FEBS Letters 478 (2000) 276–280 FEBS 23985

Incorporation of *N*-propanoylneuraminic acid leads to calcium oscillations in oligodendrocytes upon the application of GABA

Carolin Schmidt[a,*], Carsten Ohlemeyer[a], Helmut Kettenmann[a], Werner Reutter[b], Rüdiger Horstkorte[b]

[a] *Max Delbrück Centrum für Molekulare Medizin, Robert Rössle Str. 10, 13122 Berlin-Buch, Germany*
[b] *Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, Arnimallee 22, 14195 Berlin-Dahlem, Germany*

Received 24 May 2000; revised 12 July 2000; accepted 13 July 2000

Edited by Guido Tettamanti

Abstract Sialylation of glycoproteins and glycolipids plays an important role during development, regeneration and pathogenesis. It has been shown that unnatural sialylation within glial cell cultures can have distinct effects on their proliferation and antigenic profiles. These cultures metabolize *N*-propanoylmannosamine (*N*-propanoylneuraminic acid precursor = P-NAP), a synthetic non-physiological precursor of neuraminic acid, resulting in the expression of *N*-propanoylneuraminic acid in glycoconjugates of their cell membranes [Schmidt, C., Stehling, P., Schnitzer, J., Reutter, W. and Horstkorte, R. (1998) J. Biol. Chem. 273, 19146–19152]. To determine whether these biochemically engineered sialic acids influence calcium concentrations in cells of the oligodendrocyte lineage, mixed glial cultures of oligodendrocytes growing on top of an astrocyte monolayer were exposed to glutamate, histamine, adrenaline, γ-aminobutyric acid (GABA), high potassium (high K^+) and ATP. Calcium responses in P-NAP-treated oligodendrocytes were determined by confocal microscopy with the calcium indicator fluo-3 AM, and compared with control cultures. We showed that P-NAP differentially modulated the calcium responses of individual oligodendrocytes when GABA was applied. GABA induced calcium oscillations with up to four spikes per min in 60% of oligodendrocytes when treated with P-NAP.

Key words: Biochemical engineering; Glycosylation; *N*-Propanoylneuraminic acid; *N*-Propanoylmannosamine; Oligodendrocyte progenitor cell; O4

1. Introduction

N-Acylneuraminic acids or sialic acids are involved in a variety of biological functions, such as proliferation, cell–cell interaction and migration (for review, see [1]). It is conceivable that all these parameters might be affected if sialylation is altered. Alteration of sialylation is in principle possible with neuraminidases which remove sialic acid from the cell membrane or with inhibitors of sialylation. However, this strategy seems to be unsuitable for in vitro studies and even less suitable for relevant in vivo studies, on account of (i) toxicity, (ii) tissue permeability and (iii) stability of the neuraminidases or the inhibitors of sialylation. Another possible strategy for circumventing these problems is the biochemical engineering of sialic acid. This is achieved by the application of synthetic *N*-acylmannosamines which are metabolized to the respective engineered neuraminic acid and incorporated into serum and membrane glycoconjugates. These synthetically derivatized neuraminic acid precursors are generally well tolerated by all cell types tested so far, when simply added to the culture medium or even applied orally to the animal [2–7]. The expression of modified *N*-propanoylneuraminic acid by MDCK host cells prevents the infection by influenza A virus. It has been suggested that this depends on a steric hindrance of the modified sialylated receptor [8]. Furthermore, the conversion from *N*-acetyl- to *N*-glycolylmannosamine pentaacetate as biosynthetic precursor prevents the binding of myelin-associated glycoprotein to neural cells [2]. The new method for the biochemical engineering of sialic acids has been extended by the group of Carolyn Bertozzi. They used *N*-acylmannosamine in which the acyl group contained a ketone structure, namely *N*-levulinoylmannosamine. This was also converted to the respective *N*-levulinoylneuraminic acid and the unique functional group was exposed on the cell surface where the ketone may serve as a tag for chemoselective conjugation with any molecule containing a hydrazide or aminooxy group [4,9].

