AMPA-receptor-mediated excitatory synaptic transmission is enhanced by iron-induced α-synuclein oligomers

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Abstract

Aggregated α-synuclein (α-syn) is a characteristic pathological finding in Parkinson's disease and related disorders, such as dementia with Lewy bodies. Recent evidence suggests that α-syn oligomers represent the principal neurotoxic species; however, the pathophysiological mechanisms are still not well understood. Here, we studied the neurophysiological effects of various biophysically-characterized preparations of α-syn aggregates on excitatory synaptic transmission in autaptic neuronal cultures. Nanomolar concentrations of large α-syn oligomers, generated by incubation with organic solvent and Fe^{3+} ions, were found to selectivity enhance evoked α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)-receptor, but not NMDA-receptor, mediated synaptic transmission within minutes. Moreover, the analysis of spontaneous AMPA-receptor-mediated miniature synaptic currents revealed an augmented frequency. These results collectively indicate that large α-syn oligomers alter both pre- and post-synaptic mechanisms of AMPA-receptor-mediated synaptic transmission. The augmented excitatory synaptic transmission may directly contribute to nerve cell death in synucleinopathies. Indeed, already low micromolar glutamate concentrations were found to be toxic in primary cultured neurons incubated with large α-syn oligomers. In conclusion, large α-syn oligomers enhance both pre- and post-synaptic AMPA-receptor-mediated synaptic transmission, thereby aggravating intracellular calcium dyshomeostasis and contributing to excitotoxic nerve cell death in synucleinopathies.

Keywords: AMPA-receptor, oligomer, Parkinson's disease, synaptic transmission, synuclein.

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Parkinson's disease (PD) is the second most common neurodegenerative brain disorder, after Alzheimer's disease (Forman *et al.* 2005). The pathological hallmark of these neurodegenerative diseases is the formation and deposition of fibrillar aggregates of specific proteins, such as tau and Aβ in Alzheimer's disease, and α-synuclein (α-syn) in PD (Koo *et al.* 1999; Lee *et al.* 2002) and dementia with Lewy bodies (LB) (Forman *et al.* 2005). In PD, amyloid deposition is associated with loss of dopaminergic neurons in the substantia nigra and the formation of intracellular filamentous inclusions termed LB, which contain mainly aggregated α-syn (Spillantini *et al.* 1998). α-Synuclein is an abundant

pre-synaptic protein (Iwai *et al.* 1995) which under physiological conditions exists in a natively unfolded state (Lansbury 1999; Wright and Dyson 1999). The exact function of α-syn at the synapse is still not known. However, most studies support a role of α-syn in modulating vesicular pre-synaptic release (Murphy *et al.* 2000; Gureviciene *et al.* 2007). The conversion of α-syn into an accumulating misfolded protein is obviously an important factor in PD (Iwatsubo *et al.* 1996; Trojanowski *et al.* 1998; Hashimoto and Masliah 1999; Lansbury 1999). Recent evidence indicates that abnormal accumulation of misfolded α-syn also occurs at synaptic terminals and axons which may have an important pathophysiological role (Hashimoto and Masliah 1999; Lansbury 1999; Hashimoto *et al.* 2004; Kramer and Schulz-Schaeffer 2007). Moreover, previous studies support the notion that α-syn oligomers, rather than fibrils, might be the primary neurotoxic species (Conway *et al.* 2000; Kayed *et al.* 2003; Götz *et al.* 2004). Interestingly, direct transfer of α-syn aggregates from pathologically affected to neighbouring healthy neurons has been observed by a number of recent studies (Desplats *et al.* 2009; Danzer *et al.* 2011). This mechanism of extracellular spread of the disease process throughout the nervous system is reminiscent of Alzheimer's disease, and prion diseases in particular (Lee *et al.* 2010). The present study therefore analysed the effects of exogenous, well-defined, α-syn aggregates on the neurophysiology of excitatory synaptic transmission. In particular, we investigated the effect of iron-induced oligomers that have been shown previously to be associated with membrane pore formation (Kostka *et al.* 2008).

Materials and methods

Primary cell culture

Primary non-autaptic hippocampal cortical cultures were prepared as described previously (Scholz and Byrne 1987). Briefly, cortical cells were prepared from brains of newborn FvB mice (1 day) and then dissociated and resuspended in serum-free B27/neurobasal medium (Invitrogen, Carlsbad, CA, USA), plated at a minimum density of 1.25×10^5 cells/cm^2 on poly-D-lysin coated dishes.

Autaptic microisland hippocampal cultures were prepared as described previously (Rosenmund *et al.* 1993, 1995; Priller *et al.* 2009). Concisely, astrocyte feeder layers were prepared from mice pups cortex and grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose (Pan, Aidenbach, Germany), 5% fetal bovine serum (Pan), 100 IU/mL penicillin/streptomycin (Invitrogen, Karlsruhe, Germany), N2 supplement (Invitrogen), Glutamax (Invitrogen), and Mito+ serum extender (BD Biosciences, Bedford, MA, USA). Routine visual inspection of neurons in culture confirmed that synaptic connections with neighboring neurons were not present. The microisland preparation made sure that neurons only grew on the astrocyte feeder (Bekkers and Stevens 1991). Addition of 5-fluoro-2′-deoxyuridine (8.1 mM) prevented excessive proliferation of astrocytes. After 1 week, hippocampal neurons were dissected, counted and diluted to a final concentration of 10^5 cells/mL in DMEM, 10 mM HEPES and 10% horse serum (both GIBCO Life Technologies, Grand Island, NY, USA).

All cells were maintained at 37°C in the presence of 10% CO$_2$/90% air in a humidified incubator and were half-fed every week.

