C9orf72-generated poly-GR and poly-PR do not directly interfere with nucleocytoplasmic transport

Joni Vanneste1,2, Thomas Vercruysse3, Steven Boeynaems4, Adria Sicart1,2, Philip Van Damme1,2,5, Dirk Daelemans3 & Ludo Van Den Bosch1,2*

Repeat expansions in the C9orf72 gene cause amyotrophic lateral sclerosis and frontotemporal dementia characterized by dipeptide-repeat protein (DPR) inclusions. The toxicity associated with two of these DPRs, poly-GR and poly-PR, has been associated with nucleocytoplasmic transport. To investigate the causal role of poly-GR or poly-PR on active nucleocytoplasmic transport, we measured nuclear import and export in poly-GR or poly-PR expressing Hela cells, neuronal-like SH-SY5Y cells and iPSC-derived motor neurons. Our data strongly indicate that poly-GR and poly-PR do not directly impede active nucleocytoplasmic transport.

Hexanucleotide (G4C2) repeat expansions in the first intron of the C9orf72 gene are the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Healthy individuals most often have 2 to 8 of these repeats, while C9orf72-patients can have up to hundreds or thousands. C9orf72-patients show a unique pathology characterised by cytoplasmic inclusions containing dipeptide repeat proteins (DPRs). Five different DPRs arise through non-canonical translation of the sense and antisense repeat RNA, namely poly-GA (Glycine-Alanine), poly-GP (Glycine-Proline), poly-GR (Glycine-Arginine), poly-PA (Proline-Alanine) and poly-PR (Proline-Arginine). Although, DPRs are toxic in both cell culture and animal models, with the arginine containing poly-GR and poly-PR peptides as the most toxic ones (reviewed by Freibaum and Taylor, 2017), the exact pathological mechanisms by which these DPRs contribute to neurodegeneration in C9orf72-ALS/FTD patients remains disputed.

We and others have previously reported, based on both yeast and Drosophila models, that the toxicity induced in mutant C9orf72 models can be modified by genetic or pharmacological manipulation of proteins involved in nucleocytoplasmic transport (reviewed by Yuva-Aydemin and colleagues, 2018). In addition, changes in expression levels or cellular localization of nucleocytoplasmic transport proteins have been observed in mutant C9orf72-iPSC-derived motor neurons and post mortem tissue of C9orf72-patients. Furthermore, a reduced import has been measured in C9orf72-iPSC-derived motor neurons. These data argue for an important role of nucleocytoplasmic transport in the pathogenic mechanisms underlying C9orf72-ALS/FTD. However, there is currently no consensus on the mechanism(s) underlying the observed nucleocytoplasmic transport pathology. Interestingly, the poly-GR and poly-PR DPRs could be potential interactors of phenylalanine-glycine repeat-containing nucleoporines (FG Nups). FG Nups have a low sequence complexity and undergo phase separation into a dense polymer meshwork which constitute the nucleopore complex (NPC) permeability barrier. This raises the intriguing possibility that poly-GR and poly-PR causally affect motor neuron health through disturbing nucleocytoplasmic transport directly. Therefore, the aim of this study was to measure the direct effect of several DPRs, including poly-GR and poly-PR, on active nucleocytoplasmic transport.
Results

Measuring active nucleocytoplasmic transport. To measure active nucleocytoplasmic transport in intact cells, we made use of Hela Kyoto cells stably expressing the shuttle reporter NLS<sub>SV40</sub>-mNeonGreen<sub>2x</sub>-NES<sub>SPK1</sub> (Fig. 1a). This mNeonGreen-construct, fused to both a classical nuclear localization signal (NLS) and an XPO1-associated nuclear export signal (NES), allows us to measure classical importin<sub>β</sub>/α-mediated import and XPO1-mediated export in a quantitative matter. Notably, classical nuclear import is the most prevalent import pathway in the cell<sup>19</sup> and its disturbance has been suggested to underlie cytoplasmic mislocalization of TAR DNA-binding protein 43 (TDP-43)<sup>20</sup>, which is a prominent hallmark of ALS and FTD<sup>21</sup>. In addition, the presence of two connected mNeonGreen proteins limits size-dependent passive transport across the NPC<sup>22</sup>, which allows us to primarily focus on active nucleocytoplasmic transport. Due to the strong nuclear export signal, the reporter is mainly cytoplasmic under control conditions (Fig. 1a). Inducing a shift in the localization of this reporter towards the nucleus, by blocking nuclear export using leptomycin B (LMB), allows us to measure nuclear import over time. Left image represents Hela Kyoto cells in control conditions. Right image represents Hela Kyoto cells after addition of leptomycin B. Scale bar = 10 µm. (b) The concentration of mNeonGreen in the nucleus was measured by fluorescent intensity before (as a measurement for XPO1-mediated export) and after (as a measurement for classical nuclear import) addition of leptomycin B. A significant import defect was induced by the importin-β inhibitor Importazole (time 15: p < 0.0001; time 30: p < 0.0001). Data are represented as mean ± SD, Mann-Whitney U-test, ****Denotes p < 0.0001. Dots represent means of one image with 5 cells per image; n = 20 from four independent biological replicates.

