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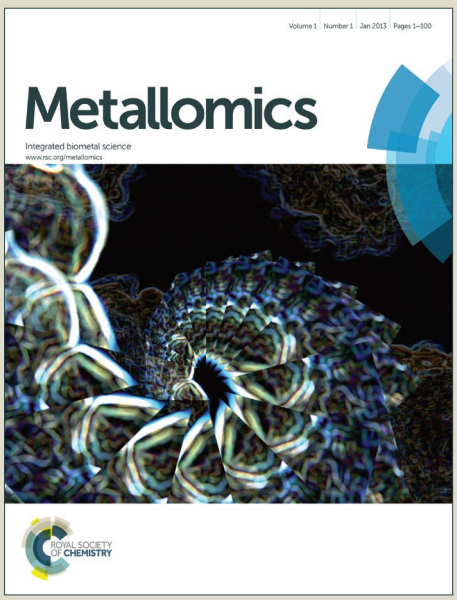

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N-cadherin-mediated cell adhesion is regulated by extracellular Zn^{2+}

N-cadherin-mediated cell adhesion is regulated by extracellular Zn^{2+}

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Synapses in the central nervous system (CNS) are highly dynamic structures that undergo reorganisation in response to synaptic activity. Dysfunctional structural synaptic plasticity is associated with impaired brain function and several neurological disorders. As response to synaptic activity, dendritic spines of excitatory synapses were reported to undergo alterations in their molecular structure and morphology leading to increased postsynaptic density size and spine volume. For these structural changes a transient activity-dependent weakening of synaptic adhesion will be necessary. Here, we report that zinc can modulate N-cadherin-mediated adhesion. Quantification of binding activity was performed using laser tweezer technique. Our results show that increased levels of zinc abolished N-cadherin binding without altering the number of N-cadherin molecules expressed at the cell surface. Furthermore, zinc directly interacted with N-cadherin and the regulatory role was found to take place under physiological zinc concentrations within minutes. Given that zinc is released at zincergic synapses in the CNS, our findings may contribute to mechanistic insights in the interplay between zinc signalling, activation of glutamate receptors and downstream pathways, and the coordination of pre- and postsynaptic changes via trans-synaptic cell adhesion complexes, all finally contributing to synaptic plasticity.

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Introduction

Zinc is the most prevalent trace metal in the brain and plays an important role in processes such as synaptic plasticity, the underlying mechanism of learning and memory. While most of the total brain zinc exists in a protein-bound state (>80%), "free" zinc as Zn^{2+}-ions can be found predominantly within synaptic vesicles [1, 2], where it is released with glutamate from glutamatergic (zincergic) presynaptic terminals [3, 4]. In particular, zinc-containing neurons are found predominantly in limbic and cerebrocortical regions [5], where the release of zinc upon synaptic activity is followed by an increase in Zn^{2+} concentration in the synaptic cleft as well as the postsynaptic intracellular compartment. Here zinc might enter mainly through NMDA and AMPA receptors, and calcium-channels [6] or is released within the postsynapse from internal zinc stores and metallothioneins (MTs) [7-9].

Within the synaptic cleft, zinc is known to bind to various neuronal ion channels and receptors, such as GPR39 [10], TrkB receptors [11], and NMDA and kainate receptors [12-14]. To that end, free zinc levels are tightly regulated, for example by zinc transporters such as Zrt-Irt-like proteins (ZIP) and the zinc transporter (ZnT) family. Intriguingly, the zinc exporter ZnT-1 was found enriched at postsynapses [15] hinting at an additional zinc extrusion mechanism directly localized to synapses. Disruption of zinc signaling is involved in several neurodegenerative and neuropsychiatric disorders such as Alzheimer's disease (AD), Autism Spectrum Disorder (ASD), and Schizophrenia (SCZ) [16]. Moreover, zinc deficient rodent animal models display impairments in memory and learning [17-19]. Thus, it is of major interest to investigate the molecular

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mechanisms by which zinc might regulate morphological and physiological changes at synapses underlying synaptic plasticity.

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There is increasing evidence that cell adhesion molecules are involved in modulation of synaptic structure and function [20-22]. Trans-synaptic cell adhesion is mediated by cadherin complexes that modify adhesive contacts, activate signal transduction pathways, and regulate synapse formation and plasticity [23]. Inhibition of N-cadherin function, the most widespread cadherin of the nervous system, has profound effects on activity-dependent spine enlargement and suppresses long term potentiation (LTP) [24-26]. Transient weakening of adhesion contacts with subsequent formation of new contacts may be one prerequisite for structural reorganization of synapses. However, how morphological changes and synaptic function are coordinately regulated has not yet been identified. In the past we have proposed a model that activity-dependent local changes of ion concentrations within the synaptic cleft during synaptic activity may initiate such a weakening of adhesion contacts. We could show that N-cadherin binding is regulated by extracellular Ca^{2+} ions as well as protons [27-29]. Thus, modulation of adhesive activity of N-cadherin by local changes of ion concentrations may be one mechanism of how N-cadherin mediates synaptic remodeling.

In this study we followed the hypothesis that transient local changes of the free zinc concentration in the synaptic cleft occurring during synaptic activity might affect N-cadherin binding activity and thereby modulates synaptic morphology and function. We found a direct regulatory interaction of zinc and N-cadherin. Thus, the interplay between zinc and N-cadherin might provide a molecular basis for the structural alterations accompanying plasticity of zincergic excitatory synapses.

Results

Zinc dependency of N-cadherin binding

To investigate if N-cadherin binding is modulated by Zn^{2+} we studied the trans-interaction of recombinant N-cadherins with endogenous N-cadherins expressed on the surface of PC-12 cells. A fusion protein of the extracellular domain of N-cadherin and the Fc-portion of human IgG (N-cad-Fc) was generated to yield functional cadherin dimers as described previously [28, 29]. N-cad-Fc-coated microbeads were allowed to settle 15 min on the cell surface of PC-12 cells leading to adherens-like junctions between microbeads and cells as shown by the recruitment of characteristic proteins of adherens junctions to bead-cell-contacts [27, 29, 30]. Subsequently the laser tweezer technique was applied to quantify the amount of beads binding to cells in dependence of the zinc concentration in the culture medium. This experimental model of measuring cadherin-binding activity at bead-cell-contacts is a very useful system since it allows exact definition of one side of the adhesion contact (i.e. the coated beads) and allows on the other hand measuring cadherin binding in a cellular context.

