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Supporting Information

Unravelling the interactions of biodegradable dendritic nucleic acids carriers and neural cells

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1) Deposition assay of Cy5-siRNAmi dendriplexes

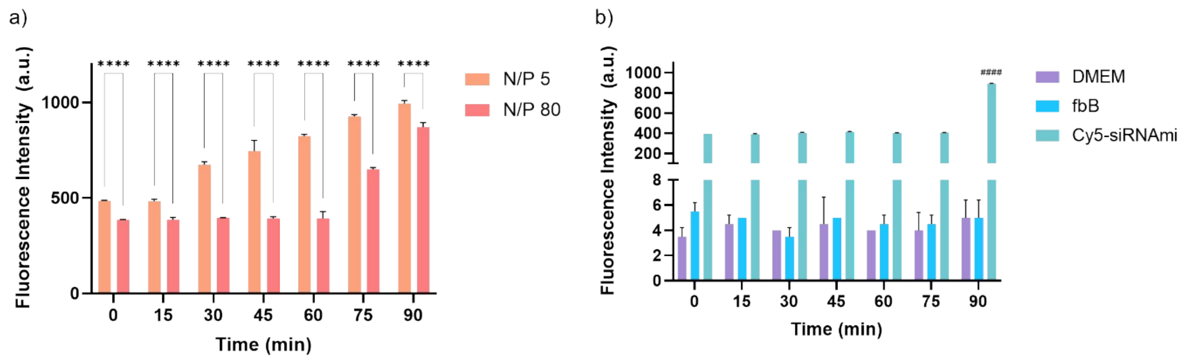

Figure S1. Deposition assay of a) Cy5-siRNAmi dendriplexes at N/P 5 and 80. Dendriplexes were incubated in medium (DMEM with GlutaMAX^{TM}) at 37 °C and the fluorescence intensity (5 x 5 spots, λ_{exc} = 633 nm, λ_{em} = 647 nm) of the well bottom was measured every 15 minutes, up to 1.5 hours. b) DMEM with GlutaMAX^{TM}, free dendrimer and Cy5-siRNAmi were used as controls. Significant differences: $^{####}$, ****p ≤ 0.0001. For free Cy5-siRNA, the symbol # denotes significant differences between times of incubation.

