Multifaceted effects of oligodendroglial exosomes on neurons: impact on neuronal firing rate, signal transduction and gene regulation

Dominik Fröhlich[1,†], Wen Ping Kuo[1,2,†], Carsten Frühbeis[1], Jyh-Jang Sun[3,6], Christoph M. Zehendner[3,7], Heiko J. Luhmann[2,3], Sheena Pinto[4], Joern Toedling[5], Jacqueline Trotter[1,2] and Eva-Maria Krämer-Albers[1,2]

[1]Molecular Cell Biology, and [2]Focus Programme Translational Neuroscience, University of Mainz, 55128 Mainz, Germany
[3]Institute of Physiology, University Medical Center, 55128 Mainz, Germany
[4]Division of Developmental Immunology, DKFZ Heidelberg, 69120 Heidelberg, Germany
[5]Institute of Molecular Biology gGmbH, 55128 Mainz, Germany
[6]Neuro-Electronics Research Flanders, 3001 Leuven, Belgium
[7]ZIM III, Department of Cardiology, Goethe University Frankfurt, 60389 Frankfurt, Germany

Exosomes are small membranous vesicles of endocytic origin that are released by almost every cell type. They exert versatile functions in intercellular communication important for many physiological and pathological processes. Recently, exosomes attracted interest with regard to their role in cell–cell communication in the nervous system. We have shown that exosomes released from oligodendrocytes upon stimulation with the neurotransmitter glutamate are internalized by neurons and enhance the neuronal stress tolerance. Here, we demonstrate that oligodendroglial exosomes also promote neuronal survival during oxygen–glucose deprivation, a model of cerebral ischaemia. We show the transfer from oligodendrocytes to neurons of superoxide dismutase and catalase, enzymes which are known to help cells to resist oxidative stress. Additionally, we identify various effects of oligodendroglial exosomes on neuronal physiology. Electrophysiological analysis using *in vitro* multi-electrode arrays revealed an increased firing rate of neurons exposed to oligodendroglial exosomes. Moreover, gene expression analysis and phosphorylation arrays uncovered differentially expressed genes and altered signal transduction pathways in neurons after exosome treatment. Our study thus provides new insight into the broad spectrum of action of oligodendroglial exosomes and their effects on neuronal physiology. The exchange of extracellular vesicles between neural cells may exhibit remarkable potential to impact brain performance.

1. Introduction

In the central nervous system (CNS), oligodendrocytes wrap axons with a multi-layered myelin sheath forming a functional axon–glia unit. Generation and maintenance of this unit requires intense communication between neurons and surrounding glia cells. Classically, intercellular communication is mediated through direct cell–cell contact via gap junctions and adhesion molecules, or secretion of paracrine factors. Recently, additional non-conventional routes of cell–cell communication have been uncovered. Extracellular vesicles (EVs) such as shedding microvesicles (MVs) and exosomes shuttle sets of proteins and RNAs to target cells (for review, see [1]). While MVs directly pinch-off from the plasma membrane, exosomes originate from the endosomal system and are significantly smaller in size (40–100 nm in diameter). They are released into the extracellular space by fusion of multivesicular bodies (MVBs) with the plasma membrane. Cells can use exosomes to dispose of obsolete proteins, but importantly, exosomes

can also influence the target cell phenotype in various ways [2]. They can protect recipient cells, affect cellular signal transduction, promote malignant transformation and facilitate tumour growth [3]. Furthermore, exosomes contribute to immune responses and spread viruses and pathogenic proteins [4,5]. Exosomal delivery of genetic material, especially mRNA and miRNA, to target cells has gained particular interest. After transfer, mRNAs are translated resulting in newly synthesized proteins in the recipient cells [6]. On the other hand, miRNAs can mediate gene silencing in target cells [7–9].

Oligodendrocytes and all major cell types of the nervous system, including neurons, astrocytes and microglia, release EVs (for review, see [10]). Upon glutamatergic stimulation, cortical neurons secrete exosomes containing the neuronal cell adhesion molecule L1 as well as the AMPA receptor subunits GluR2/3, suggesting a role in synaptic plasticity [11–13]. Exosome release from oligodendrocytes is triggered in a similar way by the neurotransmitter glutamate, which is released by electrically active neurons and acts on oligodendroglial ionotropic glutamate receptors [14]. These exosomes carry a multitude of proteins (e.g. Alix, Tsg101, tetraspanins, heat shock proteins (HSPs) and the myelin proteins PLP and CNP) as well as RNA and are endocytosed by neurons followed by the functional recovery of their cargo. Neurons supplied with exosomes exhibit increased stress tolerance resulting in an improved survival under conditions of oxidative stress or starvation [14]. In addition, oligodendroglial exosomes can act in an autoinhibitory fashion inhibiting myelin formation [15] or, alternatively, become cleared from the extracellular space by microglia in an immunologically silent manner [16]. In the peripheral nervous system, Schwann cells secrete exosomes that are internalized by axons providing local axonal support and enhancing axonal regeneration after nerve damage [17].

In this study, we analyse the versatile impact of oligodendroglial exosomes on neuron physiology using different experimental approaches. We show that exposure of neurons to exosomes increases their action potential firing rate, alters cellular signal transduction pathways, and changes their transcriptome. Moreover, in an *in vitro* model of ischaemia, oligodendroglial exosomes exert beneficial effects on neurons, potentially via the transfer of protective proteins such as catalase and superoxide dismutase (SOD).

2. Material and methods

(a) Animals and cell culture
Primary cells were prepared from the C57Bl/6-N mouse strain. Embryonic day 14 mice of either sex were used for preparation of primary oligodendrocyte (pOL) and primary cortical neuron (pCN) cultures. pOL and the cell line Oli-*neu* were prepared and cultured in Sato 1% horse serum (HS) as described before [18]. pCN were cultured in Neurobasal feeding medium (Neurobasal (Gibco); 20 ml l^{-1} B27 (Invitrogen); 0.5 mM L-glutamine; 10 ml l^{-1} 100× Pen-Strep (63.2 µg ml^{-1} Penicillin G K-salt; 135 µg ml^{-1} Streptomycin sulfate)) as described before [19]. O4-positive pOL were prepared from postnatal day 7 mice by using the Neural Tissue Dissociation Kit, gentleMACS dissociator, MACS Separator and anti-O4 MicroBeads (all obtained from Miltenyi Biotec) according to manufacturer's instructions.

(b) Antibodies and plasmids
The following antibodies were used: rat PLP (clone aa3), rabbit Catalase (Rockland), rabbit βIII-Tubulin (Tuj1; Covance), mouse α-Tub (Sigma), guinea pig doublecortin (DCX; Chemicon), rabbit GFP (green fluorescent protein; Abcam), rabbit phospho-Akt (Cell Signalling), rabbit Akt (Cell Signalling), mouse phospho-Erk1/2 (Cell Signalling), rabbit Erk1/2 (Cell Signalling). The following expression plasmids were used: pEGFP/N1, pEGFP/N1-hSOD1-wt (kind gift of Dr. Albrecht Clement, Mainz, Germany).

