Neuroprotective and Anti-Oxidative Effects of the Hemodialysate Actovegin on Primary Rat Neurons in Vitro

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Abstract The recently described therapeutic benefits of the hemodialysate actovegin on neuropathic symptoms in diabetic patients with symptomatic polyneuropathy suggest a neuroprotective activity of the drug. To elucidate the possible cellular mechanism of the pharmacological effects of actovegin, we investigated its effects on cultured primary rat neurons in vitro. Primary neurons were cultured for up to 10 days in the presence of increasing doses of actovegin (0.3–1,000 mg/l). Total cell number, dendrite length and the number of excitatory synapses, i.e., the amount of the synaptic V-Glut1 protein, were measured by immunocytochemistry followed by fluorescence microscopy. The apoptotic level in neurons after induction of apoptosis by amyloid peptide Aβ25-35 was assessed by the level of activated caspase-3. In addition, the capability of the neurons to diminish oxidative stress was assessed by measuring the cellular level of reactive oxygen species ROS in the presence of actovegin. Actovegin treatment yielded an increased maintenance of neuronal cells and total number of synapses and could lower the level of activated caspase-3 in a dose-dependent manner. Dendrite lengths were not significantly affected. In addition, actovegin reduced the cellular level of ROS in cultured neurons. The cellular effects observed suggest neuroprotective and anti-oxidative effects of the drug Actovegin®, which could at least partially explain its therapeutic benefits.

Keywords Apoptosis · Diabetic polyneuropathy · Hemodialysate · Neuroprotection · Oxidative stress

Introduction

Diabetic distal symmetric polyneuropathy (DPN) affects approximately one-third of diabetic patients and is associated with substantial morbidity including excruciating neuropathic pain and foot ulcers leading to amputation (Ziegler et al. 2008; Boulton et al. 2005). Analgesics are effective in the treatment of neuropathic pain (Dworkin et al. 2007), but do not slow down the progression of the underlying neuropathy (Boulton et al. 2005). Various therapeutic approaches to treat DPN have been developed (Cameron et al. 2001), which address the pathology of the disorder, rather than just relieve pain (Ziegler et al. 1995; Boulton et al. 2005; Chalk et al. 2007). However, despite apparent recent progress, a potent sustainable therapy of DPN still remains an unsolved medical need.

Actovegin, a deproteinized hemodialysate produced from calf blood, containing low molecular weight compounds of up to 5,000 Da, has been shown to have substantial therapeutic benefits in DPN. Recently, a randomized,
double-blind, placebo-controlled clinical trial with sequential intravenous and oral actovegin treatment of 567 patients with DPN was conducted over a period of 160 days (Ziegler et al. 2009). Actovegin treatment significantly improved neuropathic symptoms like vibration perception threshold, sensory function and quality of life of the DPN patients. The hemodialysate is approved as a drug (Actovegin®) in a number of countries and is applied to treat diabetic poly-neuropathy and other diseases (for review see: Buchmayer et al. 2011).

The goal of the present investigations was to elucidate the cellular effects, by which actovegin may exert its beneficial therapeutic effects in DPN. Well-known pharmacological actions of actovegin are the stimulation of oxygen absorption, oxygen utilization, and cellular energy metabolism (Obermaier-Kusser et al. 1989; Buchmayer et al. 2011). Furthermore, it exerts insulin-like activity, such as stimulation of glucose transport, pyruvate dehydrogenase and glucose oxidation (Jacob et al. 1996). Because of these properties, actovegin has previously been used for treatment of cerebral vascular and degenerative disorders (Kanowski et al. 1995; Herrmann et al. 1992). In addition, hemodialysates administered as infusions have shown beneficial effects on the clinical signs of dementia (Schlaffer et al. 1991; Beiswenger et al. 2008), which may indicate regenerative effects on neuronal tissue.