Electrophysiology

Cells were measured at day 18–21 *in vitro*. Whole-cell recordings were performed using an EPC 9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). The standard extracellular medium contained the following: 140 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 10 mM glucose, 4 mM CaCl$_2$, 4 mM MgCl$_2$, and 15 µM bicuculline, 300 mOsm, pH 7.3. NMDA-excitatory post-synaptic currents (EPSCs) were measured in the presence of 5 µM 6-cyano-7-nitroquinoxaline-2,3-dione and 10 µM glycine and in the absence of external Mg^{2+}. Internal pipette recording solution consisted of the following: 10 mM NaCl, 125 mM K-gluconate, 1 mM EGTA, 4.6 mM MgCl$_2$, 4 mM Na$_2$ATP, 15 mM creatine phosphate, and 20 U/mL phosphocreatine kinase, 300 mOsm, pH 7.3. Data were filtered at 2 kHz and acquired at 10 kHz using Pulse 8.5 software (HEKA Elektronik). Patch electrodes had resistances of 2–4 MΩ. Series resistance and capacity of the electrodes were compensated; only recordings with access resistance below 15 MΩ and with an initial membrane potential less than –50 mV (measured in the current-clamp mode shortly after cell access was obtained) were included in the analysis. Access resistance and cell capacities (5–25 pF) were compensated. Cells were voltage clamped at –70 mV, except for 1 ms depolarizations to 0 mV to elicit an action potential-like stimulus. Detection of spontaneous EPSCs (sEPSCs) was usually performed for at least 3×60 s (Biotrend, Köln, Germany). Data were analyzed with a template detection program (Axograph 4.1; Molecular Devices, Palo Alto, CA, USA). Threshold for detection was set to 3.5 times the baseline SD. Captured sEPSCs of individual cells were averaged to determine mean amplitude and charge per cell. Recordings and data analysis were performed blindly on each sample. 7 µM Oligomers (D0, D1, E0, E1) and monomers were diluted for electrophysiology 1 : 100 in the standard extracellular buffer, giving final concentrations of 70 nM α-syn and 100 nM Fe^{3+}.

As ferric ions were used in preparation of the α-syn aggregates, initial control experiments were carried out to exclude any detectable effect of ferric ions alone on autaptic synaptic transmission. Thus, it was confirmed that exposure of the hippocampal autaptic cultures to 100 nM Fe^{3+} alone had no effect either on EPSCs or on the frequency or amplitude of sEPSCs (Figure S1).

Expression and purification of human recombinant wild–type α–synuclein

Expression and purification was performed as described previously (Nuscher *et al.* 2004). Briefly, pET-5a α-syn wild-type (wt) plasmid was used to transform *Eschericia coli* BL21 (DE3) pLysS (Novagen, Madison, WI, USA). Expression was induced with isopropyl-D-thiogalactopyranose (Promega, Mannheim, Germany) for 4 h. Cells were harvested, resuspended in 20 mM Tris and 25 mM NaCl, pH 8.0, lysed by freezing in liquid nitrogen followed by thawing. After 30 min of boiling, the lysate was centrifuged at 17 600 *g* for 15 min at 4°C and thereafter the supernatant was filtered through a 0.22 µm filter (Millex-GV; Millipore, Bedford, MA, USA) before being loaded onto a HiTrap

Q HP column (5 mL; Amersham Biosciences, Munich, Germany) and eluted with a 25–500 mM NaCl salt gradient. The pooled α-syn fraction was desalted using a Superdex 200 HR10/30 size exclusion column (Amersham Biosciences), with 20 mM Tris and 25 mM NaCl, pH 8.0, as running buffer. The fractions were pooled and concentrated using Vivaspin columns (molecular weight cutoff: 5 kDa; Vivascience, Stonehouse, UK) and equilibrated with water. The protein concentration was determined by using a bicinchoninic acid protein-quantification kit (Pierce, Rockford, IL, USA). To separate monomeric from oligomeric α-syn, size exclusion chromatography was performed as previously described (Kostka *et al.* 2008). The monomers were separated in order to have a homogenous and consistent preparation for oligomer formation. Aliquots of purified recombinant α-syn (1 mg/mL) were stored at −80°C.

Fluorescent labeling of α–syn

Protein labeling was performed as previously described (Kostka *et al.* 2008), Briefly α-syn was labeled with Alexa Fluor-488-*O*-succinimidylester (green) and Alexa Fluor-647-*O*-succinimidylester (red) (Invitrogen, Eugene, OR, USA), respectively. Unbound fluorophores were separated by filtration steps in PD10 columns (Sephadex G25; Amersham Biosciences) equilibrated with 50 mM Tris–HCl (Roth, Karlsruhe, Germany), pH 7.0. Quality control of labeled α-syn was performed by fluorescence correlation spectroscopy measurements on an Insight Reader (Evotec-Technologies, Hamburg, Germany). Aliquots of purified recombinant fluorescently labeled monomeric α-syn were stored at −80°C.

Confocal single particle analysis

Fluorescence intensity distribution analysis (FIDA), and scanning for intensely fluorescent targets (SIFT) measurements were carried out on an Insight Reader (Evotec-Technologies) with dual color excitation at 488 and 633 nm, using a 40 × 1.2 numerical aperture microscope objective (Olympus, Tokyo, Japan) and a pinhole diameter of 70 μm at FIDA setting. Excitation power was 200 μW at 488 nm and 300 μW at 633 nm. Measurement time was 10 s. Scanning parameters were set to 100 μm scan path length, 50-Hz beam scanner frequency, and 2000 μm positioning table movement. This is equivalent to approximately 10 mm/s scanning speed. All measurements were performed at 22°C.