This technique was applied to N-cad-Fc-coated beads on the cell surface of PC-12 cells using culture medium with various concentrations of zinc. The free zinc concentration of the medium was measured by spectroscopic analysis using the zinc-dependent fluorophore FluoZin-3. Table 1 shows the results of these measurements of the culture media after addition of various amounts of $ZnCl_2$ as well as the values after chelation of zinc (TPEN) and at saturated conditions. The control culture medium (DMEM plus 1% FCS, no addition of zinc) contained about 259 pM of free Zn^{2+}. By addition of 2 µmol/l zinc chloride the free Zn^{2+} concentration was actually increased to 385 pM. Most of the added zinc ions were obviously buffered by zinc-binding molecules of the culture medium.

Table 1: Determination of the free Zn^{2+}-concentration in culture medium: $ZnCl_2$ (from isotonic $ZnCl_2$-solution) was added to DMEM containing FCS as indicated in the first column and incubated with FluoZin-3. Fluorescence was measured for three independent approaches and the free Zn^{2+}concentration was calculated for each concentration. Background and maximal fluorescence was determined by addition of TPEN and 250 µM Zn^{2+} and free Zn^{2+} levels were calculated as described above.

added substance	Fluorescence (arbitrary units)	free Zn^{2+} (pM)
TPEN	601	0
0 µM Zn^{2+}	1556±109	259
0.5 µM Zn^{2+}	1798±130	290.5
1 µM Zn^{2+}	1862±58	322
1.5 µM Zn^{2+}	2117±65	353.5
2 µM Zn^{2+}	2002±145	385
250 µM Zn^{2+}	36787	-

The percentage of N-cad-Fc-coated beads tightly bound to the cell surface (resisting displacement by the laser tweezer) was counted at different zinc-concentrations and the results are depicted in figure 1. Under control conditions, i.e. using cell culture medium without additional zinc, 43 ± 3.2% of N-cad-Fc-coated beads adhered to PC-12 cells. Specificity of bead binding was controlled by the use of Fc-coated beads as well as by measuring binding activity at Ca^{2+}-free conditions, i.e. after addition of 5 mM EGTA. Under these conditions 15 ± 3.5% and 17 ± 4.5% beads were bound, respectively, reflecting the amount of unspecific bead binding (fig. 2). Unspecific bead binding (Fc-coated beads) was not affected by addition of zinc (fig. 1). If the zinc-concentration in the medium was increased cadherin-mediated bead binding dropped to basal levels. At a free Zn^{2+}-concentration of 385 pM only 16 ± 2.5% of N-cad-Fc-coated beads were bound which represents unspecific bead binding. Half-maximal binding activity (K_D) was observed at roughly 310 pM free Zn^{2+}. This value was estimated at the concentration where the linear interpolation- curve of the data crosses the 50% difference of minimal and maximal binding activity.

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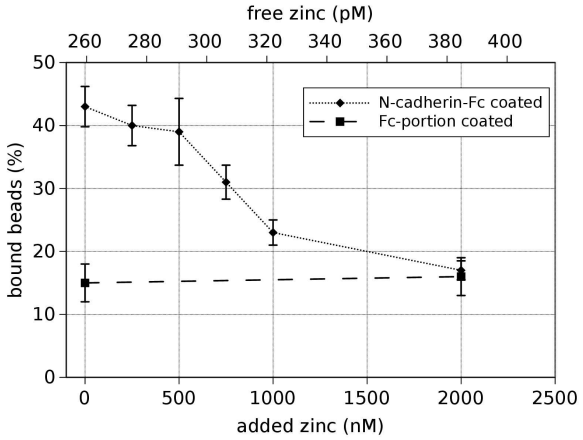

Figure 1: Zinc dependence of N-cadherin mediated adhesion. The percentage of N-cad-Fc coated microbeads adhering to the surface of PC-12 cells was measured at various extracellular Zn^{2+} concentrations using the laser tweezer technique. The free Zn^{2+} concentration is given on the x-axis as well as the amount of Zn^{2+} added to the culture medium to achieve these free Zn^{2+} levels. N-cadherin-mediated bead adhesion decreased with increasing Zn^{2+} concentration ($K_D \approx 310$ pM). Fc-coated beads showed only low binding which was independent of Zn^{2+} and resembled unspecific binding activity. Data are presented as means ± standard deviation. All data points represent at least three individual experiments with probing of 100 beads.

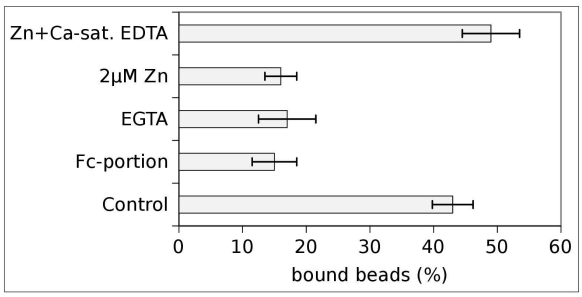

Figure 2: Control experiments demonstrating specificity of bead binding. Unspecific bead binding amounted to ~ 15% as assessed by the use of Fc-coated beads or N-cad-Fc-coated beads in the absence of Ca^{2+} (EGTA). N-cad-Fc-coated beads bound to 43% at control conditions (see also fig. 1) whereas it dropped to basal levels after addition of 2 µM total Zn^{2+} (corresponding to 385 pM free Zn^{2+}). Chelation of extracellular Zn^{2+} by addition of calcium-saturated EDTA restored bead binding confirming the Zn^{2+}-dependency of N-cadherin mediated adhesion.