2) Internalization kinetics data in neuronal cell lines

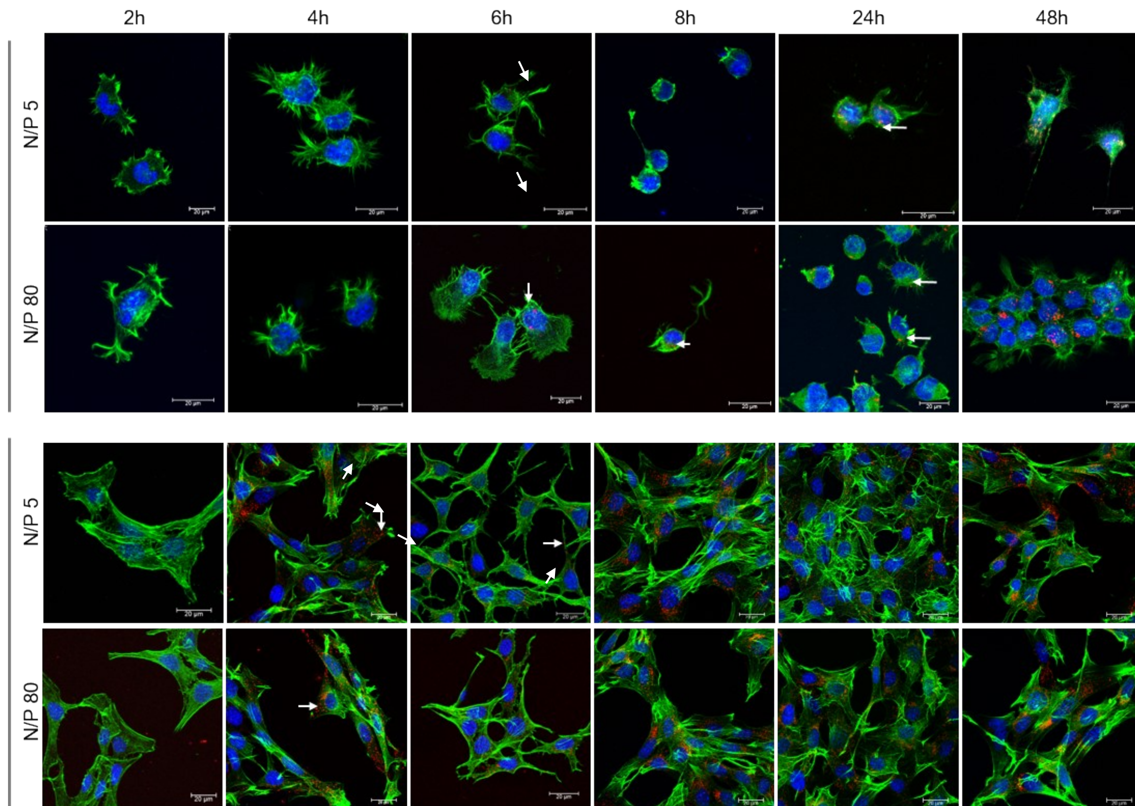

Figure S2. Confocal microscopy images of ND7/23 and HT22 cell lines after incubation with Cy5-siRNAmi-fbB dendriplexes at N/P 5 and 80 at different time points (2, 4, 6, 8, 24 and 48 hours). Nuclei stained with Hoechst 33342 (blue). Actin filaments stained with Alexa Fluor™ 488 Phalloidin (green). White arrows point to Cy5-siRNAmi dendriplexes (red). Scale bar: 20 µm.

3) Internalization kinetics data in primary cortical neurons

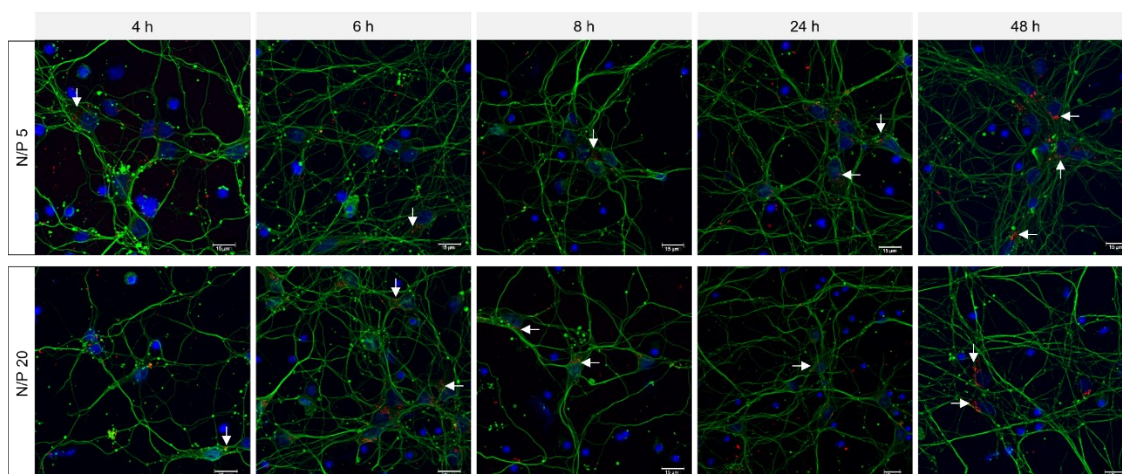

Figure S3. Confocal microscopy images of primary cortical neurons after incubation with Cy5-siRNAmi-fbB dendriplexes at N/P 5 and 80 at different time points (4, 6, 8, 24 and 48 hours). Staining: Nuclei with Hoechst 33342 (blue), βIII-tubulin (green), and Cy5-siRNAmi dendriplexes (red). Scale bar: 15 µm.