We have started to analyze the putative consequences of remodeling neuraminic acids in the central nervous system and demonstrated earlier that the behavior of glial cells is influenced. The proliferation of microglia and astrocytes is increased by *N*-propanoylneuraminic acid precursor (P-NAP) [3]. Oligodendrocytes show increased signs of a non-mature cell stage upon the application of P-NAP shown by the expression of the A2B5 epitope [3]. Oligodendrocytes which develop constitutively into myelin-forming cells in vitro and in vivo (for review, see [10]) are functionally impaired in a number of severe neurological diseases such as multiple sclerosis.

The present study was designed to investigate whether signal transduction in the nervous system is affected by modification of the side chain of sialic acids and we concentrated on the investigation of the second messenger calcium. Substances such as glutamate, histamine, adrenaline and γ-aminobutyric acid (GABA), as well as high potassium concentrations (high K^+), each of which is known to increase intracellular calcium concentrations in glial cells, were applied to P-NAP-treated oligodendrocytes in a mixed glial cell culture, and compared with control cultures.

We found a strong GABA-dependent oscillation of intracellular calcium concentrations in oligodendrocytes treated with P-NAP.

*Corresponding author. Present address: Schering AG, Müllerstr. 178, 13342 Berlin, Germany. Fax: (49)-30-46895575.
E-mail: carolin.schmidt@schering.de

0014-5793/00/$20.00
PII: S0014-5793(00)01868-8

We propose that biosynthetic modification of the side chain of sialic acid in conjunction with the activation of GABA receptors is a potent tool for modulating calcium concentrations in oligodendrocytes.

2. Materials and methods

2.1. Preparation of mixed glial cell cultures

Mixed glial cell cultures from whole brains of neonatal Wistar rats were prepared according to the method of McCarthy and de Vellis [11] and plated onto glass coverslips (11 mm diameter) coated with poly-L-lysine (P1524, Sigma, Deisenhofen, Germany; 20 μg/ml in water). Cells were maintained in basal Eagle's medium (Gibco, Karlsruhe, Germany) supplemented with 10% horse serum (Gibco). It is well established that neurons do not survive under these culture conditions. After 7 ± 2 days in culture, cells were grown to confluency until oligodendrocytes were lying on top of an astrocyte monolayer. Thereafter, cultures were maintained for 2 days in the presence or absence of *N*-acylneuraminic acid precursors (see below) at a concentration of 5 mM and 37°C, 5% CO_2, 95% humidity.

2.2. Solutions and reagents

The physiological precursor of neuraminic acid is *N*-acetylmannosamine (*N*-acetylneuraminic acid precursor or A-NAP). *N*-Acetylmannosamine was purchased from Sigma (Deisenhofen, Germany). *N*-Propanoylmannosamine (P-NAP) was synthesized as described by Kayser et al. [6]. Matrix-assisted laser desorption ionization analysis of P-NAP preparations revealed no contaminating material. All other reagents were obtained from Sigma. Solutions used for calcium imaging were freshly prepared from refrigerated stocks. The standard bathing solution for calcium imaging was permanently gassed with carbogen and contained 134 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.25 mM K_2HPO_4, 26 mM $NaHCO_3$, 10 mM glucose, pH adjusted to 7.4 with NaOH at room temperature. For a calcium-free solution, $CaCl_2$ was omitted and 0.5 mM EGTA was added. Fluo-3 acetoxymethylester (fluo-3 AM) was obtained from Molecular Probes (Eugene, OR, USA).