(c) Isolation of exosomes
Exosomes were isolated by differential centrifugation of tissue culture supernatants collected from pOL or Oli-*neu* cells according to standard procedures [20,21]. All steps were carried out at 4°C. First, dead cells and cell debris were removed by centrifugation for 10 min at 130g and for 20 min at 10 000g, respectively. Subsequently, the supernatant was centrifuged for 1 h at 100 000g to pellet the exosomes, which were resuspended in 100 µl phosphate-buffered saline (PBS) or recovered from a sucrose cushion (1.8 M sucrose in Tris-buffered saline (TBS)). Nanoparticle tracking analysis of isolated exosomes using the Nanosight LM10 system revealed an average number of 279 ± 12.3 (s.e.m.) $\times 10^6$ particles of a size between 50 and 120 nm per 1×10^6 pOL (over a collection period of 24 h).

(d) Boyden chamber co-culture
Boyden chamber co-culture was performed as described previously [14]. Briefly, 7 days old pOL (1.25×10^6 per insert) or Oli-*neu* cells (2×10^5) were co-cultured with pCN (0.7×10^6 per well) in Boyden chambers, which allow contact-free co-culture. Six-well companion plates (353502) and six-well cell culture inserts (353102) were obtained from BD Falcon. To follow exosome transfer, either Oli-*neu* cells were transfected with 10 µg PLP-EGFP and 10 µg Sirt2-EYFP plasmids, or pOL were stained with the lipophilic dye PKH67 (Sigma) according to the manufacturer's protocol. Briefly, adherent pOL (4 days *in vitro* (DIV)) were incubated with 4 µl PKH67 diluted in 1 ml Diluent C for 4 min. Subsequently, reaction was stopped by adding 1 ml PBS + 10% HS for 1 min and cells were extensively washed (five times) with complete medium to remove residual dye. One day later, medium was exchanged and co-culture was started (pOL DIV 7). Immunocytochemical staining of PKH67-labelled oligodendrocytes confirmed enrichment of the dye in the endosomal system. After 1–2 days of co-culture, pCN were either immunocytochemically stained, lysed and analysed by Western blotting, or alternatively, neuronal RNA was prepared and analysed using microarrays and quantitative reverse transcription polymerase chain reaction (qRT-PCR).

(e) Cell lysates and Western blotting
Cells were scraped off the culture dish in 10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail (Roche complete). Nuclei were pelleted by centrifugation for 10 min at 300g. Cell lysates and exosome samples were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (10 or 12% gels, Bio-Rad Mini PROTEAN electrophoresis system). Alternatively, NuPAGE pre-cast gradient (4–12%) gels (Invitrogen) with either MOPS or MES buffer were used according to the manufacturer's protocol. Western blotting onto a PVDF membrane was performed using Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell system for 3 h at 300 mA. The membrane was blocked with 4% milk powder/0.1% Tween in PBS. Proteins were detected by sequential incubation of the membrane with primary and horseradish peroxidase-coupled secondary antibodies and developed with enhanced chemiluminescence reagents (Millipore).

(f) Immunocytochemistry

Coverslips were washed with PBS and fixed for 15 min in 4% par-aformaldehyde at room temperature. After washing with PBS (3×), cells were permeabilized with 0.1% Triton X100 in PBS for 2 min and washed again with PBS. Cells were then incubated in PBS + 10% HS for at least 15 min before applying the primary antibody diluted in PBS + 10% HS for 1 h. After washing with PBS, the secondary antibody was added diluted in PBS + 10% HS for 30 min. Nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI) for 2 min. After final washing, the coverslips were briefly submerged in ddH$_2$O before mounting them in Moviol on object slides. Fluorescence images were acquired using a DM6000 fluorescence microscope (Leica).

(g) Microarray and qRT-PCR

pOL (1.25×10^6 per insert) and pCN (0.7×10^6 per well) were pre-pared and co-cultured in Boyden chambers for 2 days as described previously [14]. Four individually prepared cultures of pCN were either co-cultured with pOL or with empty inserts containing exo-some-depleted conditioned pOL medium as control. After co-culture, total RNA was prepared from pCN using the Qiagen miR-Neasy mini Kit according to the manufacturer's protocol including on-column DNA digestion. Sample preparation for the microar-rays was done using the Illumina TotalPrep RNA Amplification Kit including oligo dT primers for cDNA synthesis. For measuring gene expression, the BeadArray Mouse v2 microarray platform from Illumina was used. The microarray data were analysed using R and Bioconductor in particular the 'limma' package [22]. For validation of the microarray results, 0.7×10^6 pCN (7 DIV) were either co-cultured with 1.25×10^6 pOL (7 DIV) for 2 days or treated for 24 h with exosomes released by $8-10 \times 10^6$ pOL over a time period of 48 h. Subsequently, total RNA was prepared from pCN using the Qiagen miRNeasy mini Kit and qRT-PCR was performed with the StepOne Real-Time PCR System (Applied Biosystems) using TaqMan assays recognizing Ier3, Ccnd1, Cort, Metrn, Ldb2, Vgf, Acta2, Eif4ebp1, Chl1, Cdk1, Bdnf, Plp, Cnp, Mbp and Pgk1 (Applied Biosystems).

(h) Multi-electrode arrays

pCN were prepared from embryonic day 14 C57Bl/6 mouse brains and plated on 6-well multi-electrode arrays (MEA chips; Multi Channel Systems, Reutlingen, Germany; 2×10^5 cells per well). Each well contained 3×3 recording electrodes with 200 µm spacing. Exosomes released from 6 to 10×10^6 pOL (7 DIV) over a time period of 48 h were prepared, resuspended in 25 µl Neurobasal feeding medium, and applied to one well of pCN (DIV 14). As a control, 25 µl Neurobasal feeding medium alone was applied. The extracellular activity was recorded by using a MEA 1060-INV-BC interface. Recording of the spike activity was started 1 h before exosome treatment and lasted at least 12 h. Only experiments in which neuronal activity was recorded at three or more electrodes were further analysed for spontaneous firing rate, relative burst index and spike amplitude. For further details, see [23,24].

(i) Oxygen–glucose deprivation and analysis of neuronal viability

In Boyden chamber co-culture assays, pCN (10 DIV; 0.7×10^6/6-well) were cultured with O4-positive pOL (1×10^6/insert) for 48 h in Neurobasal/B27 or with blank inserts containing oligodendrocyte-conditioned medium. Before oxygen–glucose deprivation (OGD) exposure, the culture media was replaced by glucose-free Neurobasal and cells were transferred to an anaerobic chamber saturated with 95% N$_2$ and 5% CO$_2$ at 37°C. The co-cultures remained in an oxygen-free atmosphere for 2 h in order

to mimic an ischaemic condition. Simultaneously, control cultures were grown under normoxic conditions in glucose-containing Neurobasal/B27. To allow subsequent reoxygenation (recovery), cells exposed to the OGD were returned to glucose-containing Neurobasal/B27 and were kept under normoxic conditions for 1 h. Media exchange was equally performed in the normoxic and OGD group. Neuronal viability was determined by the MTT assay. Briefly, 0.75 mg ml^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to culture medium for 2 h. Formazan crystals formed were solubilized in a buffer containing 40% (v/v) dimethyl-formamide (Sigma), 10% (w/v) SDS and 2% (v/v) acetic acid overnight. The absorbance was measured at 562 nm using a plate reader (Tecan Infinite 2000).

