Therapeutic Potential of *N*-Acetyl-Glucagon-Like Peptide-1 in Primary Motor Neuron Cultures Derived From Non-Transgenic and SOD1-G93A ALS Mice

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Abstract Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by the death of motor neurons (MN) in the motor cortex, brain stem, and spinal cord. In the present study, we established an ALS in vitro model of purified embryonic MNs, derived from non-transgenic and mutant SOD1-G93A transgenic mice, the most commonly used ALS animal model. MNs were cultured together with either non-transgenic or mutant SOD1-G93A astrocyte feeder layers. Cell viability following exposure to kainate as excitotoxic stimulus was assessed by immunocytochemistry and calcium imaging. We then examined the neuroprotective effects of *N*-acetyl-GLP-1(7-34) amide (*N*-ac-GLP-1), a long-acting, N-terminally acetylated, C-terminally truncated analog of glucagon-like peptide-1 (GLP-1). GLP-1 has initially been studied as a treatment for type II diabetes based on its function as insulin secretagogue. We detected neuroprotective effects of *N*-ac-GLP-1 in our in vitro system, which could be attributed to an attenuation of intracellular calcium transients, not only due to these antiexcitotoxic capacities but also with respect to the increasing knowledge about metabolic deficits in ALS which could be positively influenced by *N*-ac-GLP-1, this compound represents an interesting novel candidate for further in vivo evaluation in ALS.

Abbreviations

KA	Kainate
N-ac-GLP-1	*N*-Acetyl-glucagon-like peptide-1
ALS	Amyotrophic lateral sclerosis
MN	Motor neuron
A	Astrocyte
SMI 32	Monoclonal antibody to neurofilaments, phosphorylated epitope
MAPK	Mitogen-activated protein kinase
PKA	Protein kinase A
PI3K	Phosphoinositide 3-kinase
CREB	cAMP response element-binding

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Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult onset motor neuron (MN) disease leading to rapidly progressive degeneration of upper MNs in the primary motor cortex and lower MNs in the brain stem and spinal cord. Death usually occurs within 3–5 years after disease onset due to respiratory insufficiency. So far, only marginal therapeutic options exist: the drug riluzole which has glutamate antagonistic properties was shown to prolong

survival of ALS patients by several months (Bensimon et al. 1994). The identification of mutations in the gene coding for superoxide dismutase 1 (SOD1) in familial ALS patients lead to the generation of the most commonly used and best characterized ALS animal model, the SOD1-G93A mouse which over-expresses human mutant SOD1 and closely mimics the phenotype of both familial and sporadic ALS (Rosen 2004; Gurney et al. 1994).

The definitive pathogenic mechanisms in ALS remain unclear. Among the factors that are thought to play an essential role are chronic excitotoxicity, metabolic disturbances affecting lipid and glucose metabolism, and mitochondrial dysfunction (Pasinelli and Brown 2006). A breakthrough in ALS research was the discovery that non-cell-autonomous processes due to functional dysregulation of surrounding non-neuronal cells, i.e., microglia and astrocytes, contribute to MN death (Clement et al. 2003). It was first demonstrated in vivo in chimeric mice that glial cells expressing mutant SOD1 were toxic to non-transgenic MNs and that non-transgenic glia vice versa had protective effects on mutant SOD1-expressing MNs. Further in vitro experiments in MNs derived from embryonic stem cells confirmed that mutant SOD1-G93A astrocytes reduced the survival of both non-transgenic and mutant MNs. The effect was significantly stronger on mutant SOD1-G93A MNs (Di Giorgio et al. 2007). It was therefore concluded that astrocytes are specific contributors to spinal MN degeneration in mutant SOD1-linked ALS and that they exert toxicity on MNs via release of soluble factors (Nagai et al. 2007; Mattson 2003). In spite of extensive research, no compound with substantial neuroprotective potential in human ALS patients has been identified yet. There is therefore an urgent need for novel more efficient therapeutic approaches in ALS.

Glucagon-like peptide-1 (GLP-1) is an endogenous insulinotropic peptide that controls plasma glucose levels via its action on the pancreas; specifically, via the G-protein-coupled GLP-1 receptor (GLP-1R). The peptide regulates energy metabolism by stimulating insulin synthesis and secretion from pancreatic β-cells (Kim et al. 2010). In the brain, GLP-1 receptors were found to be present exclusively in neurons of many different brain regions such as the cerebral cortex, hippocampus, and cerebellum, suggesting an important role of GLP-1 in neuronal function (Hamilton and Holscher 2009; Campos et al. 1994). Despite of the evidence that the GLP-1 receptor is present in neurons, little is known of the roles of GLP-1 in neuronal physiology. Neuroprotective effects of GLP-1 and GLP-1 receptor agonists have previously been reported (Perry et al. 2003; Li et al. 2010). GLP-1 influences a variety of central nervous system (CNS) functions, including learning and memory, kainate (KA)-induced seizures (During et al. 2003), and shows protection against excitotoxic neuronal