2.3. Calcium [Ca^{2+}]measurements

Changes in intracellular calcium were quantified according to the method described by Bernstein et al. [12]. In brief, glial cultures were rinsed in P-NAP-free medium and loaded with 2.5 μM fluo-3 AM for 30 min in bathing solution at 37°C, 5% CO_2 in the incubator. Thereafter, cultures were superfused continuously with bathing solution. For measuring changes of the intracellular calcium concentrations, a confocal laser scanning microscope (Sarastro 2000: Molecular Dynamics, Sunnyvale, CA, USA) was used to distinguish oligodendrocytes lying on astrocyte monolayers. A 200 μm pinhole was selected to adjust the thickness of the image plane from which the fluorescence was collected by the confocal system to less than 5 μm. The scanner was mounted on an upright microscope (Axioscope, Zeiss, Oberkochen, Germany) equipped with 20×magnification. The non-ratiometric Ca^{2+} indicator, fluo-3, was used to provide information about changes in relative calcium concentrations. Fluo-3 was excited at 488 nm with an argon laser, and the fluorescence was measured at an emission wavelength above 510 nm and selected with a long-pass filter. Images were acquired every 4 s. Acquisition of the fluorescence data and image analysis were performed using Imagespace (Molecular Dynamics) and standard PC evaluation software.

2.4. Immunohistochemistry

Mixed glial cell cultures were stained with mouse monoclonal antibodies directed against the O4 (Roche Molecular Biochemicals, Mannheim, Germany), a marker for cells of the oligodendrocyte lineage [13]. Non-fixed cells were stained directly after calcium imaging had been performed on the stage of the upright microscope. Thus, it was possible to correlate calcium responses with individual cells. The primary antibody was incubated for 5 min in bathing solution. After extensive superfusion with bathing solution, chain-specific Cy3-conjugated secondary antibody (Dianova, Hamburg, Germany) was applied for another 5 min.

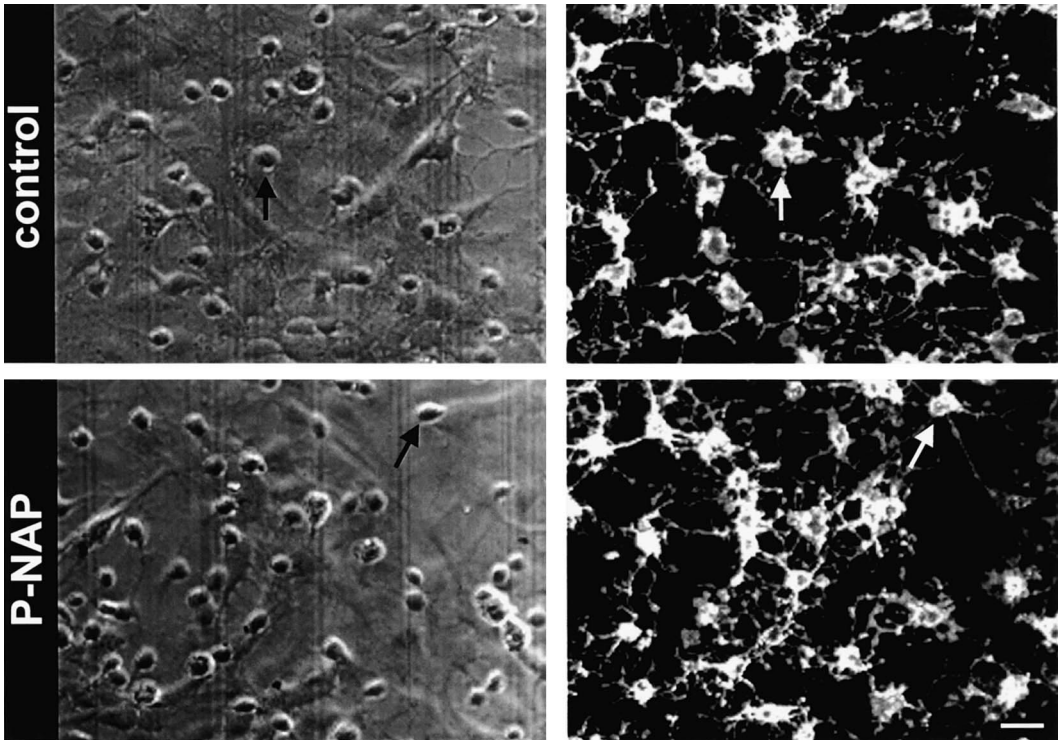

Fig. 1. Morphological characterization of oligodendrocytes of mixed glial cultures that have been maintained for 2 days in the absence (control) and in the presence of P-NAP. Left panel: phase contrast micrographs of astrocyte monolayers with oligodendrocytes growing on top of them. Right panel: O4 immunohistochemical staining of oligodendrocytes. Examples of O4-positive oligodendrocytes are marked with arrows. Bar = 12 μm.