The present experimental approach was based on earlier studies with a rat model of diabetic polyneuropathy, which showed a reduction in intraepidermal nerve fiber density in skin biopsies, as well as a decrease in the loss of intraepidermal innervation upon therapeutic interventions (Bianchi et al. 2004). Here, the cellular effects of increasing doses of the hemodialysate actovegin were assessed in a neuronal in vitro model, namely in cultures of dissociated hippocampal neurons from embryonic rat brains. Cultured embryonic rat neurons constitute an accepted and relevant model system in the scientific community and have been widely used to evaluate the effects of pharmacologic and genetic interventions. Moreover, well-described culturing protocols and procedures for phenotypic characterization of cultures of embryonal rat neurons exist (Araujo et al. 2004; Burkarth et al. 2007; Busciglio et al. 1995; Cambon et al. 2004; Maar et al. 1997; Neiiendam et al. 2004; Skibo et al. 2005; Waliczek et al. 1986). The following morphometric parameters were measured: the number of viable neurons, neurite outgrowth and synaptic connectivity. In parallel experiments, we assessed the anti-apoptotic effect of actovegin by measuring the levels of caspase-3 in response to amyloid peptide (Aβ42,40). In addition, we assessed the anti-oxidative effect of actovegin by measuring the level of reactive oxygen species (ROS).

**Materials and Methods**

**Preparation of Neuronal Cultures**

Dissociated cultures of rat hippocampal neurons were prepared according to a standard protocol (Maar et al. 1997). Hippocampal tissue from embryonic day 18 rat fetuses was collected in sterile Hanks’ balanced salt solution (HBSS buffer [Invitrogen, Karlsruhe, Germany] containing 7 mM HEPES, pH 7.3) and trypsinized using 1× trypsin–EDTA (PAA, Pasching, Austria) containing 10 mM HEPES, pH 7.3. The serum-free medium and culture conditions were tailored for the growth of neuronal cells in order to minimize the growth of contaminating other, e.g., glial cell types. Combination of nuclear staining and phase contrast microscopy ensured that the numbers of cells counted reflect changes in neuronal cell numbers. Subsequently, tissue was triturated in HBSS–HEPES buffer until no tissue clumps were visible. Cell number was determined and 2 × 10⁴ cells/well were seeded in 100 µl NMEM-B27 medium (Burkarth et al. 2007; Goetze et al. 2003) into poly-1-lysine-coated 96-well plates (Greiner Bio-One, Frickenhausen, Germany) and cultured at 37°C and 5% CO₂.

**Actovegin Treatment of Rat Hippocampal Neurons**

Rat hippocampal neurons were treated with increasing doses (0.3, 1, 3, 10, 30, 100, 300, 1,000 µg/ml) of actovegin (Actovegin®, Nycomed, Linz, Austria) by the addition of 50 µl of prediluted stock solution to the cultures, 5 h after plating. A broad dose range was tested, as it was not possible to define a dose that relates to the dose applied in the therapy of diabetic patients. Cells were cultivated at 37°C and 5% CO₂ in a humidified atmosphere. Actovegin treatment effects were explored with respect to culture maintenance and synaptic connectivity, neurite outgrowth and neuroprotection (i.e., protection against Aβ25–35-induced apoptosis). Furthermore, the protection by actovegin from oxidative stress (ROS levels) was tested in the rat neurons.

**Immunocytochemistry**

For immunocytochemical labeling of dendrites with antibodies against microtubule-associated protein 2 (MAP2; at days 3 and 6 of in vitro culture) to be used in subsequent analysis of neurite outgrowth, and excitatory synapses (Vesicular Glutamate Transporter 1 (VGlut1); day 10) for the purpose of assessment of synaptic connectivity, cultivated hippocampal neurons were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature.
Cells were washed once with PBS and then blocked and soaked with blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany) containing 0.2% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) for 1 h at room temperature. Primary antibodies against microtubule-associated protein 2 (MAP2; Sigma-Aldrich) and vesicular glutamate transporter 1 (VGlut1; Synaptic Systems, Göttingen, Germany) were diluted [1:1,000] in blocking reagent containing 0.2% Triton X-100 and incubated with the cells at 4°C overnight. Cells were washed with PBS, and primary antibodies were detected with Cy3-conjugated goat anti-mouse IgG (diluted 1:600; Dianova, Hamburg, Germany) during a 2-h incubation at room temperature. Nuclei were stained with Hoechst 33258 (1 mg/ml; Invitrogen GmbH, Darmstadt, Germany) containing 0.2% Triton X-100 and primary antibodies were detected with Cy3-conjugated goat anti-mouse IgG (diluted 1:600; Dianova, Hamburg, Germany) during a 2-h incubation at room temperature. Nuclei were stained with Hoechst 33258 (1 mg/ml; Invitrogen GmbH, Darmstadt, Germany). The working concentration of 1 µg/ml was achieved by a 1:1,000 dilution in PBS. Cells were washed and stored at 4°C until examination by fluorescence microscopy. Image acquisition was carried out on a Zeiss Axiovert 200.

