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Authors: Anja Kunze, Coleman Tylor Murray, Chanya Godzich, Jonathan Lin, Keegan Owsley, Andy Tay, and Dino Di Carlo

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Modulating motility of intracellular vesicles in cortical neurons with nanomagnetic forces on-chip†

Anja Kunze,*ad Coleman Tylor Murray,a Chanya Godzich,a Jonathan Lin,a Keegan Owsley,a Andy Taya and Dino Di Carlo*abc

Vesicle transport is a major underlying mechanism of cell communication. Inhibiting vesicle transport in brain cells results in blockage of neuronal signals, even in intact neuronal networks. Modulating intracellular vesicle transport can have a huge impact on the development of new neurotherapeutic concepts, but only if we can specifically interfere with intracellular transport patterns. Here, we propose to modulate motion of intracellular lipid vesicles in rat cortical neurons based on exogenously bioconjugated and cell internalized superparamagnetic iron oxide nanoparticles (SPIONs) within microengineered magnetic gradients on-chip. Upon application of 6–126 pN on intracellular vesicles in neuronal cells, we explored how the magnetic force stimulus impacts the motion pattern of vesicles at various intracellular locations without modulating the entire cell morphology. Altering vesicle dynamics was quantified using, mean square displacement, a caging diameter and the total traveled distance. We observed a de-acceleration of intercellular vesicle motility, while applying nanomagnetic forces to cultured neurons with SPIONs, which can be explained by a decrease in motility due to opposing magnetic force direction. Ultimately, using nanomagnetic forces inside neurons may permit us to stop the mis-sorting of intracellular organelles, proteins and cell signals, which have been associated with cellular dysfunction. Furthermore, nanomagnetic force applications will allow us to wirelessly guide axons and dendrites by exogenously using permanent magnetic field gradients.

Introduction

The role of mechanical forces in neuronal cell behavior in the central nervous system has long been neglected in neuroscience, until recent links between mechanical perturbations and axon potential firing were found.1,2 Today we know that mechanical forces or tension are involved in the formation of the cortical landscape folding,3 in neuronal cell morphology,4,5 in neurite or axonal outgrowth,6–10 in synaptic functioning,1,11 and in signal transduction12,13 through mechanically activated ion channels.14,15 A majority of these results are derived from in vitro experiments, where the mechanical stimulus was externally positioned. Applying external forces through glass micropipettes5,16,17 magnetic/optical tweezers,18–21 or stretchable cell platforms,6,11,22 impart mechanical bending, compression, or expansion on the cell plasma membrane. Within this context it was reported that stretching axons resulted in faster accumulation of synaptic vesicles at the growth cone compared to un-stretched neurons.13 But can intracellular mechanical stimulation influence vesicle transport? Answering this question will open ways to use nanoparticle-mediated biomechanical forces, which we call nanomagnetic forces, to interfere with neural pathways and their role and resolution in disease processes. To study a direct force impact on transport behavior of intracellular vesicles, we utilize chitosan-coated superparamagnetic nanoparticles (Chi-NP), which become internalized in neurons through cell intrinsic uptake mechanism such as endocytosis and phagocytosis.23 Exposing Chi-NPs to engineered magnetic field gradients on chip allows us to modulate vesicle trafficking within sub-populations of lipid vesicles in the intracellular space.

Neurobiologists hypothesize that neurons translocate membrane compartments and proteins through vesicles.24 The resulting traffic of vesicles in neurons consists of a wide variety of components and is a critical factor in the
The transport of cargos is mediated by vesicles that can be either membranous vesicles (early-/late-endosomes, to multi-vesicular endosome, lysosomes, or lipid vesicles), mRNA transporters, synaptic precursor protein vesicles, receptor vesicles, or growth factor transporting vesicles.

Second, they bind to microtubules by kinesin and dynein motors and are transported along them in anterograde and retrograde directions (away from and towards the cell nucleus), by showing a diverse set of motion patterns, e.g., fast directed transport, locally “caged” circuits, or almost immobilized “docked” behavior. The amount and type of vesicles, as well as their transport behavior, depend on the cell developmental and functional state.

Third, during neuronal development a neuron exhibits an asymmetric cell morphology resulting in the formation of an axon and multiple dendrites. This cell polarization process involves the sorting of Tau and Map2 proteins in the specific axonal and dendritic cell compartment, to build up and stabilize the cytoskeleton there. Thus, during polarization a neuron has to differentially organize its vesicles into functionally opposite cell compartments.