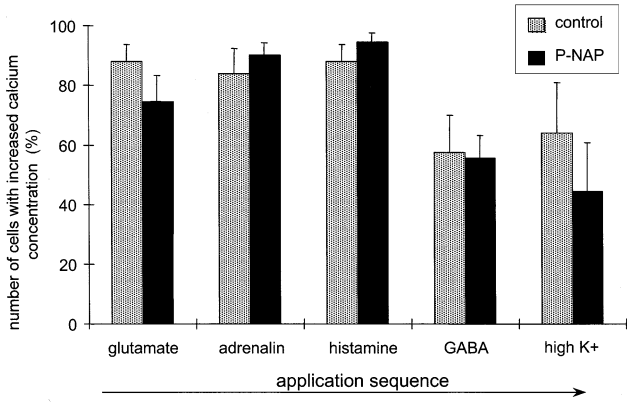

Fig. 2. Quantification of oligodendrocytes of mixed glial cultures reacting with an increase in intracellular calcium concentration upon the application of a series of substances. Cells were maintained for 2 days in the absence (control) and in the presence of P-NAP. Glutamate, adrenaline, histamine, GABA and high K^+ were applied sequentially at a concentration of 1 mM, except high K^+ which was applied at a concentration of 50 mM. Substances were applied for 30 s, except GABA which was applied for 1 min. Bars represent mean values ± S.D. from three independent experiments with 25 cells in each group.

3. Results

3.1. Morphological characterization of oligodendrocytes in the presence and in the absence of chemically engineered sialic acids

Astrocytes provide a preferable substratum for oligodendrocytes in vitro [14,15] and are also in close contact with each other in vivo [16–18]. Thus, mixed glial cultures were grown to confluency until process-bearing oligodendrocytes were growing on top of a confluent astrocytic monolayer (Fig. 1). These cultures were then maintained for 2 days in the presence and in the absence of 5 mM of *N*-propanoylmannosamine (P-NAP). It has been reported that mixed glial cell cultures incorporate and metabolize P-NAP and express it on glycoproteins of the cell membrane [3]. We used the O4 antibody for immunohistochemical identification of oligodendrocytes. The majority of all cells lying on top of the astrocytes were O4-positive with only a few processes (Fig. 1, arrows). There was no difference in the number or morphology of O4-positive cells in the presence and in the absence of P-NAP (Fig. 1), or in the presence of *N*-acetylmannosamine, the physiological precursor of neuraminic acid (*N*-acetylneurmaninic acid precursor, or A-NAP; data not shown).

3.2. P-NAP alters GABA-dependent calcium transients of oligodendrocytes

In order to functionally characterize the O4-positive oligodendrocytes, a series of mediators of signal transduction pathways were applied. Here, we concentrated on the effects on calcium responses.

We determined the calcium transients in oligodendrocytes evoked by glutamate, histamine, adrenaline, GABA and high K^+ in an application sequence in P-NAP- or A-NAP-treated and non-treated cultures. To depolarize the oligodendrocytes to the threshold for the activation of voltage-gated Ca^{2+} channels, high K^+ was applied at a concentration of 50 mM. All other substances were applied at 1 mM. Oligodendrocytes cultured in the presence or absence of P-NAP responded to all of the applied substances with an increase of intracellular calcium concentration (Fig. 2). During one experiment, an apparent decreased responsiveness to the applied substances was observed at the end of each application sequence, indicating a decrease of the number of reactive cells (Fig. 2). However, each substance led to an increase of the intracellular calcium concentration in up to 90% of the oligodendrocytes when applied first (data not shown). The number of cells reacting with an increase in intracellular calcium concentration was not significantly different between cultures maintained in the absence or in the presence of P-NAP (Fig. 2). In control experiments under calcium-free conditions, only very few oligodendrocytes reacted to GABA (data not shown).