Further control experiments were performed to guarantee specificity of bead binding and to rule out any unspecific effects due to changes of extracellular zinc. As already described above unspecific bead binding was determined by the use of Fc-coated beads and Ca^{2+}-free conditions. To demonstrate that the observed effect of inhibition of cadherin-mediated bead binding after addition of 2µM Zn^{2+} is really mediated by zinc, Zn^{2+} was specifically chelated after 15 min of incubation by addition of 10 µM Ca^{2+}-saturated EDTA for another 15min. EDTA has a higher affinity for zinc than for calcium resulting in chelating of zinc without lowering the free calcium concentration. Under this condition bead binding was restored and even increased to 49 ± 4.5% showing that the observed effect was really mediated by extracellular Zn^{2+} (fig 2). It has to be emphasised that EDTA cannot enter the cell and thus effects on the cytosolic zinc-concentration can be ruled out. Removal of Zn^{2+} from a cell by depletion of extracellular Zn^{2+} would take place on a totally different time scale. Thus the extracellular Zn^{2+} is responsible for the observed effects.

Cellular uptake of Zn^{2+} is negligible

In order to see whether significant amounts of Zn^{2+} were taken up by the PC12-cells under the conditions investigated, cells were loaded with the membrane permeable Zn^{2+}-indicator FluoZin-3 AM prior to exposure to extracellular Zn^{2+}. The uptake was quantified by measuring the corresponding fluorescence over time. The results are depicted in Figure 3.

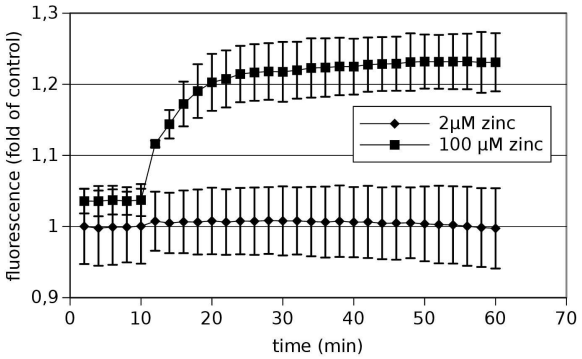

Figure 3: Uptake of Zn^{2+} into PC12 cells. PC-12 cells were loaded with the Zn^{2+}-selective fluorescent probe FluoZin-3. After 10 minutes recording of the baseline, the indicated final concentrations of Zn^{2+} were added. Data are shown as means from n=3 independent experiments

Clearly no significant amounts of zinc were taken up within one hour under the conditions used for the cadherin binding assays, i.e. 2 µM total added zinc (corresponding to 385 pM free Zn^{2+}). If an unphysiological high Zn^{2+}-concentration of 100 µM was added, a significant increase could be observed demonstrating that the method is in principle applicable. Thus the described effects of Zn^{2+} on N-cadherin mediated adhesion were mediated by the extracellular Zn^{2+} and not by elevated intracellular zinc levels.

Cellular growth and viability are not changed by Zn^{2+}

To test if cellular growth was impaired by the addition of Zn^{2+} in the range used, PC-12 cells were grown in normal culture medium supplemented with increasing concentrations of Zn^{2+} in the range from 0 to 2 µM (added total concentration) for 24h and 72h, respectively. The total number of cells was determined by flow cytometry and the amount of viable cells was determined by using a MTT assay. At concentrations up to 2 µM of added Zn^{2+} no significant alteration of the number of total or viable cells could be observed (data not shown).

Zinc does not change N-cadherin expression

Next we checked whether the amount of N-cadherin expressed on the cell surface was altered by the addition of Zn^{2+}. For that purpose cells were treated with trypsin in the

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absence of Ca^{2+}, resulting in proteolysis of the cadherin extracellular domain if cadherins were inserted in the plasma membrane. Figure 4A shows a Western blot of cell lysates of PC-12 cells incubated in culture medium without or with additional zinc (2 µM) and after treatment with trypsin or without.

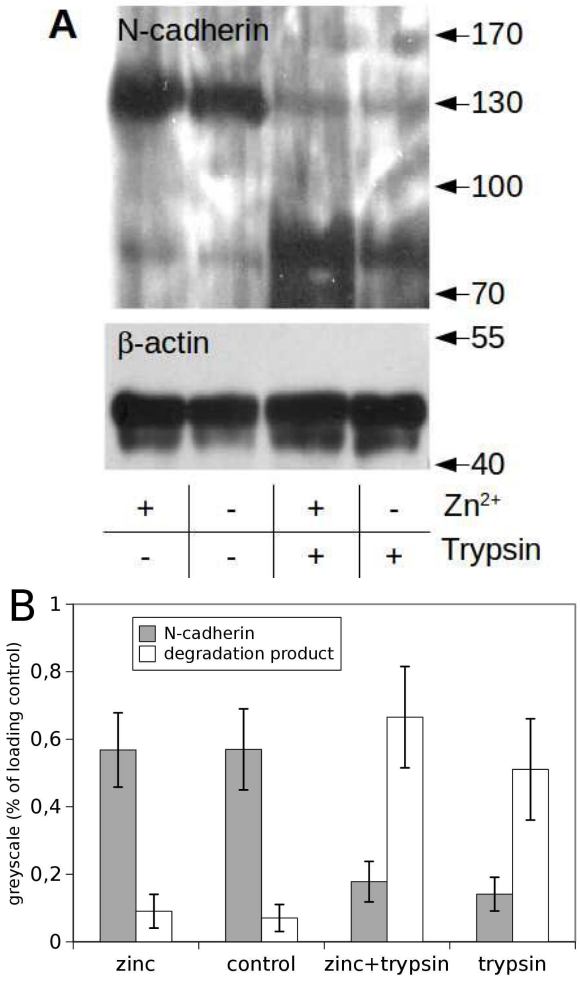

Figure 4: N-cadherin levels in PC12-cells with and without zinc treatment. PC-12 cells were exposed to culture media with (+) or without (-) additional Zn^{2+} and treated subsequently with trypsin resulting in proteolysis of the extracellular domain of N-cadherin if cadherins are inserted in the plasma membrane. A representative Western blot (A) shows that trypsin treatment resulted in a nearly complete proteolysis of N-cadherin independent of Zn^{2+} indicating nearly complete surface expression of N-cadherin. Equal amounts of protein were loaded onto each lane; β-actin served as loading control. The greyscale analysis of three independent blots is shown in (B)