damage (Perry et al. 2002) when delivered directly into the CNS, suggesting both neurotrophic and neuroprotective effects mediated through neuronal GLP-1 receptors. Under the hypothesis that GLP-1 could be a potential treatment for neurodegenerative diseases, we have now assessed the neuroprotective effects of N-acetyl-GLP-1(7-34)amide (N-ac-GLP-1), a long-acting, N-terminally acetylated, C-terminally truncated analog of GLP-1 (John et al. 2008; Gilman et al. 2003) on primary MNs derived from either transgenic SOD1-G93A ALS mice or from non-transgenic animals. Due to the known influence of surrounding non-neuronal cells on the survival of MNs in ALS (Clement et al. 2003; Nagai et al. 2007), we tested the effects of N-ac-GLP-1 not only in MNs alone but also in a MN-astrocyte co-culture system. In our study, excitotoxicity was induced by KA which induces Ca^{2+} influx through KA–AMPA glutamate receptor channels (Carriedo et al. 1996) and subsequent mitochondrial Ca^{2+} loading and ROS generation downstream from Ca^{2+} entry (Carriedo et al. 2000). As it has previously been described that GLP-1 modulates calcium responses to glutamate and membrane depolarization in hippocampal neurons (Gilman et al. 2003), and as calcium plays important roles in neuronal plasticity and neurodegenerative diseases, we have further examined the effects of N-ac-GLP-1 on intracellular calcium regulation in transgenic SOD1-G93A and non-transgenic MNs.

Methods

Primary Culture

Isolation and in vitro cultivation of MNs was performed as previously described (Wiese et al. 2010). Lumbar ventral spinal cords were dissected from individual mouse embryos (gestational age: E12/13). G93A transgenic familial ALS mice (high copy number; B6SJLTg (SOD1-G93A)1Gur/J) (Gurney et al. 1994) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). These mice over-express the human mutant SOD1 allele containing the $Gly_{93} \rightarrow Ala$ (G93A) substitution. We maintained the transgenic G93A hemizygotes by mating transgenic males with B6SJLF1/J hybrid females. Transgenic offspring was genotyped by PCR assay of DNA obtained from the cerebellum of the embryos. After tissue dissociation, MNs were enriched by a p75[NTR]-antibody(Abcam, Cambridge, UK)-based immunopanning technique. Culture medium (Neurobasal medium, Gibco Invitrogen, Darmstadt, Germany) with 2 % horse serum; 2 % B27-supplement (Gibco Invitrogen, Darmstadt, Germany); 0.5 mM glutamax-I; 5 ng/ml rHuBDNF (Peprotech, Hamburg, Germany); and 5 ng/ml rHuCNTF (Peprotech, Hamburg, Germany) was

added to the resulting pellet. Highly enriched MNs were seeded on glass coverslips either pre-incubated first with polyornithin (diluted 1:1,000, Sigma, Steinheim, Germany) and then with laminin (2.5 μg/ml, Invitrogen, Steinheim, Germany) or pre-incubated first with poly-L-lysine (diluted 1:1,000, Sigma, Steinheim, Germany) and then plated with primary astrocytes prepared from non-transgenic or mutant G93A transgenic neonatal mouse, as previously described (Kotsiari et al. 2010). MNs were seeded in an average density of 2.0×10^4 cells/cm^2 (monoculture) or 1.0×10^4 cells/cm^2 (co-culture).

Toxicity Experiments and Measurement of Cell Viability by MTT Assay

MN-rich cell fractions derived from either non-transgenic or mutant G93A transgenic ALS mice (non-tg MN/G93A MN) were seeded on laminin (Sigma, Steinheim, Germany) or on glial feeder layers of non-transgenic or transgenic neonatal mouse astrocytes (non-tg A/G93A A) at an average density of 2.0×10^4 cells/cm^2 (monoculture) or 1.0×10^4 cells/cm^2 (co-culture). After 7 days in vitro (DIV 7), monocultures of non-transgenic MNs were incubated for 24 h with different concentrations of KA (100, 300, and 600 μM, respectively) to test for dose-dependent neurotoxic effects of KA.

Cell viability in non-transgenic astrocytes after KA exposure was also assessed via the MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenylte-trazolium bromide) assay (Sigma, Steinheim, Germany), which measures the ability of cells to reduce MTT to formazan. After acid isopropanol extraction, formazan absorbance was quantified at 570 nm with a reference wavelength of 630 nm. 300 μM KA together with N-ac-GLP-1 (Curatis Pharma GmbH, Hannover, Germany) in different concentrations (10 and 100 nM, respectively) was applied to MNs in monoculture and in the different MN–astrocyte co-culture conditions, to test for dose-dependent effects of N-ac-GLP-1 on MN survival.

Immunocytochemistry and Assessment of MN Survival

Cells were fixed with 4 % *para*-formaldehyde (PFA). Glial feeder layers were stained overnight with an antibody against glial fibrillary acidic protein (GFAP) (1:300; Sigma, poly-clonal, Dako Cytomation, Glostrup, Denmark) to identify astrocytes. A secondary anti-rabbit Alexa Fluor 488 antibody (1:300; anti-IgG; Invitrogen, Darmstadt, Germany) was added for 45 min. Neurons were immunolabeled overnight with an antibody against β-III tubulin (1:140, Abcam, Cambridge, UK); secondary antibody: anti-mouse Alexa Fluor 488 antibody, 1:300; anti-IgG; (Invitrogen, Darmstadt, Germany). MNs were stained overnight with an antibody

against SMI 32 (1:1,000; monoclonal, Cambridge, UK) which is specific for MNs derived from embryonic spinal cord. A secondary anti-mouse Alexa Fluor 555 antibody (1:300; anti-IgG; Invitrogen, Darmstadt, Germany) was added for 45 min. MNs were also incubated with an antibody against the GLP-1 receptor (anti-GLP1R antibody, 1:100; Rabbit polyclonal IgG; Abcam; Cambridge, UK); Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Darmstadt, Germany). Equal percent-ages of astrocytes and MNs in each co-culture preparation was ascertained by double staining of astrocytes (GFAP) and MNs (SMI 32), equal purity of both MN monocultures and co-cultures was assessed by double staining of all neurons (β-III tubulin) and MNs only (SMI 32). MN survival was quantified by cell counts in five visual fields of each coverslip in a total of 8–16 different preparations for each condition.