For FIDA the fluorescence data were evaluated by using the FCSPP® evaluation software version 2.0 (Evotec-Technologies). For SIFT analysis the software tool 'SIFT-2D' (Evotec-Technologies) was used. In general, for FIDA (Kask *et al.* 2000) and SIFT analysis (Bieschke *et al.* 2000), fluorescence from the two different fluorophores was recorded simultaneously with two single-photon detectors. Photons were summed over time intervals of constant length (bins) using a bin length of 40 μs. Detailed quantitative FIDA analysis of aggregate brightness (Q2) for the experiment shown in Fig. 1(c) was done exemplarily in channel 1. This provides the results of α-syn labeled with Alexa488 (green). Brightness (Q) of α-syn monomers were obtained from control measurements containing neither dimethyl sulfoxide (DMSO) nor Fe^{3+}. These data were used for 2-component FIDA analysis in samples containing aggregates (aggregate brightness Q2). In regard to the SIFT histograms shown in Fig. 1(a), the frequency
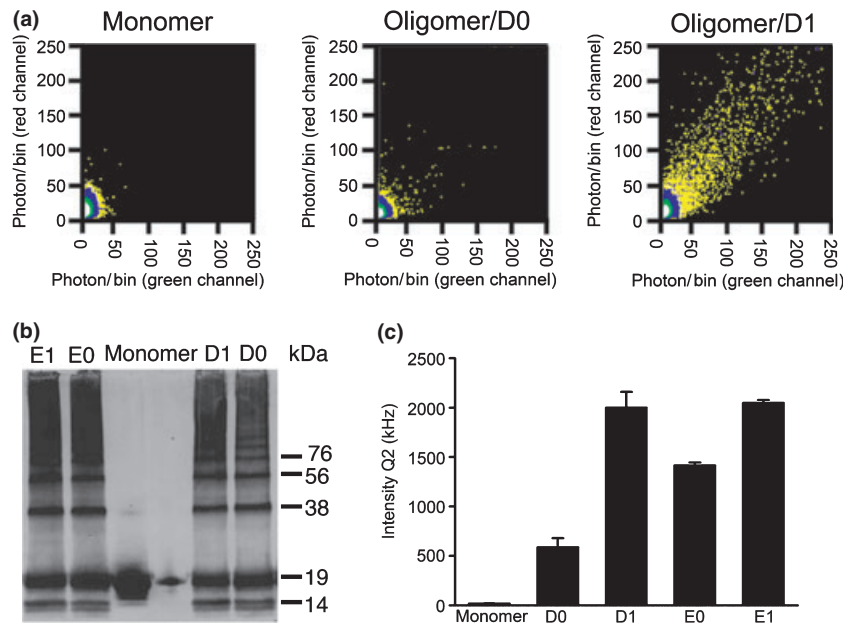

Fig. 1 Fe^{3+} ions induce the formation of higher-order α-syn oligomers. (a) Two-dimensional intensity distribution histograms obtained in an independent experiment show that the synergistic action of DMSO and Fe^{3+} (D1) results in the formation of much larger α-syn aggregates than found with DMSO alone (D0). (b) SDS–PAGE and immunoblot analysis of *in vitro* cell-free aggregation of α-syn shows the generation of dimers, trimers, tetramers, pentamers, and hexamers using the various aggregation protocols (D0, D1 and E0, E1). Monomeric α-syn is visible as a predominant band at 14 kDa. (c) In FIDA analysis, the intensity of photons relates to the size of the oligomers. Data were obtained in the same experiment as (a) and (b). The graph shows clearly that E1 and D1 preparations contain similarly very large aggregates (monomers: $n = 3$; D0: $n = 6$, D1: $n = 6$; E0: $n = 3$; E1: $n = 3$).

of specific combinations of 'green' (α-syn labeled with Alexa488) and 'red' (α-syn labeled with Alexa647) photon counts, derived from monomeric control or aggregation measurements respectively, was recorded and presented exemplarily in two-dimensional intensity distribution histograms, as described previously (Bieschke et al. 2000).

Preparation of α–syn–oligomers

Long incubation protocol (types E0 and E1)

These α-syn oligomers were prepared and maintained as described previously (Danzer et al. 2007). Briefly, type E0 and E1 oligomers were prepared by dissolving lyophilized protein (at a final concentration of 7 μM) in 50 mM sodium phosphate buffer, pH 7.0, containing 20% ethanol. In case of type E1 oligomers, 10 μM $FeCl_3$ (J.T. Baker, Griesheim, Germany) was also added, whereas type E0 oligomers were prepared without $FeCl_3$. After 4 h of shaking (GFL, Burgwedel, Germany), oligomers were re-lyophilized and re-suspended in 50 mM sodium phosphate buffer, pH 7.0, containing 10% ethanol. Open shaking for 24 h (stage 5, Thermomixer 5436; Eppendorf, Wesseling-Berzdorf, Germany) at 22°C evaporated the residual ethanol. After 6-day incubation with closed lids, oligomers were characterized.

Short incubation protocol (types D0 and D1)

D1 oligomers were prepared similarly to type E1 oligomers by dissolving lyophilized protein (at a final concentration of 7 μM) in 50 mM Hank's balanced salt solution, pH 7.0, containing 1% DMSO (v/v). 10 μM $FeCl_3$ was also added, whereas type D0 oligomers were formed in the absence of Fe^{3+} ions. After 4 h of shaking, both oligomer types were ready for use.

Alexa-488/Alexa-647-labeled oligomers were prepared in the same manner as non-labeled oligomers, by using the Alexa-488/Alexa-647-conjugated monomers. Oligomers (D0, D1, E0, E1) and monomers were diluted 1 : 100 in the standard extracellular buffer used for electrophysiology. Measurements were done in triplicates.

Native polyacrylamide gel electrophoresis

Native polyacrylamide gel electrophoresis was performed using the NativePAGETM Novex® Bis-Tris Discontinuous Gel System, from Invitrogen. A 3–12% Bis–Tris Gel and NativePAGE™ Running Buffer were used for separation of the α-syn aggregation mix. Samples were prepared in NativePAGE™ Sample Buffer and incubated at 65°C for 10 min before loading onto the gel. Electrophoresis was performed by applying 60 V at 22°C for 1.5 h, following which the gel was transferred onto nitrocellulose membranes with a semi-dry blotting system. For immunoblotting, polyvinylidene fluoride membrane (Millipore, Watford, UK) were incubated with primary monoclonal) synuclein-antibodies (15G7) (1 : 250) overnight at 4°C. The membranes were then incubated with alkaline phosphatase-conjugated secondary antibody (1 : 1000) for 1 h at 22°C. The visualization was performed with a secondary antibody coupled to alkaline phosphatase (polyclonal goat anti-mouse and anti-rabbit Ig/alkaline phosphatase; Dako, Carpinteria, CA, USA) and chromogene nitroblue tetrazolium salt with 5-bromo-4-chloro-3-indolylphosphate. Western blots were scanned with a high-resolution flatbed scanner, and the density of bands was determined using Total Lab version 2.01 software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK).