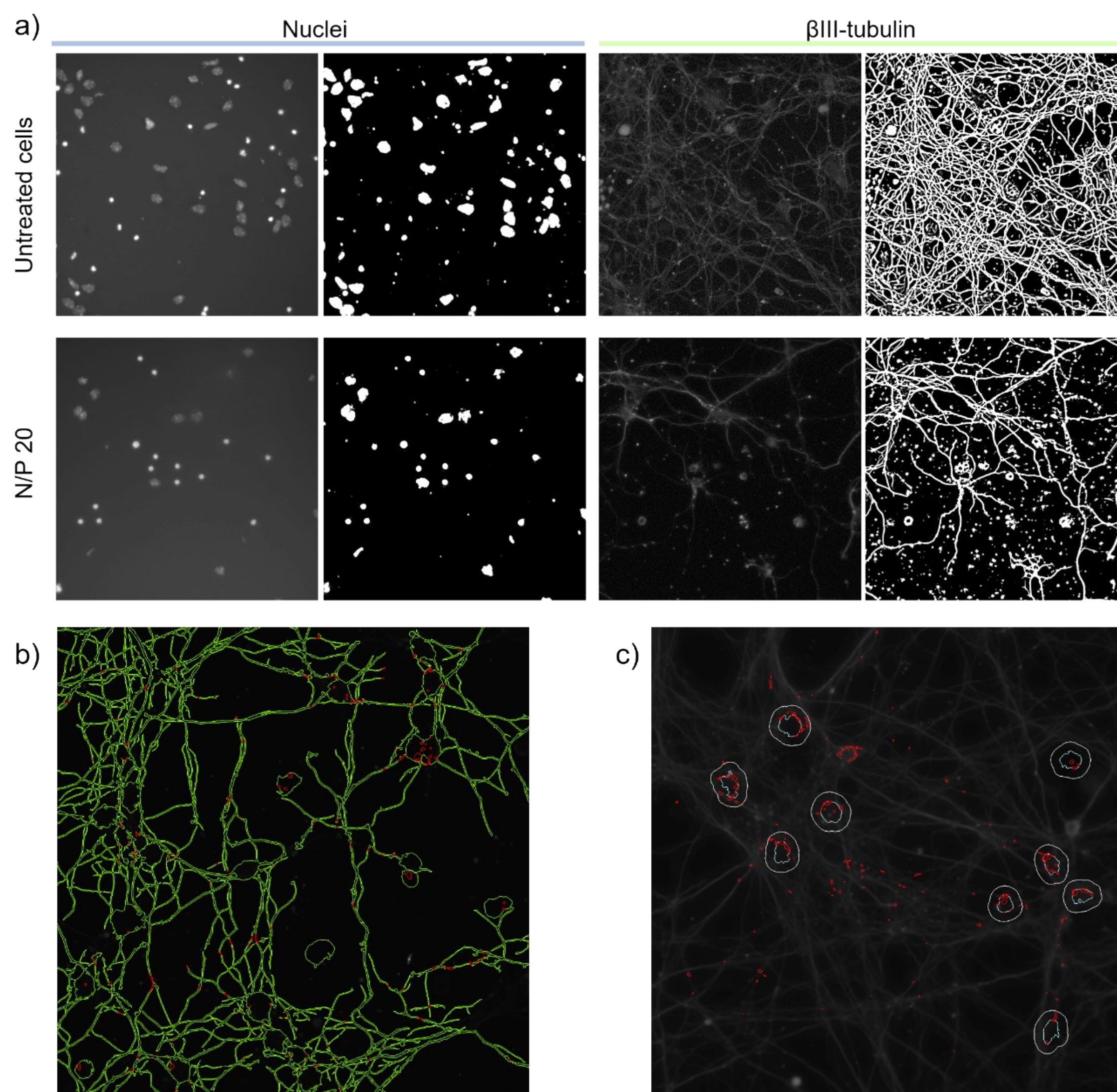

Figure S4. Representative images used in the analysis of internalization kinetics in primary cortical neurons. Cells were incubated with Cy5-siRNAmi-fbB dendriplexes at N/P 5 and 20 at different time points (4, 6, 8, 24 and 48 hours). Untreated cells were used as control. Staining: Nuclei with Hoechst 33342, βIII-tubulin, and Cy5-siRNAmi dendriplexes. Acquired images were a) segmented and then Cy5 fluorescence intensity (red) was quantified in b) neuronal extension (green) and in c) perinuclear area (white).

4) Internalization mechanism in neuronal cell lines

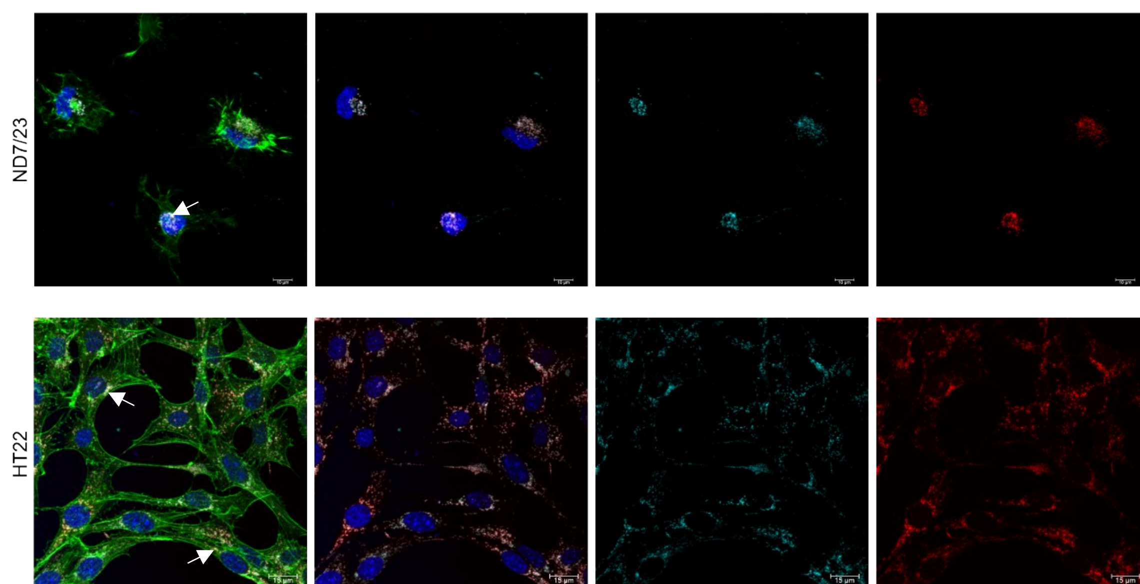

Figure S5. Confocal microscopy images of ND7/23 and HT22 cells after incubation with Cy5-siRNAmi-fbB dendriplexes at N/P 5 for 24 hours. Staining: nuclei with Hoechst 33342 (blue), actin filaments with Alexa Fluor™ 488 Phalloidin (green), Clathrin (cyan), and Cy5-siRNAmi dendriplexes (red). Scale bar: 15 µm.

