 9$; Figs. 3B and C). Interestingly, the sum of currents activated by the HNK-1 Ab and baclofen applied after the Ab administration was not different from the current activated by baclofen in control conditions without Ab administration (50.6 ± 3.7 pA vs 55.7 ± 3.8 pA, $P > 0.05$) [Fig. 3C; (7-4) and (2-1)]. This is in line with the observation that the HNK-1 Ab acts at perisomatic but not dendritic inhibitory synapses (Saghatelian et al., 2000) and that baclofen can activate somatic as well as dendritic GABA_BR. Additionally, occupancy of postsynaptic GABA_BR by spontaneously released GABA could be lower as compared to the bath applied baclofen. Notably, Ab administration is likely to induce a permanent activation of perisomatic postsynaptic GABA_BR, because even 20 min after the end of Ab application the holding current remained high (31.4 ± 5.1 pA, $n = 9$) [Fig. 3B; bar (6-4) in Fig. 3C].

Elevation of extracellular K⁺ mimics and occludes the effects of the HNK-1 antibody

Since application of HNK-1 Ab leads to the activation of postsynaptic GABA_BR and K⁺ channels and since this activation is necessary for presynaptic downregulation of GABA release in the same synapse (Saghatelian et al., 2000), we reasoned that there should be a retrograde signal between CA1 pyramidal cell and perisomatic inhibitory contacts. Usually, a release of retrograde signals requires an increase in intracellular Ca²⁺ that can be blocked by intra-

cellularly applied chelators of Ca^{2+} (Lenz and Alger, 1999). We therefore performed HNK-1 Ab injection experiments including 10 mM [ethylenebis(oxyethylenitrilo)]-tetraacetic acid (EGTA) into the patch pipette solution. Chelation of Ca^{2+} did not interfere with the effects produced by the HNK-1 Ab ($56.0 \pm 6.6\%$, $n = 3$). Thus, retrograde signaling mechanisms are likely to be independent of postsynaptic intracellular Ca^{2+} concentration. They also appeared to be independent of nitric oxide (NO)-mediated signaling since preincubation of slices with the inhibitor of NO synthetase L-NNA ($50 \mu\text{M}$, 4 h of preincubation) did not block the reduction of pIPSCs by HNK-1 Ab ($60.0 \pm 9.1\%$, $n = 3$). Since the activation of K^+ channels produces a long-lasting outflow of K^+ from CA1 pyramidal cells, this could increase extracellular K^+ concentration in the synaptic cleft of perisomatic synapses. Perfusion of slices with a solution containing elevated (7.5 mM) K^+ concentration significantly decreased the amplitude of pIPSCs ($70.6 \pm 3.3\%$, $n = 4$), thus mimicking the effects of the HNK-1 Ab (Fig. 4A). Injection of the HNK-1 Ab under conditions of elevated extracellular K^+ concentration did not inhibit pIPSCs (Fig. 4A) and did not affect PPR (0.78 ± 0.09 and 0.81 ± 0.07 before and after HNK-1 Ab application, respectively). Elevation of extracellular concentration of K^+ to 7.5 mM after Ab injection did not reduce pIPSCs, showing an occlusion of effects of the HNK-1 Ab by high K^+ (Fig. 4B). Recordings of pIPSCs at several concentrations of extracellular K^+ revealed that the inhibitory effects of the HNK-1 Ab strongly depended on the K^+ concentration (Fig. 1A; Fig. 4A and C–E) and the magnitude of GABA_BR -activated K^+ current (Fig. 4F). The maximal inhibitory effects was observed under perfusion with nominally K^+ -free solution. Thus, our results show that elevation of extracellular K^+ concentration mimics and occludes the effects of the HNK-1 Ab.

The HNK-1 carbohydrate decreases GABA_BR -activated K^+ conductance

Having shown that application of the HNK-1 Ab induced a constitutive activation of GABA_BRs by probably disrupting a link between the HNK-1 carbohydrate and GABA_BRs , we hypothesized that the HNK-1 carbohydrate may decrease the GABA_BR -activated K^+ conductance. We tested the synthetic HNK-1 carbohydrate (Kornilov et al., 2000) and a peptide mimetic for the HNK-1 carbohydrate, which has been shown to be functionally fully equivalent to the HNK-1 carbohydrate isolated from nerve tissue (Simon-Haldi et al., 2002). Application of the HNK-1 peptide mimetic ($100 \mu\text{g}/\text{ml}$) did not induce any detectable changes in the GABA_BR -activated K^+ current in pyramidal cells (data not shown). Since the effect of exogenously applied HNK-1 peptide mimetic could be occluded by endogenously expressed HNK-1 carbohydrate that may constitutively block GABA_BRs , we investigated the effect of the HNK-1 carbo-