Viability measurements (MTT)

Cytotoxicity was assessed using a tetrazolium-based colorimetric assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay] (Mosmann 1983). Primary cortical neurons were cultured in a 96-well flat-bottomed tissue culture plate as described. In brief, cortices were dissected from new born FvB mice pubs in Hank's balanced salt solution and digested in DMEM solution containing 0.2 mg/mL cysteine, 100 mM $CaCl_2$, 50 mM EDTA, 10 U/mL papain (Roche, Mannheim, Germany) at 37°C. Digestion was stopped after 30 min and tissue was gently triturated using fire-polished Pasteur pipettes. Cell suspensions, diluted to 5–8×10^4 cells/mL, were then added to a 96-well flat bottom plate. After 18 days in culture, cells were maintained at 37°C in the presence of solvent control, or α-syn monomers; D0 and D1 α-syn oligomers were added to the cultured neurons at a final concentration of 70 nM, with 100 nM Fe^{3+}, for 5 days. In addition, increasing amounts of glutamate (5 μM, 10 μM, 25 μM, 50 μM or 100 μM) were added and cell viability was analysed after incubation for 6 h. For this purpose MTT (5 mg/mL) was added to each culture well. After incubation at 37°C for another 4 h, the formazan crystals were dissolved by adding 100 μL DMSO, and the plates were shaken vigorously to ensure complete solubilization. Formazan absorbance was assessed at 560 nm by an absorbance microtiter-plate reader (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany). Values are expressed as percentage of viable cells.

Results

Biophysical characterization of α–syn oligomeric species generated by different aggregation protocols

Previous studies have shown that different species of α-syn oligomers (Kostka et al. 2008) exhibit differential neurotoxicity (Danzer et al. 2007). In order to investigate the pathophysiological effects of aggregates on synaptic transmission, we essentially used two sets of α-synuclein oligomer preparations: one set of oligomers were triggered by incubating with either DMSO or EtOH alone, while in the second preparation low micromolar Fe^{3+} ions were included with solvent. For initial biophysical characterization of these preparations, a single-particle approach was applied using fluorescently-labeled α-syn. This approach has been successfully applied in our lab for defining various oligomeric proteins (Giese et al. 2004; Bertsch et al. 2005; Levin et al. 2005; Danzer et al. 2007). All aggregated samples were diluted 1 : 100 before measurement. Thus, incubation of 7 μM α-syn monomer at 22°C for 4 h resulted in no detectable aggregation (Fig. 1a, 'Monomer'). The addition of solvent, like 1% DMSO (D0) or 10% EtOH (E0), triggered oligomeric aggregation into low-molecular-weight structures (Fig. 1a, 'Oligomer/D0'; for E0 vide Danzer et al. 2007). Further addition of 10 μM Fe^{3+} with the solvent generated significantly larger α-syn aggregates (D1, E1) than found with DMSO or ethanol alone. The synergistic action of solvent and Fe^{3+} on the formation of larger α-syn aggregates, that were detectable as high-intensity signals in

scanned measurements by SIFT analysis, is shown in Fig. 1(a) (Oligomer/D1). Immunoblot analysis of the D0/D1 and E0/E1 α-syn preparations confirmed oligomer formation, with appearance of multiple bands at molecular weight levels consistent with α-syn dimers, trimers, tetramers, and pentamers (Fig. 1b). Analysis by FIDA confirms that indeed the E1 and D1 oligomers exhibit significantly higher molecular aggregate brightness when compared to E0 and D0 oligomers (Fig. 1c), implying larger supra-molecular structures.

Taken together, our results from single-particle confocal analysis and immunoblotting characterization strongly indicate that the main effect of Fe^{3+} ions consists in triggering the formation of higher-order aggregates from preformed low-molecular-weight building blocks.

Evoked excitatory neurotransmitter release is enhanced by iron-induced α-syn oligomers

To determine whether α-syn oligomeric species alter neurotransmitter release, patch-clamp recordings were performed on cultured primary hippocampal neurons. Micro-island cultures were used to induce the formation of autaptic synaptic connections on individual neurons (Bekkers and Stevens 1991; Priller *et al.* 2006). Synaptic transmission could then be evoked by briefly depolarizing a voltage-clamped neuron, while using the same patch pipette to measure the resulting EPSCs. The advantage of this method over analysis of synaptic transmission in conventional hippocampal neuronal cultures or brain slice preparations is that this approach results in the stimulation of all synapses of a single neuron. The evocation of the post-synaptic current is therefore not dependent on the stimulus intensity at all, and the synaptic response does not need to be normalized to stimulus intensity. This in turn gives a more accurate measurement of the synaptic strength of single neurons. Using this approach, we concentrated our analysis on glutamatergic neurons because they are much more abundant than inhibitory GABAergic cells in autaptic cultures.

We thus recorded α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA)-receptor and NMDA-receptor (NMDAR)-mediated EPSCs from 20 to 30 neurons, at day 18–21 *in vitro*, prepared from newborn FvB mice. Action potential-evoked neurotransmitter release was induced by a brief somatic depolarization (1 ms depolarization from −70 mV holding potential to 0 mV). Synaptic responses were measured as peak inward current a few ms after action potential induction (Fig. 2a, inserts). The amplitude of AMPA-mediated EPSCs was significantly increased following the application of α-syn oligomers formed in the presence of Fe^{3+} ions and solvent (D1 and E1, Fig. 2a). Conversely, α-syn monomers and oligomers formed by incubation with solvent alone did not affect the amplitude of evoked excitatory synaptic currents (E0, Fig. 2a). Thus, large

higher-order synuclein aggregates, formed in the presence of solvent and Fe^{3+}, enhance evoked AMPA-receptor-mediated excitatory post-synaptic currents.

Factors possibly affecting this change include an increase in post-synaptic responsiveness, an alteration in pre-synaptic release parameters and/or a higher synapse number. In order to determine whether the latter was the case, we analysed NMDAR-mediated synaptic currents, whilst blocking AMPA receptors using 6-cyano-7-nitroquinoxaline-2,3-dione (5 µM). In contrast to the effect of α-syn preparations with Fe^{3+}-induced D1 oligomers on AMPA-receptor-mediated synaptic currents, NMDA receptor currents were not affected at all by the aggregates (Fig. 3a). This indicates that large α-syn oligomers do not enhance the number of functional synapses, but rather enhance the number of post-synaptic AMPA receptors, or their responsiveness.