Figure 1. Active nucleocytoplasmic transport assay. (a) Hela Kyoto cells stably express the reporter NLS<sub>SV40</sub>-mNeonGreen<sub>2x</sub>-NES<sub>SPK1</sub>. The classical nuclear localization signal (NLS) is recognized by an importin α/β heterodimer (Impα/β), which results in the translocation of the reporter into the nucleus. The nuclear export signal (NES) of the reporter construct is recognized by exportin1 (XPO1), which is subsequently exported into the cytoplasm. Due to the strong nuclear export signal, the mNeonGreen-reporter is mainly cytoplasmic under control conditions. Addition of the XPO1-inhibitor leptomycin B (LMB) induces a shift in the localization of this reporter towards the nucleus, which allows us to measure nuclear import over time. Left image represents Hela Kyoto cells in control conditions. Right image represents Hela Kyoto cells after addition of leptomycin B. Scale bar = 10 µm. (b) The concentration of mNeonGreen in the nucleus was measured by fluorescent intensity before (as a measurement for XPO1-mediated export) and after (as a measurement for classical nuclear import) addition of leptomycin B. A significant import defect was induced by the importin-β inhibitor Importazole (time 15: p < 0.0001; time 30: p < 0.0001). Data are represented as mean ± SD, Mann-Whitney U-test, ****Denotes p < 0.0001. Dots represent means of one image with 5 cells per image; n = 20 from four independent biological replicates.

GR<sub>20</sub> and PR<sub>20</sub> poly-dipeptides do not block active nuclear import or export. In order to investigate the direct effect of poly-GR or poly-PR on active nucleocytoplasmic transport, we added 10 µM of peptides containing 20 repeats of either GR or PR (GR<sub>20</sub> and PR<sub>20</sub>, respectively) to the Hela Kyoto reporter cell line. GR<sub>20</sub> and PR<sub>20</sub> are cell-penetrating peptides that are taken up immediately<sup>24</sup>, which we confirmed by immunocytochemistry (Fig. 2a). We did not observe an increased nuclear intensity of the reporter construct after two hours of incubation with GR<sub>20</sub> and PR<sub>20</sub> (Fig. 2b – before LMB). This suggests that the poly-dipeptides GR<sub>20</sub> and PR<sub>20</sub> do not obstruct XPO1-mediated export. Likewise, addition of LMB, which allows us to measure classical nuclear import, did not reveal a decreased nuclear import in the presence of both GR<sub>20</sub> and PR<sub>20</sub> (Fig. 2b – 15 and 30 min). Furthermore, no impeded transport was observed with a higher concentration of GR<sub>20</sub> and PR<sub>20</sub> (30 µM) or after a longer incubation with PR<sub>20</sub> (24 hours; Fig. 2c,d).

Next to TDP-43 mislocalization, cytoplasmic mislocalization of the Fused in sarcoma (FUS) protein has recently been suggested as a common phenotype of ALS<sup>23</sup>. Therefore, we investigated whether poly-GR or poly-PR peptides could reduce FUS import through a direct interaction. Hela Kyoto cells expressing a shuttling reporter construct containing the same NLS as FUS were used to answer this question (supplementary Fig. S1A). FUS carries a non-classical proline-tyrosine NLS (PY-NLS) and is imported via transportin 1<sup>26</sup>. Hence, transfected cells expressing the transportin 1 inhibitor M9M<sup>27</sup> had significantly reduced import of the reporter.
construct (p < 0.0001; Supplementary Fig. S1B). Similar to previous results, GR20 and PR20 did not induce disturbed transportin 1 import (Supplementary Fig. S1C).