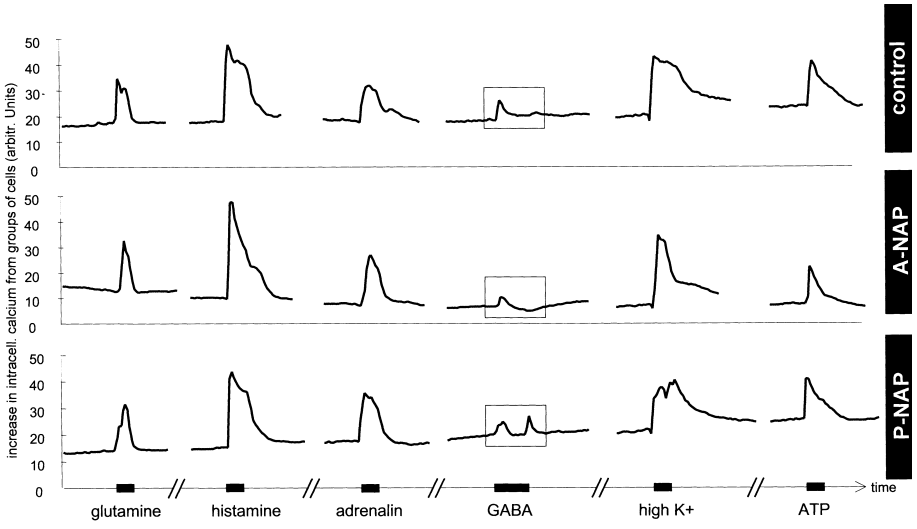

Fig. 3. The calcium signals of a group of oligodendrocytes (22–25 cells) subjected to a series of substances are shown in one representative set of experiments. Cultures of oligodendrocytes growing on top of astrocyte monolayers were maintained for 2 days in the absence (control) or presence of A-NAP or P-NAP. Substances were applied for 30 s, except GABA which was applied for 1 min. Between each application, cultures were allowed to recover for 10 min (//). Glutamate, adrenaline, histamine and GABA were applied at a concentration of 1 mM, and K^+ at 50 mM. To test the viability of the cultures, 100 μM ATP was applied. Note the prominent additional and elevated calcium spike upon the application of GABA in P-NAP-treated cultures, compared with the control and A-NAP-treated cultures (boxes).

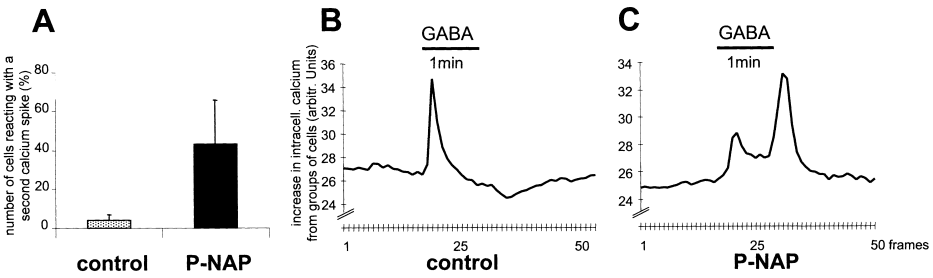

Fig. 4. (A) Quantification of oligodendrocytes reacting with a second increase of intracellular calcium concentration upon the application of GABA for 1 min. Cultures were maintained in the absence (control) and in the presence of P-NAP for 2 days. Bars represent mean values ± S.E.M. from three independent experiments. (B,C) Representative set of experiments showing the sums of calcium traces of groups of oligodendrocytes (22–25 cells) in the absence (B) and in the presence of P-NAP (C). Note the elevated second calcium spike in P-NAP-treated oligodendrocytes. The fluo-3 signals were recorded every 4 s (frames).