(j) Phosphorylation arrays

Exosomes released by 1.5×10^6 O4-positive pOL (5 DIV) over a time period of 48 h were collected by differential centrifugation, resuspended in PBS, and applied to 0.7×10^6 pCN (7 DIV) on a 3 cm culture dish for certain time periods (0 min, 15 min, 30 min, 1 h, 2 h, 15 h and 24 h) before lysis in buffer supplemented with Halt Phosphatase Inhibitor Cocktail (Thermo Scientific). Con-trol neurons (0 min) were treated with PBS. When exosome treatment of pCN was combined with oxidative stress or nutrient deprivation (ND), pCN were co-cultured with pOL in Boyden chambers for 48 h in neurobasal/B27 and compared to control cul-tures treated with pOL-conditioned, exosome-depleted medium as described previously [14]. Briefly, oxidative stress was induced by exposing neurons to 25 µM H$_2$O$_2$ for 1 h after 48 h of co-culture with pOL and before lysis. For ND, the pCN–pOL co-culture was grown in neurobasal without B27 supplement for 48 h before neurons were lysed. For further analysis, pCN lysates were sub-jected to SDS-PAGE and Western blotting. To examine the influence of exosome treatment on kinase activation in stressed neurons, 1.4×10^6 pCN (7 DIV) were treated with purified oligo-dendroglial exosomes obtained from 3×10^6 O4-positive pOL for 15 h and subsequently challenged with 25 µM H$_2$O$_2$ for 1 h before lysis. Control cells were treated with PBS before the exposure to H$_2$O$_2$. pCN lysates were subjected to protein determination using the BCA Protein Assay Kit (Novagen). One hundred micro-grams of total protein was applied onto array membranes of the Proteome Profiler Human Phospho-MAPK Array kit (R&D Sys-tems) according to the manufacturer's instructions.

3. Results

(a) Oligodendroglial exosomes influence neuronal activity

One of the most basic physiological functions of neurons is to generate action potentials in order to transmit information and communicate with other neurons. Previously, it has been shown that microglia-derived MVs can influence neur-onal electrical activity [25]. To test whether oligodendroglial exosomes also have the potential to alter neuronal activity, *in vitro* MEA recordings were performed. pCNs were cultured on 6-well MEA chips (nine electrodes per well) for 14 days (figure 1a) to allow establishment of neuronal circuits and acquisition of spontaneous electrical activity. Recordings of neuronal spike activity over 6 h are depicted in figure 1b–e. Exo-somes derived from cultured pOLs were applied 1 h after recording was started. Subsequently, the relative firing rate (figure 1c), burst index (figure 1d), spike amplitude (figure 1e) and the average channel number per well (figure 1b) were cal-culated. The results were only taken into account if the average number of active channels (electrodes) was higher
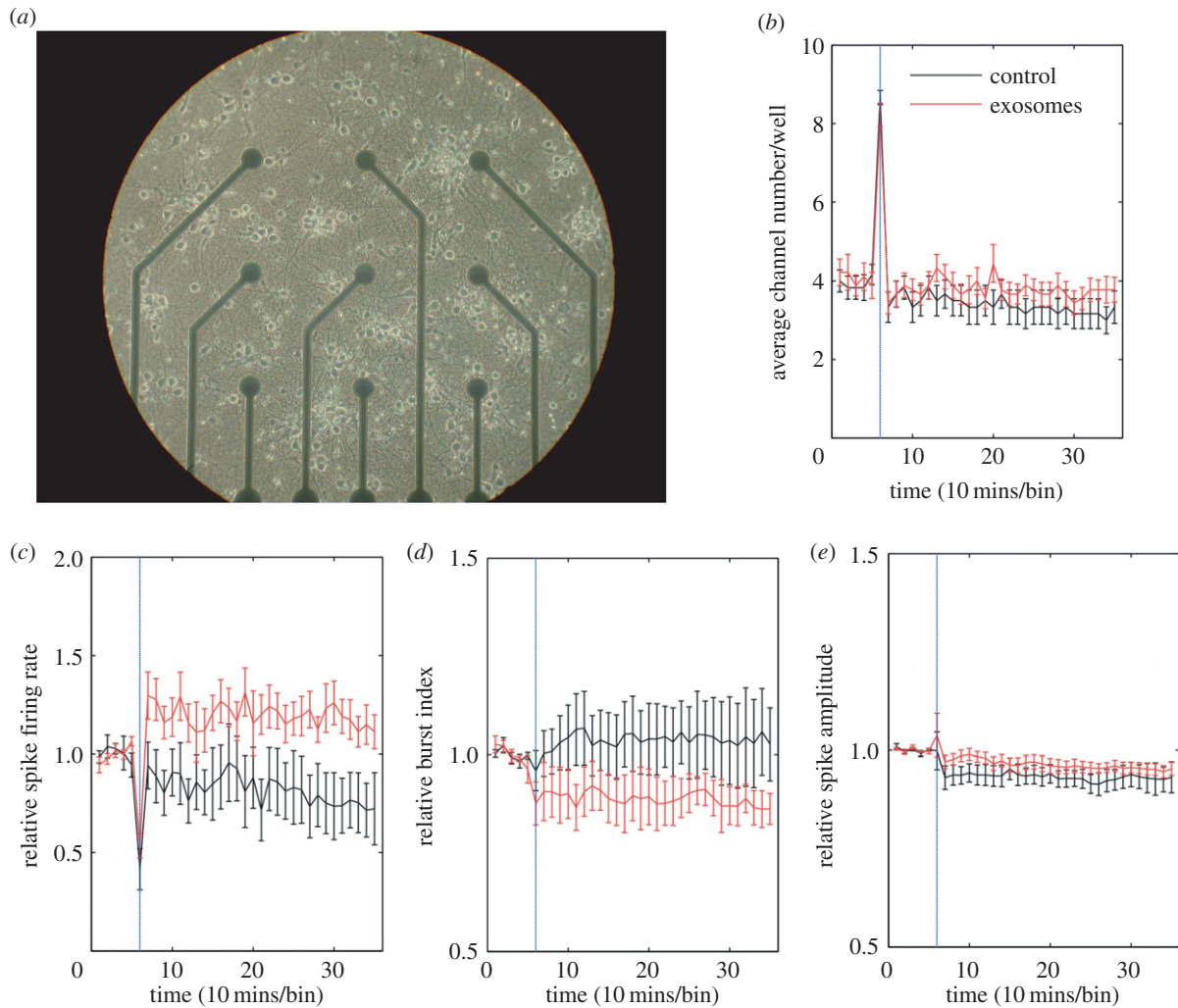
Figure 1. Impact of oligodendroglial exosomes on neuronal electrical activity. (*a*) Image of a 14 DIV pCN culture grown in a 9-channel MEA. Electrode spacing is 200 μm. (*b*–*e*) Recordings were performed at DIV 14. The blue vertical line indicates the application of pOL exosomes (red graph, $n = 9$) or sham control (black graph, $n = 6$) after 1 h of recording. Average channel number/well (*b*), relative spike firing rate (*c*), relative burst index (*d*) and relative spike amplitude (*e*) are depicted over 6 h of recording. Error bars $\pm$ s.d. (*$p < 0.05$; Students *t*-test).

than three during the recording. While the relative spike amplitude was not changed after application of oligodendroglial exosomes, the relative firing rate increased significantly and immediately (figure 1*c*). In contrast to the firing rate, the relative burst index decreased slightly after exosome treatment (figure 1*d*). The relative burst index is a measurement for the synchronicity of the neuronal network activity. A lower burst index indicates that neurons fire in a less synchronized fashion [23,24]. In summary, administration of oligodendroglial exosomes resulted in an increased number of action potentials with unaltered spike amplitude. Concomitantly, exosome-treated neurons fired less synchronously.