Impaired vesicle transport can lead to mis-sorting of proteins resulting in developmental disorders or neurodegeneration. Considering the example of the protein Tau during axonal development, the more developed the cytoskeleton, the more vesicles can interfere with Tau proteins potentially slowing down transport. An overexpression of Tau in neuroblastoma cells has been shown to impair the transport of mitochondria in neurites. The same group proposed that Tau differentially regulates the attachment and detachment

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**Fig. 1** Controlling vesicle trafficking through magnetic forces. (a) Magnetic forces can establish intracellular protein gradients in cortical neurons, probably due to an altered intracellular trafficking mechanism. While cellular response to a chronic stimulus might be delayed, we want to study here the impact of magnetic forces below 24 h within cortical neurons immediately after applying the magnetic force stimulus. (b) Legend for schematic. (c1 and c2) Schematic demonstrates the altered trafficking hypothesis, where fMNPs get transported within vesicles along microtubules using based on directed (molecular motor driven), or diffusive processes and interfere with superimposed stalling forces under the application of a permanent magnetic fields. (c1) Without magnetic forces vesicle motion follow anterograde and retrograde routes. (c2) With magnetic field gradients local vesicle trafficking can get blocked (perpendicular), or de-/accelerated in a specific direction (parallel) due to the mechanical interplay of vesicles filled with fMNPs.
but not the speed of kinesin motors,\textsuperscript{27} which leads to a Tau gradient with a low concentration in the soma and high concentration near the growth cone.\textsuperscript{30,33} Dixit \textit{et al.} proposed that an inverted Tau concentration gradient with high concentrations of Tau near the soma might regulate degeneration processes in neurons. Thus, through engineering Tau concentrations in neurons we may prevent neurons from degenerating their networks, especially, when no other growth cues are available. In this context, we have shown previously that a nanomagnetic force stimulus 5–70 pN, applied over 24 hours, locally modulated Tau distribution in cortical neurons.\textsuperscript{34} While the translocation of Tau was related to the nanomagnetic force strength, how vesicle transport gets impacted under the application of nanomagnetic forces remains an open question.

Here we investigate how intracellular nanomagnetic forces can impact the transport pattern of lipophilic dye labeled vesicles in cortical neurons over short time scales (Fig. 1). Using previously developed arrays of magnetizable permalloy elements,\textsuperscript{34,35} we applied localized forces on nanoparticle-laden vesicles to explore the effect of force on the motility of vesicles in a highly-parallelized manner (Fig. 2a1–a3).

**Experimental**

**Neuronal cell culture and endosomal labeling**

Rat cortical hemispheres were dissected from whole embryonic rat brains (E18, BrainBits) and dissociated with 10% (v/v) papain (\textit{Carica papaya}, Roche) in Hibernate®-E (BrainBits) at 35 °C for 15 min. After dissociation, cortical neurons were centrifuged (6 min, 600 rpm, at room temperature) and seeded at a cell concentration of $2 \times 10^6$ cells per ml, drop wise on-chip (150–300 μl). Unbound cells were removed after 2 h incubation through a gentle washing step and incubated overnight (95% air, 5% CO\textsubscript{2}, 37 °C) in Neurobasal serum free with 2% (v/v) serum free B-27®, 1% (v/v) GlutaMAX\textsuperscript{TM} and 1% (v/v) Pen Strep. Pre-differentiated, mixed population neurons derived from human induced pluripotent stem cells (human iPSC line, XCL-1) were thawed drop-wise in 37 °C neuronal medium (Neuro Kit, XN-001-S-NH, Xcell Science) and counted on a hemocytometer following resuspension. Cells were then seeded at $2 \times 10^6$ cells per ml density by placing 250 μl on-chip and cultured in complete neuronal medium for the remainder of the study. To visualize different intracellular vesicle types in rat cortical neurons we used CellLight® Late Endosomes-RFP, BacMam 2.0 to fluorescently label Rab7a.
proteins in late endosomes, and two lipophilic dyes: Vybrant® DiD (1,1'-dioctadecyl-3,3',3',3'-tetramethylindodicarbocyanine 4-chlorobenzensulfonate salt) and DiI (1,1'-dioctadecyl-3,3',3'-tetra-methylindodicarbocyanine perchlorate) cell-labeling solution to highlight intracellular lipid vesicles and LysoTracker® Deep Red to stain acidic lysosomes. RFP late endosomal transfection was combined with DiD (far-red fluorescent) vesicle staining or Deep Red LysoTracker staining and with DiI (red-fluorescent) with DiD or LysoTracker. Baculovirus transfection of Rab7a was started 16 h prior to nanoparticle exposure in neurons at day one in culture with 50 particles per cell. LysoTracker labeling was optimal when loaded 2 h prior to imaging at a final concentration of 100 nM in 5 ml. DiD and DiI staining was achieved following vendor protocol. Prior to nanoparticle incubation, 100 μl of a 0.5% (v/v) DiI or DiD/media solution was administered to neurons on-chip, incubated up to 1 h and subsequently gently washed with pre-warmed culture media (2×).