However, when GABA was applied, P-NAP-treated cultures reacted differently compared with control cultures with respect to relative intracellular calcium concentrations. When GABA was applied for 1 min in the presence of external calcium, a second calcium spike appeared in P-NAP-treated oligodendrocytes. (Fig. 3, compare boxes and Fig. 4C, another independent experiment showing calcium traces of groups of oligodendrocytes). The additional prominent spike was not present in the control experiments in the absence (Figs. 3 and 4B) or presence of A-NAP (Fig. 3). 43 ± 23% single oligodendrocytes in P-NAP-treated cultures showed this characteristic second spike compared with 4 ± 3% of the non-treated controls (Fig. 4A) or A-NAP-treated controls (data not shown). In P-NAP-treated cells, the second calcium spike induced by GABA was often even more pronounced than the first rise in intracellular calcium (Figs. 3 and 4C, and see also the calcium trace of a single oligodendrocyte in Fig. 5A).

3.3. *Increased calcium spiking efficacy with prolonged application of GABA in oligodendrocytes pretreated with P-NAP*

Our findings suggest that GABA leads to an oscillation of intracellular calcium concentrations in P-NAP-treated cultures. When GABA was applied for longer periods in the presence of external calcium to cultures that had been incubated with P-NAP, an increase in homogeneous calcium spiking and heterogeneous calcium spiking, i.e. multipeak calcium spiking, was observed. Fig. 5A shows a representative example of an oligodendrocyte which was subjected to 1 and 3 min GABA, showing 1–4 spikes/min. The calcium oscillation could also be extended to 4 min with the application of GABA (data not shown). However, frequencies and amplitudes of the calcium responses were heterogeneous from experiment to experiment. 60 ± 21% of all oligodendrocytes in P-NAP-treated cultures show 2–4 spikes per min when GABA was applied. Only 23 ± 7% of the non-treated control cells showed these kinds of calcium traces upon the application of GABA (Fig. 5B). No comparable calcium oscillations were observed in those few cells which reacted towards GABA under calcium-free conditions (data not shown).

Calcium responses were in general immediate. Only a few cells reacted with a delay of 24 ± 5 s. In all cultures, spontaneous calcium spiking, i.e. the generation of spikes not directly related to the application of any substance, was the same (data not shown).

4. Discussion

In this study, we demonstrate that the signal transducer calcium is differently modulated in P-NAP-treated oligodendrocyte cultures compared with cultures grown in the absence of P-NAP or in the presence of the natural precursor of neuraminic acid A-NAP. We observed a prominent oscillation of intracellular calcium concentration in P-NAP-treated oligodendrocytes when GABA was applied.

Glial cells play an active part during information process-

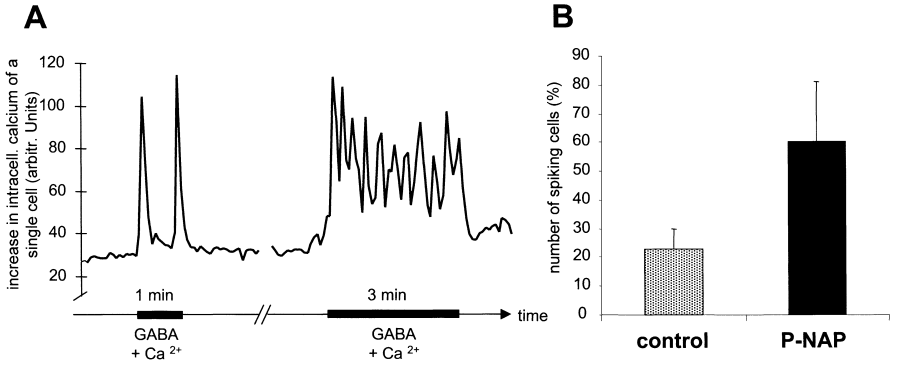

Fig. 5. (A) GABA was applied in the presence of extracellular calcium (GABA+Ca^{2+}) for 1 and 3 min to an oligodendrocyte of a mixed glial culture that had been incubated with P-NAP for 2 days. Note the increase of homogeneous calcium spiking with prolonged incubation time. (B) Quantification of oligodendrocytes that showed spiking during the application of GABA (≥two spikes). Bars represent mean values ± S.E.M. from three independent experiments.