Iron-induced α-syn oligomers enhance spontaneous neurotransmitter release

As an indicator of post-synaptic responsiveness, we next examined the frequency and the amplitude of sEPSCs. As shown in Fig. 2(b), the frequency of sEPSCs is significantly enhanced after the application of preparations with large, iron-induced α-syn oligomers (D1 and E1) but not with α-syn monomers or small, solvent-induced α-syn oligomers (E0; Fig. 2b). Data obtained with the D0 preparation lead to similar results as the E0 preparation (data not shown). The mean of the sEPSC amplitude, however, remained unchanged (Fig. 2c). Moreover, as above, the frequency of NMDAR-mediated spontaneous synaptic current was not differentially affected by the α-syn aggregate preparation, as *both* monomers and D1 oligomers in fact increased the frequency (Fig. 3b). In other words, this phenomenon is obviously not specific to the α-syn aggregates.

Short-term synaptic plasticity in cultured hippocampal neurons is not affected by synuclein aggregates

Repetitive stimulation gives insights into the nature of the release probability (P_r) and the size of the readily releasable vesicle pool in pre-synaptic boutons (Schneggenburger *et al.* 1999). Trains of action potentials lead to facilitation in synapses with low initial P_r but to depression in synapses with high initial P_r. By applying paired pulses with 25–500 ms intervals to the cultured neurons, the paired-pulse ratio, defined as relative amplitude of the second EPSC normalized to the first EPSC, was obtained and compared between hippocampal neurons treated with 100 nM monomers, D1, E0, E1 and their respective controls. No significant differences in the paired-pulse ratio were observed between neurons incubated with the different α-syn oligomer preparations and their controls, both at high extracellular $[Ca^{2+}]$, leading to paired pulse depression, and at low extracellular $[Ca^{2+}]$, leading to paired pulse facilitation (Fig. 4).
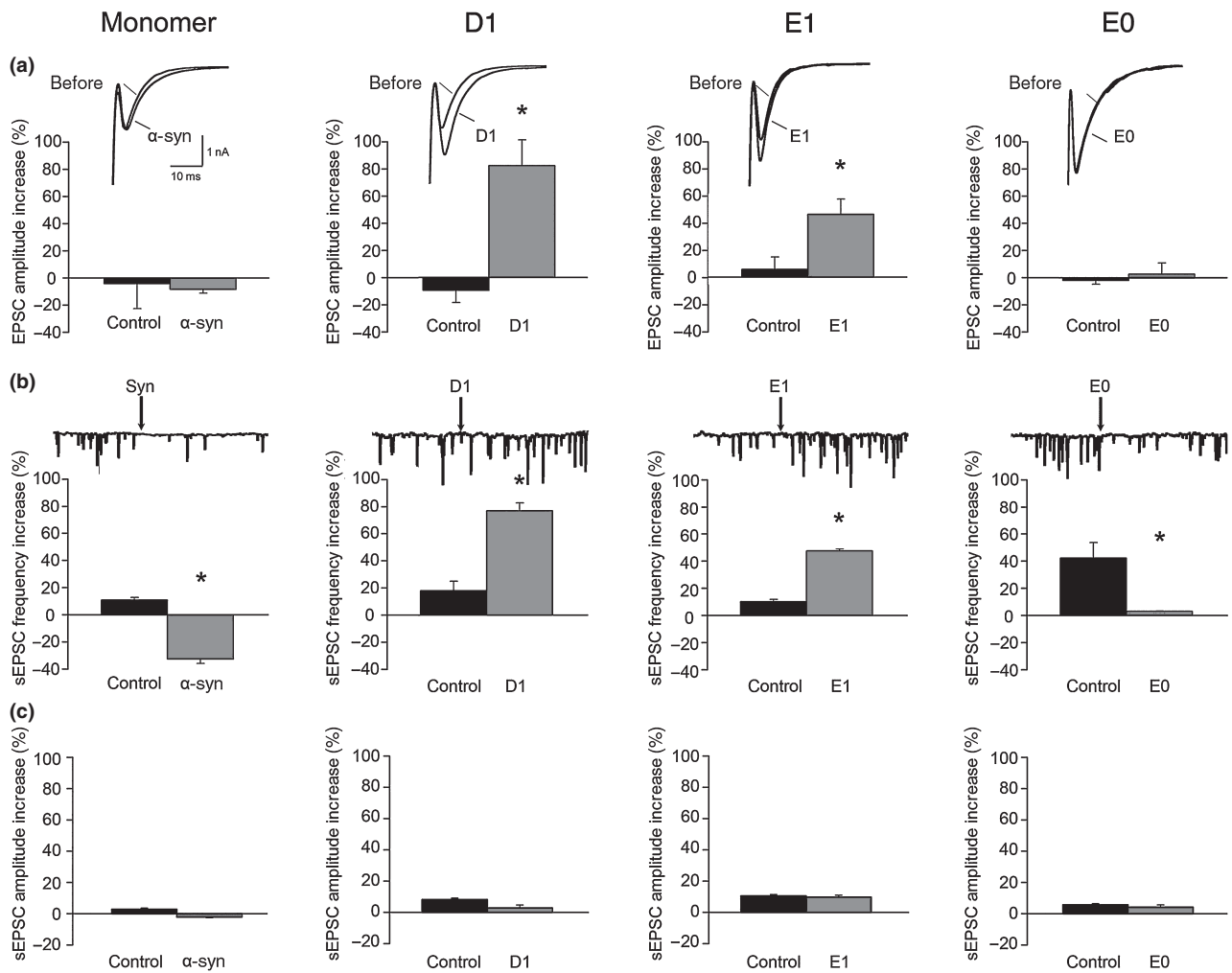
Fig. 2 Effect of α-syn oligomers on AMPAR-mediated synaptic transmission. (a) Effect of α-syn preparations (monomers, D1, E1, E0; 70 nM) on the maximal amplitude of AMPA-receptor-mediated EP-SCs. (b) Effect of α-syn preparations (monomers, D1, E1, E0; 70 nM) on the AMPA-receptor-mediated mEPSC frequency. (c) Effect of α-syn preparations (monomers, D1, E1, E0; 70 nM) on the mean AMPA-receptor-mediated mEPSC amplitude. Graphs illustrate the effect of synuclein aggregates in comparison to the application of solvent control (*$p < 0.05$). Insets show representative traces after application of α-syn monomer ($n = 26$), D1 ($n = 25$), E1 ($n = 21$) and E0 ($n = 24$) preparations.