**Highly parallelized on-chip nanomagnetic force induction**

To impose an intracellular mechanical force, cortical neurons were incubated with 30 μg ml⁻¹ chitosan or dextran bi-conjugated green fluorescent iron oxide nanoparticles (super-paramagnetic, ḳ_{d,vendor} = 100 nm, nano-screenMAG/G-Chitosan or -D, Chemicell) for six hour prior to experimental manipulation. Unconjugated iron oxide nanoparticle were shown to induce cytotoxic effects in neuronal-like cells to the use of superparamagnetic nanoparticles. Local magnetic field gradients were generated through ferromagnetic neodymium magnet (Apex Magnets) was placed on the top right side of the imaging platform and immersed into CO₂-independent pre-warmed Hibernate E low fluorescence imaging media (BrainBits). Prior to magnetic force induction vesicle motion was recorded and individual cell position saved for additional experimental procedures (no magnetic force experiment). A neodymium magnet _B_ = 150 mT (½ inch × ½ inch × ½ inch, Apex Magnets) was placed on the top right side of the neuromagnetic chip to expose the culture to a permanent magnetic field and to allow phase contrast microscopy. Vesicle movement was monitored with a delay of 1–2 min after magnetic field and force induction (with magnetic force experiment).

Within magnetic field gradients a mechanical force imposes on the superparamagnetic nanoparticles. Local magnetic field gradients were generated through ferromagnetic iron nickel alloys called magnetic elements (MEs) on chip within a permanent magnetic field. The force range for different cluster sized fMNPs has been characterized previously using Stoke’s and Faxen’s law and were re-confirmed here through COMSOL simulation. Briefly, 4 μm × 8 μm × 4 μm (H × L × T) MEs generated a magnetic field gradient of ~20 A m⁻² within a permanent magnetic field (_B_ = 150 mT, _d_ = 1.5 mm, ½ in. × ½ in. × ½ in., Apex Magnets). Correlation between magnetic element size and particle volume dependent magnetic forces was previously estimated. Nanoparticle clusters of _r_ = 400 nm, 490 nm and 750 nm resulted in _F_ = 6.1–19.2 pN, 11.3–35.3 pN and 40.1–125.7 pN, respectively. The resulting magnetic force stimulation area opened with the use of an array of six MEs (2 × 3, 4 μm × 8 μm), 16 μm spaced in _x_-direction and 4 μm spaced in _y_-direction; or of three MEs in one column 16 μm spaced. The cell adhesion pattern was in symmetry above the ME array covering a surface of 20 μm × 20 μm. A pattern line (7.5 μm wide and 50–100 μm long) connected the cell pattern region with MEs with an ME empty cell region. The resulting magnetic field strength and magnetic field gradient pattern near the magnetic elements are shown in Fig. S1 in ESI.

**Biochemically blocking and activating liposomal motion**

Biochemically altering vesicle transport was achieved through blocking and activating vesicle movement using monastrol and insulin. For blocking, we prepared 1 mM monastrol (Sigma, M8515) stock solution by dissolving lyophilized powder (≥98%, HPLC) in DMSO. An aliquot of 100 μl was dissolved in 1 ml Hibernate E and chips were immersed into imaging solution and let incubated for 10 min prior to imaging. Vesicle transport was accelerated through the addition of 10% (v/v) human insulin (Sigma, I9278, 10 mg ml⁻¹ stock), to media in the imaging platform, where neurons were incubated for at least 20 min prior imaging. Note: insulin concentration was intentionally chosen high above physiological condition to activate a cells response within few minutes.

**Image acquisition and analysis**

Life cell imaging of fluorescently labeled vesicles in cortical neurons were semi-automated captured using a programmable stage (MV-2000 Te/Ti 2000 He, ASLimaging) on a Nikon microscope (Eclipse Ti, DAPI, FITC, TRITC, CY-5 filters, 60× oil, NA = 1.4 objectives) with a CCD camera (QuantEM:512SC EM, Photometrics) operated at fixed gain. Cell position was captured and referenced with magnets applied in positive-X-axis pointing towards right. Channel exposure times were set to CY-5: 800 ms, TRITC: 800 ms and FITC: 500 ms. Multi-channel image acquisition was programmed with 3–6 s time intervals to capture 3 channels/time point. Multi-channel time stacks, which contained vesicle position _x_(_p_), _y_(_p_), _z_(_p_) with _n_ number of vesicles and _p_ position at multiple time points _t_(_p_), were further processed as 8-bit color image sequence, corrected for transformative shift with StackReg plugin (ImageJ) in case of occurrence and histogram corrected for auto bleaching. Subsequent, moving vesicle dots were tracked using TrackMate (DOG detector, 0.8 μm blob diameter; filters: median intensity, estimated diameter and signal/noise ratio; linking: 1.6 μm) and exported into .xml files. A MATLAB script excludes tracks shorter than 108 s, generated star plots for
tracks \(\Delta t_{\text{max}} = 2 \text{ min}\), computes mean square displacement \(^{47}\)

\[
\text{MSD, } \Delta t = 57 \text{ s}, r = 3 \text{ s}, \text{ MSD} = \frac{1}{P - r} \sum_{t = r}^{P - 1} \left( \frac{X_{t+1} - X_t}{r} \right)^2, \text{ averaged}
\]