ing, growth, differentiation and regeneration in the nervous system. Oligodendrocytes express cell adhesion molecules such as the neural cell adhesion molecule, neurotransmitter receptors such as GABA receptors and also specific glycolipids such as A2B5 on their cell surfaces. One common component of all these molecules is *N*-acetylneuraminic acid, the terminal monosaccharide of most glycoconjugates. Biochemical engineering of neuraminic acids via the unnatural precursor P-NAP and the incorporation of *N*-propanoylneuraminic acid instead of the natural *N*-acetylneuraminic acid in all glycoconjugates has been shown to modulate several oligodendrocyte functions [3].

The observation of calcium oscillations in intact tissues and the recognition of calcium-dependent processes that are adapted to frequency-modulated oscillatory calcium signals point to a physiological significance of this phenomenon [19]. Calcium oscillations in astrocytes are thought to represent a highly plastic signaling system that underlies the reciprocal communication between neurons and astrocytes [20]. It has been shown that glutamate induces calcium spikes in astrocytes [21,22] with a frequency of 4–7 spikes per min. Our results show that the frequency of GABA-induced spikes in oligodendrocytes was in the range of up to four spikes/min.

It has been reported that platelet-derived growth factor (PDGF)-BB induces both oscillatory and non-oscillatory Ca^{2+} responses in cultured oligodendrocytes, grown either as O-2A progenitors or differentiated oligodendrocytes [23]. It was concluded that intracellular levels of sphingosine and sphingosine 1-phosphate differentially modulate calcium signaling triggered by PDGF receptor stimulation in these cells [24]. The functional consequences of oscillatory and non-oscillatory calcium responses are not yet clear. They may reflect the complex action of signal transducers, such as growth factors and neurotransmitters in oligodendrocytes. Differential signal processing may provide a mechanism whereby calcium controls diverse cellular functions via different signaling pathways, as shown for neuronal cells where the mode of calcium entry determines distinct gene expression [25].

Since our understanding of glial–glial interactions is only sparse, it remains unclear whether the observed calcium responses in oligodendrocytes growing on astrocytes are direct or indirect. Astrocytes are in general also responsive to the applied substances [12,26,27]. Thus, it is possible that the stimulation of the underlying astrocytes leads to an indirect effect: the application of the substances (i.e. via intracellular calcium increase) might release a second effector which, in turn, stimulates the oligodendrocyte lying on top of the astrocytes. However, the immediacy of the oscillatory calcium transients suggests a direct response of the oligodendrocytes.

In a number of cases, we observed a second increase in intracellular calcium in P-NAP-treated cells which was even more prominent than the immediate first answer after the external stimulus GABA. It remains to be further analyzed whether this might be due to a P-NAP-dependent activation of voltage-operated calcium channels.

Ionotropic GABA receptors are sialylated glycoproteins, as shown by lectin binding studies and neuraminidase treatment [28,29]. We propose a modulation of neurotransmitter-induced calcium transients in oligodendrocytes after unnatural sialylation of ionotropic GABA receptors and possibly other glycoconjugates using the biochemically engineered P-NAP. It remains to be elucidated whether an altered sialylation of GABA receptors might directly account for the intracellular calcium signals observed during the application of GABA. P-NAP-dependent alteration in the sialylation of glycoproteins may provide a means for intracellular or intercellular information coding.

Acknowledgements: The authors thank B. Hunger for excellent technical assistance, Dr. M. Mickeleit for synthesizing P-NAP, Dr. A. Scott for improving the English and Dr(s). A. Verkhratsky and J. Schnitzer for helpful discussion and comments. This work was supported by the Deutsche Forschungsgemeinschaft as a part of the priority program 'Functions of Glial Cells' (Schn 346/4-1), the Sonnenfeldstiftung and the Fonds der Chemischen Industrie.