**Intracellularly expressed poly-GR and poly-PR do not impede active transport.** It has been shown that the length of DPRs has a clear effect, with the longer repeats being more toxic\[^{12,28}\]. Therefore, we used lentiviral vectors (LVs) expressing 100 repeats of four different DPRs, namely mCherry-PA100, mCherry-GA100, mCherry-GR100 and mCherry-PR100. The Hela-reporter cell line was transduced 96 hours before measuring nucleocytoplasmic transport. As reported before\[^{28}\], poly-PA was diffusely present throughout the cell, poly-GA formed cytoplasmic inclusions, poly-GR was mainly cytoplasmic and poly-PR was mainly localized in the nucleoli (Fig. 3a). None of the intracellularly expressed DPRs affected nuclear export (Fig. 3a – before LMB) or

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**Figure 2.** GR20 and PR20 poly-dipeptides do not block classical nuclear import and XPO1-related export. Nucleocytoplasmic transport was measured in the reporter Hela Kyoto cells in the presence of GR20 or PR20 peptides. (a) Cellular uptake of GR20 or PR20 was confirmed by immunocytochemistry, making use of antibodies directed against the peptides. Left two images were stained with an anti-poly-GR antibody (Millipore). Images on the right were stained with a homemade anti-poly-PR antibody. Scale bar = 10 µm. (b) Both manual (left graph) as automated (right graph) analyses were performed to measure active nucleocytoplasmic transport after two hours of incubation with 10 µM of GR20 or PR20. mNeonGreen fluorescent intensity was measured in the nucleus and cytoplasm, before and after addition of leptomycin B (LMB) (15 and 30 min). GR20 or PR20 peptides did not induce transport defects. A small but significant increase in import was observed for GR20 in the automated analysis (time 15: p = 0.0019). Neither a higher concentration (30 µM, automated analysis) (c) nor a longer incubation time (24 hours, automated analysis) (d) resulted in transport defects. Data are represented as mean ± SD, non-parametric one-way ANOVA followed by Dunn’s multiple comparison test, **Denotes p < 0.01. Manual analysis: dots represent means of one image with 5 cells per image; n = 19–20 from four independent experiments. Automated analysis: dots represent means of one well with 1499–4408 cells per well; n = 9 from three independent experiments.**
import (Fig. 3a – 15 and 30 min). On the contrary, there was a small but significant (time 15: \( p = 0.007 \) – time 30: \( p = 0.0173 \)) increase of import observed in mCherry-PR100 expressing cells.

To confirm that the obtained levels of poly-GR and poly-PR induced toxicity, we measured the effect on protein translation, as it was previously shown that poly-GR and poly-PR can induce toxicity by disturbing protein synthesis\(^{24,29–32}\). Indeed, reduced levels of protein translation was observed in poly-GR and poly-PR expressing cells (Fig. 3b,c).
SY5Y cells with indicated constructs and the reporter construct NLSSV40-mNeonGreen2x-NESpki. Significant \( \alpha \) domain of importin-\( \beta \) nuclear import without blocking export. As a positive control, overexpression of the importin-\( \beta \) (Fig. S2A). Impeded import will shift the fluorescent signal to the cytoplasm, allowing us to measure disturbed transport defects were observed in contrast to the data published by Zhang and colleagues 43. Indeed, when we co-transduction of SH-SY5Y cells (Fig. 4a) or motor neurons (Fig. 5a,b) with LVs expressing a mCherry-DPR construct as well as the NLS SV40-mNeonGreen2x-NESpki reporter. We could confirm that poly-GR or poly-PR did not induce a blockade of nucleocytoplasmic transport (Figs 4b and 5c). Moreover, no disturbed transport was observed in poly-PA expressing cells (Figs 4b and 5c), which is in line with the non-toxic characteristics of poly-PA (reviewed by Freibaum and Taylor, 2017). On the other hand, significant export (Fig. 5c - time 0: \( p < 0.0001 \)) and import (Fig. 4b: \( p < 0.0001 \)) defects were observed in poly-GA expressing cells, as reported previously36,37.