qRT-PCR

RNA was isolated from spinal cord tissue of both non-trans-genic ($n = 6$) and SOD1-G93A transgenic mice ($n = 5$). Homogenization was done in Trizol reagent (Invitrogen, Carlsbad, CA). Genomic DNA elimination was done using a DNase kit (Stratagene, La Jolla, CA). Reverse transcription of total RNA (10 μg) was performed using random primers (Invitrogen). For quantification of mRNA expression levels of GLP-1 receptor the SYBR-Green method with Power SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used. Cyclophilin (Ppia) was taken as a reference (Housekeeping) gene. Primer pairs were synthesized by MWG as follows: mouse GLP-1 receptor: (forward: CAG GGC TTG ATG GTG GCT ATC; reverse: CGC TCC CAG CAT TTC CG). Complementary DNA was used in a con-centration of 0.25–20.0 ng/μL corresponding to mRNA. SYBR-green qRT-PCR was performed with the StepOnePlus instrument (Applied Biosystems) under the following ampli-fication conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The relative amount of each gene was calculated via the comparative Ct method as pre-viously described by K. Livak (Applied Biosystems User Bulletin #2, 2001). The expression of the gene of interest was normalized against the reference (housekeeping) gene in all samples and gene expression was analyzed by $2^{-\Delta\Delta Ct}$ method (Thau et al. 2012).

Calcium Imaging

For calcium imaging studies, MNs in monoculture were incubated for 20 min at room temperature with the mem-brane permeable ester form of the high-affinity ratio-metric calcium dye FURA 2 AM (4 μM, Sigma, Steinheim, Germany). Fluorescent images were obtained at temporal and spatial resolution (5 Hz, TILL Vision Imaging System

Non-Transgenic Astrocytes have Protective Effects on MNs

In MN–astrocyte co-cultures, there were larger neuronal networks when compared to monocultures. After KA exposure (300 μM), significant reduction of MN numbers was observed in each co-culture condition (Fig. 2). Vulnerability to KA toxicity was slightly but not significantly higher in G93A transgenic MNs compared to the non-transgenic ones when in co-culture with transgenic astrocytes (Fig. 2). Comparing MN counts in different co-culture conditions without addition of KA, significantly larger numbers of both non-transgenic and transgenic MNs were counted when they were surrounded by non-transgenic as compared to transgenic astrocytes (Fig. 2). After KA exposure, we observed significantly lower numbers of non-transgenic and transgenic MNs when they were surrounded by SOD1-G93A instead of non-transgenic astrocytes (Fig. 2). This indicates a protective effect of non-transgenic astrocytes on both non-transgenic and G93A transgenic MNs.

GLP-1 Receptors are Expressed on MNs and Mediate Protective Effects Against KA Toxicity in Monoculture and Different Co-culture Conditions