Cell Number, Neurite Outgrowth and Synaptic Connectivity of Rat Hippocampal Neurons

Average neurite lengths of MAP2-labeled neuronal cultures were determined using the ImageJ-Plugin Neurite Average (Natural and Medical Sciences Institute, Reutlingen, Germany), which employs the principles of stereology (Ronn et al. 2000). Determination of the number of synaptic connections of VGlut1-labeled neuronal cultures and counting of cell numbers was carried out using ImageJ’s Analyze Particles function: http://rsb.info.nih.gov/ij/.

Briefly, Neurite Average projects a virtual grid onto micrographs of MAP2-labeled neuronal cultures and determines the number of intersections of this grid with MAP2-positive dendritic structures. The number of these intersections corresponds to the degree of dendritic arborization at a given time point. Micrographs of either Hoechst 33258- or VGlut1-stained neuronal cultures were thresholded and binearized and the resulting signals were counted.

Aβ_{25–35} Treatment and Apoptosis Induction of Rat Hippocampal Neurons

Rat hippocampal neurons were cultivated until day 8, and cell culture medium was aspirated; 100 µl of Aβ_{25–35} stock solution (Bachem Distribution Services GmbH, Weil am Rhein, Germany; 225 µM in cell culture medium) was added to each well, and desired actovegin concentrations were adjusted by the addition of 50 µl of a prediluted actovegin stock solution (final Aβ_{25–35} concentration, 150 µM). Plates were further cultivated until day 10, the time point for the assessment of apoptosis induction.

Quantitation of apoptotic levels was carried out using the Caspase-Glo® 3/7 Assay (Promega GmbH, Mannheim, Germany) after induction of apoptosis with synthetic Aβ_{25–35}. The cell culture medium was aspirated at day 10, and 50 µl PBS was added to each well. Cells were lysed by addition of 50 µl Caspase-Glo® 3/7 reagent and incubation for 30 min at room temperature. Luminescence signals (which were proportional to total caspase-3/7 activity) were recorded using a Pherastar microplate reader (BMG Labtech, Offenburg, Germany). For normalization purposes, cell numbers were quantitated in cultures set up and treated with Aβ_{25–35} peptides and actovegin in parallel using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Briefly, medium was aspirated at day 10 and 50 µl PBS was added to each well. Cells were lysed by the addition of 50 µl CellTiter-Glo® Reagent and a 10-min incubation at room temperature. Luminescence signals were recorded using a Pherastar microplate reader. Activated caspase-3 levels were determined on a per-cell basis; data are reported as activated caspase-3 per cell. Each iteration of activated caspase-3 determination was normalized to three independent determinations of cell number.

Measurement of Oxidative Stress

Levels of oxidative stress, i.e., the level of ROS, in rat hippocampal neurons were determined at day 8 after tertiary-butyl hydroperoxide (TBHP) treatment to induce ROS production experimentally or day 10 after actovegin treatment, respectively. In the case of TBHP treatment, cell culture medium was replaced at day 8 by 100 µl of fresh NMEM-B27 and different concentrations of TBHP were added to the cell culture medium by addition of 50 µl of prediluted TBHP stock solution in NMEM-B27 to achieve final concentrations of 0.1, 0.2, 0.3, 0.5, 0.8, 1, 2 and 3 mM, respectively. Cells were incubated at 37°C and 5% CO_2 for one hour before being subjected to ROS measurements.

For determination of cellular ROS levels, neuronal cultures, TBHP or actovegin treated, were washed twice with prewarmed HBSS buffer, and 50 µl of a 15-µM c-H_{2}DCFDA stock solution was added after the last washing step (c-H_{2}DCFDA concentration, 5 µM per well). Cells were incubated for 25 min at 37°C and 5% CO_2, and excess c-H_{2}DCFDA was washed off with prewarmed HBSS. Fluorescence intensity (proportional to total ROS content) was measured with a Pherastar microplate reader using a 485/520 nm filter set. In order to calculate ROS content on a per-cell basis, cell numbers were quantified in the same plate, directly following recording of the fluorescent signal using the CellTiter-Glo® Luminescent Cell Viability Assay described above.