Iron–induced α–syn oligomers enhance neuronal excitotoxicity

Having observed that D1 oligomeric aggregates specifically increase AMPA-receptor-mediated synaptic transmission, we finally wanted to determine whether they would promote glutamate-induced cell death in cultured neurons. Primary cortical neurons were thus exposed to α-syn monomers, small (D0) or large (D1) oligomers for up to 5 days. Neuron viability was monitored using the MTT assay. As shown in Fig. 5, the D1 preparation significantly enhanced glutamate excitotoxicity, already visible at 1 day after the start of the incubation. Whereas 10 μM glutamate was not toxic under control conditions, or upon incubation with small α-syn oligomers (D0) up to 4 days of incubation, the same

glutamate concentration significantly reduced the survival of D1-treated neurons (Fig. 5a and b). Analyzing the dose-response correlation between increasing concentrations of extracellular glutamate (5–100 μM) and the toxicity of D1 oligomers, we observed a strong correlation already 6 h after incubation (Fig. 5c).

Discussion

We have carried out a comprehensive study on the neuro-physiological effects of biophysically-characterised α-syn oligomer preparations on excitatory synaptic transmission in autaptic hippocampal neurons. Our major finding is that large α-syn aggregates, formed in the presence of organic solvent
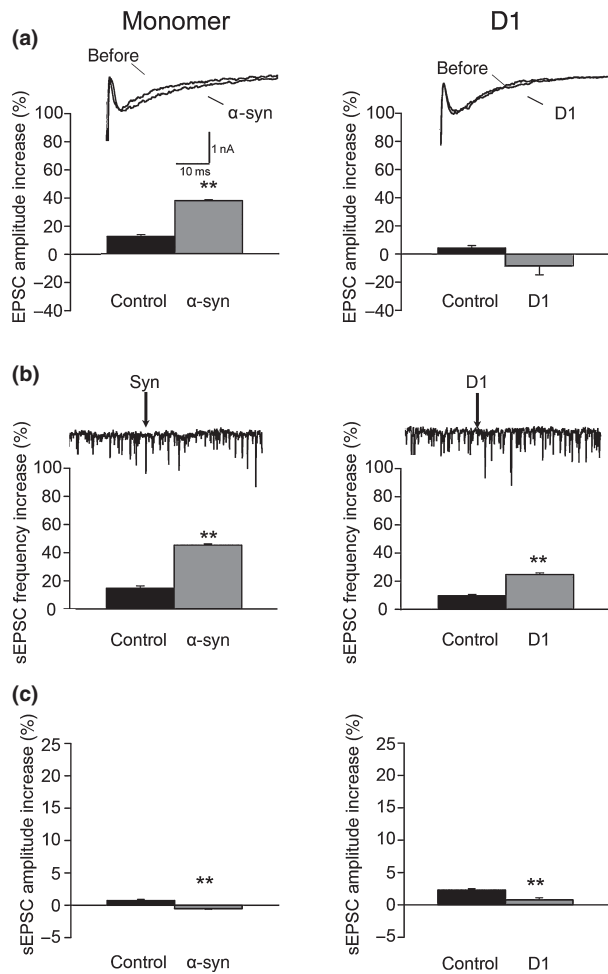
Fig. 3 Effect of α-syn oligomers on NMDAR-mediated synaptic transmission. (a) Effect of α-syn preparations (monomers and D1; 70 nM) on the maximal amplitude of NMDA receptor-mediated EPSCs. (b) Effect of α-syn preparations (monomers and D1; 70 nM) on the NMDA receptor-mediated mEPSC frequency. (c) Effect of α-syn preparations (monomers, D1; 70 nM) on the mean NMDA receptor-mediated mEPSC amplitude. Graphs illustrate the effect of synuclein aggregates in comparison to the application of solvent control (**$p < 0.05$). Insets show representative traces after application of α-syn monomer ($n = 15$), D1 ($n = 13$).

pathway, mossy fiber-cortical area (CA) 3 pathway, and Schaffer collaterals-CA1 pathway (Abeliovich *et al.* 2000; Steidl *et al.* 2003; Gureviciene *et al.* 2007, 2009; Watson *et al.* 2009). However, very different effects have been observed: short-term synaptic plasticity studies of corticostriatal slices from both α-syn over-expressing mice and α-syn knock-out mice showed that only elevated amounts of human α-syn reliably induced paired-pulse facilitation in the dorsolateral region of the striatum (Watson *et al.* 2009). In contrast, other reports showed paired-pulse depression or reduced paired-pulse facilitation in either the dentate gyrus perforant pathway or the mossy fiber-CA3 pathway of hippocampus from transgenic mice expressing human α-syn or a variant α-syn with the A30P mutation (Steidl *et al.* 2003 and Gureviciene *et al.* 2007). The inconsistent results obtained for the effects of αsyn on short-term synaptic plasticity may be ascribed to the experimental models used and the brain regions investigated. It seems therefore difficult to speculate about why paired-pulse facilitation remained unaffected in our conditions.