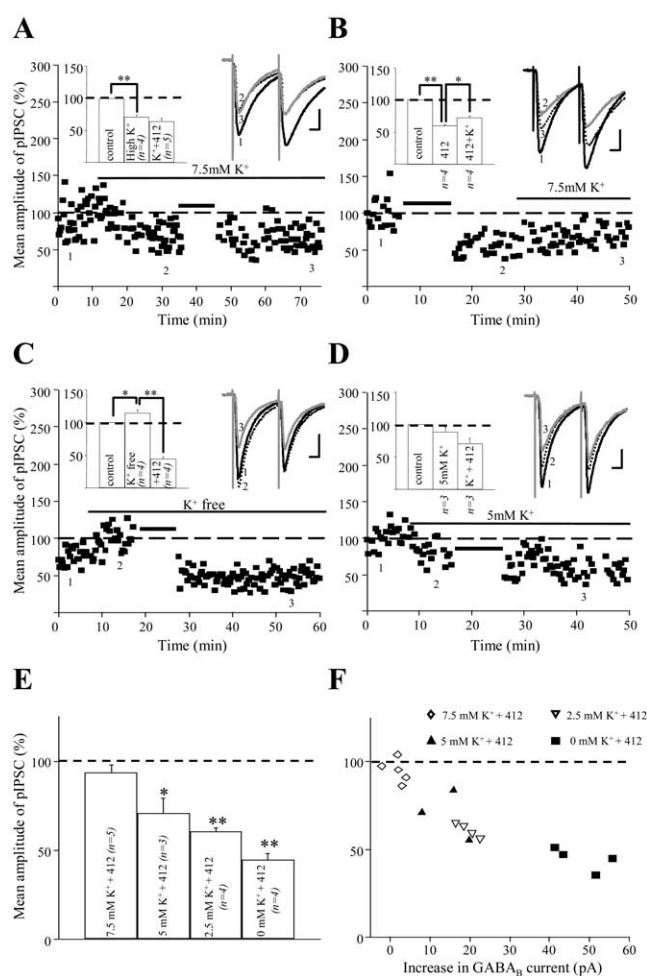


Fig. 4. Dependence of effects evoked by the HNK-1 antibody on extracellular K^+ concentration. A–D, Individual experiments and statistical summaries (bars) demonstrating the interplay between HNK-1 Ab (412; $50 \mu\text{g}/\text{ml}$) and extracellular K^+ concentration. The time periods of application of bath solutions with extracellular K^+ concentrations different from 2.5 mM are shown by thin black lines. Data are presented as normalized pIPSC amplitudes. The periods of Ab application are shown by bold black lines. Insets show the corresponding averaged pIPSCs in periods marked by numbers 1–3, n indicates the number of cells. Scale bars for all experiments are 25 pA and 10 ms. E and F, Summary of experiments demonstrating a dependence of pIPSC inhibition by the HNK-1 Ab on the extracellular K^+ concentration (E) and the magnitude of GABA_BR -activated K^+ currents (F). Stars show significant differences ($*P < 0.05$; $**P < 0.01$; paired t -test).

hydrate, peptide mimetic and the HNK-1 Ab on GABA_BRs coupled to Kir3.1/2 channels heterologously expressed in HEK293 cells. GABA applied to these cells expressing $\text{GABA}_B\text{R1}$ and $\text{GABA}_B\text{R2}$ heteromers together with Kir3.1/2 channels induced a large inward current (I_{Kir} ; $-460 \pm 101 \text{ pA}$, $n = 7$) with an extracellular K^+ concentration of 25 mM at a holding potential of -90 mV . After stabilization of I_{Kir} in the presence of $10 \mu\text{M}$ GABA, application of the HNK-1 Ab did not affect GABA_BRs coupled to Kir3 currents (Fig. 5E), implying (i) that these cells do not express HNK-1 carrying molecules involved in the