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Statistical Analysis

Experimental data sets were analyzed with the help of Statview 5.0 software (SAS-Institute Inc.) using one-way analysis of variance and Fisher’s PLSD (protected least significant difference) post hoc tests. At least three replicates were performed for all experiments. All results are presented as mean values ± SEM. Data were considered to be significantly different at $P$ values $<0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Results

Maintenance of Rat Primary Neurons

Following incubation of primary rat neurons for 10 days in the presence or absence of actovegin, cell numbers in actovegin-treated cultures (as quantified using nuclear staining) were significantly elevated up to 2.4-fold compared with untreated cultures ($P < 0.001$ at concentrations of actovegin greater than 10 μg/ml; Fig. 1a). The increase

Fig. 1 Evaluation of a cell number via nuclei staining and b total excitatory synapse number via vesicular glutamate transporter 1 (VGlut1) staining on day 10 in vitro following incubation of rat primary neurons with increasing concentrations of Actovegin® (Act.; 0.3–1,000 μg/ml) compared with untreated cells. Each plotted point represents the mean value obtained from six replicate wells; the error bars represent the SEM. ***$P < 0.001$, **$P < 0.01$; Fisher-PLSD test (comparison with untreated cells). c–f Show corresponding micrographs of representative cultures at day 10 in vitro treated with 1,000 μg/ml Actovegin® or left untreated (scale bar: 200 μm [C], 50 μm [E]). g–n: Representative phase contrast micrographs and manual determination of neurons in untreated (g–j) and Actovegin® 300 μg/ml-treated (k–n) primary neuronal cultures at day 7 and day 9 in vitro, respectively (scale bar in Fig. 1c: 200 μm; scale bars in Fig. 1e, g, k: 50 μm; DIV = days in vitro)
in the cell number observed was dose dependent. Micro-
graphs of representative nuclear and synaptic staining of
actovegin-treated and untreated cultures on day 10 are
presented in Fig. 1c–f. Cultures that were inspected at day
7 and 9 in vitro using phase contrast microscopy revealed
an improved maintenance of neuronal cell numbers and an
increase in the density of the dendritic network in actove-
gin-treated compared with untreated cultures; this result
was verified by manual counting of neuronal cells
(Fig. 1g–n).

Synaptic Connectivity of Rat Primary Neurons

An antibody against VGlut1 (a marker protein for excit-
atory presynaptic terminals) was used in indirect immu-
nocytochemistry to label excitatory synapses, the major
synaptic type in the cultures analyzed. After 10 days of
culture, the numbers of synaptic contacts in cultures of
rat primary neurons were significantly elevated up to 3.6-
fold in response to the administration of increasing doses
of actovegin when compared to untreated cultures
\((P < 0.001\) at actovegin doses greater than \(300\ \mu g/ml)\
(Fig. 1b). Although the total number of synaptic contacts
increased significantly as a result of an overall elevated
neuronal cell number, the occurrence of synaptic contacts
was not significantly increased by actovegin on a per-cell
basis when compared to untreated cultures (Fig. 2).
There was a decrease at a concentration of \(1\ \mu g/ml\) of
actovegin.

Neurite Outgrowth of Rat Primary Neurons

Neurite outgrowth of primary neurons treated with acto-
vegin was assessed to test for a possible growth-promoting
action. Addition of different doses of diluted actovegin to
cultivated rat hippocampal neurons resulted in no signifi-
cant change in neurite length compared with untreated
cells, when measured after 3 and 6 days of culture (data not
shown).

Neuroprotection of Rat Primary Neurons

We employed \(A\beta_{25-35}\) peptides for the induction of apop-
tosis in primary embryonic rat neurons to test for a
potential anti-apoptotic effect of actovegin. \(A\beta_{25-35}\) rep-
resents the core fragment of Morbus Alzheimer-associated
\(A\beta_{1-40}\) and \(A\beta_{1-42}\) amyloid peptides and is attributed to the
latter’s neurotoxicity (Klementiev et al. 2007; Pike et al.
1995). Additionally, intra-cerebroventricular (i.c.v.) injec-
tion of \(A\beta_{25-35}\) into rodent brain results in neurotoxic
effects that have been also observed after administration of
full-length \(A\beta_{1-42}\) (Maurice et al. 1998; Stepanichev and
Moiseeva et al. 2003a, b, Trubetskaya et al. 2003; Cheng
et al. 2006). Most interestingly, an occurrence of \(A\beta_{25-35}\)
as a cleavage product of \(A\beta_{1-40}\) in the brains of Alzheimer
patients has been described recently (Kubo et al. 2002).