The most likely explanation for enhanced AMPA-receptor-mediated synaptic transmission is that the large α-syn oligomers alter the permeability of pre- and post-synaptic membranes to calcium ions. A slight increase of $[Ca^{2+}]_i$ both in the pre- as well as the post-synaptic compartment could account for all the neurophysiological phenomena described above. Indeed, the formation of ion-permeable pores by Fe-induced α-syn oligomers has already been shown in previous studies (Kayed *et al.* 2004; Danzer *et al.* 2007; Tsigelny *et al.* 2007; Kostka *et al.* 2008). Based on these studies, an enhanced calcium influx has been claimed to be the primary toxic event mediating neuronal cell loss in PD. However, the oligomer concentrations shown to be neurotoxic in the latter studies were very high, highlighting the problem of whether these pores may actually have a true pathophysiological role in PD. The present work, using nanomolar oligomer concentrations, can provide novel insight as to how calcium influx via a small number of α-syn-induced membrane pores may still offer a valid explanation for synapse loss and neuronal cell death in synucleinopathies. Indeed, a low-level pre- and post-synaptic calcium influx is sufficient to explain the effects of large Fe-induced α-syn oligomers on AMPA-receptor-mediated synaptic transmission. Moreover, the constant calcium influx could significantly contribute to the toxicity of these oligomer species in excitatory neurons. The augmented excitatory synaptic transmission further increases calcium influx via voltage-dependent calcium channels, a positive-feedback mechanism that exacerbates the already high cytosolic calcium concentration within the post-synaptic compartment. Eventually, this may cause an enhanced internalization of AMPA receptors, as a critical mechanism underlying inhibition of synaptic efficacy such as long-term depression and finally dendritic spine loss (Beattie *et al.* 2000; Biou *et al.* 2008).

and iron, do selectively enhance AMPA-receptor-mediated synaptic transmission via pre- and post-synaptic mechanisms. This enhancement includes both an increase in the strength of stimulated EPSCs as well as an increased frequency of sEPSCs. On the other hand, alterations in paired-pulse facilitation were not observed. Indeed, a number of *in vitro* and *in vivo* studies using α-syn null and over-expression models have indicated that α-syn may participate in the modulation of short-term synaptic plasticity (Cheng *et al.* 2011). The role of α-syn in synaptic plasticity was investigated in various brain regions, including the nigrostriatal pathway, corticostriatal pathway, dentate gyrus perforant
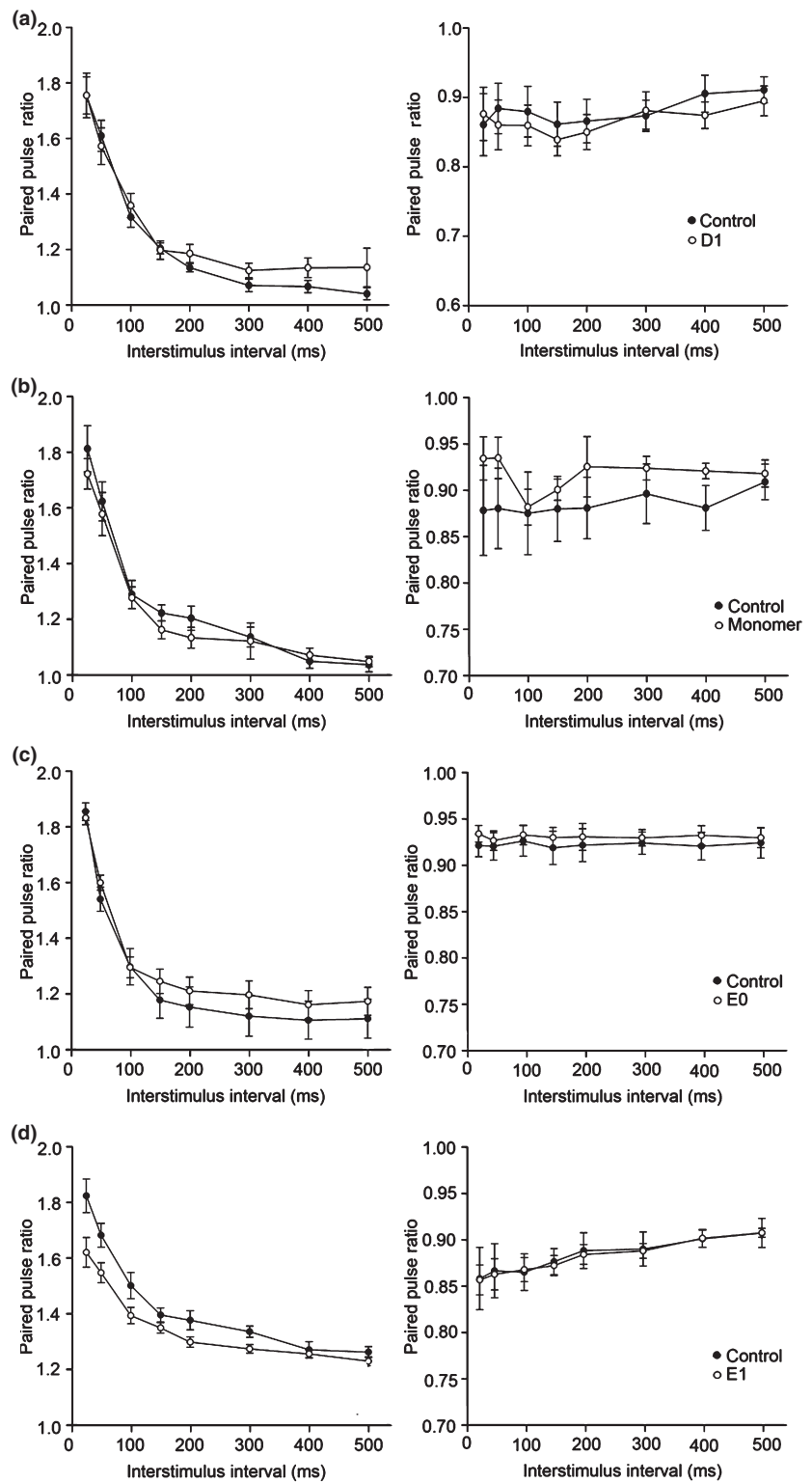
Fig. 4 Paired-pulse ratio of hippocampal neurons in the presence and absence of α-syn oligomers. LEFT Average EPSC amplitudes of hippocampal neurons exposed to (a) D1 ($n = 13$), (b) monomers ($n = 12$), (c) E0 ($n = 14$) and (d) E1 ($n = 11$) α-syn preparations, in response to different stimulus intervals at 1 mM extracellular [Ca^{2+}] normalized to the size of the first response. RIGHT Average EPSC amplitudes of hippocampal neurons exposed to (a) D1 ($n = 8$), (b) monomers ($n = 7$), (c) E0 ($n = 5$) and (d) E1 ($n = 5$) α-syn preparations, in response to different stimulus intervals at 4 mM extracellular [Ca^{2+}] normalized to the size of the first response.