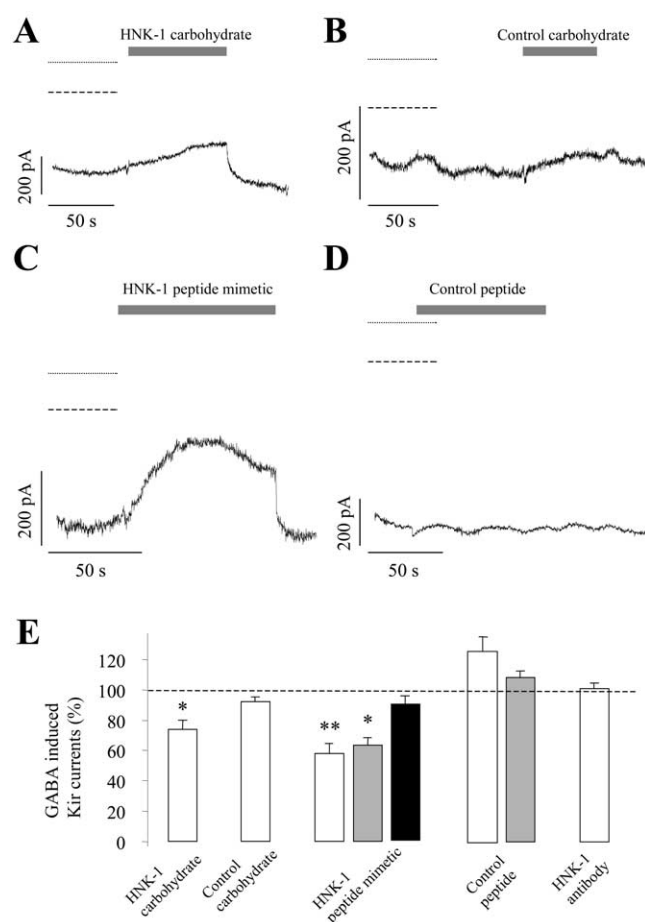


Fig. 5. Effects of the HNK-1 carbohydrate, peptide mimetic and HNK-1 antibody on GABA_BR1/R2 heteromers heterologously expressed in HEK293 cells. A, The HNK-1 carbohydrate (50 μ g/ml) reduced GABA-induced inward K⁺ currents in HEK293 cells expressing GABA_BR1b/R2a and Kir3.1/2 heteromers. B, A control carbohydrate applied at the same concentration as HNK-1 did not evoke any effect. C and D, Effects of the HNK-1 peptide mimetic and scrambled peptide applied at 100 μ g/ml. Horizontal bars above the current traces show the time of application. All experiments were done in the continuous presence of 10 μ M GABA at [K⁺]_{out} = 25 mM and V_{hold} = -90 mV, dotted and broken lines above traces show current levels in normal rat Ringer solution and high external K⁺. E, Summary of the effects of the tested compounds on GABA_BR1/R2 mediated Kir currents. Empty, grey and black bars show the effects on cells expressing GABA_BR1b/R2, GABA_BR1a/R2 and mGluR2, respectively. Bars represent mean normalized amplitudes + SEM of Kir currents (the amplitudes of GABA-induced Kir currents before application of the tested compounds were taken as 100%, the basal Kir currents as 0%, $n = 3-8$). Stars show significant differences (* $P < 0.05$; ** $P < 0.01$; paired t -test).

effects in hippocampal slices and therefore provide the possibility to study the direct inhibitory action of the HNK-1 carbohydrate on GABA_BRs and (ii) that the HNK-1 Ab itself does not affect GABA_BRs.

Application of the synthetic HNK-1 carbohydrate induced a significant reduction of inward currents to $73 \pm 6\%$ ($n = 3$; $P < 0.05$) of control levels (Fig. 5A and E). A control carbohydrate did not show any significant effect ($92 \pm 3\%$, $n = 3$; Fig. 5B and E). Further, the HNK-1 peptide mimetic induced—as the carbohydrate itself—an inhibition

of I_{Kir} to $58 \pm 6\%$ ($n = 5$; $P < 0.01$) of control levels. We then investigated whether the so-called sushi domains at the N-terminus of the GABA_BR1a splice variant (Bettler et al., 1998) may influence the effect of HNK-1 on I_{Kir}. The sushi domains are found in selectins that are known to bind to the HNK-1 carbohydrate (Needham and Schnaar, 1993). For this purpose we expressed GABA_BR1a, together with GABA_BR2 and Kir3.1/2 channels in HEK293 cells. The HNK-1 peptide mimetic reduced I_{Kir} to $63 \pm 5\%$ ($n = 7$, $p < 0.05$) of control (Fig. 5C and E), indicating that the HNK-1 effect is not influenced by the sushi repeats. A scrambled peptide applied at the same concentration as the HNK-1 peptide mimetic did not reduce I_{Kir} in cells transfected with GABA_BR1a/GABA_BR2/Kir3.1/2 ($127 \pm 10\%$, $n = 7$) and in cells transfected with GABA_BR1b/GABA_BR2/Kir3.1/2 ($108 \pm 4\%$, $n = 2$) (Fig. 5D and E). The HNK-1 peptide mimetic failed to significantly inhibit I_{Kir} ($92 \pm 5\%$, $n = 6$) in control experiments using HEK293 cells transfected with Kir3.1/2 channels and metabotropic glutamate receptor mGluR2, which, like GABA_B receptors, is a class III G protein-coupled receptor. These results demonstrate that the HNK-1 peptide mimetic specifically decreases GABA_BR-activated K⁺ conductance. They also rule out the possibility that the HNK-1 peptide mimetic acts on downstream molecules, such as K⁺ channels.