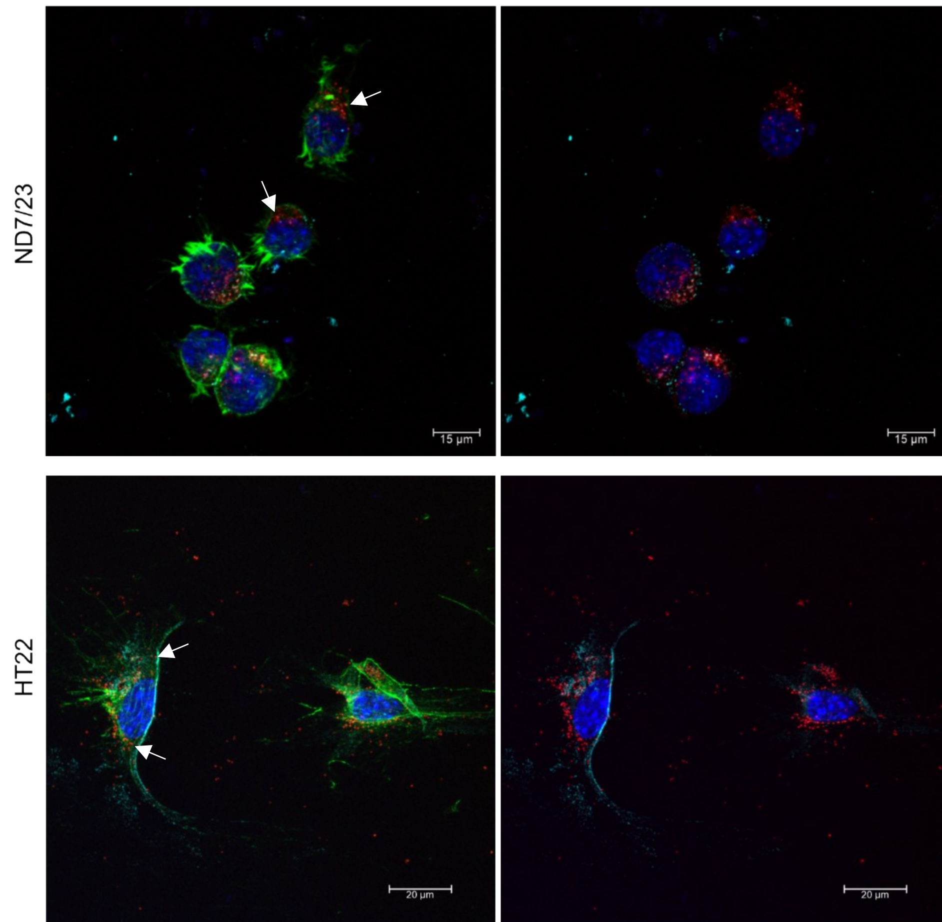

Figure S6. Confocal microscopy images of ND7/23 and HT22 cells after incubation with Cy5-siRNAmi-fbB dendriplexes at N/P 5 for 24 hours. Staining: nuclei with Hoechst 33342 (blue), actin filaments with Alexa Fluor™ 488 Phalloidin (green), Caveolin-1 (cyan), and Cy5-siRNAmi dendriplexes (red). Scale bar- ND7/23: 15 µm; HT22: 20 µm.