Without trypsin treatment an immunoreactive band for N-cadherin could be seen at the expected height of ~130 kDa independent of the Zn^{2+} concentration. A greyscale analysis of three blots is shown in figure 4B. This analysis indicates that the total amount of N-cadherin was not affected by zinc. Trypsin treatment led to nearly complete proteolysis of cadherins at both conditions (with/without addition of Zn^{2+}). The band at 130 kDa disappeared and a degradation product at about 80 kDa became dominant. A Western blot for actin served as loading control. Thus, the amount of N-cadherin localised in the plasma membrane was not altered by elevated zinc-concentrations within the investigated time window of up to 30 minutes.

Binding of $Zn^{2+}$to N-cadherin

To investigate if reduction of N-cadherin binding activity by Zn^{2+} is mediated by a direct binding of Zn^{2+} to N-cadherin molecules or by any indirect effects the amount of zinc bound to N-cad-Fc was measured by atomic absorption spectrometry (AAS). 100 µg of N-cad-Fc were incubated with or without 10 µM of zinc in 100µl HBSS. Then the proteins were washed three times with zinc-free buffer by using Centricon centrifugation filters. After that the samples were subjected to AAS to determine the total amount of zinc contained. The concentration of zinc in the N-cad-Fc sample preincubated with zinc was 4.32 µM in comparison to 0.56 µM in the sample without prior zinc incubation. To rule out zinc binding to the Fc-portion of the fusion protein control experiments with Fc instead of N-cad-Fc were performed. No significant changes of the zinc content of the Fc-portion depending on prior zinc incubation could be detected. Thus, zinc can directly bind to the extracellular domain of N-cadherin. Assuming a molecular weight of N-cad-Fc of 130 kD, one finds that about one zinc-ion per cadherin molecule is bound.

In an alternative approach direct Zn^{2+}-binding was proven using a FluoZin-3 based fluorescence spectroscopy approach which confirmed the interaction and led to a stoichiometric ratio of about 2 Zn^{2+}-ions per cadherin molecule. While the direct binding of Zn^{2+} to the extracellular domain of N-cadherin could be shown by these approaches beyond doubt, only a lower limit of the stoichiometry can be given. This is due to the fact that the experimental procedure, especially the washing steps which take quite some time, allow the dissociation of formerly bound ions.

Interference of Zn^{2+} and Ca^{2+}-binding to N-cadherin

Clearly Zn^{2+} directly binds to N-cadherin and thereby alters cadherin trans-interaction. How this functions on the molecular level has to be determined. As cadherins are calcium dependent adhesion proteins, it is tempting to assume that Zn^{2+}-binding could interfere with the Ca^{2+}-binding, which is known to be vital for cadherin function. To test for this hypothesis, affinity chromatography was performed on a N-cadherin-functionalised column and a BSA-functionalised column for control. The columns were initially equilibrated with Ca^{2+} in the presence and absence of Zn^{2+} and then eluated with Ca^{2+}-free buffer. Then the Ca^{2+}-concentration in the different eluation-fractions was determined photometrically. A typical result is shown in Figure 5. What can clearly be seen is that from the N-cadherin column Ca^{2+} is eluated rather slowly. Up to 1.5 minutes after the bulk of Ca^{2+} from the exclusion volume of the column has passed, free Ca^{2+} ions are still detectable, representing the release of the bound Ca^{2+} from the

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immobilised N-cadherin molecules. This is in contrast to the BSA-functionalised column where Ca^{2+} drops down simply exponentially, i.e. a streight line can be observed in the logarithmic representation. After 160s of eluation time no Ca^{2+} could be detected in the eluate from the BSA-column with our detection method. In the presence of Zn^{2+} however, there is no such difference between BSA and N-cadherin functionalised columns. This indicates that Zn^{2+} binding to N-cadherin somehow impaired the Ca^{2+}-binding very fast as the presence of Zn^{2+} in the Ca^{2+}-containing equilibration buffer instantaneously prevents Ca^{2+}-binding. When instead of Zn^{2+} Cd^{2+} was used, no change in Ca^{2+}-elution could be observed (data not shown) indicating a Zn^{2+}-specific effect.

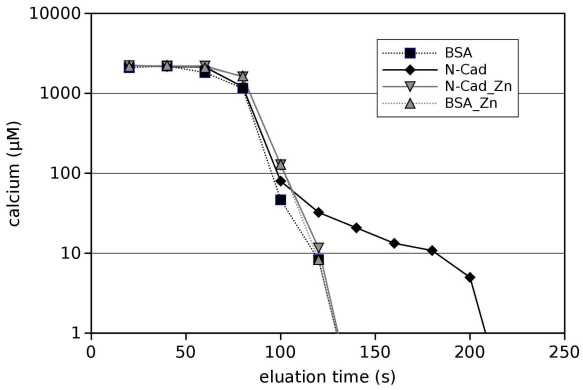

Figure 5: Ca^{2+}-eluation from protein columns in the presence and absence of Zn^{2+}. Prior to eluation with a Ca^{2+}-free buffer the columns packed with either N-cadherin (solid lines) or BSA (dashed lines) were equilibrated with Ca^{2+} in the presence (grey lines) or absence (black lines) of Zn^{2+}. The free Ca^{2+}-concentration was determined by measuring the transmission at 600nm of an Eriochrome Black T- indicator solution.