(b) Stress protective function of oligodendroglial exosomes

Our previous work revealed that oligodendroglial exosomes have the potential to protect recipient neurons from oxidative stress and ND [14]. To investigate the supportive effects of oligodendroglial exosomes in a diseased background, we used OGD, which is a widely accepted *in vitro* stroke model. OGD mimics ischaemia resulting in apoptotic and necrotic cell death [26]. pOLs and pCNs were co-cultured in Boyden chambers, allowing a constant supply of the neurons with oligodendroglial exosomes. Control neurons were cultured without oligodendrocytes, but in the presence of oligodendrocyte-conditioned medium depleted of exosomes, to exclude effects mediated by soluble factors. Co-cultures were subjected to OGD followed by a recovery phase and neuronal viability was assessed in relation to normoxic cultures by MTT assay (figure 2*a*). Intriguingly, neurons grown under ischaemic conditions but supplied with oligodendroglial exosomes revealed a significantly higher metabolic activity compared with control cells (increase of $21.5 \pm 7.6\%$). This indicates that exosomes protect neurons under ischaemic conditions.

A possible mechanism of protection may be that exosome-mediated transfer of enzymes assists the relief of cell stress. Since oxygen radicals form especially during the reperfusion phase [27] after ischaemia, transferred enzymes such as catalase and SOD may reduce oxidative stress. To this end, we investigated the exosome-mediated transfer of these two enzymes to neurons. hSOD1-EGFP expressed in the oligodendroglial cell line Oli-*neu* is present in exosomes derived from these cells and can be detected in lysates of pCNs co-cultured in Boyden chambers (figure 2*b*), indicating delivery of hSOD1-EGFP to neurons via oligodendroglial exosomes. Furthermore, catalase can be detected in exosomes isolated from pOLs and catalase protein levels in co-cultured neurons were elevated by $22 \pm 5\%$ in coincidence with PLP/DM20 appearing in neurons as a result of exosome transfer (figure 2*c*,*d*). By
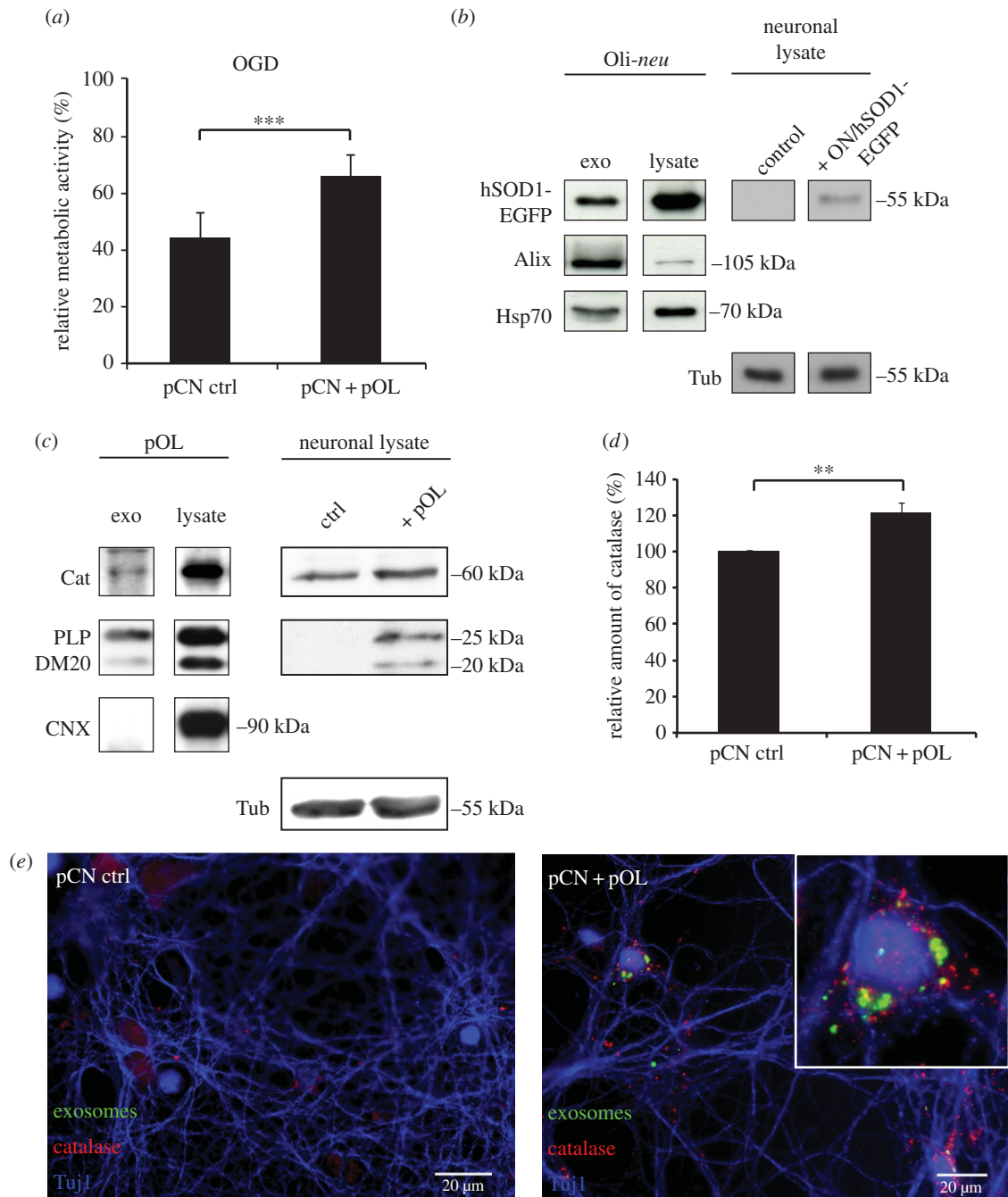
Figure 2. Neuroprotection mediated by exosomes. (*a*) pCN exposed to pOL exosomes in Boyden chamber co-cultures and subjected to OGD compared to control conditions (pCN ctrl, pOL-conditioned medium depleted of exosomes). After OGD followed by reoxygenation, neuronal metabolic activity was determined by MTT assay. The relative metabolic activity correlates OGD co-cultures to respective cultures grown under normoxic conditions. Error bars, s.e.m. (***$p < 0.001$; $n = 5$, Students t-test). (*b*–*d*) Western blot analysis of pCN lysates after co-culture with Oli-*neu* cells (ON) or pOL. (*b*) Presence of hSOD1-EGFP in isolated exosomes (left) and transfer of hSOD1-EGFP from transfected ON cells to pCN (right). Control pCN were co-cultured with untransfected ON. Alix and Hsp70 identify exosomes. (*c*) Western blotting of isolated pOL-derived exosomes demonstrates presence of catalase (Cat, left). PLP/DM20 serves as an oligodendrocyte-specific exosome marker and calnexin (CNX) as a contamination marker. Catalase levels determined after co-culture of pCN with pOL (right). Detection of PLP/DM20 in pCN indicates exosome transfer. (*d*) Quantification of catalase normalized to tubulin (Tub) as derived from Western blot signals in (*c*) (right). Error bars, s.e.m. (**$p < 0.01$; Wilcoxon test, $n = 10$). (*e*) Images of pCN co-cultured with pOL (right panel), which were stained with PKH67 to label pOL-derived exosomes (green). Control pCN were cultured in absence of pOL (left panel). pCN were immuno-stained for catalase (red) and the neuronal marker Tuj1 (blue). Scale bar, 20 μm.