5) Internalization mechanism in primary cortical neurons

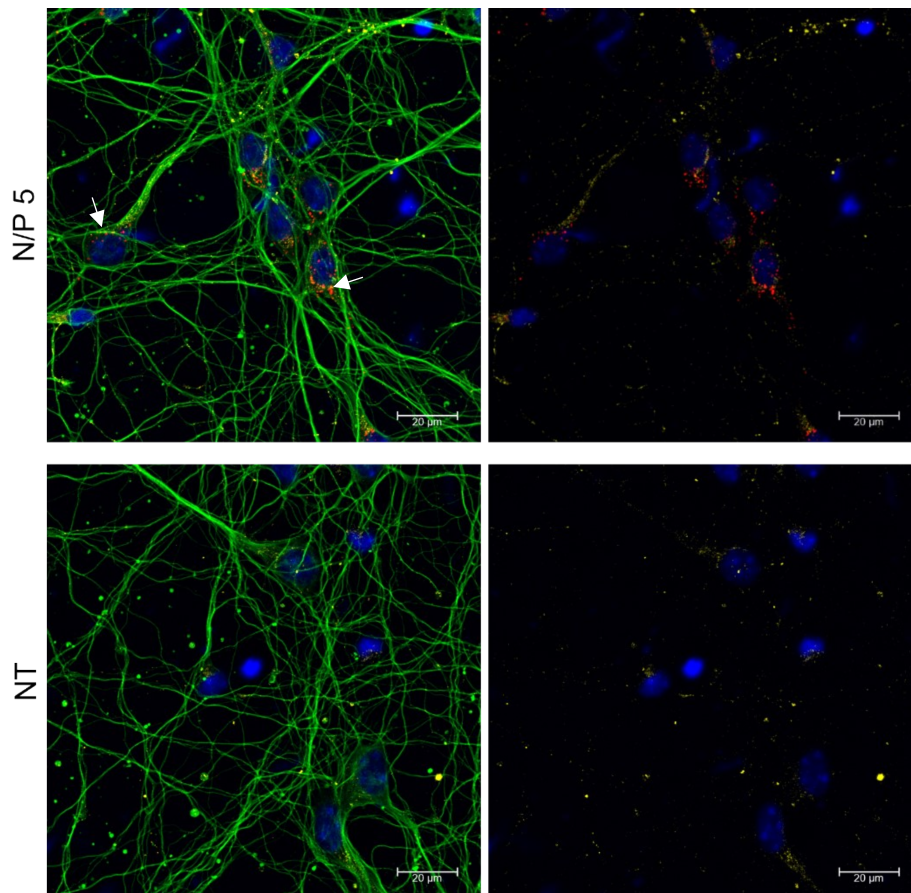

Figure S7. Confocal microscopy images of primary cortical neurons after incubation with Cy5-siRNAmi-fbB dendriplexes at N/P 5 for 24 h. Untreated cells (NT) were used as control. Staining: nuclei with Hoechst 33342 (in blue), βIII-tubulin (in green), Clathrin (in yellow), and Cy5-siRNAmi dendriplexes (in red). Scale bar: 20 µm.

6) Intracellular path in neuronal cell lines (late endosomes)

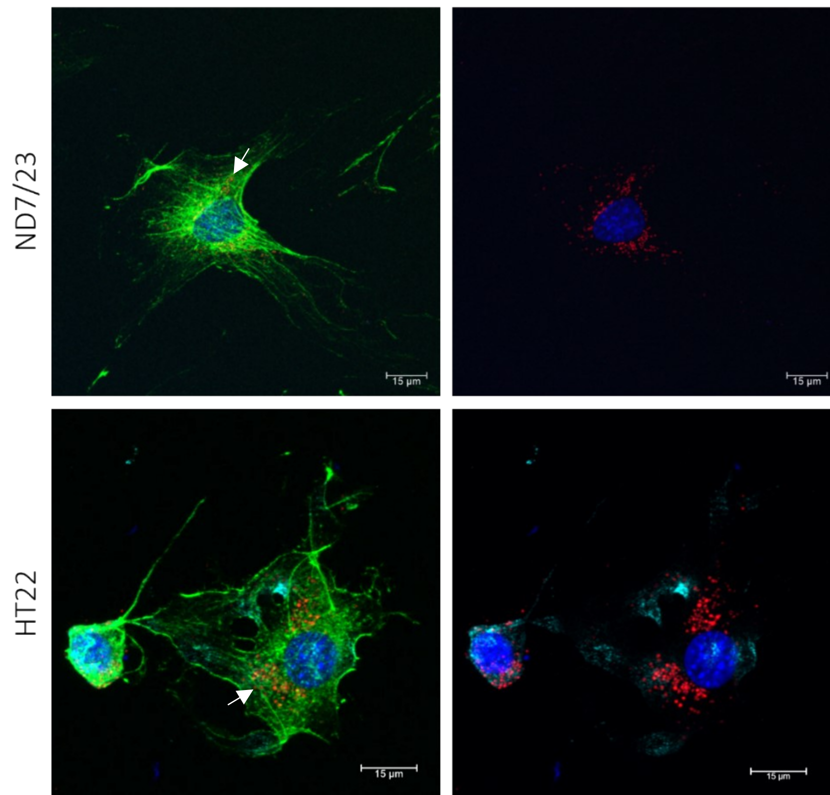

Figure S8. Confocal microscopy images of ND7/23 and HT22 cells after incubation with Cy5-siRNAmi/fbB dendriplexes at N/P 5 for 24 h. Staining: nuclei with Hoechst 33,342 (in blue), actin filaments with Alexa Fluor™ 488 Phalloidin (green), LAMP-1 (cyan), and Cy5-siRNAmi dendriplexes (red). Scale bar: 15 µm.