A large proportion of MNs expressed the GLP-1 receptor. Expression was identified in both non-transgenic and transgenic MNs by immunostaining (Fig. 3A). GLP-1 receptor gene expression was further studied in spinal cord tissue of non-transgenic and transgenic mice by quantitative real time PCR which revealed no significant difference between non-transgenic and mutant SOD1-G93A transgenic mice (Fig. 3B). To assess the neuroprotective effects of N-ac-GLP-1 on MNs in monoculture and in different co-culture conditions, 300 μM KA was applied together with N-ac-GLP-1 at DIV 7 for 24 h. Dose-dependent effects of N-ac-GLP-1 on MNs were assessed in monoculture and the four different co-culture conditions, using two different concentrations of N-ac-GLP-1 (10, 100 nM). Immunocytochemical analysis showed that N-ac-GLP-1 dose dependently protected both non-transgenic and
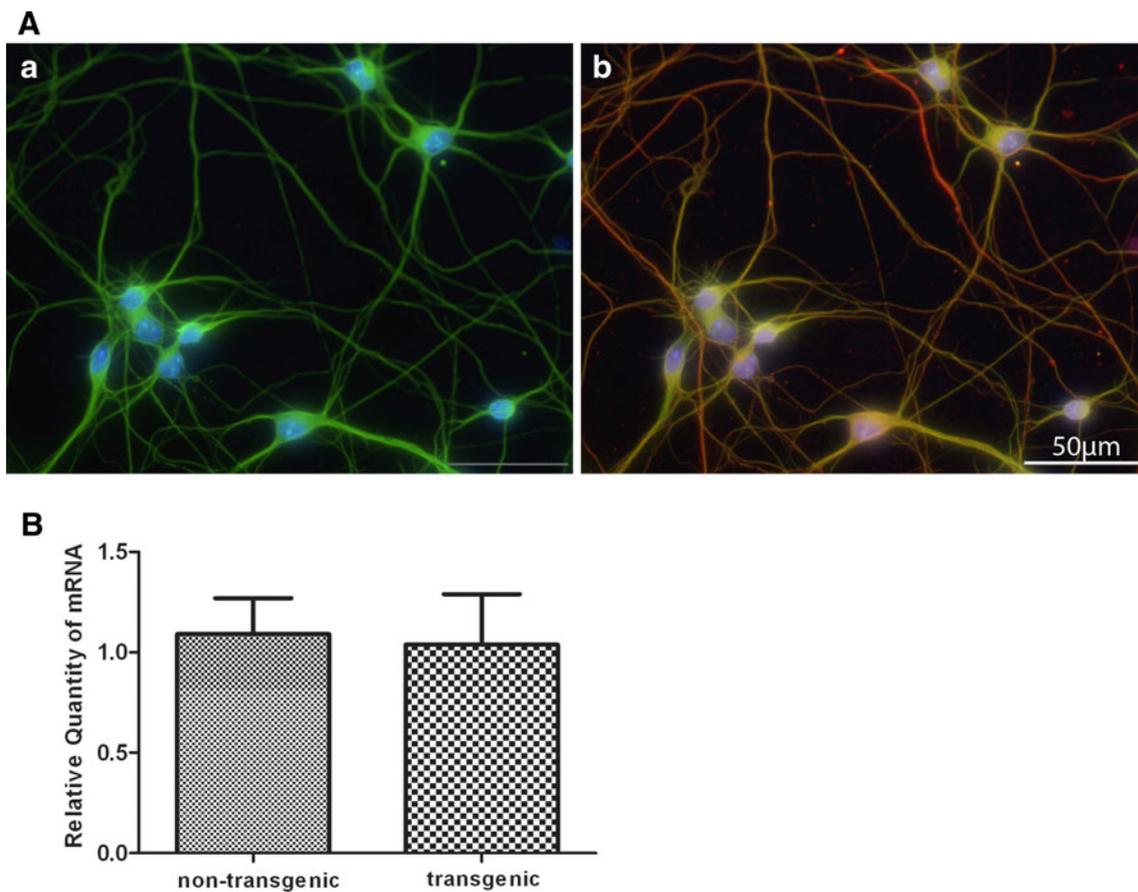
Fig. 3 A Detection of GLP-1 receptor on MNs. Immunostaining using an anti-GLP1R antibody showed the presence of the GLP-1 receptor (*green*) (*a*). In (*b*), MNs are labeled by SMI 32 staining (*red*). Scale bar 50 μm. **B** Quantification of GLP-1 receptor mRNA expression in non-transgenic and SOD1-G93A mice. The mRNA expression of GLP-1 receptor was compared in spinal cord tissue of both non-transgenic ($n = 6$) and mutant SOD1-G93A transgenic ($n = 5$) mice and showed no significant difference. Values are given as mean ± SEM

transgenic MNs in monoculture as well as in co-culture with astrocytes against KA toxicity (Fig. 4). Treatment of MNs with 300 μM KA caused a twofold loss in SMI-positive cell numbers which was significantly ameliorated by N-ac-GLP-1 treatment (Fig. 4B).

There was an independent significant neuroprotective effect of 100 nM N-ac-GLP-1 in each co-culture condition (Fig 4C). Significantly higher numbers of MNs were counted after combined KA-/N-ac-GLP-1 exposure when MNs were surrounded by non-transgenic as compared to transgenic astrocytes but the proportion of surviving MNs did not differ in each condition. Incubation with N-ac-GLP-1 alone (Fig. 4, striped columns) did not have a significant effect on MN survival per se: again, both for non-transgenic and transgenic MNs, significantly higher numbers were counted in co-culture with non-transgenic as compared to transgenic astrocytes (Fig. 4C).

N-ac-GLP-1 Induces a Rapid and Transient Elevation of the Concentration of Cytosolic Calcium

To further analyze the mechanism underlying the neuroprotective effects of N-ac-GLP-1 against KA-excitotoxicity at the physiological level, calcium imaging experiments were performed (Fig. 5). Non-transgenic MNs in monoculture younger than DIV 10 were observed to be non-spontaneously active, i.e., they did not show occurrence of spontaneous intracellular Ca^{2+} transients, but Ca^{2+} transients could be elicited by application of KA. Calcium transients could be elicited by a pulse-wise application of N-ac-GLP-1 in cultures <DIV 10. Non-spontaneously active cultures were exposed to two 3 s pulses of 100nM N-ac-GLP-1, during which a short stimulation with N-ac-GLP-1 led to an excessive rise in intracellular Ca^{2+} levels and MN cell death (Fig. 5a).

N-ac-GLP-1 Reduced KA-Induced Intracellular Calcium Transients in MNs

A 170 s pulse of 30 μM KA-induced Ca^{2+} transients in MNs <DIV10. After 70 s of KA application, 10nM N-ac-GLP-1 was applied for 70 s. This led to a decrease of the Ca^{2+} influx which then returned to baseline when the application of N-ac-GLP-1 together with KA was stopped (Fig. 5b). Under continued KA exposure after termination of N-ac-GLP-1 application, all cells showed an intracellular Ca^{2+} overload which could indicate neuronal cell death (Fig. 5c). Starting at DIV 10, cells were spontaneously active and showed quite regular Ca^{2+} transients. In these spontaneously active cells, N-ac-GLP-1 application did slightly reduce but not significantly alter spontaneous Ca^{2+} transients regarding frequency and shape (Fig. 5d).