**Intracellularly expressed poly-GR and poly-PR do not impede active transport in neuronal-like SH-SY5Y cells and iPSC-derived motor neurons.** As it has been shown that NPC proteins are cell-type specific34, we further investigated the effect of DPRs on nucleocytoplasmic transport in neuronal-like SH-SY5Y cells (Fig. 4) and in iPSC-derived motor neurons (Fig. 5). Healthy donor-derived iPSCs were differentiated into motor neurons as we published before35. The nucleocytoplasmic transport assay was performed 96 hours after co-transduction of SH-SY5Y cells (Fig. 4a) or motor neurons (Fig. 5a,b) with LVs expressing a mCherry-DPR construct as well as the NLS SV40-mNeonGreen2x-NESpki reporter. We could confirm that poly-GR or poly-PR did not induce a blockade of nucleocytoplasmic transport (Figs 4b and 5c). Moreover, no disturbed transport was observed in poly-PA expressing cells (Figs 4b and 5c), which is in line with the non-toxic characteristics of poly-PA (reviewed by Freibaum and Taylor, 2017). On the other hand, significant export (Fig. 5c - time 0: \( p < 0.0001 \)) and import (Fig. 4b: \( p < 0.0001 \)) defects were observed in poly-GA expressing cells, as reported previously36,37.

**Discussion**

To investigate the previously observed link between nucleocytoplasmic transport and mutant C9orf729–12,14,15,38 in more detail, we measured the effect of poly-GR and poly-PR on active nucleocytoplasmic transport. Overall, our data do not support a directly induced impediment of nucleocytoplasmic transport by the most toxic DPRs, poly-GR or poly-PR.

Three alternative, not mutual exclusive, explanations for the observed association between mutant C9orf72 and nucleocytoplasmic transport are possible. First, it has been demonstrated that various cellular stresses can induce perturbation of conventional nucleocytoplasmic transport pathways and this through several mechanisms, including post-translational modifications and relocalization and degradation of transport factors (reviewed by Kose and Imamoto, 201439). Interestingly, multiple mechanisms driving poly-GR/PR toxicity have been identified, such as reduced translation and mitochondrial stress (reviewed by Balendra and Isaacs, 201940). This suggests that the previously observed interaction between nucleocytoplasmic transport and poly-GR or poly-PR-induced toxicity9,10,12 could be caused by an indirect stress-induced mechanism. For example, expression of poly-GR and poly-PR peptides can induce the formation29,41 and influence the composition of stress granules (SGs)42. These induced SGs can subsequently recruit nucleocytoplasmic transport factors, which results in nucleocytoplasmic transport deficits43. We rarely observed SG formation in poly-GR100 expressing cells and in less than 15% of poly-PR100 expressing cells (Supplementary Fig. S3A). The low number of SGs could explain why no transport defects were observed in contrast to the data published by Zhang and colleagues43. Indeed, when we only take mCherry-PR100 expressing Hela Kyoto cells that contain SGs into account, significant nucleocytoplasmic transport defects are observed (Supplementary Fig. S3B,C).

Second, a combination of other disease-related factors such as TDP-43 aggregates14,15,44, GA aggregates36,37, RNA foci45, cytoskeletal dysfunction16 and aging17 might explain the observed nucleocytoplasmic transport...
pathology in C9orf72-patients. We observed poly-GA induced nucleocytoplasmic transport deficits in SH-SY5Y cells and iPSC-derived motor neurons (MNs) and were subsequently cultured up to 32 days. On day 28, motor neurons were co-transduced with LVs expressing a mCherry-DPR construct (PA100, GA100, GR100, PR100) or mCherry alone and a LV expressing NL3SY2x-mNeonGreen2x-NESa. Nucleocytoplasmic transport was measured on day 32. (b) Images represent 32-day old motor neurons expressing the indicated constructs 96 hours after transduction. Scale bar = 10 µm. (c) Nucleocytoplasmic transport measured at day 32. Significant disturbed transport observed for poly-GA expressing motor neurons (time 0: p < 0.0001; time 15: p < 0.0001; time 30: p = 0.0334). Data are represented as means ± SD, non-parametric one-way ANOVA followed by Dunn’s multiple comparison test. Dots represent means of one image with ± 5 cells per image; n = 30–40 from four independent biological replicates. Means ± SD. *Denotes p < 0.05; ****Denotes p < 0.0001.