Discussion

Cadherins are localised at synaptic sites bordering the active zone [31-33]. As mentioned above cadherins have various functions at synapses, such as in synaptogenesis and in synaptic plasticity at mature synapses [34]. They affect synaptic structure and function, for example formation of LTP. Several mechanisms how cadherins regulate synaptic plasticity are possible, which are not necessarily mutually exclusive. Trans-synaptic interactions might activate intracellular signalling pathways thereby influencing synaptic strength. Furthermore, LTP of synapses is accompanied by an increase in synaptically localised cadherin such as N-cadherin [24]. Despite the fact that cadherins are Ca^{2+}-dependent adhesion molecules and Ca^{2+}-binding to the extracellular domain is necessary for their proper function, little attention has been drawn to the possibility that binding of Ca^{2+} or other ions to the extracellular domain might be one important mechanism to regulate the strength of adhesion. Interestingly, adhesive activity of N-cadherin is regulated between 0.2 and 1.3 mM Ca^{2+} (K_D = 0.7 mM) [27]. In addition, N-cadherin binding has been shown to be regulated by the extracellular concentration of protons [29]. This is of special interest because transient changes of ion concentrations (such as Ca^{2+} and H^+) in the synaptic cleft will occur during high synaptic activity and thus, regulation of N-cadherin binding under such conditions might be one mechanism by which cell adhesion is regulated and synaptic plasticity is influenced.

Here we report Zn^{2+} binding to N-cadherin and regulation of N-cadherin binding activity by extracellular Zn^{2+}. Zinc was already shown to play an important role in synaptic transmission and serves as an endogenous neuromodulator. Moreover, zinc is an important factor regulating dynamic processes mediating the stability of the post synaptic density (PSD) [8, 35]. Zinc is selectively stored in, and co-released with neurotransmitters from glutamatergic presynaptic vesicles [6]. Thereby, vesicular zinc transiently elevates local concentrations in the synaptic cleft. However, the exact amount of such release is not known and controversially discussed. Total Zn^{2+} in human cerebrospinal fluid is 150 nM [36], and a basal concentration of 38.6 nM extracellular Zn^{2+} has been measured by *in vivo* microdialysis in the hippocampi of rats [37]. As a result of Zn^{2+}-buffering by proteins and low molecular weight ligands, the concentration of free Zn^{2+} in biological fluids is usually several orders of magnitude lower than the total amount. Resting free extracellular Zn^{2+} is close to the detection limit of most experimental techniques for its quantitation, and is probably in the picomolar concentration range, maybe even as low as 1 pM [3]. However, others reported free baseline Zn^{2+}-concentrations in the nanomolar range [37]. Upon synaptic transmission, Zn^{2+} is transiently released, and the concentration of extracellular free Zn^{2+} is elevated to nanomolar or even micromolar values [38-40]. Thus, the exact values of the free Zn^{2+}-concentrations in the brain are not known and might vary within the synaptic cleft. But, baseline concentrations within the picomolar range and nanomolar to micromolar concentrations after synaptic activity seem to be most realistic. In this paper, we could show that N-cadherin responds to zinc in a low concentration, displaying high affinity. Half maximal binding was already reached at a concentration of about 310 pM free zinc. This argues for a basal binding under physiological conditions with few free zinc ions present and decreased binding upon synaptic activity with high amounts of locally released free zinc. Non-physiological conditions such as zinc deficiency might stabilise N-cadherin further, impairing structural changes necessary for the implementation of synaptic plasticity. In this context it is interesting to mention that reduction of zinc attenuates hippocampal LTP induction and cognitive memory [41].

Moreover, we could show that zinc directly binds to the extracellular domain of N-cadherin. A sequence analysis of N-cadherin using Metaldetector v2.0 software [42] revealed no typical zinc-binding motifs. However, in this approach only the primary sequence was analysed. There might be intermolecular zinc-binding sites (within the cadherin-dimer) or binding sites formed by different subdomains of the protein. Thus, a detailed investigation of the 3D-structure would be necessary to identify the exact location

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of the interaction site. A hint comes from the experiments showing that Zn^{2+} can prevent Ca^{2+}-binding to N-cadherin, while Cd^{2+} or Mg^{2+} can not. If this is due to a competition of Zn^{2+} and Ca^{2+} for the same binding site or if Zn^{2+} binds to another site leading to conformational or electrostatic effects rendering Ca^{2+}-binding impossible can not be concluded from the results available so far. This has to be determined in future studies. However, Zn^{2+} is released from synapses during neuronal activity physiologically and elevated Zn^{2+}-levels in the physiological range dramatically modulate N-cadherin binding.

Experimental

Cell culture and recombinant N-cadherin-Fc

PC12 rat pheochromocytoma cells (ECACC 88022401), endogenously expressing N-cadherin, were cultured in DMEM containing 5% FCS and 10% horse serum (Biochrom, Berlin, Germany), 2 mM glutamine, 50 U/ml penicillin-G and 50 µg streptomycin at 37°C in a humidified atmosphere (5% CO_2). N-cadherin-Fc fusion protein (mouse N-cadherin extracellular domain fused to Fc-portion of human IgG1, called N-cad-Fc in the following) was generated and purified as described previously [29]. Briefly, Chinese hamster ovary cells were stable transfected, and the secreted N-cadherin-Fc was purified from the culture supernatants by affinity chromatography using protein A agarose (Merck, Darmstadt, Germany).

Coating of polystyrene beads

N-cad-Fc was coupled to protein A-coated superparamagnetic polystyrene microbeads (Dynabeads, diameter 2.8 µm, Dynal, Oslo) as described [27, 29]. Briefly, after vortexing 10 µl bead solution were washed 3x in 100 µl of buffer A (100 mM Na-phosphate, pH 8.1) by sedimenting the beads via a magnet and reuptake in buffer A. The washed beads were suspended in 100 µl buffer A containing 10 µg of cadherin-Fc protein and allowed to react for 30 min at room temperature under permanent slow overhead rotation. After washing 3x in 100 µl buffer B (200 mM triethanolamine, pH 9.0) beads were incubated for 45 min in 100 µl buffer B containing 0.54 mg dimethyl pimelimidate ○ 2 HCl (DMP, Pierce, Rockford, USA) at RT. Free DMP was blocked by two washes for 30 min at 37°C in 100 µl 100 mM Tris pH 8.0. Finally the beads were washed 3x in Hanks Balanced Salt Solution (HBSS, Gibco, Karlsruhe). Beads were stored for up to 7 days in HBSS containing 0.1% BSA and 0.02% sodium azide at 4°C at slow overhead rotation to avoid sedimentation.