\(A\beta_{25-35}\) peptide-induced apoptosis in primary neurons
cultured in the presence or absence of actovegin was
determined by measuring the levels of activated caspase-3.
Cell numbers were determined in cultures treated in par-
allel by using the CellTiter-Glo\textsuperscript{®} assay. The aim was to
normalize the levels of activated caspase-3, taking into
account the increase in cell numbers observed with acto-
vegin treatment. In the absence of \(A\beta_{25-35}\) peptides, levels
of apoptosis induction in cells treated with increasing doses
of actovegin were not consistently altered compared with
untreated cells (Fig. 3). In the presence of \(A\beta_{25-35}\ pep-
tides, rat primary neurons treated with increasing amounts
of actovegin exhibited a significant decline in the induction
of apoptosis (a decrease in the levels of activated caspase-3
per cell; \(P < 0.001\) at concentrations of actovegin greater
than \(300\ \mu g/ml\)). This was apparent at the mid- to high-
dose range and followed a trend toward dose dependency
(Fig. 3).

Oxidative Stress in Rat Primary Neurons

Preliminary experiments were carried out to test the
suitability of a fluorescence-based assay system using
c-H\textsubscript{2}DCFDA (5-[and-6]-carboxy-2'-7'-dichlorodihydro-
fluorescein diacetate, Sigma-Aldrich) as a fluorogenic
indicator to detect ROS in the cultures of primary rat

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig_2}
\caption{Evaluation of vesicular glutamate transporter 1 (VGlut1)/cell
ratio of rat primary neurons as calculated from VGlut1 and nuclear
stainings on day 10 in vitro following incubation with increasing
concentrations of Actovegin\textsuperscript{®} (Act.; 0.3–1,000 \(\mu g/ml\)). Signals were
compared with those of untreated cells. Each plotted point represents
the mean value obtained from six replicate wells; the error bars
represent the SEM. \,**P < 0.01; Fisher-PLSD test (comparison with
untreated cells)\end{figure}
neurons used. Treatment of neurons with increasing amounts of tert-butyl hydroperoxide (TBHP), a common inducer of ROS production, resulted in significantly elevated levels of intracellular ROS ($P < 0.001$ at concentrations of TBHP greater than 0.2 mM; Fig. 4a). The ability of TBHP to induce oxidative stress was dose dependent, with higher concentrations of TBHP resulting in higher levels of ROS generation. Thus, the cell-permeant fluorescent indicator of intracellular oxidative status c-H2DCFDA was confirmed as a suitable marker in the determination of levels of ROS in the neuronal culture system used.

With respect to actovegin treatment, levels of ROS were determined in neuronal cultures grown for 10 days in vitro under the influence of actovegin to test for a possible effect on oxidative stress in neuronal cells. A significant reduction in ROS content (on a per-cell basis) was detected in cells cultivated in the presence of increasing amounts of actovegin ($P < 0.001$ at concentrations of actovegin greater than 0.3 $\mu$g/ml; Fig. 4b).

**Discussion**

In the present study, we used hippocampal neurons cultured from embryonic rat brains as an in vitro model to study drug effects on neuronal tissues. The neurons were treated with increasing actovegin concentrations in order to identify possible cellular effects, which may explain the beneficial therapeutic effects in patients with symptomatic diabetic polyneuropathy (DPN).

Briefly, we found that the hemodialysate actovegin has substantial dose-dependent protective effects on rat neurons over a wide range of concentrations. It significantly supported the maintenance of cultured neurons by increasing cell survival and by reducing apoptotic levels when challenged with the neurotoxic peptide $\alpha$-$\beta$25-35. In addition, it increased the total number of synaptic connections and decreased the burden of oxidative stress in the neurons. No significant effect of actovegin, however, was observed with respect to neurite outgrowth. The protective effects of actovegin observed in the in vitro model may in concert contribute to the therapeutic benefits of the drug.
actovegin, which was observed in a clinical trial (Ziegler et al. 2009). In the respective randomized, double-blind, placebo-controlled clinical trial, it was recently shown that sequential parenteral and oral treatment with actovegin was associated with a positive effect on neuropathic symptoms, vibration perception threshold, sensory nerve function and mental health-related quality of life in patients with type 2 diabetes and symptomatic polyneuropathy. Since nerve ischemia and hypoxia are believed to play a role in the pathogenesis of diabetic neuropathy, actovegin as a drug may protect against hypoxic cell injury, as previously observed in cultured hepatocytes (de Groot et al. 1990). The results of the clinical study (Ziegler et al. 2009) are in line with the data obtained in vitro presented herein. The increased survival and synaptic density of neurons cultured in vitro in the presence of Actovegin® suggest that protection and enhanced maintenance of neuronal networks may occur under conditions such as diabetic polyneuropathy or following trauma and stroke. The anti-apoptotic effect of Actovegin® observed in vitro suggests a neuroprotective effect in neurodegenerative disorders, such as Alzheimer’s disease (Schlaffer et al. 1991; Saletu et al. 1990; Semlitsch et al. 1990; Ziegler 2008).