“caging” diameter \(^{48}\)

\[
\text{CD, } \Delta t = 114 \text{ s}, \text{ CD}_{\text{avg}} = \frac{1}{k} \sum_{t = 1}^{k} \text{ CD} \left( t \right),
\]

total traveled length

\[
L, \Delta t = 114 \text{ s}, L_{\text{real}} = \sum_{t = 1}^{k} L_t \text{ and averaged}
\]

velocity

\[
V_{\text{avg}} = \frac{1}{P} \sum_{t = 1}^{P - 1} \frac{L_t}{t + 1 - t - 1} \text{ and outputs frequency}
\]

for categorized vesicle behavior based on CD and \(L\). MSD of vesicle tacks were then compared with MSD model \(^{47}\) (2D case) of stationary processes for free diffusion \(\text{MSD}_{\text{diff}} = 4Dt\), intracellular free diffusion \(\text{MSD}_{\text{cell}} = 4k_{\text{cell}}D_{l,H_2O}(\tau^2)\) and confined diffusion \(\text{MSD}_{\text{conf}} = R_c^2(1 - e^{-4k_{\text{cell}}D_{l,H_2O}(\tau)/R_c^2})\), with \(k_{\text{cell}} = 0.05\), Stokes–Einstein: \(D_{l,H_2O} = 37^°\).

Statistical analysis

Velocity and length distributions were tested against normality distribution appropriate ANOVAs were chosen based on parametric and non-parametric test routines (OriginPro 9, \(p < 0.001\) or otherwise indicated). Histogram distribution of CD–L categories were compared using Chi-Square test \(^{19}\) and were visualized based on \(p > 0.05\) (not significant) and \(p \leq 0.05\) (significant different).

Results & discussion

Chi-NP prefer to internalize into DiI labeled vesicles in primary cortical neurons

To study magnetic forces on intracellular lipid vesicle motion pattern in 48 h cultured primary cortical neurons (E18, rat), chitosan coated superparamagnetic nanoparticles (Chi-NPs) were incubated to enter the neuronal cytosol through cell intrinsic uptake mechanism. We chose chitosan coated functionalized superparamagnetic nanoparticles (Chi-NP) due to their neuroproective effect. \(^{50,51}\) Nanoparticles can enter the neuronal cytosol through active, or passive uptake mechanisms, or diffusive through the cell membrane. Because the surface charge and the hydrodynamic radius highly influences the uptake mechanism, \(^{52}\) we quantified the surface charge through the zeta-potential and the hydrodynamic radius. The Chi-NP surface charge was cationic in aqueous solution with a hydrodynamic diameter \(h_{d,H_2O} = 210 \text{ nm}\) (Zetasizer). In neuronal medium, however, we found that Chi-NPs cluster, most-likely formed a protein corona, \(^{53}\) which modified their zeta-potential \(\zeta\) from \(\zeta_{H_2O} = 11.4 \pm 6.0 \text{ mV}\) to \(\zeta_{\text{Neuro}} = -9.7 \pm 0.3 \text{ mV}\) and their diameter up to \(h_{d,\text{Neuro}} = 630 \text{ nm}\) after 24 h (Fig. 2b), which was also reported in other studies. \(^{34}\) Thus, primary cortical neurons most-likely respond to cationic nanoparticle clusters.

Based on active, or passive uptake mechanism nanoparticles may end up in early- and late endosomes, lysosomes or other vesicle types (e.g. synaptic vesicles, multi-vesicular endosomes, Fig. 2a2). The association of Chi-NPs with intracellular vesicles in primary cortical neurons was visualized through functionalized green fluorescence (Em: 476 nm, Ex: 490) Chi-NPs co-labeled with four different vesicles markers (late endosome, lysosome, lipophilic dye DiI and DiD labeled intracellular vesicles).

After, six hour incubation with the Chi-NP (30 g ml\(^{-1}\)), we analyzed 709 fluorescent vesicles in 22 individual neurons from five different neuron preparations. Based on the green signal from the Ch-NPs and the fluorescent signal from labeled vesicles (DiI and DiD), a late-endosomal marker (CellLight Bacmam, Rab7a-RFP) or a lysosomal marker (LysoTracker FarRed), we found Chi-NPs overlapping with 30.0% fluorescent lysosomes, with 44.5% DiI, with 12.7% DiD labeled vesicles and with 15.5% late endosomes (Fig. 2c1, c2 and d). When comparing DiI and DiD co-labeled images we observed that most DiI spots do not co-position with DiD spots (ESI,† Fig. S4). In contrast, late endosomes overlapped with 90% of the DiD spots (Fig. 2c1) and 43% of the lysosomes overlapped with DiI vesicles. This observation suggests that DiD and DiI labeled intracellular vesicles are not the same type of vesicles. Because we found most Chi-NPs to be located in DiI labeled vesicles, we decided to study the nanomagnetic force effect in neurons in DiI vesicles and to compare our findings to DiD labeled vesicles.