Laser tweezer experiments

A home-built laser tweezer setup was used as described before [27]. The beam of a Nd:YAG laser (1064 nm) was expanded to fill the back aperture of a high NA-objective (Plan Neofluar, 100x1.3 oil, Zeiss), coupled through the epiillumination port of an Axiovert 40 inverted microscope (Zeiss, Oberkochen, Germany) and reflected to the objective by a dicroic mirror (FT510, Zeiss).

Zn^{2+} was adjusted in the decomplemented DMEM containing 5% FCS by addition of increasing volumes of an isotonic, i.e. 0.3 M $ZnCl_2$ solution that 0-2µmol/l of Zn^{2+} were added in total. Decomplementation of the serum was carried out at 56° C for 60 min. Protein coated polystyrene microbeads were allowed to interact with the cell surface of monolayers for 15 min. Then in each experiment 100 beads were probed during the following 5-10 min by the laser tweezer. At least three experiments with different batches of cells and N-cad-Fc-coated beads were performed for each experimental condition investigated.

Specificity of cadherin-Fc binding was investigated by Ca^{2+}-depletion of the culture medium (addition of 5 mM of EGTA). Furthermore, control beads were coated with human Fc fragment only and these beads were applied to the cells and probed by the laser tweezer.

Free Zn^{2+} measurements in cell culture medium

Free Zn^{2+} was measured using FluoZin-3 [43, 44]. Different amounts of $ZnCl_2$ were added to DMEM + 5% FCS covering the range used for the laser tweezer experiments. The medium was incubated for 30 min at 37°C, corresponding to the incubation time in the laser tweezer experiment and then subjected to a fluoroscopic determination of the free Zn^{2+}-concentration. FluoZin-3 (tetrapotassium salt, Invitrogen, Germany) was added to a final concentration of 1 µM and incubated for 15 min. 100µl of each sample were transferred into 96-well plates. The resulting fluorescence *F* was recorded on a Tecan Ultra 384 fluorescence well plate reader (Tecan, Crailsheim, Germany) at room temperature. Free zinc concentrations were calculated using the formula by Grynkiewicz et al. [45]:

$$[Zn]_{free} = K_D \left[\frac{F - F_{min}}{F_{max} - F}\right]$$

with a dissociation constant for the Zn^{2+}/FluoZin-3 complex of K_D=8.9 nM. For the determination of the background fluorescence and the maximal fluorescence F_{min} and F_{max}, the medium was incubated with 10 µM of the Zn^{2+}-chelator N,N,N',N'-tetrakis (TPEN) or $ZnSO_4$ (250 µM), respectively. All measurements for Zn^{2+}-determination were carried out using three independent samples (n=3).

Trypsin treatment of cultured cells

PC-12 cells grown in Petri dishes were incubated for 15 min in DMEM + 5% FCS with 2µmol/l added $ZnCl_2$. After that cell layers were either scratched from the petri dish with a rubber policeman in Laemmli sample buffer or treated with 0.125% trypsin and 2 mM EDTA in PBS for 7 min at 37°. After addition of 200 µl FCS to stop trypsin activity cells were washed three times in PBS and dissolved in Laemmli sample buffer. These samples were subjected to SDS-PAGE and Western blotting.

Electrophoresis and Western blotting

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Cell lysates dissolved in Laemmli sample buffer and protein content was determined by amido black staining [46, 47]. Immunoblots were performed with equal amount of protein per lane after separation with SDS-PAGE (7.5% polyacrylamide). Membranes were blocked with 5% low-fat milk for 1 h at room temperature in phosphate-buffered saline containing 0.05% (v/v) Tween 20 and incubated with the respective primary antibody overnight at 4°C. The antibody ab18203 against N-cadherin (Abcam, Cambridge, UK) was used at a dilution of 1:1500 and the β-actin antibody CLONE AC-15 (Sigma, St. Louis, MS) at a dilution of 1:5000. As secondary antibodies horseradish peroxidase (HRP)-labeled goat anti-rabbit and goat anti-mouse IgG (Jackson Immunoresearch,West Grove, PA) both diluted 1:3000 were used. Bound antibodies were detected with the enhanced chemiluminescence technique (Amersham). Grey-scale analysis was performed using on the blots which were scanned using 8-bit grey-scale digitalization by employing the image software Gimp 2.8.

Measurement of intracellular free zinc

Cells were loaded with 1 µM FluoZin-3 AM (Invitrogen, Karlsruhe, Germany) in loading buffer (25 mM HEPES, pH 7.35, 120 mM NaCl, 5.4 mM KCl, 5 mM glucose, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 0.3% bovine serum albumin) for 30 min at 37°C. Subsequently, cells were washed with culture medium and transferred into a 96 well plate at a density of 1 x 10^6 cells per ml. The resulting fluorescence was recorded on a Tecan Ultra 384 fluorescence well plate reader (Tecan, Crailsheim, Germany) using an excitation wavelength of 485 nm and measuring the emission at 535 nm.

MTT assay

Cells were seeded onto 96 well plates and incubated with different Zn^{2+}-concentrations. Subsequently, cells were treated for three hours with 0.01 % (w/v) MTT (Methylthiazolyldiphenyl-tetrazolium bromide, Sigma) in normal culture medium, lysed in isopropanol and the absorption determined on a Tecan Sunrise well plate reader (Crailsheim, Germany) at 570 nm, using a reference wavelength of 700 nm.

