The Uptake, Retention and Clearance of Drug-Loaded Dendrimer Nanoparticles in Astrocytes - Electrophysiological Quantification

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Supplementary Material

Methods:

CMCht/PAMAM dendrimer nanoparticle synthesis and functionalization: *CM*Cht/PAMAM dendrimer NPs were produced as previously reported.[13] In brief, Starburst PAMAM-carboxylic acid-terminated dendrimers (PAMAM-CT) (generation 1.5, 20% w/v in methanolic solution) with an ethylenediamine core were purchased (Sigma, Germany) and a series of step-by-step reactions were performed. First, the PAMAM-CT generation was doubled and a PAMAM-methyl ester terminated dendrimer was produced. Subsequently, a condensation reaction between the methyl ester and amine groups of PAMAM and *CM*Cht took place. Finally, the non-reacting methyl ester groups were converted into carboxylic groups. MP was incorporated in the *CM*Cht/PAMAM dendrimer NP combining a *CM*Cht/PAMAM dendrimer NP aqueous solution with an ethanolic MP solution with a final concentration of 5×10^{-4} M (w/w), under vigorous agitation. Saturated sodium carbonate solution (Na_2CO_3, Aldrich, Germany) and acetone (Pronalab, Portugal) were added to precipitate the mixture. The precipitate was collected by filtration and dispersed in ultrapure water to undergo dialysis (cellulose tubing, benzoylated for separating compounds with a molecular weight ≤1200; Sigma, Germany) for 48 h. MP-loaded *CM*Cht/PAMAM dendrimer NPs were obtained by freeze-drying (Telstar-Cryodos-80, Spain). To label the NPs, FITC (Sigma, Germany) was added and a covalent bonding reaction was carried out between the amine group of *CM*Cht and the isothiocyanate group from FITC. The resulting molecules were freeze-dried (Telstar-Cryodos-80, Spain).

Electrophysiological experiments: Cell-attached high-resolution membrane capacitance measurements were performed using a two-phase lock-in amplifier (SWAM IIB; Celica, Slovenia) incorporated into a patch-clamp amplifier. For the electrophysiological recordings, the cells-seeded coverslips were bathed in extracellular solution (ECS) containing NaCl (130 mM), KCl (5 mM), $MgCl_2$ (1 mM), $CaCl_2$ (2 mM), Na-HEPES (10 mM) and D-glucose (10 mM) at pH 7.4. Fire-polished 3–5 MΩ glass pipettes were coated with Sylgard (Midland, USA). Signals were filtered (10 Hz, −3 dB, low pass, Bessel 4-pole)

and acquired (at 200 Hz) with an analogue-to-digital converter (National Instruments BNC-2110, National Instruments, USA) using custom software (Cell, Celica, Slovenia). Membrane patches were voltage clamped at a holding potential of 0 mV, to which a sine wave voltage (111.1 mV rms) was applied (800 Hz). Discrete steps were resolved by progressive filtering of records.

Astrocytes transfection with the plasmid DNA: we used the plasmid DNA encoding NPY tagged with NPY-mCherry (a kind gift from Dr. Ronald W. Holz, University of Michigan, USA). The cells were incubated in lipofection medium (DMEM, sodium pyruvate (1 mM), L-glutamine (2 mM)) and mixed with transfecting reagent (Fugene, Promega, USA) with of plasmid DNA (1 μg) for 15 min at 37°C. Then the culture medium was added and supplemented with UltraSerG serum (3%) (Life Technologies, USA) and the expression of NPY-mCherry was observed after incubation for 24 h.
Live confocal microscopy images were acquired with an inverted microscope (Zeiss LSM META 510, Germany) equipped with a 63× oil-immersion objective. FITC molecules bonded to the NPs were excited by a 488-nm argon laser, and the emission was collected through a bandpass filter (505–530 nm). Alexa Fluor 546 labelling on the vesicles was excited with the He/Ne laser (543 nm) and the emission light was filtered with a long-pass filter, with the cut-off below 560 nm. To eliminate possible bleed through, green and red emission fluorescence were acquired sequentially. Time series images were recorded in 20-s intervals over 45 min. All recordings were performed in 300 mOsm ECS.