In summary, poly-GR and poly-PR induced toxicity has previously been linked to nucleocytoplasmic transport. However, a better understanding of the underlying mechanisms is urgently needed to translate these findings in new therapeutic strategies. In this paper, we did not observe a directly induced obstruction of nucleocytoplasmic transport by poly-GR or poly-PR in Hela Kyoto cells, SH-SY5Y cells and human-derived motor neurons. This strongly suggests that a direct effect of poly-GR or poly-PR on nucleocytoplasmic transport is not the primary factor in the destructive pathway observed in C9orf72-patients. Our data rather support the hypothesis that indirect mechanisms underlie the previously observed modifying effect of nucleocytoplasmic transport factors on poly-GR or poly-PR induced toxicity.
Material and Methods

Cell culture. Hela Kyoto cells were grown in DMEM with high glucose and L-glutamine (ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS) and gentamycin (20 µg/ml). SH-SY5Y cells (Sigma, 94030304) were maintained in DMEM-F12 medium (ThermoFisher Scientific) supplemented with 10% FBS and penicillin/streptomycin (100 µg/ml). Maintenance of human iPSCs (Takara Bio, Göteborg Sweden) and motor neuron differentiation was performed as described before.

Hela Kyoto reporter cell lines. The reporter construct NLSc-myc-GFP2x-NESiK2 was integrated in the AAVS1 locus of Hela Kyoto cells under a CMV promotor using the CRISPR technique. Hela Kyoto cells co-transfected with three plasmids were selected with 1 µg/ml puromycin and single-cell cloned. The following plasmids were used: a pcDNA3.1 plasmid containing the insert CMV-NLSc-myc-GFP2x-NESiK2 followed by a puromycin-resistance gene (Puro9), a donor plasmid containing Cas9 plus a gRNA targeting the AAVS1 locus (GCCAGTCACCATCTGTCCCTAAGTGG) and a donor plasmid containing a gRNA (GCCAGTCACCCAAAAAGGGGG). A similar approach was used for the Hela Kyoto cell line expressing the reporter NESpki2-mNeonGreen2x-NLSFUS reporter construct NLS SV40-mNeonGreen2x-NES pki2 was integrated in the AAVS1 locus of Hela Kyoto cells under a CMV promotor using the CRISPR technique.

Lentiviral vector design. Flag-tagged DPR100 plasmids were a kind gift of Dr. Daisuke Ito (Department of Neurology, Keio University, Tokyo, Japan). These plasmids were used as a template to generate the Lentiviral vectors. HEK293T cells were co-transfected with vectors psPAX2 (packaging vector), pMDG.2 (envelope vector) and one of the DPR-plasmids, using X-tremeGene9 transfection reagent (Roche). At 24 hours after transfection, the medium was changed to serum-free, high BSA growth medium (DMEM, 1% BSA, 100 µg/ml penicillin/streptomycin). Virus-containing medium was harvested 48 hours after transfection and stored at −80°C.

Transduction of Hela Kyoto and SH-SY5Y cells was carried out with the aid of polybrene 96 hours before analysing nucleocytoplasmic transport. Transduction of iPSC-derived motor neurons was carried out without polybrene at day 28 of the differentiation protocol and 96 hours before measuring nucleocytoplasmic transport.

GR20 and PR20 poly-dipeptides. The GR20 and PR20 peptides were synthesized by Pepsan (Lelystad, The Netherlands). Peptides were dissolved in milli-Q water and stored at −80°C. Hela Kyoto cells were treated with 10 μM or 30 μM of GR20 or PR20 for 2 hours (serum-free medium) or 24 hours before measuring nucleocytoplasmic transport. As a result of the short half-life of GR20 (20–30 min), this peptide was added once more after 1 hour.

Nucleocytoplasmic transport assay. Nucleocytoplasmic transport assays were performed on Hela Kyoto cells stably expressing the report construct NLSc-myc-mNeonGreen2x-NESiK2 and SH-SY5Y cells/iPSC-derived motor neurons constitutively expressing the reporter construct NLSc-myc-GFP2x-NESiK2 after transduction. Cells were incubated with GR20/PR20 peptides (2 hours, 24 hours) or transduced with indicated constructs (96 hours) before fixation with 4% paraformaldehyde (PFA) in PBS, before (0 min) and after (indicated time points) addition of 45 nM leptomycin B (LMB; InvivoGen, Toulouse, France). As positive control, Hela Kyoto cells were treated for 2 hours with Importazole (50 μM; Selleckchem, Munich, Germany) before the addition of LMB.