Vesicle trafficking base on the Stokes–Einstein theory depends on the vesicle diameter. Chi-NPs clusters in DiI labeled vesicles and lysosomes presented an average spot diameter of 0.63 \(\mu\text{m}\) (range 0.3–1.7 \(\mu\text{m}\)). NP clusters in the few late-endosome and DiD-labeled vesicles had average spot diameter of 0.7 \(\mu\text{m}\) (range of 0.3–1.4 \(\mu\text{m}\)). Comparing area distribution of DiI and DiD vesicles in neurons incubated with and without Chi-NPs (ESI,† Fig. S4b and c) did not result in a significant increase of 0.5 \(\mu\text{m}^2\) area sized DiI spots for 0.87 \(\mu\text{m}\) NP clusters and even decreased the amount of 0.4 \(\mu\text{m}^2\) sized DiD spots. Thus, the size of DiI labeled vesicle was not impacted through the uptake or association with Chi-NPs.

In summary, although possessing a large hydrodynamic size, Chi-NPs sequester into the endo-lyosomal network and show highest co-positioning with DiI labeled intracellular vesicles after six hour of incubation without impacting significantly the size of intracellular vesicles in primary cortical neurons.

Magnetic forces preferentially alter lipophilic dye, DiI, stained vesicle motion

Assuming magnetic forces impact vesicle motion locally, we analyzed the position of DiI and DiD labeled vesicle spots in image sequences after six hour nanoparticle exposure before applying and with the magnetic field. While size distribution of uptaken Chi-NP clusters cannot be controlled during experiments, nanomagnetic forces were ensured to
remain below 100 pN, by a fixed magnetic element (MEs) design using a previously developed neuromagnetic chip in combination with an externally applied 150 mT (B_{max}) permanent magnet. A poly-L-lysine pattern restricted cell attachment of neurons adjacent to the MEs such that the applied magnetic forces are localized peripheral to the cell nucleus (Fig. 2a3).

Fig. 3a shows a representative image sequence of change in position of DiI vesicles carrying Chi-NPs without an intracellular magnetic force stimulus. Co- positioned Chi-NPs

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**Fig. 3** Dil-stained lipid vesicles respond to Chi-fMNP induced magnetic forces. (a) Image sequence of Dil labeled cortical neuron (E18, 2 DIV) highlights an active moving vesicle without superimposed magnetic forces (no M). P_0: start position = arrow at t = 0 s. Green arrow indicates time specific current position. (b) Image sequence of the same neuron applied to magnetic fields (w M) inducing magnetic forces on Chi-fMNP-loaded vesicles (yellow/orange due to red – Dil and green – fMNP co-localization). (c1) Image region of interest above 6 pattern MEs used for tracking vesicle motion. (c2) Vesicle track map of automatic detected vesicles and generated tracks. (d) Averaged velocities (box-plot) of DiI and DiD-labeled vesicles were monitored with and without superimposed magnetic forces. (e1-e3) Centered vesicle trajectories of tracked DiD and Dil vesicles were extracted from the same cortical neuron (e1) with (w M), (e2) without magnetic forces (no M), and in (e3) shown as XOR plots. (f1 and f2) Mean-square displacement diagram (MSD) shows MSD values for individual tracks (exp. data) in comparison with free diffusion (outside the cell), diffusion within the cytoplasm (diffusion in cell), and confined diffusion within the cytoplasm (confined).
move in the same direction as Dil vesicles indicating association or even co-localization, see ESI†. The same field of view was then exposed to an external magnetic field \((B_{\text{max}} = 150 \text{ mT, stationary})\), which immediately altered the vesicle motion pattern due to applied nanomagnetic forces (Fig. 3b, ESI† Video V1 and V2). Vesicle displacement was monitored over a 180 s time period at 3 s intervals within a region that included the cell body and neurites \((40 \mu\text{m} \times 40 \mu\text{m})\), from which vesicle tracking plots were generated using TrackMate† (Fig. 3c1 and c2).

From the individual vesicle tracks we analyzed the average vesicle speed (Fig. 3d), space orientation of vesicle tracks (Fig. 3e1–e3, star plots) and quantified the mean square displacements (MSDs) over a time lag \((r)\) of 90 s and compared them against MSDs of five potential models: (1) average-sized spherical vesicle, \(d_v = 300 \text{ nm}\), free diffusive, no viscous restriction; (2) same as (1), with viscous restriction \(k_{\text{cell}} = 0.05\), \(\eta_{\text{cell}} \approx 0.05 \eta_{\text{sub}}\), (3) same as (1), confined restriction; (4) same as (2) for a \(d_v = 180 \text{ nm}\); and (5) same as (2) for larger intracellular vesicles \(d_{\text{h,spect}} = 800 \text{ nm}\) (Fig. 3f1 and f2).