7) Intracellular path in primary cortical neurons (late endosomes and autophagosomes)

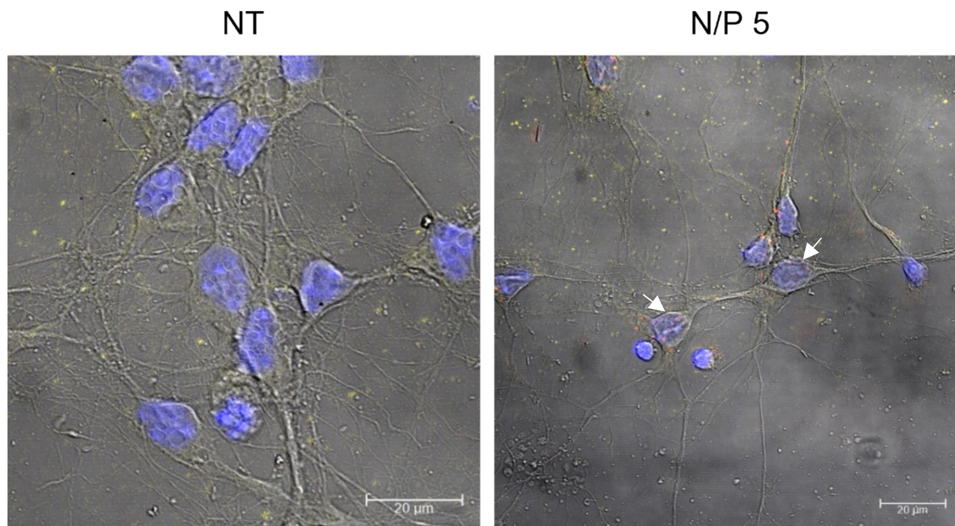

Figure S9. Confocal microscopy images of primary cortical neurons after incubation with Cy5-siRNAmi/fbB dendriplexes at N/P 5 for 24 hours. Untreated cells (NT) were used as control. Staining: nuclei with Hoechst 33342 (blue), late endosomes – LAMP-1 (yellow), and Cy5-siRNAmi dendriplexes (red). Scale bar: 20 µm.

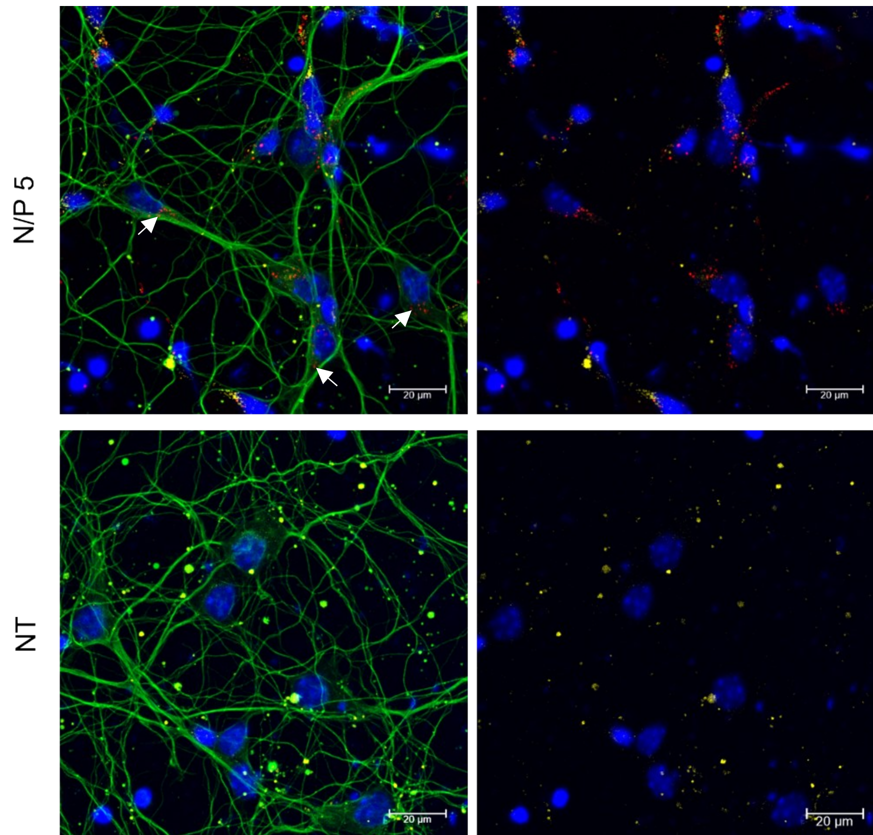

Figure S10. Confocal microscopy images of primary cortical neurons after incubation with Cy5-siRNAmi-fbB dendriplexes at N/P 5 for 24 hours. Untreated cells (NT) were used as control. Staining: nuclei with Hoechst 33342 (blue), autophagosomes - LC3 (yellow), and Cy5-siRNAmi dendriplexes (red). Scale bar: 20 µm.