immunocytochemical staining of neurons co-cultured with oligodendrocytes that were labelled with the dye PKH67, we observed that the signal for catalase was increased, in particular in those neurons that have internalized PKH67-labelled exosomes, suggesting that an exosome-mediated transfer of catalase from oligodendrocytes to neurons may have occurred (figure 2*e*). However, it is also possible that higher catalase levels in target neurons are due to upregulation of its expression. Taken together, these results suggest that exosomes deliver antioxidant enzymes such as catalase and SOD1, conferring enhanced stress resistance to exosome-recipient neurons.

(c) Activation of signalling pathways by oligodendroglial exosomes

To further elucidate the mechanisms underlying exosome-mediated neuroprotection, we investigated the activation of pro-survival signalling pathways such as MEK/Erk and

PI3K/Akt [28–31] in neurons in response to oligodendroglial exosomes under normal and stress conditions. We first aimed to elucidate the kinetics of kinase activation after exosome addition. Hence, pCNs were treated with purified oligodendroglial exosomes for different time periods (figure 3a,b). Subsequent analysis of neuronal lysates by Western blotting revealed the activation of Erk1/2 after 15 min and Akt after 30 min, reaching the maximal phosphorylation level at 15 min and 2 h, respectively (figure 3b). Whether activation of the signalling pathways occurs immediately after binding of exosomes to the cell surface or later during the internalization process will critically influence the activation kinetics.

To test the activation of both pathways under stress, we analysed phosphorylation levels in neurons co-cultured with pOLs in Boyden chambers, which were exposed to oxidative stress or ND (figure 3c,d). Control neurons were cultured without oligodendrocytes, but in oligodendrocyte-conditioned culture supernatant depleted of exosomes. Neurons challenged with either stress exhibit activation of Akt and Erk1/2 when co-cultured with oligodendrocytes (figure 3d). These results suggest that stimulation of Akt and Erk signalling pathways may play a role in exosome-dependent neuroprotection.

To identify additional kinases phosphorylated in neurons due to exosome treatment, we used the Phospho-MAPK (Mitogen Activated Protein Kinases) Array. pCNs were treated with purified oligodendroglial exosomes or vehicle for 15 h and subsequently subjected to oxidative stress for 1 h. The assay confirmed Akt activation and revealed an exosome-mediated increase in the phosphorylation state of CREB, GSK-3α/β, GSK-3β and JNK, while ERK did not exhibit robust activation under these conditions (figure 3e; highlighted in colours). Thus, oligodendroglial exosomes promote phosphorylation of distinct proteins involved in signalling.

(d) Influence of exosomes on neuronal gene expression

Having shown that exosomes modulate distinct signalling pathways in recipient cells, we analysed potential downstream effects on gene expression. One effect of signalling may be the activation or repression of certain genes, for instance by phosphorylation of the transcription factor cAMP response element-binding protein (CREB; figure 3e) [32,33]. Additionally, exosomes contain distinct mRNAs and miRNAs, which may exhibit pronounced effects on neuronal gene expression [6,7]. To test whether neuronal gene expression is altered by exposure of neurons to oligodendroglial exosomes, we performed a microarray analysis. pCNs were co-cultured in Boyden chambers with pOLs for 2 days. As a control, neurons were cultured in the absence of oligodendrocytes in oligodendrocyte-conditioned, exosome-depleted medium providing all soluble factors released by oligodendrocytes excluding exosomes. Subsequently, neuronal mRNA was prepared and differentially expressed genes were evaluated by microarray analysis. The quality of the raw microarray data was primarily assessed by looking at the spatial distribution of raw intensities on the array surface and by comparing the distribution of bead intensities between the individual arrays (data not shown). The expression data were normalized between the individual samples using the quantile normalization, and the data were analysed for differential gene expression using the approach of the R-package limma [34]. A complete list of the most significant differentially regulated genes is displayed in the electronic supplementary material, table S1 (False-Discovery Rate (FDR) ≤ 20%).

A number of selected candidates (table 1) that appeared promising in the context of CNS function were further validated by qRT-PCR in independent biological replicates (figure 4a). Validation revealed significantly altered expression of the following genes: immediate early response 3 (Ier3), cortistatin (Cort), VGF nerve growth factor inducible (Vgf), actin alpha 2 (Acta2), cell adhesion molecule with homology to L1CAM (Chl1), cyclin-dependent kinase 1 (Cdk1) and brain-derived neurotrophic factor (Bdnf). Moreover, the oligodendrocyte-specific transcripts of PLP, CNP and MBP were detectable in pCNs after co-culture with pOLs. Since the mRNAs of PLP and MBP are present in oligodendroglial exosomes (data not shown), these transcripts may be transferred to neurons.

To confirm that the observed effects can be attributed to exosomes, isolated oligodendroglial exosomes were applied to pCNs and the expression of candidate genes was determined by qRT-PCR (figure 4b). Comparison of the qRT-PCR results derived from the co-cultured and exosome-treated neurons revealed a proportion of likewise regulated genes with statistical significance. Ier3, Vgf and Bdnf are significantly downregulated, while Plp is significantly upregulated in both experimental settings, indicating a robust, exosome-mediated effect. Taken together, these results indicate that oligodendroglial exosomes alter neuronal gene expression and furthermore identify Ier3, Vgf and Bdnf as candidate target genes silenced in response to horizontal exosome transfer from oligodendrocytes to neurons.