We next asked whether the HNK-1 carbohydrate interacts directly with the GABA_BR. Equimolar quantities of membrane fractions of cells expressing GABA_BR1a/GABA_BR2 or GABA_BR1b/GABA_BR2 as well as negative control membrane fraction of homogenates of cells not expressing GABA_BRs were spotted from serial dilutions on a nitrocellulose filter and incubated with either HNK-1 synthetic carbohydrate (12.5 μ g/ml) (Fig. 6A) or HNK-1 peptide mimetic (85 μ g/ml) (Fig. 6B). Strong signals were detected up to a 128fold diluted membrane fraction from GABA_BR1a/GABA_BR2 (Fig. 6, lines 1 and 5) and GABA_BR1b/GABA_BR2 (Fig. 6, lines 2 and 6) expressing cells, whereas a weak signal was detected only from a 2-fold diluted sample of the control membrane fraction not expressing GABA_BR (Fig. 6, lines 4 and 8). In control experiments, we demonstrated that the HNK-1 synthetic carbohydrate and peptide mimetic interacted with laminin (Fig. 6, lines 3 and 7), a known receptor for the HNK-1 carbohydrate (Hall et al., 1995). These results demonstrate that HNK-1 carbohydrate binds to GABA_BRs.

We then studied whether the HNK-1 binding site on GABA_BRs may be the agonist binding site. The HNK-1 peptide mimetic (100 μ g/ml) did not significantly displace the GABA_BR agonist [³H]-CGP27492 ([³H]-3-APPA) from native GABA_BRs or the radiolabeled antagonist [¹²⁵I]-CGP64213 from recombinant GABA_BRs (data not shown). Inhibition of GABA_B-mediated I_{Kir} by the HNK-1 peptide mimetic was therefore not due to a competitive action of the peptide at the GABA binding site.

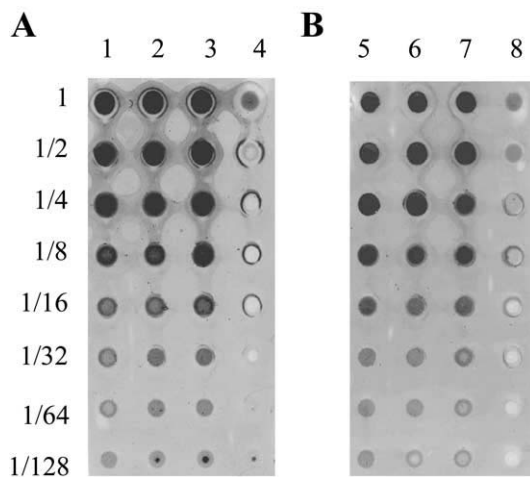


Fig. 6. Binding of the HNK-1 carbohydrate to the GABA_B receptors. Serially two-fold diluted (up to 128 fold) and immobilized cell membrane fractions were first incubated with synthetic HNK-1 carbohydrate (A) or HNK-1 peptide mimetic (B), then with HNK-1 Ab and finally with IRD-800 labelled anti-rat Ig secondary Ab. Columns 1, 5 and 2, 6: membranes from cells with stable expression of GABA_BR1a/ GABA_BR2a and GABA_BR1b/ GABA_BR2a, respectively; columns 3, 7: laminin, and columns 4, 8: negative control membranes from parental non-transfected cells not expressing GABA_BRs. Protein concentrations for cell membranes and laminin were 670 μ g and 100 μ g, respectively, in the starting material.

Discussion

We have demonstrated that the strength of perisomatic inhibitory transmission is under endogenous control of the recognition molecule associated HNK-1 carbohydrate that inhibits postsynaptic GABA_BR activation. We further demonstrated that disruption of HNK-1/GABA_BRs interaction by HNK-1 Ab induces persistent outflow of K⁺ via GABA_BR coupled K⁺ channels that retrogradely affects GABA release from presynaptic terminals. Our results indicate that the HNK-1 carbohydrate controls the efficacy of inhibitory transmission and highlights a novel mechanism of GABAergic modulation that links recognition molecules with metabotropic receptors.

HNK-1 carbohydrate-mediated mechanism of reduction of perisomatic inhibition via activation of postsynaptic GABA_BR

Our pharmacological experiments with antagonists and an agonist of GABA_BRs demonstrates that these receptors mediate the effects of the HNK-1 Ab. How can activation of postsynaptic GABA_BRs by the HNK-1 Ab modulate GABA_AR-mediated currents? Several possible mechanisms could be deduced from published data. First, GABA_BRs can affect both the desensitization and the conductance of GABA_ARs through G protein-mediated interactions via phosphorylation by the adenylyl cyclase-protein kinase A pathway (Hahner et al., 1991; Si et al., 1997; Xi et al., 1997; Kawaguchi and Hirano, 2000). Second, activation of