Results

Table 1. Relative proportion of exocytotic vesicle types

	Average vesicle fusion type (%)		SEM	n/N
	Transient	Full fusion		
CTRL	72.3	27.7	9.0	117/10
NP	78.7	21.3	4.1	120/7
NPinc	87.7	12.3	1.8	139/3

Proportion (in %) of occurrence of exocytosis type: transient or full-fusion exocytotic events in three different experimental conditions: cultured in regular medium (CTRL), cultured in regular medium and exposed to NP in the patch-pipette solution during the recording (NP) and incubated for 24 h with NP and recorded in ECS (NPinc). SEM, standard error of mean; n, number of events; N, number of cells analysed.

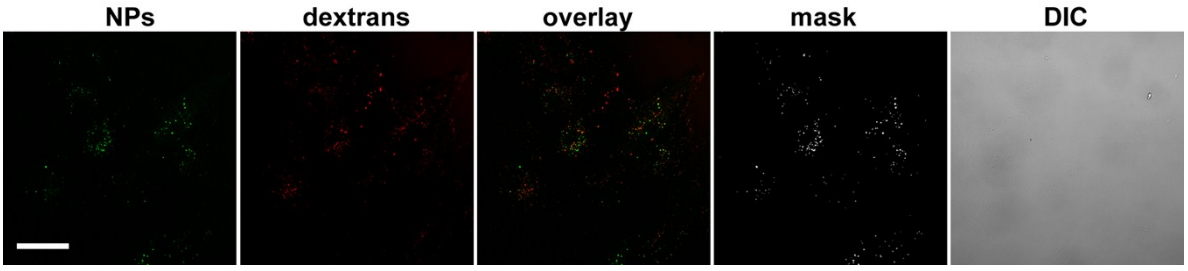

Figure S4. Co-localization of NPs with endocytotic dextran-labelled compartments in astrocytes after 24h of incubation with NP. Representative confocal micrographs of live astrocytes after Alexa Fluor 546-dextran labelling of macropinosomes (dextrans, red) and incubation with FITC-labelled *CM*Cht/PAMAM dendrimer NPs (24h, NP, green). Co-localization can be seen in yellow (overlay) and was extracted as white pixels (mask). Transmission light image of cells is presented in the last panel. Scale bars represent 50 μm. Relative co-localization of NPs with the dextran-labelled macropinosomes relative to all above threshold pixels of fluorescent NPs in all cells in the image together was 22.2 %. Threshold was set to 20%.

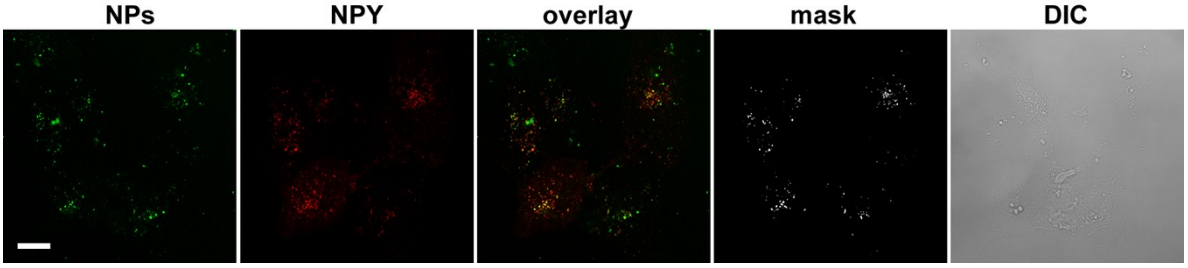

Figure S5. Co-localization of NPs with exocytotic mCherry-NPY-labelled vesicles in astrocytes in astrocytes after 24h of incubation with NP. Representative confocal micrographs of live astrocytes: labelled exocytotic vesicles with mCherry-NPY (NPY, red) and incubated with FITC-labelled *CM*Cht/PAMAM dendrimer NPs (24h, NP, green). Co-localization can be seen in yellow (overlay) and was extracted as white pixels (mask). Transmission light image of cells is presented in the last panel. Scale bar represents 20 μm. Relative co-localization of NPs with the the NPY-labelled exocytotic vesicles relative to all above threshold pixels of fluorescent NPs in all cells in the image was 43.7 %. Threshold was set to 20%.