8) Endocytosis study with inhibitors

Table S1. Inhibitors of endocytosis (dependent on clathrin or caveolin) tested and the concentrations used by us in ND7/23 and HT22 cells. Despite the effects recorded on these cells, the use of these inhibitors is described in many other cells (mostly, non-neuronal cells).

<table>
<tr><th>Inhibitor</th><th>Endocytic mechanism</th><th>Concentrations tested</th><th>Effect in cells</th><th>Cells already tested</th><th>References</th></tr>
<tr><td>Chlorpromazine</td><td>Clathrin</td><td>2, 4, 6, 8 and 10 μg/mL</td><td>90-100% of cells died in less than 10 mins</td><td>HeLa, A549, 1321N1, COS-7, Vero, HuH-7, ARPE-19, D407, CIK, SK-Hep1, HEK293</td><td>dos Santos et al., 2011; Vercauteren et al., 2010; Rejman et al., 2005; Wang et al., 2016; Maddila et al., 2021; Itoh et al., 2008</td></tr>
<tr><td>Phenylarsine oxide</td><td>Clathrin</td><td>0.05, 0.1, 1 and 5 μM</td><td rowspan="3">~90% of cells died in less than 30 mins</td><td>H358, Calu-3, SNU-1327, H1703, adrenal chromaffin cells</td><td>Prichard et al., 2021; Kim et al., 2021</td></tr>
<tr><td>Genistein</td><td>Caveolin</td><td>50, 100, 200 and 400 μM</td><td>D407, Vero, COS-7, HuH-7, ARPE-19, A549, HCT116, COLO205, Sf1Ep</td><td>Vercauteren et al., 2010; Horibe et al., 2018; Singh et al., 2012</td></tr>
<tr><td>Filipin III</td><td>Caveolin</td><td>0.5, 1, 2, 5 and 10 μg/mL</td><td>A549, CIK, Sf1Ep, SK-Hep1</td><td>Rejman et al., 2005; Wang et al., 2016; Singh et al., 2012; Madilla et al., 2021</td></tr>
<tr><td>Methyl-β-Cyclodextrin</td><td>Caveolin</td><td>20, 250, 500, 1000 and 5000 μM</td><td>90-100% of cells died in less than 10 mins (concentrations above 1000 μM). At 20-500 μM, no uptake differences were observed</td><td>D407, Vero, COS-7, HuH-7, ARPE-19, A549, BY-2, HEK293, Sf1Ep</td><td>Vercauteren et al., 2010; Chen et al., 2018; Itoh et al., 2008; Singh et al., 2012; Rejman et al., 2005</td></tr>
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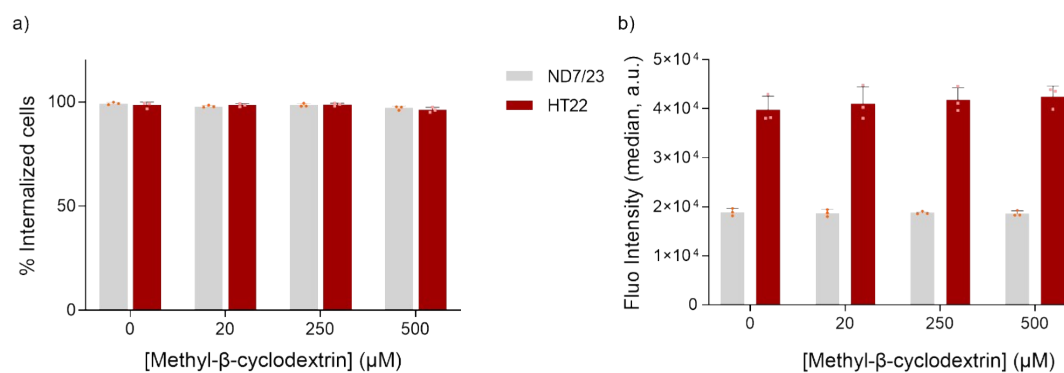

Figure S11. Cellular interaction of N/P 5 Cy5-siRNAmi dendriplexes by cell lines pre-treated with a caveolin-mediated endocytosis inhibitor. ND7/23 and HT22 cells were incubated for 10 minutes with methyl-β-cyclodextrin (MBCD, 20-500 μM), and then incubated for 6 hours with dendriplexes carrying Cy5-siRNAmi ($[\text{Cy5-siRNAmi}]_f = 100$ nM). Characterization by flow cytometry: a) Percentage of cells with fluorescence signal of Cy5-siRNAmi dendriplexes and b) Intensity of fluorescence in cells. Results are shown as mean ± SD of three independent experiments (n=3). Two-way ANOVA tests were used for statistical analysis. No significant differences between the non-treated cells (0 μM) and the MBCD-treated cells were observed.