(e) Transfer of oligodendroglial exosomes decreases neuronal doublecortin expression

Oligodendroglial exosomes may influence neuronal gene expression by delivery of miRNAs mediating gene silencing. Thus, we investigated the presence of specific miRNAs in oligodendroglial exosomes that had been previously reported in oligodendrocytes [35]. By qRT-PCR, we detected the miRNAs miR-9 and miR-19a in exosomes derived from the oligodendroglial cell line Oli-neu (data not shown). Both miRNAs were predicted to bind DCX (algorithm from www.microRNA.org). DCX is a microtubule-stabilizing protein [36], which is downregulated during neuronal differentiation. To test whether exosome-mediated transfer of these miRNAs influences neuronal DCX expression, we applied oligodendroglial exosomes to cultured pCNs and analysed neuronal DCX levels by Western blotting. Application of exosomes derived from the oligodendrocyte precursor cell line Oli-neu to pCNs (figure 5a,d) or Boyden chamber co-culture of pCNs with Oli-neu cells (figure 5b–d), likewise resulted in a decreased DCX expression in recipient neurons. To label the glial exosomes, Oli-neu cells ectopically expressed the exosomal proteins Sirt2-EYFP and PLP-EGFP, which were also detectable in neuronal lysates and thus served as indicators for exosome transfer (figure 5a,b). To the contrary, co-culture with mature pOLs had the tendency to increase neuronal DCX expression (figure 5c,d). Taken together, exosomes derived from an oligodendroglial precursor cell line but not from mature oligodendrocytes resulted in decreased neuronal DCX expression in vitro.

4. Discussion

This study provides evidence that oligodendroglial exosomes exert a variety of effects on recipient neurons and influence a
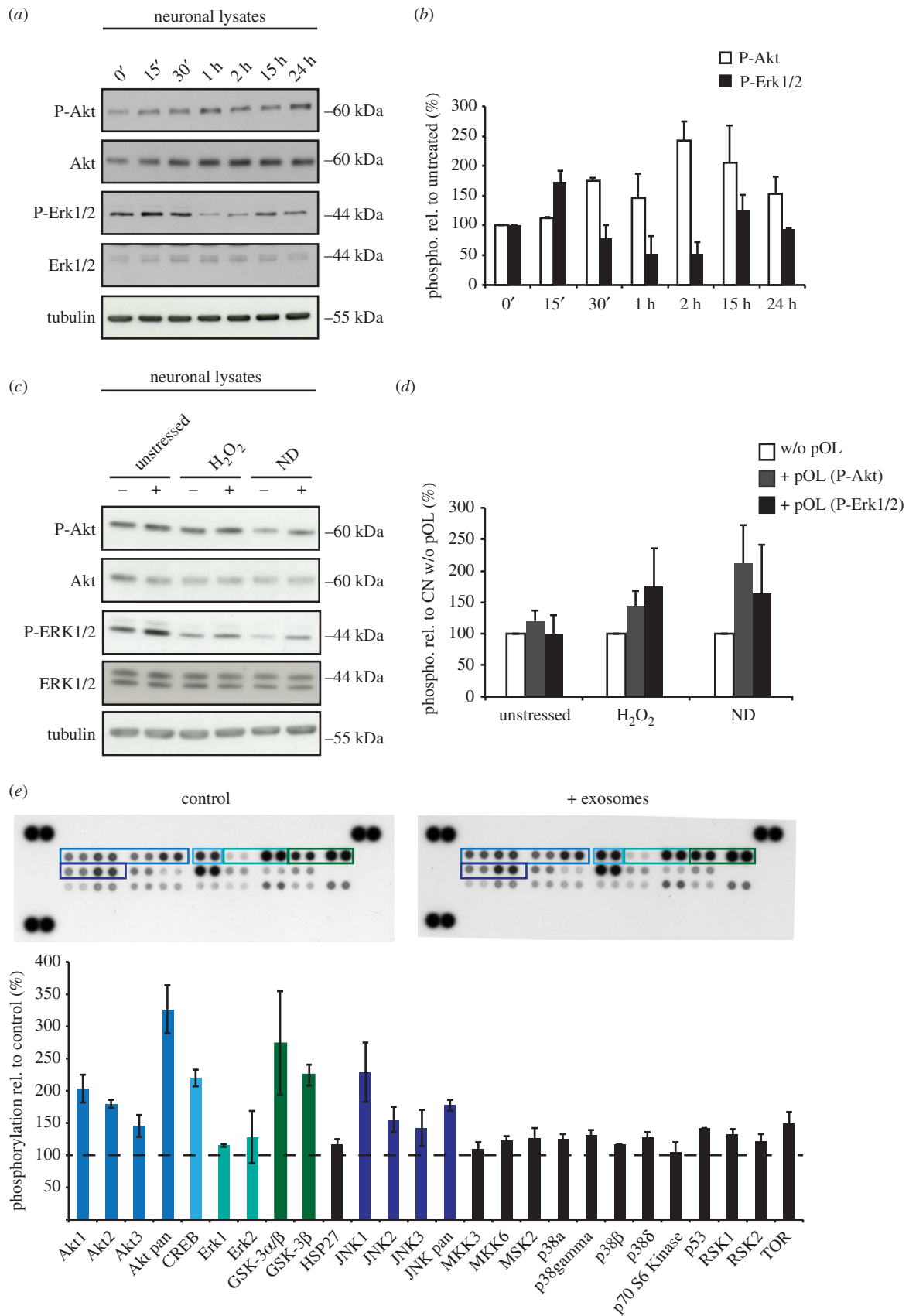
Figure 3. Analysis of exosome-dependent signalling pathways in neurons. (*a,b*) Purified oligodendroglial exosomes were applied to pCN for 0 min, 15 min, 30 min, 1 h, 2 h, 15 h and 24 h. P-Akt and P-Erk1/2 levels determined by Western blotting were normalized to total Akt or Erk and expressed in relation to untreated pCN at timepoint 0 min ($n = 2$). (*c,d*) Analysis of exosome signalling in recipient neurons under stress conditions. pCN co-cultured in Boyden chambers with ($+$) or without ($-$) pOL for 48 h were subjected to oxidative stress (25 μM H_2O_2 for 1 h before lysis) or ND (culture in absence of B27 supplement during co-culture) followed by Western blot analysis compared to unstressed cells. (*d*) Relative P-Akt and P-Erk1/2 levels in pCN are expressed in relation to control cells ($-$) of each condition to selectively visualize exosome-dependent kinase activation ($n = 3$). (*e*) Phospho-MAPK Array for phosphorylated proteins detected in neuronal lysates. pCN were either treated with PBS (control) or purified oligodendroglial exosomes and subjected to oxidative stress ($n = 4$). Abundance of phosphorylated proteins was quantified by densitometry and compared to PBS-treated pCN (dashed line). Proteins showing a higher phosphorylation level compared to control are highlighted by coloured boxes. Error bars, s.e.m.

Table 1. Neuronal genes regulated in exosome-dependent fashion. Selection of differentially regulated genes detected by microarray analysis (down to a false-discovery rate (FDR) of 20%; $n = 4$), which were further subjected to qRT-PCR validation. The logFC value is the logarithmic representation of the fold change (FC) value. For a complete list of regulated genes, see the electronic supplementary material, table S1.