Discussion

Our present findings show that KA-mediated neurotoxicity in primary MNs is dose dependent. In addition, we found significant effects of the co-culture condition on the survival of MNs: in our MN/astrocyte co-culture system, MN viability was reduced and KA-induced neuronal death was potentiated in the presence of SOD1-G93A transgenic astrocytes as compared to non-transgenic astrocytes. This is in line with the previous literature on the non-cell-autonomous character of MN death in ALS: normal MNs degenerated in the presence of mutated non-neuronal cells, and vice versa non-transgenic non-neuronal cells were neuroprotective (Di Giorgio et al. 2007; Clement et al. 2003; Nagai et al. 2007). Several studies indicate that neuro-astroglial interaction is region specific and that astrocytes from different anatomical regions have distinct properties (Lee et al. 1994; Westergaard et al. 1991; Barbin et al. 1988). Neurons differentiate and survive better when co-cultured with astrocytes from the same region than with astrocytes from another region (Chamak et al. 1987). Although this region specificity was not implemented in our present study, the astrocytes obtained from non-transgenic mouse brain already showed significant protective effects on G93A transgenic MNs from spinal cord. Our co-culture system, therefore, presents a valuable in vitro tool to screen for novel neuroprotective compounds in ALS.

Previous studies have already shown neuroprotective effects of GLP-1. It can completely protect cultured rat hippocampal neurons against glutamate-induced apoptosis (Gilman et al. 2003). Treatment with GLP-1 receptor agonists was shown to increase progenitor cell proliferation in the brain, to reduce infarction size and improve functional outcome in stroke models and to be protective in cellular and animal models of Parkinson's and Alzheimer's disease (Li et al. 2009; Bertilsson et al. 2008; McClean et al. 2011; Hamilton et al. 2011). A very recent study showed that the GLP-1 receptor agonist exendin-4 was protective against hydrogen peroxide- and staurosporine-induced toxicity in vitro and reduced cell death in the spinal cord in transgenic ALS mice (Li et al. 2012). Compared to previously described GLP-1 receptor agonists such as exendin-4, N-ac-GLP-1 has the advantage of 100 % amino acid sequence identity to endogenous human GLP-1(7-34) which prevents antigenicity and adverse effects. N-terminal acetylation warrants resistance to proteolytic degradation by dipeptidyl peptidase IV (DPP IV) and, therefore, ascertains long lasting biological activity (John et al. 2008). Whether these advantages of N-ac-GLP-1 will indeed provide a clinical benefit regarding efficacy and tolerability will require further in vivo evaluation.

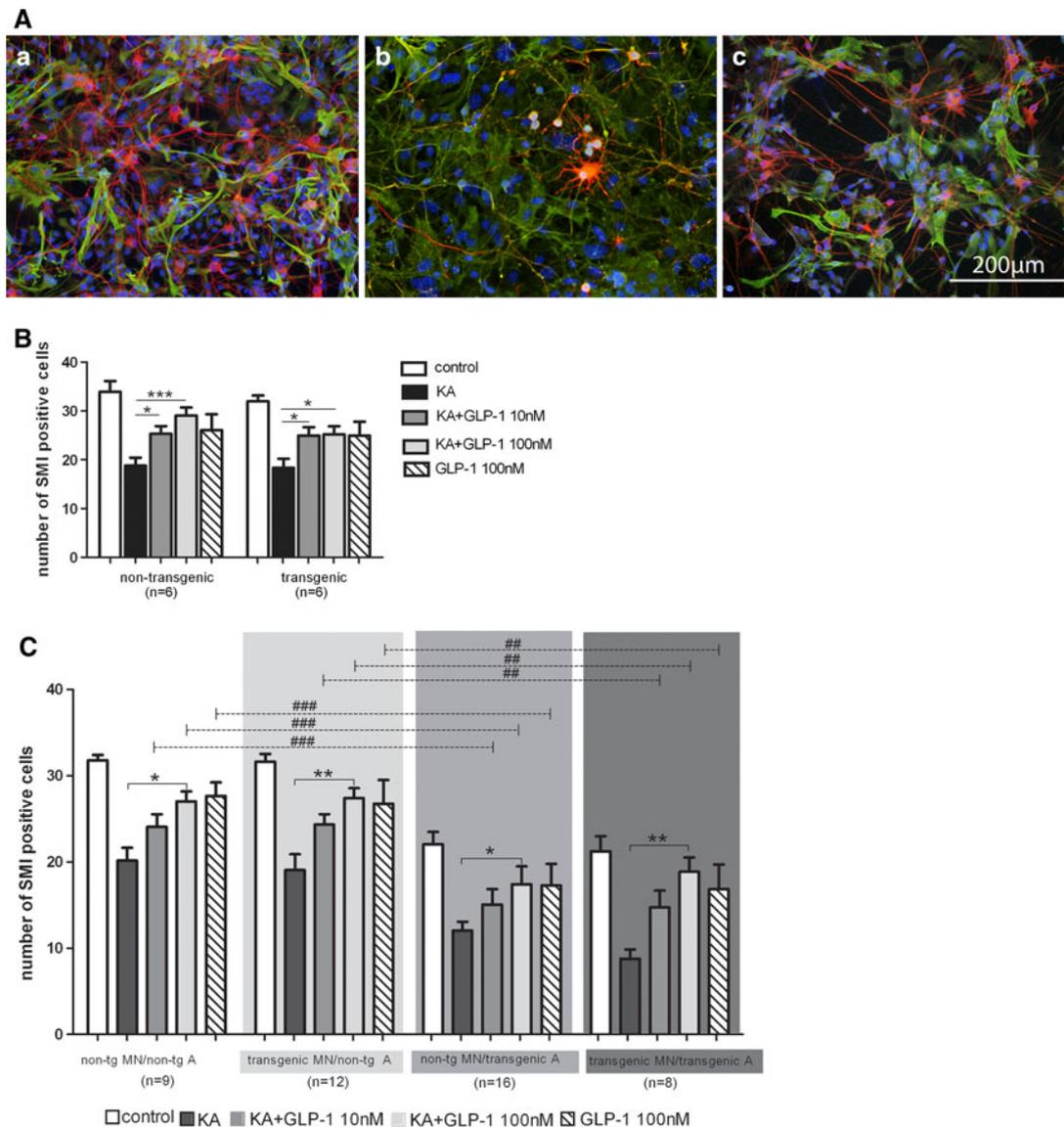While incubation with N-ac-GLP-1 did not significantly protect MNs against the toxicity that was mediated by