GABA_BRs reduces the number and/or affinity of GABA_AR binding sites through second messenger systems that remain to be defined (Kardos and Kovacs, 1991). Third, a G-protein linked mechanism has been shown to inhibit GABA_AR clustering and/or recycling through alteration of cytoskeletal anchoring proteins (Meyer et al., 2000). Fourth, an alteration in K⁺ concentration in the synaptic cleft of perisomatic synapses may change the reversal potential for Cl⁻ ions, thus affecting the driving force and conductance of IPSCs (Ling and Benardo, 1995; Thompson and Gahwiler, 1989a, 1989b). Fifth, decrease in the input resistance following activation of GABA_BR coupled Kir3 channels might affect the amplitude of IPSCs. Finally, elevation of extracellular concentration of K⁺ via activation of postsynaptic GABA_BRs may retrogradely affect neurotransmitter release (Weight and Erulkar, 1976; Malenka et al., 1981). Our previous work (Saghatelian et al., 2000) suggests that the first five hypotheses are unlikely. In that study we have shown that modulation of IPSCs by the HNK-1 Ab occurs via the HNK-1 carried by the extracellular matrix molecule tenascin-R. This could render HNK-1 capable to interact with pre- and/or postsynaptically localized receptors and, thus, to affect pre- and/or postsynaptic processes. However, postsynaptic parameters of IPSCs appeared to be intact after application of the HNK-1 Ab: it did not change reversal potential for pIPSCs, shape of pIPSCs or amplitudes of miniature IPSCs. The increased frequency of miniature IPSCs, number of failures and coefficient of variation of pIPSC amplitudes, and the reduced mean and maximal number of GABA quanta released were indicative that the HNK-1 Ab exerts presynaptic effects (Saghatelian et al., 2000). Additionally, the present study demonstrates that the HNK-1 Ab does not affect intracellular signaling mechanisms in pyramidal neurons, since inhibition of the GABA_BR-mediated K⁺ conductance by intracellular Cs⁺ or extracellular Ba²⁺ blocks and/or reverses the Ab effect. Thus, HNK-1 Ab induced effects on pIPSCs have postsynaptic induction (activation of K⁺ channels coupled to GABA_BRs) and presynaptic expression (reduction of transmitter release).

Elevation of extracellular concentration of K⁺ mimicked and occluded the effect induced by HNK-1 Ab. However, it should be noted, that rises in extracellular K⁺ following HNK-1 Ab application may be rather local and may not spread to nearby synapses/cells. By contrast, artificial elevation of extracellular K⁺ concentration would not only occlude HNK-1 Ab induced effects, operating at the level of individual synapses, but are likely to activate also other mechanisms that may exert different and even opposite functions. One of the major consequences of elevation of extracellular K⁺ concentration is an overall increase in the excitability of entire neuronal networks. Thus, although 7.5 mM external K⁺ was the experimental concentration that blocked the effect of HNK-1 Abs, it must not be the actual concentration that occludes the HNK-1 Ab mediated effect.

Blocking the effect of HNK-1 Ab by elevation of extra-

cellular K^+ concentration suggests that K^+ could be the missing link between pre- and postsynaptic effects of HNK-1 Ab. Recently, long-lasting depression of GABAergic transmission has been demonstrated after activation of postsynaptic $GABA_B$ Rs (Kotak et al., 2001) and a long-lasting outflow of K^+ via postsynaptic K^+ channels activated by $GABA_B$ Rs has been shown to elevate extracellular K^+ concentration (Obrocea et al., 1998). This is interesting since K^+ has already been considered as a putative retrograde messenger in long-term potentiation (Bliss and Collingridge, 1993) and in basal synaptic transmission, where elevation of extracellular K^+ concentration associated with activity in postsynaptic elements increased the excitability of presynaptic afferent fibers (Malenka et al., 1981), resulting in downregulation of presynaptic transmitter release (Weight and Erulkar, 1976). Thus, it is conceivable, that application of HNK-1 Ab induces elevation of extracellular K^+ concentration via activation of postsynaptic $GABA_B$ Rs that may alter the excitability of presynaptic terminals and lead to the modulation of GABA release. Alternatively, decrease in pIPSCs after HNK-1 Ab application might be a consequence of vesicle depletion due to an increase in the rate of spontaneous transmitter release following depolarization of presynaptic terminals by elevated extracellular K^+ concentration. Although currently available tools do not allow to measure and specifically manipulate K^+ concentrations in the synaptic cleft of perisomatic inhibitory synapses, the combined observations point to a novel mechanism (for a summary scheme, see Fig. 7) dependent on K^+ signaling and linking functions of postsynaptic metabotropic receptors with those of the tenascin-R associated HNK-1 carbohydrate. Regulation of perisomatic inhibition by the HNK-1 Ab thus appears to involve a chain of events, which starts from the activation of postsynaptic $GABA_B$ Rs and K^+ channels and results in reduction of evoked GABA release and increase of asynchronous spontaneous GABA release.

A retrograde messenger different from K^+ has been implicated in depolarization-induced suppression of inhibition (Alger and Pitler, 1995). Recently, endocannabinoids have been identified to play this role (Wilson et al., 2001). In contrast to our observations, however, depolarization-induced suppression of inhibition slightly decreased the frequency of miniature IPSCs, did not require activation of postsynaptic K^+ channels sensitive to internal Cs^+ and was affected by chelation of intracellular Ca^{2+} (Morishita et al., 1997; Lenz and Alger, 1999). This raises the question whether multiple retrograde signals may shape perisomatic inhibition in the hippocampus.