Determination of direct Zn^{2+}-Cadherin-binding using atomic absorption spectrometry (AAS)

N-cad-Fc or pure Fc-fragments at a concentration of 1mg/ml in HBSS were incubated for 15min with 10µM $ZnCl_2$. Then the proteins were washed 5 times with HBSS using Centricon centrifugation filters. One centrifugation step for removal of excess liquid took approximately 3-5min. Then these samples were subjected to AAS. As negative control the proteins were washed the same way but the incubation step with $ZnCl_2$ was omitted. All reagents for atomic absorption were of appropriate quality for trace element analysis (TraceSelect, Fluka, Germany). Samples were diluted 1:10 in ultrapure water containing 0.2% (v/v) HNO_3 and were analysed by flame AAS on a Perkin Elmer AAnalyst 800 instrument and quantified with a standard curve between 0 and 1 mg/l zinc in 0.2% (v/v) HNO_3. Acetylene flow was set to 2.0 l/min and oxidant (air) to 17.0 l/min, measuring the absorption at 213.9 nm (slit 0.7 nm).

Confirmation of direct Zn^{2+}-Cadherin-binding using FluoZin-3A

As alternative approach to proof that Zn^{2+} directly binds to N-cad-Fc 100µl samples of HBSS containing 1µM $ZnCl_2$ were incubated with increasing amounts of N-cad-Fc from 0 to 1µM. Then 1µM of FluoZin-3A was added and the free Zn^{2+}-concentration was determined as described above.

Ca^{2+}-binding to N-cadherin in the presence and absence of Zn^{2+}

CNBr-activated sepharose (100 mg; Sigma, Taufkirchen, Germany) was allowed to swell for 45 min at 4 °C in 1 mM HCl (10 mL). The swollen sepharose (~1 ml) was transferred to a column (diameter 5 mm) and washed with 100 mL of 1 mM HCl, followed by 3 ml of distilled water. The column was equilibrated with 1 ml of coupling buffer (100 mM $NaHCO_3$, 500 mM NaCl, pH 8.4) and then loaded with 1 ml of coupling buffer containing a mixture of 0.8 mg/mL N-cadherin-Fc and 0.8 mg/ml bovine serum albumin (BSA) and allowed to react for 2 h at RT under slow overhead rotation. Afterwards the column was washed once with 3 ml coupling buffer, followed by a wash with 300 ml blocking buffer (200 mM glycine, pH 8.0) and then subjected to incubation for 3 h at RT in blocking buffer. After three washes with 3 ml acetate buffer (100 mM acetate, 500 mM NaCl, pH 4.5), the column was washed and equilibrated with 10 ml of HBSS containing 2 mM Ca^{2+}. For control experiments a second column was packed identically to the above described but only BSA was coupled to the sepharose.

For determination of Ca^{2+}-binding the N-cadherin- as well as the BSA-functionalised column were equilibrated with 5 ml of a Ca^{2+}-containing HEPES-buffer (5mM HEPES pH7.4, 150mM NaCl, 2mM $CaCl_2$, ± 1µM $ZnCl_2$). Then the column was eluated with the HEPES-buffer lacking Ca^{2+} and Zn^{2+} with a flow velocity of about 1.1 ml/min. Fractions of 348 µl (5 drops) were collected. The Ca^{2+}-concentration in the fractions was determined photometrically using the Ca^{2+}-sensitive chromophore Eriochrome Black-T. For this 500µM Eriochrome Black-T were dissolved in 100 mM Tris (pH 11) and 100 µl of the fractions were added to 500 µl of the chromophore solution. If the chromophore was saturated, the measurement was repeated but only 10 µl of the fraction were added to the indicator solution. The Ca^{2+}-concentration was determined photometrically by measuring the transmission at 600 nm. Calibration was done using samples of defined Ca^{2+}-concentration.

Conclusions

Here, we show that zinc binds to the extracellular domain of N-cadherin and modulates N-cadherin-mediated adhesion. Increased levels of zinc abolished N-cadherin binding ($K_D \approx$ 310 pM). Our results imply a model, where synaptic activity

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leads to an increase in the extracellular zinc concentration within the synaptic cleft which leads, among other effects, to reduced N-cadherin adhesion, thereby opening a short time window for structural changes that might accompany processes such as synaptic plasticity and LTP.

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1 T. B. Cole, H. J. Wenzel, K. E. Kafer, P. A. Schwartzkroin and R. D. Palmiter, *Proc Natl Acad Sci U S A,* 1999, **96,** 1716-1721.

2 R. D. Palmiter, T. B. Cole, C. J. Quaife and S. D. Findley, *Proc Natl Acad Sci U S A,* 1996, **93,** 14934-14939.

3 C. J. Frederickson and A. I. Bush, *Biometals,* 2001, **14,** 353-366.

4 C. J. Frederickson and D. W. Moncrieff, *Biol Signals,* 1994, **3,** 127-139.

5 C. J. Frederickson and G. Danscher, *Prog Brain Res,* 1990, **83,** 71-84.

6 C. J. Frederickson, J. Y. Koh and A. I. Bush, *Nat Rev Neurosci,* 2005, **6,** 449-462.

7 A. M. Grabrucker, *Dev Neurobiol,* 2014, **74,** 136-146.

8 S. Grabrucker, L. Jannetti, M. Eckert, S. Gaub, R. Chhabra, S. Pfaender, K. Mangus, P. P. Reddy, V. Rankovic, M. J. Schmeisser, M. R. Kreutz, G. Ehret, T. M. Boeckers and A. M. Grabrucker, *Brain,* 2014, **137,** 137-152.

9 J. Y. Lee, J. H. Kim, R. D. Palmiter and J. Y. Koh, *Exp Neurol,* 2003, **184,** 337-347.

10 E. Chorin, O. Vinograd, I. Fleidervish, D. Gilad, S. Herrmann, I. Sekler, E. Aizenman and M. Hershfinkel, *J Neurosci,* 2011, **31,** 12916-12926.