Fig. 4 A Immunocytochemical analysis of the MN-astrocyte co-cultures. Photographs show representative MN cultures at DIV 7 on astrocyte feeder layers. *a* Non-transgenic MNs without any treatment. *b* Reduction of SMI 32 positive MNs following KA exposure. *c* Increase in MN survival due to *N*-ac-GLP-1 co-incubation. MNs were stained by an antibody against SMI 32 (red). Astrocytes were immunopositive for GFAP (green). Stained nuclei of cultured cells appear in blue (DAPI). Scale bar 200 μm. **B, C** Protective effects of *N*-ac-GLP-1 against KA toxicity in MN monoculture and different co-culture conditions. Assessment of the neuroprotective effect of *N*-ac-GLP-1 in the presence of KA. MN monocultures (**B**) and MN-astrocyte co-cultures (**C**) were incubated for 24 h with 10 or 100 nM of *N*-ac-GLP-1 and 300 μM KA at DIV 7 and surviving MNs were quantified by immunocytochemistry. *N*-ac-GLP-1 protected MNs from toxicity induced by KA. Higher numbers of surviving MN were counted following incubation with 100nM instead of 10nM of *N*-ac-GLP-1. The protective effect of 10nM *N*-ac-GLP-1 was significant in MN monoculture (*$p < 0.05$, **A**). The protective effect of 100nM *N*-ac-GLP-1 was significant in monoculture and all co-culture systems: non-tg MN (***$p < 0.001$), tg MN (*$p < 0.05$), non-tg MN/non-tg A (*$p < 0.05$), transgenic MN/non-tg A (**$p < 0.01$), non-tg MN/transgenic A (*$p < 0.05$), transgenic MN/transgenic A (**$p < 0.01$). Comparing the different co-culture conditions (*dashed lines*), with both 10nM *N*-ac-GLP-1 (*dark gray columns*) and 100nM *N*-ac-GLP-1 (*light gray columns*), significantly higher MN numbers were counted when they were surrounded by non-transgenic as compared to transgenic astrocytes. Exposure to *N*-ac-GLP-1 alone (*striped columns*) did not affect MN survival; similar to untreated cultures (Figs. 2, 4C, *white columns*), significantly higher MN numbers were counted in co-culture with non-transgenic as compared to transgenic astrocytes (###$p < 0.001$, ##$p < 0.01$). Values represent mean ± SEM, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ###$p < 0.001$, ##$p < 0.01$, two-way ANOVA with Bonferroni post-tests