ID	symbol	description	logFC	FDR (*p*-value)
ILMN_2737200	Mbp	myelin basic protein	0,87	1.79×10^{-7}
ILMN_1216764	Ier3	immediate early response 3	−0,52	1.48×10^{-4}
ILMN_1221503	Ccnd1	cyclin D1	−0,52	1.48×10^{-4}
ILMN_2601471	Ccnd1	cyclin D1	−0,47	3.10×10^{-3}
ILMN_2955725	Cort	cortistatin	−0,44	3.10×10^{-3}
ILMN_1226607	Cort	cortistatin	−0,42	3.10×10^{-3}
ILMN_2739843	Metrn	meteorin, glial cell differentiation regulator	−0,42	3.10×10^{-3}
ILMN_2754435	Ldb2	LIM domain binding 2	0,38	1.44×10^{-2}
ILMN_2925923	Vgf	VGF nerve growth factor inducible	−0,36	2.52×10^{-2}
ILMN_2693895	Acta2	actin, alpha 2, smooth muscle, aorta	0,60	2.92×10^{-2}
ILMN_2670398	Eif4ebp1	eukaryotic translation initiation factor 4E binding protein 1	−0,35	4.64×10^{-2}
ILMN_2644008	Chl1	cell adhesion molecule with homology to L1CAM	0,34	4.72×10^{-2}
ILMN_2657844	Cdk1	cyclin-dependent kinase 1	−0,32	7.45×10^{-2}
ILMN_3105417	Bdnf	brain-derived neurotrophic factor	−0,33	1.28×10^{-1}

broad spectrum of neuronal physiology. While this is not an in-depth analysis of individual exosome functions, the study indicates that exosomes are multifunctional entities in the brain and identifies putative molecular pathways of their action in neurons. Exosomes carry a complex assortment of different biomolecules with distinct activities that become integrated by target cells, most probably producing distinct functional outputs. We show that oligodendroglial exosomes modify neuronal electrical activity, activate certain signalling pathways, influence neuronal gene expression and deliver supportive molecules increasing neuronal stress tolerance under distinct stress conditions such as OGD. These effects may vary depending on the brain region where the transfer takes place and, moreover, on the status of the cells. Exosomes released by oligodendrocytes under conditions of cell stress may differ in composition from exosomes released by unstressed oligodendrocytes. It has been shown before that exosomes secreted from mast cells exposed to oxidative stress vary in their mRNA content to those from unstressed cells and increase resistance of recipient mast cells to oxidative stress [37]. Similarly, exosomes released from astrocytes subjected to heat stress are altered in their composition and are specifically enriched in protective HSP 70 [38]. On the other hand, the status of the recipient cells may further influence the effect of transferred exosomes. Neuronal subpopulations may differ in their internalization of exosomes and vary in their usage of exosomal cargo. Moreover, during disease, pathogens may influence the composition and effect of transferred exosomes. Exosomes can be hijacked by pathogens in the CNS and facilitate their spreading. Exosomes can carry and transfer α-synuclein, PrPSc (the scrapie and disease-specific form of the prion protein), amyloid precursor protein (APP) and several APP cleavage products including the Aβ peptide, phosphorylated tau and SOD, which are involved in Parkinson's disease, prion disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS), respectively [4,39].

(a) Oligodendroglial exosomes influence neuronal activity

Here, we demonstrate that treatment of cultured neurons with oligodendroglial exosomes enhances spontaneous neuronal electrical activity. Cultured neurons with already established synaptic connections exposed to exosomes generate more action potentials but at the same time fire in a less synchronized fashion. In general, these findings are consistent with the idea that oligodendrocyte-derived exosomes support the energy metabolism of neurons enabling an increased firing rate. However, the impact of exosomes on neuronal excitability *in vivo* remains to be proven. An excitatory effect has been reported for microglia-derived MVs that increase spontaneous and evoked excitatory transmission in hippocampal neurons [25]. Microglia-derived MVs act at the presynaptic site of the excitatory synapse by increasing the release probability of synaptic vesicles through induction of sphingolipid metabolism. A similar mechanism could be responsible for the increase of neuronal activity by oligodendrocyte-derived exosomes.

(b) Stress protective function of oligodendroglial exosomes

We have demonstrated previously that oligodendroglial exosomes ship protective cargo to neurons increasing their resistance against oxidative stress and starvation [14]. Here, we add further evidence that exosomes are also protective during OGD, which is a well-established *in vitro* model of cerebral ischaemia [40]. During brain ischaemia, the extracellular glutamate concentration increases, which can lead to neuronal cell death [40,41]. Exosome release from mature oligodendrocytes is actually triggered by glutamate [14], which in fact may help to prevent neuronal cell death during stroke, confining the ischaemic insult. Putative
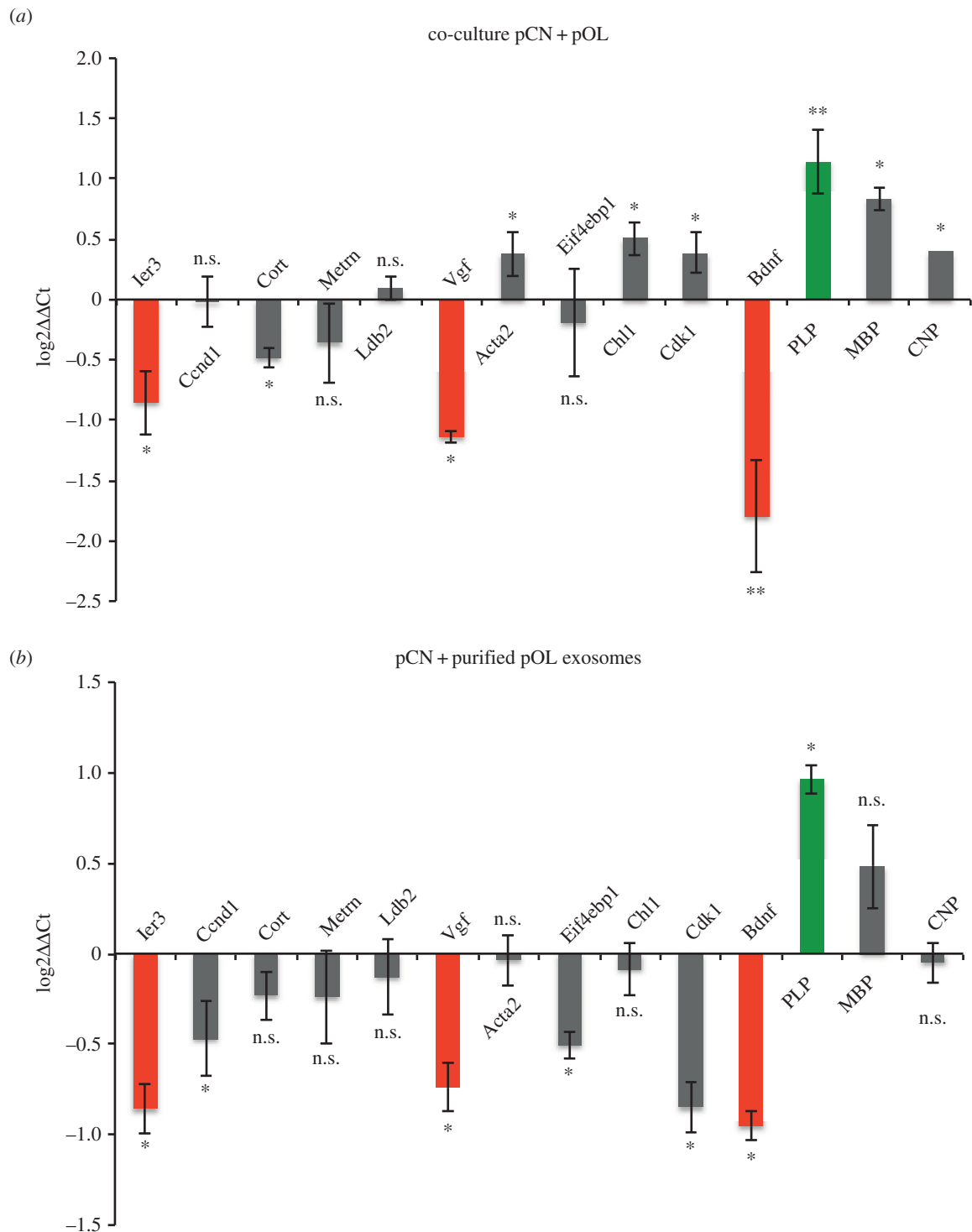
Figure 4. Validation of exosome-dependent neuronal gene regulation. qRT-PCR analysis of candidate genes selected from microarray analysis. (a) pCN co-cultured in Boyden chambers with pOL compared to control pCN ($n = 6$; BDNF and PLP, $n = 14$). (b) pCN treated with isolated pOL-derived exosomes compared to untreated pCN ($n = 7$; BDNF, PLP, MBP and CNP, $n = 5$). Pgk1 was used as normalization standard. Log2 values of the $\Delta\Delta$Ct values are displayed. Genes validated in both paradigms as downregulated or upregulated are indicated by red bars (dark grey in print) and green bars (light grey in print), respectively. Error bars, s.e.m. (n.s., not significant; *$p < 0.05$; **$p < 0.01$; Wilcoxon test). (Online version in colour.)