The HNK-1 carbohydrate directly interacts with $GABA_B$ Rs

In this study we have shown that the HNK-1 carbohydrate binds to $GABA_B$ Rs and that application of synthetic HNK-1 carbohydrate or HNK-1 peptide mimetic suppresses

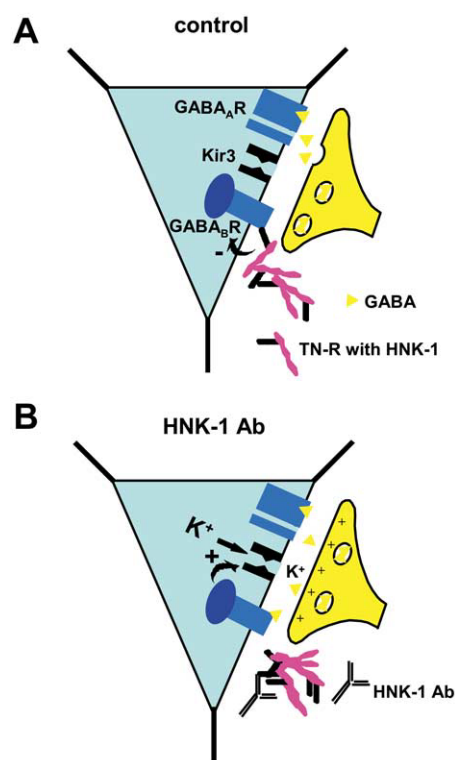


Fig. 7. Proposed model for the functional consequences of the HNK-1 antibody application to the CA1 region of the hippocampus. A: The HNK-1 carbohydrate carried by the extracellular matrix glycoprotein tenascin-R (TN-R) interacts directly with postsynaptic $GABA_B$ Rs on the soma of a pyramidal neuron. This interaction inhibits the activation of $GABA_B$ Rs by the inhibitory neurotransmitter GABA released by perisomatic interneurons onto pyramidal cells in the CA1 region of the mouse hippocampus. Under these conditions, GABA (Δ) released from presynaptic terminals of these inhibitory interneurons activates postsynaptic $GABA_A$ Rs but not $GABA_B$ Rs. B: Application of monoclonal antibody directed against the HNK-1 carbohydrate neutralizes the inhibition of postsynaptic $GABA_B$ Rs by the HNK-1 carbohydrate. As a consequence, tonic GABA release activates postsynaptically both $GABA_A$ Rs and $GABA_B$ Rs. Activation of postsynaptic $GABA_B$ Rs leads to an increase in K^+ conductance and probably to accumulation of extracellular K^+ . This, in turn, might depolarizes the presynaptic terminals (+), elevates the rate of spontaneous asynchronous vesicle release and decreases the evoked GABA release.

the $GABA_B$ R-activated K^+ currents in HEK293 cells heterologously expressing $GABA_B$ Rs coupled to Kir3.1/2 channels. Interaction of the HNK-1 carbohydrate with $GABA_B$ Rs was not mediated via the sushi domains at the N-terminus of a $GABA_B$ Rs. Moreover, the blocking effect of the HNK-1 peptide mimetic is not due to displacement of GABA at $GABA_B$ Rs. It is noteworthy in this respect that a non-competitive mGluR1 antagonist (CPCOEt) has been found to bind to an extracellular surface domain of a transmembrane segment of this receptor and inhibits receptor signaling without affecting glutamate binding (Litschig et al., 1999). A sequence with homology to the HNK-1 carbohydrate binding site in the A-chain of laminin-1 and -2 (Hall et al., 1995; 1997) is present in the vicinity of the second transmembrane domain of $GABA_B$ Rs (sequence between amino acids 626–640 in the splice variant

GABA_BR1a). Whether this sequence represents the HNK-1 binding site, will be the topic of further investigations.

In vivo relevance of HNK-1 mediated regulation of perisomatic inhibition

Recently, we have shown that application of HNK-1 Ab increases long-term potentiation (LTP), whereas injection of HNK-1 peptide mimetic reduced the level of LTP in the CA1 region of the hippocampus (Saghatelian et al., 2000). Since application of the HNK-1 peptide mimetic was effective only during high frequency stimulation, but not under conditions of basal synaptic transmission, it was hypothesized that high frequency stimulation by itself could neutralize the HNK-1 inhibitory action on GABA_BRs via proteolysis or conformational modification of the HNK-1 carrying glycoprotein. Proteolysis of adhesion molecules has been shown to accompany LTP (Fazeli et al., 1994; Hoffman et al., 1998) and removal of HNK-1 carrying glycoprotein during high frequency stimulation would allow the HNK-1 peptide mimetic to act by binding to GABA_BRs, thus replacing the endogenous HNK-1 carbohydrate. If long-term modifications of synaptic transmission are influenced by the HNK-1 carbohydrate, then removal of glycoproteins carrying this carbohydrate in genetically modified animals should result in the modifications of LTP. Indeed, we have shown that the deficiency in tenascin-R carrying the HNK-1 carbohydrate resulted in impaired LTP and reduced perisomatic inhibition, accompanied by an increase in postsynaptic GABA_BRs activity (Saghatelian et al., 2001).