mutant SOD1-G93A–astrocytes, we observed significant protective effects against KA-excitotoxicity both in transgenic and non-transgenic MNs. Glutamate receptor mediated excitotoxicity has long been recognized as a major pathomechanism in ALS (Bogaert et al. 2010). An imbalance between excitatory and inhibitory neurotransmission
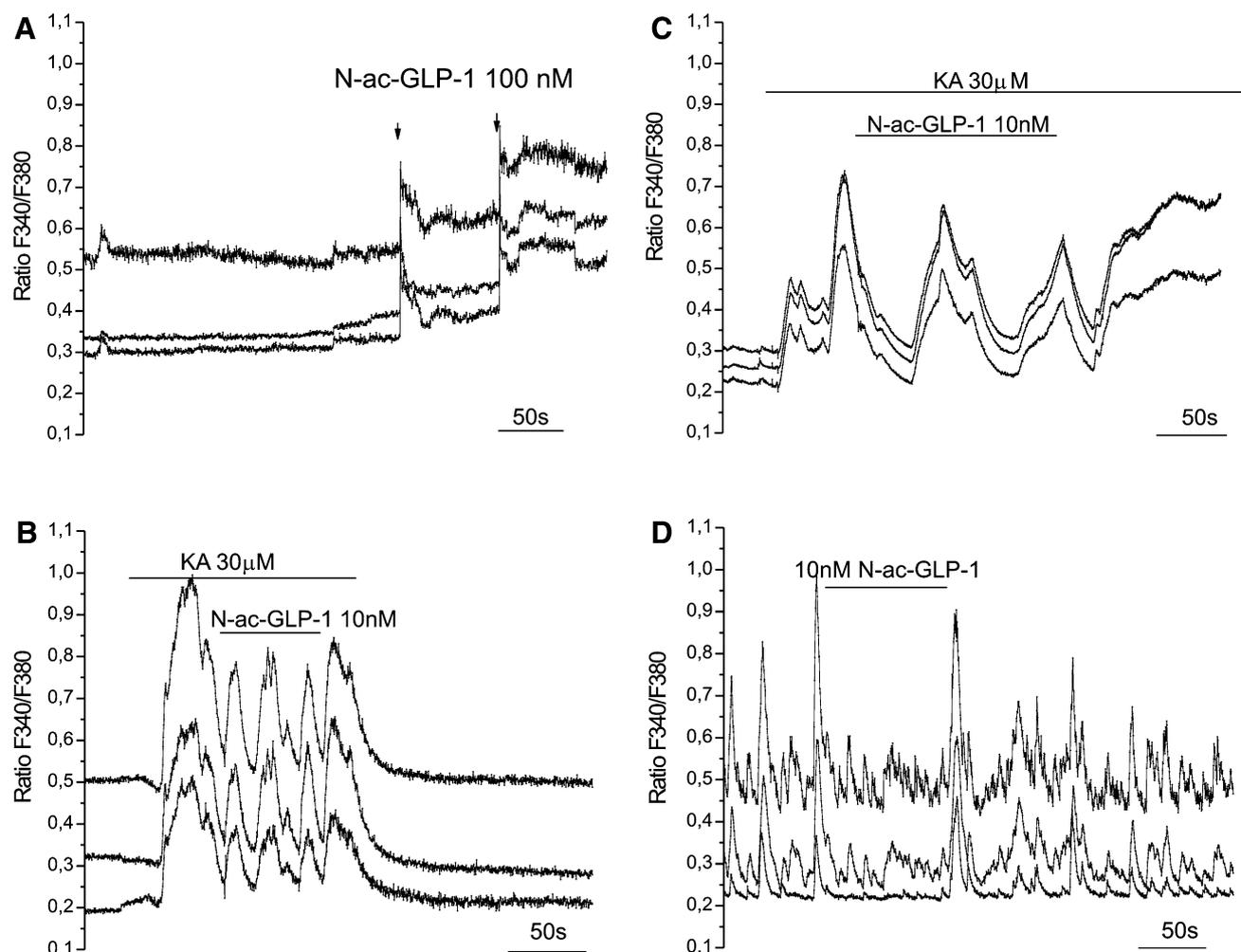
Fig. 5 Microfluorometric Ca^{2+} measurements monitoring cytosolic calcium transients. Non-spontaneously active cultures (<DIV 10, **a**–**c**) were exposed to long pulses of 30 μM KA. Upon that, Ca^{2+} transients were observed in all regions of interests (cytosol, nucleus and neurites). *N*-ac-GLP-1 induces a rapid and transient elevation of $[Ca^{2+}]i$ (**a**). After 70 s of KA application, 10nM*N*-ac-GLP-1 was applied for 70 s. The influx of Ca^{2+} became smaller following *N*-ac-GLP-1 application (**b**). When the application of both KA and *N*-ac-GLP-1 was stopped, the intracellular Ca^{2+} levels returned to baseline (**b**). When KA was continuously applied, all regions of interest showed an intracellular Ca^{2+} overload when *N*-ac-GLP-1-application was terminated which could indicate neuronal cell death (**c**). Starting at DIV 10, large cells (diameter >20 μM), which were considered to be MNs, became spontaneously active and showed quite regular Ca^{2+} transients. In spontaneously active cells, a slight reduction but no significant effect of the *N*-ac-GLP-1 application on calcium transients was visible (**d**). Cytosolic calcium concentrations are presented as ratios of the fluorescence signals obtained at 340 and 380 nm (F340/F380)

causes overactivation of glutamate receptors and disruption of cellular calcium homeostasis via Ca^{2+}-influx through AMPA receptor channels. This leads to perturbation of physiological cellular functions and ultimately cell death (Cleveland and Rothstein 2001; Grosskreutz et al. 2007; Lau and Tymianski 2010). To further assess this mechanism in our culture system, we performed calcium imaging experiments with and without KA and/or *N*-ac-GLP-1 exposure. A previous study showed that GLP-1 induces a rapid and transient elevation of $[Ca^{2+}]i$ in hippocampal neurons via stimulation of cAMP production and activation of cAMP response binding protein (Gilman et al. 2003). Consistent with this observation, we also found that application of

N-ac-GLP-1 alone induced cytosolic calcium elevation in MNs. This is in line with the phenomenon that GLP-1 stimulates calcium influx in pancreatic β-cells and that increased calcium influx into mitochondria with subsequent ATP production is considered to underlie its insulinotropic effect (Baba et al. 1999; MacDonald et al. 2002). In our cultures, in cells younger than DIV 10, calcium influx was elicited by KA as previously described (Ragancokova et al. 2009). The magnitude of the calcium current was significantly lower in all measured regions of interest (cytosol, nucleus, and neurites) after 10 nM *N*-ac-GLP-1 treatment compared with the current before or after *N*-ac-GLP-1 application as control. A study in hippocampal neurons