11 Y. Z. Huang, E. Pan, Z. Q. Xiong and J. O. McNamara, *Neuron,* 2008, **57,** 546-558.

12 D. D. Mott, M. Benveniste and R. J. Dingledine, *J Neurosci,* 2008, **28,** 1659-1671.

13 J. Veran, J. Kumar, P. S. Pinheiro, A. Athane, M. L. Mayer, D. Perrais and C. Mulle, *Neuron,* 2012, **76,** 565-578.

14 A. M. Vergnano, N. Rebola, L. P. Savtchenko, P. S. Pinheiro, M. Casado, B. L. Kieffer, D. A. Rusakov, C. Mulle and P. Paoletti, *Neuron,* 2014, **82,** 1101-1114.

15 C. Sindreu, A. Bayes, X. Altafaj and J. Perez-Clausell, *Mol Brain,* 2014, **7,** 16.

16 S. Pfaender and A. M. Grabrucker, *Metallomics,* 2014, **6,** 960-977.

17 D. F. Caldwell, D. Oberleas, J. J. Clancy and A. S. Prasad, *Proc Soc Exp Biol Med,* 1970, **133,** 1417-1421.

18 K. A. Keller, A. Grider and J. A. Coffield, *Physiol Behav,* 2001, **72,** 339-348.

19 G. Martel, C. Hevi, N. Kane-Goldsmith and G. P. Shumyatsky, *Behav Brain Res,* 2011, **223,** 233-238.

20 D. L. Benson, L. M. Schnapp, L. Shapiro and G. W. Huntley, *Trends Cell Biol,* 2000, **10,** 473-482.

21 G. W. Huntley, O. Gil and O. Bozdagi, *Neuroscientist,* 2002, **8,** 221-233.

22 S. Murase, E. Mosser and E. M. Schuman, *Neuron,* 2002, **35,** 91-105.

23 J. Arikkath and L. F. Reichardt, *Trends Neurosci,* 2008, **31,** 487-494.

24 O. Bozdagi, W. Shan, H. Tanaka, D. L. Benson and G. W. Huntley, *Neuron,* 2000, **28,** 245-259.

25 O. Bozdagi, X. B. Wang, J. S. Nikitczuk, T. R. Anderson, E. B. Bloss, G. L. Radice, Q. Zhou, D. L. Benson and G. W. Huntley, *J Neurosci,* 2010, **30,** 9984-9989.

26 L. Tang, C. P. Hung and E. M. Schuman, *Neuron,* 1998, **20,** 1165-1175.

27 W. Baumgartner, N. Golenhofen, N. Grundhofer, J. Wiegand and D. Drenckhahn, *J Neurosci,* 2003, **23,** 11008-11014.

28 W. Baumgartner, A. Osmanagic, M. Gebhard, S. Kraemer and N. Golenhofen, *Synapse,* 2013, **67,** 705-715.

29 W. M. Heupel, W. Baumgartner, B. Laymann, D. Drenckhahn and N. Golenhofen, *Mol Cell Neurosci,* 2008, **37,** 548-558.

30 W. Baumgartner, G. J. Schutz, J. Wiegand, N. Golenhofen and D. Drenckhahn, *J Cell Sci,* 2003, **116,** 1001-1011.

31 B. Bartelt-Kirbach, K. Langer-Fischer and N. Golenhofen, *Cell Commun Adhes,* 2010, **17,** 75-82.

32 A. M. Fannon and D. R. Colman, *Neuron,* 1996, **17,** 423-434.

33 N. Uchida, Y. Honjo, K. R. Johnson, M. J. Wheelock and M. Takeichi, *J Cell Biol,* 1996, **135,** 767-779.

34 M. B. Dalva, A. C. McClelland and M. S. Kayser, *Nat Rev Neurosci,* 2007, **8,** 206-220.

35 H. H. Jan, I. T. Chen, Y. Y. Tsai and Y. C. Chang, *J Neurochem,* 2002, **83,** 525-534.

36 C. O. Hershey, L. A. Hershey, A. Varnes, S. D. Vibhakar, P. Lavin and W. H. Strain, *Neurology,* 1983, **33,** 1350-1353.

37 C. J. Frederickson, L. J. Giblin, A. Krezel, D. J. McAdoo, R. N. Mueller, Y. Zeng, R. V. Balaji, R. Masalha, R. B. Thompson, C. A. Fierke, J. M. Sarvey, M. de Valdenebro, D. S. Prough and M. H. Zornow, *Exp Neurol,* 2006, **198,** 285-293.

38 S. Y. Assaf and S. H. Chung, *Nature,* 1984, **308,** 734-736.

39 G. A. Howell, M. G. Welch and C. J. Frederickson, *Nature,* 1984, **308,** 736-738.

40 A. R. Kay, *J Neurosci,* 2003, **23,** 6847-6855.

41 A. Takeda, S. Takada, M. Ando, K. Itagaki, H. Tamano, M. Suzuki, H. Iwaki and N. Oku, *Neuroscience,* 2010, **171,** 443-450.

Metallomics Accepted Manuscript

42 A. Passerini, M. Lippi and P. Frasconi, *Nucleic Acids Res,* 2011, **39,** W288-292.
43 H. Haase, S. Hebel, G. Engelhardt and L. Rink, *Anal Biochem,* 2006, **352,** 222-230.
44 L. M. Plum, A. Brieger, G. Engelhardt, S. Hebel, A. Nessel, M. Arlt, J. Kaltenberg, U. Schwaneberg, M. Huber, L. Rink and H. Haase, *Metallomics,* 2014, **6,** 1277-1287.
45 G. Grynkiewicz, M. Poenie and R. Y. Tsien, *J Biol Chem,* 1985, **260,** 3440-3450.
46 A. Dieckmann-Schuppert and H. J. Schnittler, *Cell Tissue Res,* 1997, **288,** 119-126.
47 W. Heinzel, A. Vogt, E. Kallee and W. Faller, *J Lab Clin Med,* 1965, **66,** 334-340.

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