Interestingly, we have observed a decrease rather than an increase in LTP, as it would be expected from an inhibitory action of the HNK-1 carbohydrate on LTP. However, a reduced perisomatic inhibitory transmission in the mutant mice could facilitate the induction of long-term plasticity in the living animals, before preparation of slices for *in vitro* recordings, leading to an increased level of basal excitatory transmission and consequently to an occlusion of LTP. In agreement with this hypothesis, we have found that deficiency in tenascin-R results in the upregulation of excitatory synaptic transmission that strongly occluded LTP (Saghatelian et al., 2001). Moreover, recent observations suggest that tenascin-R deficient mice show an abnormal number of perforated synapses in the CA1 region of the hippocampus (A. Nikonenko and M. Schachner, unpublished data), formation of which is known to accompany long-term modifications in synaptic transmission (for review, see Luscher et al., 2000). Also, mice deficient in the HNK-1 sulfotransferase exhibit reduced LTP (Senn et al., 2002). Analysis of the water maze task showed that the HNK-1 sulfotransferase deficient mice had a poorer performance as compared to wild-type littermates in the acquisition of the relearning task, but not of the learning task. Moreover, these mutants did not show selectivity for the target area when tested after a 72-h interval, while they did when tested after a 1-h

interval. Thus, our observations suggest an important role of tenascin-R and its associated HNK-1 carbohydrate in hippocampal synaptic plasticity via GABA_BR-mediated regulation of inhibitory transmission both in *in vitro* and *in vivo*.

Experimental methods

The HNK-1 antibody, carbohydrate and peptide mimetic

Monoclonal antibody (Ab) 412, a rat IgG, recognizing both the sulfated and non-sulfated epitopes of the HNK-1 carbohydrate (Kruse et al., 1984) was purified from hybridoma cell culture supernatants obtained under serum free conditions by affinity chromatography on a protein G Sepharose 4 fast flow column (Pharmacia Biotech, Uppsala, Sweden) in phosphate buffered saline (pH 7.2). Ab was eluted with a 0.1 M glycine buffer, pH 2.7, and quickly neutralized with 1 M Tris-HCl buffer, pH 9.

Synthesis, purification, analysis of structure and purity of the HNK-1 pentasaccharide 2-aminoethyl glycoside (3-SO₃⁻-GlcAβ-3Galβ-4GlcNAcβ-3Galβ-4Glcβ-C₂H₄NH-PAA) coupled to polyacrylamide (40 kD) was carried out as described (Kornilov et al., 2000). The control carbohydrate was the β-D-glucose 2-aminoethyl polyacrylamide derivative (Syntesome, Munich, Germany). Since the natural or synthetic HNK-1 carbohydrate is difficult to obtain in sufficient amounts, we also used in this work an HNK-1 peptide mimetic previously isolated by phage display techniques using the 412 Ab for selection (Simon-Haldi et al., 2002). The HNK-1 peptide mimetic functionally substitutes for the HNK-1 carbohydrate in all aspects tested: It competed for the interaction of the HNK-1 Ab with HNK-1 glycolipids, bound to laminin-1 and motor neurons, and promoted neurite outgrowth from motor neurons, but not sensory neurons *in vitro*. The HNK-1 peptide mimetic (FLHTRLFV) and the corresponding scrambled control peptide (TVFHFRL) were purchased from Eurogentec (Seraing, Belgium).

Slice preparation

Transverse slices of hippocampi (350 μm thick) were prepared for patch-clamp recordings from 14- to 20-day-old C57BL/6J mice. After halothane anaesthesia, decapitation and removal of the brain, the hippocampi were cut with a Vibroslice (Campden Instruments, Loughborough, UK) in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 250 sucrose, 25 NaHCO₃, 25 glucose, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂ (pH 7.3). The slices were kept at room temperature in carbogen-bubbled ACSF, containing 125 mM NaCl instead of 250 mM sucrose, for at least 2 h before the start of recordings.