similarly showed that calcium response to membrane depolarization is attenuated in hippocampal neurons treated with 10 nM GLP-1 (Gilman et al. 2003). The precise mechanism how GLP-1 antagonizes pro-apoptotic stimuli in diverse cell types is unclear. It is likely that the inhibitory effects of N-ac-GLP-1 on excitotoxicity and calcium transients are not directly mediated by interaction of N-ac-GLP-1 with ion channels but by activation of cAMP and subsequent activation of downstream kinases and transcription factors (Perry et al. 2002). By immunohistochemistry, we detected expression of GLP-1 receptors on MNs, and qRT-PCR revealed equal GLP-1 receptor mRNA expression in the spinal cord of non-transgenic and transgenic mice, providing an avenue for direct signaling. The GLP-1 receptor is a 7-transmembrane protein that belongs to the class B1 G-protein-coupled receptor family (Fortin et al. 2010). It is associated with adenylyl cyclase that, upon activation, increases intracellular cAMP levels. Downstream of cAMP, many GLP-1 actions have been associated with activation of protein kinase A (PKA), phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK), (Lovshin and Drucker 2009). GLP-1 could suppress glutamate-induced currents and calcium currents either by decreasing levels of expression of the channel proteins or by modulating their activity. Previous studies have shown that GLP-1 can affect the expression of glutamate receptor subunits, most likely via activation of MAPK and cAMP response element-binding (CREB) signaling pathways (Brandoli et al. 1998; Mattson et al. 1995). Spontaneous activity of MNs occurred after DIV 10. MNs in general display a significantly higher occurrence of spontaneous Ca^{2+} influx than non-MNs suggestive of increased expression of Ca^{2+} permeable AMPA/KA receptors (Jahn et al. 2006). This influx of spontaneous calcium transients was not significantly altered after N-ac-GLP-1 treatment regarding frequency and shape. As supported by our cell viability and calcium imaging data showing inactivation of KA-induced calcium transients by N-ac-GLP-1, together with the previous literature on GLP-1 mediated neuroprotection, the antiexcitotoxic effect of N-ac-GLP-1 certainly is a major component of its protective potential. The exact elucidation of the mechanisms underlying GLP-1 effects on calcium channels is beyond the scope of this study but clearly requires further investigations. The only FDA-approved drug for neuroprotective treatment of ALS, riluzole, also acts on glutamate toxicity, mainly via blockade of sodium-channels, inhibition of glutamate release from synaptic terminals and via upregulation of astrocytic glutamate uptake (Bensimon et al. 1994; Martin et al. 1993; Carbone et al. 2012). Treatment with both riluzole and N-ac-GLP-1 together may lead to additive effects of two drugs with distinct mechanisms of action against glutamate toxicity and, therefore, increase of the protective efficacy.

Even though N-ac-GLP-1 in vitro was mainly protective against excitotoxicity and not efficient against the deleterious effects of mutant-SOD1-expressing astrocytes, further in vivo evaluation of its therapeutic capacities is of particular interest in ALS. An impairment of cellular energy homeostasis, related in parts to mitochondrial dysfunction, is an important pathogenic mechanism in both familial and sporadic ALS. Mitochondrial swelling and vacuolization are among the earliest pathological features in SOD1-G93A mice (Wong et al. 1995). Mitochondrial dysfunction affects the electron transport chain and leads to ATP depletion, which contributes to cell death. Compounds improving mitochondrial function such as creatine have been shown to be neuroprotective in ALS transgenic mice (Klivenyi et al. 1999). Partial restoration of the deficient energy homeostasis with increased energy expenditure/hypermetabolism in transgenic ALS mice by a high caloric diet also increased survival and improved motor function (Dupuis et al. 2004). In human ALS patients, it was shown that hyperlipidemia is associated with longer survival (Dupuis et al. 2008; Dorst et al. 2011). Moreover, Browne et al. (2006) have shown that glucose uptake and ATP production was reduced in corticospinal and bulbospinal motor tracts in SOD1-G93A mouse (Dupuis et al. 2011), and reduced glucose tolerance with an increase in free fatty acids as a marker for insulin resistance was recently shown in ALS patients (Pradat et al. 2010). Bioenergetic deficits therefore appear to play an important role in ALS pathogenesis and represent a novel therapeutic target worth further investigation. It has been hypothesized that in ALS and other neurodegenerative diseases, affected neurons slightly start to deviate from the optimal intraneuronal biochemical conditions such as intracellular pH, concentrations of oxygen, glucose, ATP, and Ca^{2+} ions until they finally exceed a life-threatening limit. It is possible that a long-standing unfavorable living condition might make neurons consume their energy insidiously and after many years lead to neuronal death (Kanazawa 2001). Beyond its antiglutamatergic properties, the metabolic effects of N-ac-GLP-1 might therefore be relevant as well but can only be further clarified by long-term systemic evaluation in in vivo models.

In conclusion, our evaluation of the neuroprotective effects of N-ac-GLP-1 in our in vitro system revealed that N-ac-GLP-1 provides protection against cell death induced by KA neurotoxicity. This confirms the data of Perry et al. (2002), where cultured hippocampal neurons were protected by GLP-1 against glutamate-induced apoptosis. Besides this direct antiexcitotoxic effect, N-ac-GLP-1 is of particular interest for further in vivo evaluation in ALS as it might also be able to counteract metabolic deficits which appear to be another important pathomechanism. Our observation that most relevant neuroprotective effects were

observed in SOD1-G93A transgenic MNs when these were surrounded by non-transgenic astrocytes suggests that further in vivo evaluation should also test combination treatment of GLP-1 together with therapies aiming to restore glial function.

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Acetyl-glucagon-like-peptide-1-(7-34)-amide = curaglutide