References

[1] Varki, A. (1992) Glycobiology 2, 25–40.
[2] Collins, B.E., Fralich, T.J., Itonori, S., Ichikawa, Y. and Schnaar, R.L. (2000) Glycobiology 10, 11–20.
[3] Schmidt, C., Stehling, P., Schnitzer, J., Reutter, W. and Horstkorte, R. (1998) J. Biol. Chem. 273, 19146–19152.
[4] Mahal, L.K., Yarema, K.J. and Bertozzi, C.R. (1997) Science 276, 1125–1128.
[5] Keppler, O.T., Stehling, P., Herrmann, M., Kayser, H., Grunow, D., Reutter, W. and Pawlita, M. (1995) J. Biol. Chem. 270, 1308–1314.
[6] Kayser, H., Zeitler, R., Kannicht, C., Grunow, D., Nuck, R. and Reutter, W. (1992) J. Biol. Chem. 267, 16934–16938.
[7] Kayser, H., Geilen, C.C., Paul, C., Zeitler, R. and Reutter, W. (1992) FEBS Lett. 301, 137–140.
[8] Keppler, O.T., Herrmann, M., von der Lieth, C.W., Stehling, P., Reutter, W. and Pawlita, M. (1998) Biochem. Biophys. Res. Commun. 253, 437–442.
[9] Yarema, K.J., Mahal, L.K., Bruehl, R.E., Rodriguez, E.C. and Bertozzi, C.R. (1998) J. Biol. Chem. 273, 31168–31179.
[10] Cameron, R.S. and Rakic, P. (1991) Glia 4, 124–137.
[11] McCarthy, K.D. and de Vellis, J. (1980) J. Cell Biol. 85, 890–902.
[12] Bernstein, M., Lyons, S.A., Moller, T. and Kettenmann, H. (1996) J. Neurosci. Res. 46, 152–163.
[13] Sommer, I. and Schachner, M. (1981) Dev. Biol. 83, 311–327.
[14] Sakurai, Y., Nishimura, D., Yoshimura, K., Tsuruo, Y., Seiwa, C. and Asou, H. (1998) J. Neurosci. Res. 52, 17–26.
[15] Kachar, B., Behar, T. and Dubois-Dalcq, M. (1986) Cell Tissue Res. 244, 27–38.
[16] Aloisi, F., Agresti, C., D'Urso, D. and Levi, G. (1988) Proc. Natl. Acad. Sci. USA 85, 6167–6171.
[17] Noble, M. and Murray, K. (1984) EMBO J. 3, 2243–2247.
[18] Butt, A.M., Colquhoun, K., Tutton, M. and Berry, M. (1994) J. Neurocytol. 23, 469–485.
[19] Berridge, M.J., Bootman, M.D. and Lipp, P. (1998) Nature 395, 645–648.
[20] Pasti, L., Volterra, A., Pozzan, T. and Carmignoto, G. (1997) J. Neurosci. 17, 7817–7830.
[21] Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S. and Smith, S.J. (1990) Science 247, 470–473.
[22] Cornell-Bell, A.H. and Finkbeiner, S.M. (1991) Cell Calcium 12, 185–204.
[23] Fatatis, A. and Miller, R.J. (1997) J. Biol. Chem. 272, 4351–4358.
[24] Fatatis, A. and Miller, R.J. (1996) J. Biol. Chem. 271, 295–301.
[25] Bading, H., Ginty, D.D. and Greenberg, M.E. (1993) Science 260, 181–186.
[26] Verkhratsky, A. and Kettenmann, H. (1996) Trends Neurosci. 19, 346–352.
[27] von Blankenfeld, G. and Kettenmann, H. (1991) Mol. Neurobiol. 5, 31–43.
[28] Sweetnam, P.M. and Tallman, J.F. (1986) Mol. Pharmacol. 29, 299–306.
[29] Sweetnam, P., Nestler, E., Gallombardo, P., Brown, S., Duman, R., Bracha, H.S. and Tallman, J. (1987) Brain Res. 388, 223–233.