Without nanomagnetic forces (no magnetic field, or no fMNPs) the average speed of both Dil and DiD intracellular vesicles was found around 12.5 nm s\(^{-1}\) and falls in the category of slow “axonal” (= neurite) transport,\(^{56}\) in contrast to fast single kinesin motor driven, fast axonal transport of 800 nm s\(^{-1}\).\(^{57}\) With magnetic forces, Dil vesicle speed resulted in an averaged 7 nm s\(^{-1}\) (Fig. 3f, ANOVA for non-normally distributed data \(p < 0.001\)). The reduction of speed can be explained though oppositely acting nanomagnetic forces against the direction of vesicle displacement. The differences in speed reduction between Dil and DiD labeled vesicles can be explained by the difference in association with Chi-NPs. We recall here that 44.5% of all Dil vesicles throughout the neuronal cell body co-positioned with Chi-NPs, but only 12.7% of DiD vesicles (Fig. 2). To test the impact of directed nanomagnetic forces on uni-directional vesicle displacement we compared vesicle motion patterns with and without magnetic field exposure. Fig. 3e1–e3 show the motion pattern in star plots for Dil and DiD vesicles (maximal tracking time of \(t_{\text{max}} = 114 \pm 6 \text{ s}\) ) loaded with Chi-NPs both with and without an applied magnetic field. We observed that the plot of Dil vesicle tracks altered under the application of a magnetic field. In contrast, DiD vesicle star plots remain very similar (Fig. 3e3).

Furthermore, MSD curves of intracellular Dil vesicles being exposed to Chi-NPs indicate that vesicle motion can be classified as confined diffusive under high viscosity (in cell, Fig. 3f1 and f2). Averaging all MSD curves shows a super-diffusive trend for vesicles being exposed to Chi-NPs in contrast to vesicles without nanoparticles (ESI† Fig. S5).

In summary, we can conclude that nanomagnetic forces differentially act on DiD and Dil labeled intracellular vesicles in cortical neurons. Specifically, DiD vesicles motility was slowed down significantly due to superimposing nanomagnetic forces blocking the displacement of vesicles unidirectionally.

### Nanomagnetic forces outrun biochemical effects on Dil labeled vesicle motion

Lipid vesicles are very important endosomes in neuronal cells when it comes to taking up extracellular components, transporting cargos or exchanging cargos.\(^{48}\) During these processes vesicles are assumed to be in different motional states, such as totally immobile “docked”, weak diffusive “primed”, or transporting “mobile”, and forces might impact these categories.\(^{11,13,22}\) Therefore, the next question we addressed, is, if we can observe different categories of vesicle motion, and if in our case intracellular forces impact the amount of vesicles in one particular category. To classify and distinguish between “immobile”, “primed” and different “mobile” types of vesicles in our experiments we used a trajectory metric recently introduced by Nofal et al. called “caging” diameter.\(^{48}\) The “caging” diameter (CD) is described as the maximum displacement reached by a vesicle within a specific time frame. Nofal et al. used the CD plots versus time plots to identify fluctuation patterns, which they used to discriminate between three types of vesicle motion: “primed” (nearly immobile, or stationary), “caged” (confined diffusion, perpetual motion), and “directed” motion (long-distance).\(^{58}\) In our experiment, the time length of 114 s includes fluctuations in speed due to changes between categories, e.g. a mobile vesicle stops and then resumes moving.\(^{22}\) These fluctuations are best seen when the total traveled length \(L\) is also taken into account. Scatter plots of CD versus \(L\) demonstrate a larger diversity of molecular movements, which we classified here into five categories: (i) primed, (ii) caged, (iii) primed-transported, (iv) caged-transported and (v) directed transport based on their trajectory appearance and corresponding threshold values for CD and \(L\) (Fig. 4d).

Under conditions with Chi-NPs but no applied magnetic field (Fig. 4f, N: w/Chi-NP, w/o M), we found 70% of our vesicles motion falling under category I, which corresponds to immobile or docked motion. Furthermore, we found 9% of vesicles in caged motion (category II), 13% in primed-transport motion (category III), 4% with caged and directed motion (category IV) and 5% in directed motion (category V). By applying magnetic forces, ranging from 6 pN to 126 pN \((B_{\text{max}} = 0.15 \text{ T, } \sim 20 \text{ A m}^{-2}, 4 \mu\text{m} \times 8 \mu\text{m} \text{ ME, } h_\text{d} = 0.8 \mu\text{m} \text{ and } 1.5 \mu\text{m})\) the number of vesicles shifted within the different motion categories (Fig. 4f). Different motion categories, however, were affected differently. The number of vesicles in direct motion reduced to 0.5%, caged-directed motion reduced to 4.5%, primed-directed motion to 2% and caged motion to 1.3% 67%, which corresponds to a decrease about 90%, 95%, 85% and 67%, respectively. Faster transport categories such as directed and caged-directed were more strongly affected by the magnetic force impact.

### Insulin-stimulated vesicles potentiate magnetic force driven orientation of vesicle movement

To further evaluate the link between liposomal vesicle mobility and applied magnetic forces, we altered vesicle
motility in two ways: (i) by indirectly activating and (ii) by inhibiting vesicle transport (Fig. 5a). First, we aimed to promote the activation of DiI vesicle dynamics through the addition of 50 nM insulin to the 2 day old cortical neurons in culture (Fig. 5a1). Insulin has been reported to impact the AMPA pathway,59,60 and Rab4 pathway. In particular, the former pathway seems to foster internalization of extracellular liquid, or components,61–63 potentially signaling in cortical neuron to recruit more vesicles for endocytosis and to accelerate the transport of vesicles to ensure increased exocytosis.42 In Fig. 5b1 the star plot, for tracked vesicles upon exposure to insulin and internalized Chi-NP, presents longer trajectories for the same time interval, which indicates an increase in vesicle activity due to faster transport. Looking closer into the different motion categories shows that adding insulin to the neuron culture impacted caged and primed-directed motion most significantly (67–80%) and increased directed motion about 25% (Fig. 5c1, experiment: T1 (no NPs), Chi-Square test = p < 0.05, h0: A = N, A: activated, N: no treatment). On top of it, adding Chi-NP