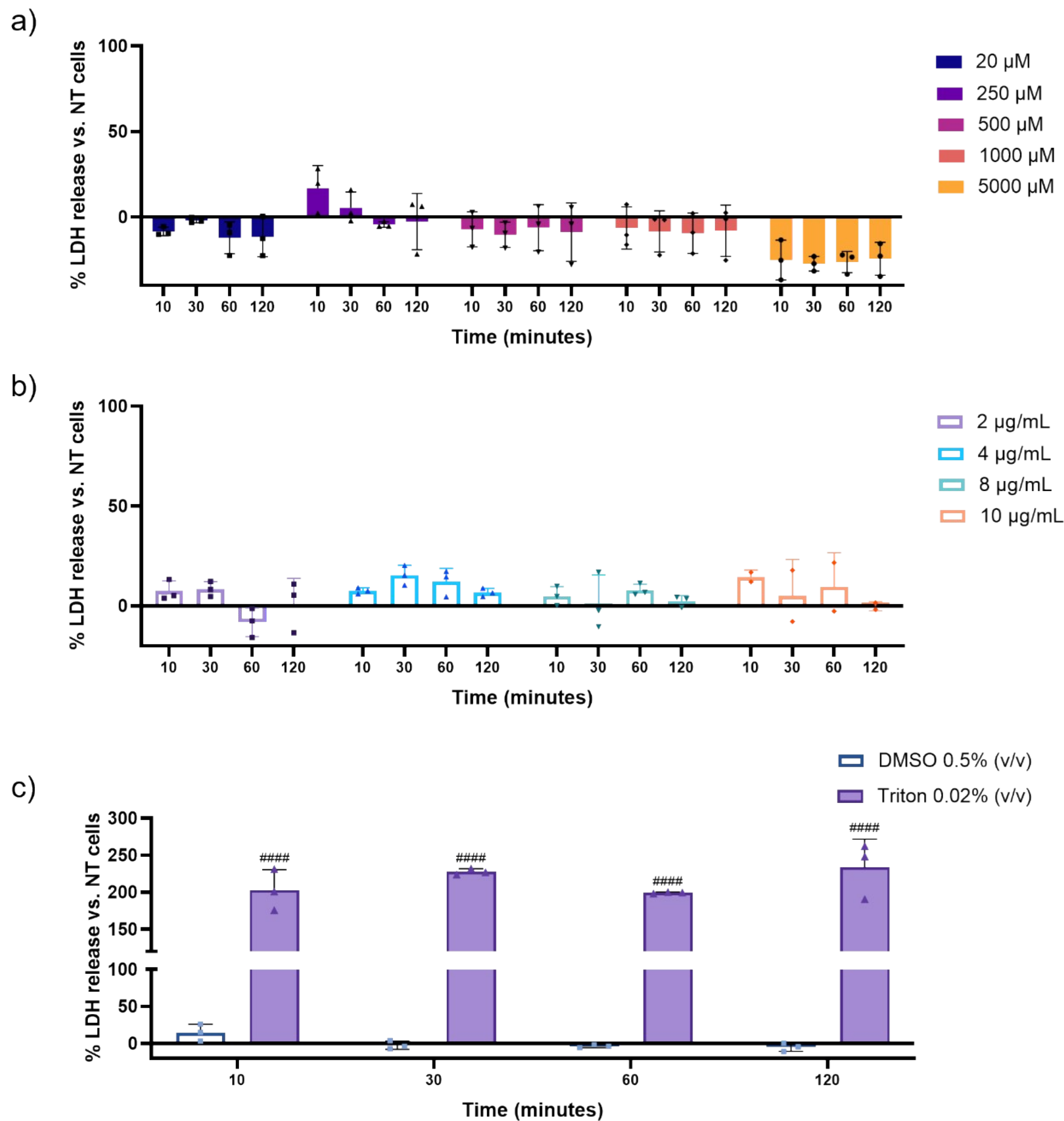

Figure S12. Cytotoxic profile evaluation in primary cortical neuron cultures. Plasma membrane integrity assessed by LDH assay, after exposure of the cultures to: a) methyl-β-cyclodextrin (MBCD) or b) chlorpromazine. Non-treated (NT) cells, cells treated with dimethylsulfoxide (DMSO, 0.5% (v/v)) and Triton™ X-100 (0.02% (v/v)) were used as controls. Results are represented as mean ± SD of three independent experiments (n = 3). Two-way ANOVA tests were used for statistical analysis. Significant differences: #### $p \leq 0.0001$. The symbol # indicates significant differences versus the NT cells.