Hela Kyoto cells containing the reporter construct NLSc-myc-mNeonGreen2x-NLSFUS were fixed with 4% PFA 96 hours after transduction with indicated constructs. As positive control, cells were transected with Lipofectamine P3000 (Thermo Fisher Scientific) according to the instructions of the manufacturer with a plasmid expressing an importin β binding domain (pBiT2.1-EF1α-IBB-mRFP-NESbio) and fixed after 24 hours.

Hela Kyoto cells containing the reporter construct NESpki2-mNeonGreen2x-NLSFUS were fixed with 4% PFA after incubation with GR20/PR20 peptides. As positive control, cells were transfected with Lipofectamine P3000 (Thermo Fisher Scientific) according to the instructions of the manufacturer with a plasmid expressing the transportin 1 and 2 import inhibitor M99 (pBiT2.1-EF1α-Dmr-M99) and fixed after 24 hours.

Cells were imaged with a Leica SP8 confocal microscope. The fluorescent intensity of reporters in the nucleus and cytoplasm was analyzed with ImageJ. A minimum of 25 cells (Hela Kyoto), 40 cells (SH-SY5Y, iPSC-derived...
motor neurons) per condition per experiment were measured. A minimum of 5 different images of minimum two different coverslips was used. Automated analysis were performed with the use of the CellInsight CX5 high content screening platform (Thermo Scientific). A minimum of 5600 cells from three different wells per experiment were measured.

**SUnSET assay.** To examine the production of newly translated proteins, a SUnSET assay was performed as previously described[55]. In short, Hela Kyoto cells were grown in 24-well plates on glass coverslips and transduced with indicated constructs. After 96 hours, cells were incubated with fresh medium containing 1 μg/ml puromycin and fixed after 1 hour to perform immunostaining. Cells were imaged with a Leica SP8 confocal microscope and analyzed automatically with Cellprofiler. A total of 15 images from three different coverslips were used per experiment. 141–622 cells were measured per experiment. Data from three independent experiments were pooled.

**Immunofluorescence.** Cells plated on coverslips were fixed in 4% paraformaldehyde for 20 min at room temperature and were washed with PBS. Permeabilization and blocking was done using PBS containing 0.1% Triton X-100 (Acros Organics) and 5% donkey serum (Sigma) for 1 hour. Cells were incubated overnight at 4°C in blocking buffer (2% donkey serum) containing the different primary antibodies against poly-GR (1/500, EMB Millipore, MABN778), poly-PR (homemade with PR15 as antigen, rabbit, affinity purification), β-Tubuline (1/500; Abcam; ab7751), anti-G3BP (1/500; Abcam; ab56574) or anti-puromycin (1/1000, Millipore, MABE342). After washing with PBS, cells were incubated with corresponding secondary antibodies (1/2500, Invitrogen) for 1 hour at room temperature. Nuclei were stained using NucBlue Live Cell Stain reagent (Invitrogen). Fluorescent images were taken with Leica SP8 confocal microscope.

**Sodium arsenite treatment.** Hela Kyoto cells were treated with 0.5 mM of sodium arsenite for 1 hour before fixation with 4% paraformaldehyde.

**Statistical analysis.** Data of minimum three biological independent experiments were pooled in all analyses, of which each experiment contributed the same n. The mean of one image (manual analysis or Cellprofiler) or well (automatic analysis with CellInsight CX5 high content screening platform) was seen as one data point. Statistical analyses were performed on the log transformations for the nucleocytoplasmic transport assay. D’Agostino-Pearson omnibus normality test was used to test data for normality. Parametric tests were used on normally distributed data. Non-parametric tests were used on non-normally distributed data. Mann-Whitney U-test was used to determine statistical differences in the Importazole experiment. (Non-)parametric one-way ANOVA followed by Dunn’s multiple comparison/Dunnett’s multiple comparison test was used for the other experiments to determine statistical differences between groups. Data values represent mean ± SD. Graphpad Prism version 7 was used to perform statistical analyses.

**Data availability**

All data generated or analysed during this study are included in this published article (and its Supplementary Information files) and are available from the corresponding author on reasonable request.

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Author contributions
J.V. carried out all the experiments. A.S. assisted with motor neuron differentiation. J.V. and T.V. performed statistical analysis. J.V. wrote the manuscript. J.V., T.V., S.B., A.S., P.V.D., D.D. and L.V.D.B. made substantial contribution to acquisition and/or interpretation of data. All authors critically read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to L.V.D.B.

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