As the neuronal cultures were derived from rat hippocampal tissue, it is possible that non-neuronal cell types such as astrocytes were present and may have contributed to the increase in cell numbers observed here. However, neuronal cells could be readily identified by phase contrast microscopy owing to their morphology, and an expansion of glial cells was not observed either in actovegin-treated or untreated cultures. Moreover, it is unlikely that the much higher cell numbers observed after actovegin treatment were due to an increase in numbers of non-neuronal cells, as there was no evidence for a change (i.e., reduction) in synaptic contacts on a per-cell basis as could have been caused by an increase in the number of non-neuronal cells.

In order to shed light on the nature of the active properties of Actovegin®, a number of experiments were conducted to assess the effects of treatment of rat primary neurons with increasing amounts of fetal calf serum (FCS), which is also derived from calf blood like actovegin. Here, a slight but statistically insignificant trend toward increased cell numbers and synaptic connections at submaximal doses of FCS was observed. This may argue for a stronger beneficial effect of Actovegin® due to an enrichment of the active physiological substance(s) during the production of Actovegin®.

Although cell numbers were found to be maintained in the presence of Actovegin®, experiments that explored the induction of apoptosis suggested that there was no effect of Actovegin® per se on reducing apoptosis when assessed on day 10 of culture in the absence of apoptosis-inducing agents like amyloid peptides. This apparent discrepancy suggests that loss of neuronal cells may occur through mechanisms other than apoptosis.

While analgesic drugs, such as antidepressants, anticonvulsants and opioids, are used for the symptomatic treatment of painful diabetic polyneuropathy, other compounds, such as antioxidants (alpha-lipoic acid) and aldose reductase inhibitors, have been developed to address the putative pathogenetic mechanisms of this condition (Boulton et al. 2005; Ziegler et al. 2004, 2006). Treatment with alpha-lipoic acid has been shown to reduce oxidative stress in vitro, to prevent nerve dysfunction in experimental diabetic neuropathy, and to reduce neuropathic symptoms and deficits in patients with diabetes (Cameron et al. 1998). Thus, the potential of Actovegin® to reduce oxidative stress levels in vitro as demonstrated here may at least partially explain its beneficial effect in patients with diabetes and symptomatic polyneuropathy (Ziegler et al. 2009). Results from previous clinical studies have demonstrated positive therapeutic effects of Actovegin® on age-associated memory impairment and dementia (Schlaffer et al. 1991; Saletu et al. 1990; Semlitsch et al. 1990; Ziegler et al. 2008). Compared with placebo, Actovegin® significantly provided better outcomes in assessments at the psychopathological, psychometric and behavioral levels. The experiments employing amyloid peptides described in the current study not only demonstrate the benefits of Actovegin® in protection against Alzheimer’s disease, in which the accumulation and neurotoxic action of amyloid peptides in the brain plays a role in the development of this neurodegenerative disorder. Interestingly, an increased level of reactive oxygen species (ROS) has also been observed after experimental addition of these peptides in earlier studies using Alzheimer’s models. Although not specifically tested in the present setting, it is likely that the reduction in apoptosis in the rat neurons is triggered by a reduction in ROS through Actovegin® (Dumont and Beal 2011; Nunomura et al. 2001).

It should, however, be kept in mind that, despite its clarity, the relevance of the current in vitro study for the in vivo beneficial effects of Actovegin® observed in patients with diabetic polyneuropathy is limited and needs to be further clarified by experimental in vivo approaches including animal and human studies. It has to be noted that the highest experimentally used concentrations of Actovegin®, which are in the supra-physiological range, have shown a tendency to be less effective in respect to VGlut-1 expression and cell numbers.

In conclusion, the neuroprotective effects observed in vitro are in line with the results from previous clinical trials (Ziegler et al. 2009; Saletu et al. 1990; Semlitsch et al. 1990) and indicate that Actovegin® could offer a potential treatment option for neurodegenerative disorders.
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