![Fig. 4 Classifying vesicle motion based on a “caging diameter” exhibits a more diverse range of motion patterns in contrast to the MSD. (a) Vesicle example and definition of caging diameter. (b) One set of centered Dil vesicle tracks. (c) Scatter plot of averaged “caging” diameter (CD) over total traveled length (L) within s. (d) CD–L plot used to categorize vesicle tracks based on five characteristic trajectory paths separated by numerical thresholds (horizontal and vertical lines). (e) Five different vesicle track categories corresponding to the black dots in (d): Cat I: docked, Cat II: caged, Cat III: mixed transport, Cat IV: caged with directed motion, Cat V: directed transport. (f) Bar histogram plot of relative counts per category for three independent experiments. Zero: no motion/totally immobilized. N_{traj} = total number of trajectories per ROI, w M with magnetic field, no M no magnetic field.](image-url)
significantly increased the amount of directed motion (about 270%, Fig. 5c1, T6).

Similar to normal conditions, adding magnetic forces to our activated vesicle motion resulted in 62% more primed vesicle motion (Fig. 5c1, T10). In our opinion, the observed stalling effect is caused by mechanical barriers (the cell membrane) and the imposing of stalling nanomagnetic forces. Assuming an average neurite growth rate of 1 μm min⁻¹ and perpendicular acting nanomagnetic forces explains well our observation of primed motion within our experimental time frame (114 s). Further indication for the mechanical barrier effect can be found in Fig. 5b1 (w insulin, w M), where vesicle trajectories show a higher organization of movement directionality following the orientation of the magnetic force...
vectors. While activation is one way to study interactions between vesicle movement and magnetic forces, another way is to block molecular motors. Using monastrol we aimed to inhibit vesicle motion based on the assumption of inhibiting anterograde axonal transport mostly involved in neural migration and axonal development. Under monastrol exposure (100 μM, B) without the addition of Chi-NP vesicle motion did not change significantly compared to untreated (normal) conditions (Fig. 5c1, T2 Chi-Square \( p > 0.05 \), ESI† Video V3–V5). This finding is not surprising as we reported earlier that without nanoparticles MSD was confined diffusive. With the addition of Chi-NPs, however, we observed more vesicles with caged-direct and direct transport motion than without nanoparticles under normal condition (Fig. 5c1, T6, Chi-Square \( p < 0.05 \)). The treatment with monastrol then significantly altered the proportion of vesicle motion categories (Fig. 5b2 and c1, T8, Chi-Square \( p < 0.05 \)). With the addition of magnetic forces on monastrol treated vesicles the proportion of primed vesicles increased slightly from 86% up to 92%, and resulted in zero directly transported vesicles (Fig. 5b2 and c1, T8, Chi-Square \( p < 0.05 \)). Thus, the applied magnetic forces interfere with vesicle motion independently of intracellular biochemical alterations, but show different effect strengths depending on activated or blocked vesicle motion. Furthermore, our findings are in agreement with biophysical observations in vitro, where individual kinesin molecules exhibit 5–6 pN stalling force. As we are operating at a force range above 6 pN, it seems that we may modify motor activity enabling us to switch from one motion categories to another and mechanically guiding vesicle transport direction.

To demonstrate control of vesicle transport directionality, we designed a three ME pattern (Fig. 6a1 and a2) such that it produces different force gradients and directions but similar overall forces (Fig. 6a1 versus a2) in comparison to the 6 ME pattern. For both patterns, we found that directed vesicle motion run parallel to the magnetic force axis (Fig. 6c1 and c2). We observed no changes in the proportion of movement categories between the two designs and the proportions of movement categories was independent of the neuron’s position relative to the MEs (Fig. 6d1 and d2, e: T3, e: T6, Chi-Square, \( p > 0.05 \)). Therefore, these findings indicate that with a given force magnitude, the number of motion categories remain the same, however, the orientation of vesicle motion can be engineered by modulating the magnetic force vector direction.