This hypothesis may explain the pathophysiology of dendritic spine loss in patients with dementia with LB described previously (Kramer and Schulz-Schaeffer 2007), as well as underscore the role of iron and mitochondria in PD. The stability of dendritic spines is highly dependent on calcium homeostasis at the post-synaptic compartment as well as on mitochondrial activity. Indeed, calcium dyshomeostasis at the dendritic spine as well as altered mitochondrial activity have both been shown to induce dendritic spine loss (Zempel *et al.* 2010). Healthy neurons easily compen-
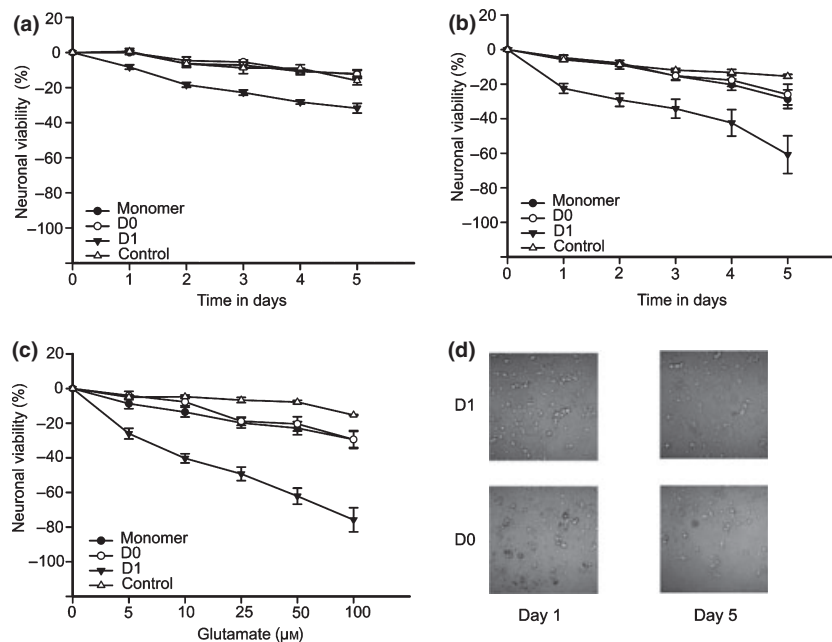
Fig. 5 Cytotoxic effects of D1 oligomers on cortical neurons. Cortical neurons from wild-type (FvB) mice were incubated with solvent control, α-syn monomer, D0 or D1 in serum-free NBA medium. Neuron viability was monitored by counting apoptotic cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results correspond to means ± SEM of at least three independent experiments. (a) Viability of cortical neurons over 5 days, with the addition of solvent control, 70 nM synuclein monomer, 70 nM D0 or 70 nM D1 aggregates. (b) Viability of cortical neurons over 5 days grown in the presence of 10 μM glutamate, and with the addition of solvent control, 70 nM synuclein monomer, 70 nM D0 or 70 nM D1 aggregates. (c) Viability of cortical neurons after 6 h of culture in the presence of 5–100 μM glutamate, and with the addition of solvent control, 70 nM synuclein monomer, 70 nM D0 or 70 nM D1 aggregates. (d) Images show representative murine cortical neurons (day 18 in culture) after treatment with D1 or D0 oligomer preparations (70 nM) for 1 or 5 days.

sate an abnormally high cytosolic calcium concentration; however, under conditions in which the intracellular buffering of calcium is compromised this protective mechanism may not be enough (Chan *et al.* 2009). Neurons in brain regions with high iron concentrations, like the substantia nigra, are predominantly affected in PD (Rhodes and Ritz 2008; Snyder and Connor 2009). In such conditions, the formation of large oligomer species that we have found to be formed in the presence of Fe may be thus promoted. Mitochondria, as a primary source of ATP, are essential for the activity of sarco/endoplasmic reticulum Ca^{2+}-ATPase pumps within the endoplasmic reticulum (ER) membrane that deliver Ca^{2+} from the cytosol, against a high concentration gradient, into the ER (Thayer *et al.* 2002). Reduced mitochondrial function, occurring with aging or because of mitochondrial defects in certain familial PD cases (Hatano *et al.* 2009), may therefore uncover the chronically enhanced cytosolic calcium concentration resulting from calcium permeable α-syn membrane pores and enhanced excitatory synaptic transmission.

With regards to the rationale for using extracellular applications of α-syn, it is pertinent to point out that although α-syn aggregates are typically found intracellularly, there is an emerging consensus that these aggregates can be exocytosed and exchanged between neurons. Interneuronal spread of α-syn aggregates has been suggested by the striking finding that α-syn is directly transmitted from neuronal cells over-expressing α-syn to transplanted embryonic stem cells both in tissue culture and in transgenic animals, with consequent host-to-graft transfer of PD pathology (Desplats *et al.* 2009). *In vitro* studies have confirmed that α-syn is secreted by living neurons into the extracellular medium, whilst α-syn has also been detected in the CSF and plasma of patients with PD (El-Agnaf *et al.* 2006; Mollenhauer *et al.* 2008). Moreover, it has recently been conclusively demonstrated that a heterogeneous population of α-syn oligomers, ranging from dimers to ∼ 30-mers, are present in the extracellular medium of a neuronal culture. These oligomers are taken up and transported in a retrograde fashion to the neuronal soma, with damaging effects to the neurons (Danzer *et al.* 2011). Our data strongly support the potential neurotoxicity of these extracellular oligomers, and suggest a potential excitotoxic mechanism involving calcium dyshomeostasis. Presumably, intracellular α-syn oligomers would also be expected to be able to form calcium-permeable pores by interacting with the inner side of the neuronal membrane.

In conclusion, large α-syn aggregates, formed in the presence of organic solvent and iron, selectively enhance AMPA-receptor-mediated synaptic transmission via pre- and

post-synaptic mechanisms. This augmented excitatory synaptic transmission may further aggravate intracellular calcium dyshomeostasis because of the formation of calcium permeable pores, ultimately contributing to synapse loss and nerve cell death in synucleinopathies.

Acknowledgements

Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. 100 nM Fe^{3+} has no effect on synaptic transmission of hippocampal autaptic neurons.

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