Patch-clamp recordings in hippocampal slices

Slices were continuously superfused in a recording chamber with carbogen-bubbled ASCF (2–3 ml/min). The conventional patch-clamp technique (Blanton et al., 1989) was used for intracellular recording of synaptic currents. Cells were kept at a holding potential of -60 mV. Patch pipettes were filled with the intracellular solution containing (in mM): 125 K-gluconate, 20 KCl, 10 HEPES, 0.2 EGTA, 2 Mg-ATP, 0.2 Na-GTP and 10 glucose. The osmolarity was 310–315 mOsm, pH was 7.2. To measure basal synaptic transmission, pulses of 20–50 μ A, 0.2 ms were delivered through glass electrodes with a tip resistance of 2 M Ω using a stimulus isolator (WPI, Sarasota, USA). The currents were evoked by stimulation close to the *stratum pyramidale* to activate perisomatic interneurons. To isolate pIPSCs, glutamate receptor antagonists CNQX (25 μ M, Tocris, Bristol, UK) and AP5 (50 μ M, Tocris) were bath-applied. To activate or block GABA_B receptors, baclofen (40 μ M, Tocris) or 2(OH)-saclofen (20 μ M, Tocris) and CGP54626A (200 nM, a gift from Dr. Froestl, Novartis, Basel, Switzerland), respectively, were used. Whole-cell voltage clamp recordings were performed using an EPC-9 amplifier (HEKA, Lambrecht, Germany). Experiments where serial resistance, cell resistance or capacitance were changed by more than 15% were discarded. Application of the HNK-1 Ab extensively dialyzed against ACSF was performed by injecting it into the slices through a glass pipette, 20 μ m in diameter, using an oocyte nanoliter injector (WPI). Application lasted for 10 min with totally 0.46 μ l being injected in each experiment at 2.3 nl every 3 sec.

Electrophysiological recordings in transfected HEK293 cells

HEK293 cells were plated on poly-L-lysine-coated glass coverslips and transiently transfected with cDNA coding for rGABA_BR1a or rGABA_BR1b, rGABA_BR2a, mGluR2, Kir3.1/2 concatamer and enhanced green fluorescent protein using Fugene kit (Roche Bioscience, Mannheim, Germany) at standard conditions. Two to five days after transfection, whole-cell voltage clamp experiments were done using an Axonpatch 200A amplifier and pClamp 6 software (Axon Instruments, Foster City, USA). Currents were recorded at a holding potential of -90 mV with a sampling rate of 10 Hz. Cells were perfused continuously with normal rat Ringer's solution, containing (in mM): 135 NaCl, 5.4 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES, pH 7.2. For recording of Kir-mediated currents, extracellular K⁺ concentration was raised to 25 mM and Na⁺ concentration reduced accordingly. Borosilicate pipettes with tip resistances between 4 and 8 M Ω were filled with intracellular solution containing (in mM): 140 KCl, 2 MgCl₂, 10 EGTA, 10 HEPES, 2 ATP-Na, 0.3 GTP-Na, pH 7.2. GABA (10 μ M) was applied using a multi-barrel pipette. Because of the restricted amount of peptides

available they were directly applied to the bath while continuous perfusion was stopped.

Binding of the HNK-1 carbohydrate to GABA_BRs

Serially 2-fold diluted samples of a crude membrane fraction of HEK293 cells stably expressing GABA_BR1a/R2a and GABA_BR1b/R2a (670 μ g protein of starting material prepared as described by Kaupmann et al., 1998) were blotted onto nitrocellulose membranes under vacuum during 10 min. A crude membrane fraction of parental cells without GABA_BRs (670 μ g) and laminin (100 μ g) that has been shown to bind to the HNK-1 carbohydrate (Hall et al., 1995), were used as negative and positive controls, respectively. The membranes were rinsed twice in a A100 buffer containing (in mM): 100 KCl, 10% glycerol, 20 HEPES-Na, 0.2 EDTA, 10 2- β -mercaptoethanol and 0.5 protease inhibitors (PMSF), then incubated in Odyssey blocking buffer (ScienceTec, Les Ulis, France) for 30 min at room temperature. After addition of the synthetic HNK-1 carbohydrate (12.5 μ g/ml) or HNK-1 peptide mimetic (85 μ g/ml) the incubation was continued overnight at room temperature. Then, membranes were rinsed in phosphate buffered saline (PBS, pH 7.3) for 5 min, incubated in blocking buffer for 1 h and, after addition of rat HNK-1 Ab (final concentration 25 ng/ml), the incubation was continued in Odyssey blocking buffer containing 0.1% Tween 20. The membranes were washed again in PBS containing 0.1% Tween 20 for 5 min and incubated with 1:2000 diluted infrared dye (IRD-800) labeled secondary anti-rat Ig antibodies (LI-COR) in Odyssey blocking buffer for 1 h. The membranes were again washed in PBS containing 0.1% Tween 20 for 5 min and then in PBS for another 5 min. Fluorescence signals were detected with an Odyssey imager (LI-COR) equipped with lasers exciting in the infrared region (780 nm), providing a higher signal-to-noise ratio in comparison to other fluorescence detection methods, thus allowing to detect target molecules with a higher sensitivity.

Binding of the HNK-1 peptide mimetic to the agonist binding site of GABA_BRs

To test binding of the HNK-1 mimetic and control peptides to the agonist binding site of GABA_BRs, we used a crude membrane fraction from rat cortex and CHO cells stably expressing GABA_BR1b/R2a heteromers. Binding experiments were performed as described (Kaupmann et al., 1997, 1998).

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