Surface functionalization of nanoparticles impacts intracellular vesicle motion pattern

Nanoparticles are entering mammalian cells through a cellular membrane using a diverse range of uptake mechanisms such as phagocytosis, micropinocytosis, clathrin- or caveolae-mediated endocytosis, or direct penetration. Anionic nanoparticle clusters have been reported to utilize caveolae-mediated endocytosis with a high proportion getting sorted
into lysosomes in epithelial cells. Further, the amount of surface charge seems to have an impact on the uptake mechanism in neuroblastoma cells and intracellular particle location. To test the impact of magnetic forces on vesicle motion induced by nanoparticles with different surface charges, we incubated the 2 day old cortical neurons with starch-coated, fluorescently-labeled, superparamagnetic nanoparticles (D-NP, $\zeta_{\text{Neuro}} = -0.03 \pm 0.3 \text{ mV}$) for six hour. It was reported previously that starch-coated nanoparticles are less likely to pass the neuronal cell membrane. Thus, we assumed that DiI vesicle motion remains unaffected when exposed to starch-coated nanoparticles. The difference in vesicle motions between Chi-NPs and D-NPs are shown in star plots in Fig. 7. Without a magnetic field DiI vesicles show similar movement patterns independent on the surface charge of the nanoparticles. Only the effect of increase in velocity of DiI vesicles (Fig. 3) when exposed to Chi-NPs was not observable for D-NPs. With magnetic field, the weaker anionic D-NPs have the same potential to generate magnetic forces, however, we observed only 24% reduction in vesicle motion, which can also be explained by a slight alteration in vesicle motion categories induced by the magnetic field in the absence of fMNPs (Fig. 5c1, T3, Chi-Square: $p < 0.05$ $H_0$: no M = w M).

In control studies (no Chi-NPs) performed in primary rat cortical neurons we observed a small but significant increase in primed and caged vesicles solely attributed to the application of the magnetic field (Fig. 6c1 and c2, e: $T_{3,4}$, e: $T_{8,9}$, Chi-Square, $p < 0.05$). A possible explanation can come from the “tug of war” model, which proposes a diffusive state, when none of the molecular motors are bound to microtubules. Forces much below the pico-newton range could in such a diffusive state locally trap or guide vesicle motion. During an unbound state, a magnetic Lorentzian force, due to the charged nature of the vesicle, may induce circular vesicle motion, which we observe as trapped vesicle motion. Alternatively, it has been suggested that cortical neurons of rodents might carry iron oxide content derived from dissociation reactions of iron content, which can potentially interact with a bulk magnetic field. To test if cortical neurons from other species exhibit similar behavior we cultured human neuronal cells derived from human induced pluripotent stem cells (hiPS) on our neuromagnetic platform and compared their vesicle motion after 2 days in vitro to the one in rat cortical neurons under magnetic gradient exposure (no nanoparticles, see ESI† for Fig. S2, Video V6 and V7). Without fMNPs human neurons showed a significant difference in vesicle motion, specifically for directed transport (Cat V, ESI† Fig. S2) compared to rat neurons. Furthermore, the application of a magnetic field on human neurons resulted in no significant changes of overall vesicle motion behavior.

**Conclusions**

In conclusion, we have designed a method to alter the motion of lipophilic dye labeled vesicles in rat embryonic
cortical neurons by applying magnetic forces on cell-internalized chitosan coated superparamagnetic nanoparticles. We provide evidence that our chitosan coated nanomagnets preferentially associate with Dil labeled intracellular vesicles after six hour of incubation and impact their motion pattern. Using our novel micromagnetic chip, we align cortical neurons to embedded MEs using protein patterns, and study the effects of localized nanomagnetic forces ranging from 6 to 120 pN on lipid vesicle dynamics in primary rat cortical neurons. We observed vesicle transport by tracking vesicle trajectories and utilized a caging diameter metric in combination with total traveled length to quantify transport activity. Applying these new metrics to our experimental data, we were able to define five distinguishable categories of vesicle movement, which can be compared to previously reported classifications: primed, docked, primed-directed, docked-directed and directed movement patterns. Super-imposing the nanomagnetic-mediated physical stalling force or re-directing force on vesicles out-run the biochemically stimulation effect through chemical activation and inhibition of vesicle movement. Beyond this point, we observed that the type of surface charge at the shell of the nanomagnets has an impact on the location of where the nanomagnetic forces act and therefore impacted the force effect on vesicle motion patterns. Controversially, in rat cortical neurons vesicle motion seems to be sensitive to bulk magnetic fields (150 mT), without the presence of nanomagnets, but not in human excitatory neurons. Having shown a link between applied magnetic forces and vesicle dynamics, now opens the possibility to connect magnetic forces to protein formation, cytoskeletal changes and intracellular signaling pathways in neurons in the future. As a consequence, future study can be targeted to spatially and temporally control transport and signaling events in cortical neurons such as inhibiting the propagation of tangles of the microtubules-associated protein Tau, or re-orienting cytoskeleton structure after injury. In a broader perspective, controlling vesicle motion locally and intracellularly by magnetic forces can bring many benefits to pharmacological treatments, where temporal and spatial cell signal administering are crucial.

Author contributions
A. K. designed, performed, analyzed chips and experiments, interpreted data, wrote and revised the manuscript. C. M. fabricated chips, wrote and revised the manuscript. C. G. assisted with experiments wrote and revised supplementary data. K. O. developed and performed numerical simulations, and wrote the manuscript. J. L. provided differentiated human neurons, wrote the manuscript. A. T. characterized nanoparticle uptake. D. D. interpreted data, wrote and revised the manuscript.

Competing financial interests
The authors of this manuscript declare no competing financial interest.

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