candidates exhibiting supportive function when transferred via exosomes may include HSPs as well as proteins involved in the oxidative stress response, like catalase and SOD. The role of HSPs in neuroprotection is well established and it has been previously shown that they can be supplemented from adjacent glial cells [42–45]. Our recent findings indicate that Hsc/Hsp70 [14], catalase and SOD1 are present in oligodendroglial exosomes and transferred to neurons. Since catalase mediates the breakdown of H_2O_2

to water and oxygen, this enzyme supplied to neurons by adjacent oligodendrocytes may protect neurons from H_2O_2-induced oxidative stress. Catalase is a peroxisome-resident protein and it may be interesting to note that intact peroxisomes in oligodendrocytes are required to maintain axon integrity *in vivo* [46]. SOD is another important enzyme involved in the antioxidant defence acting upstream of catalase by catalysing the dismutation of superoxide into oxygen and H_2O_2. It has been demonstrated previously that
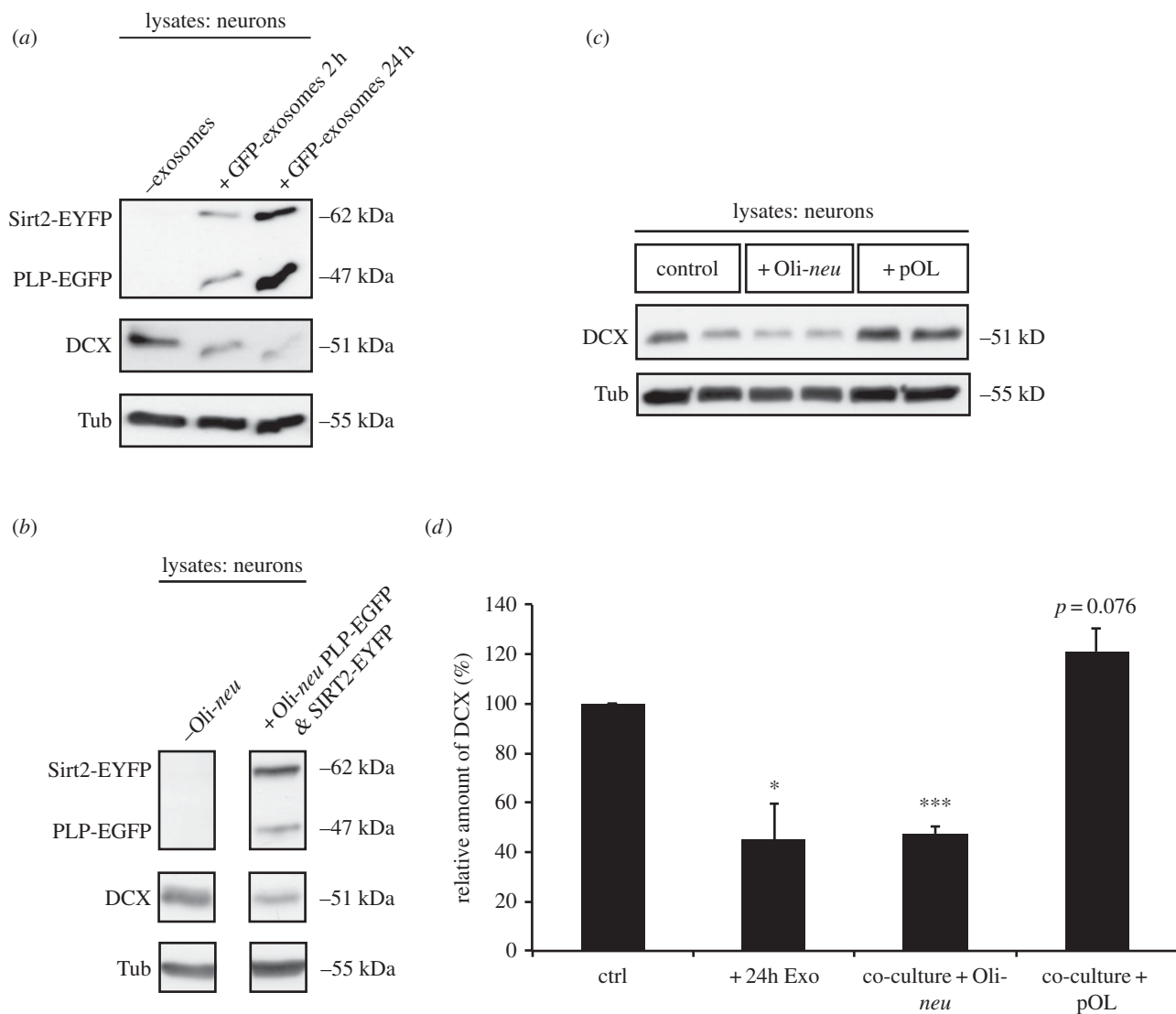
Figure 5. Downregulation of DCX in neurons in response to exosomes derived from oligodendrocyte precursor cells. (a−c) Western blot analysis of neuronal lysates. (a) pCN were treated with exosomes isolated from Oli-neu cells ectopically expressing Sirt2-EYFP and PLP-EGFP for 2 and 24 h, respectively (n = 6). (b,c) Boyden chamber co-culture of pCN either with Oli-neu cells ectopically expressing Sirt-EYFP and PLP-EGFP (b, n = 4) or with pOL and Oli-neu (c, n = 6) for 2 days. (d) Doublecortin (DCX) expression levels normalized to tubulin (Tub) as quantified from Western blots. Control pCN (untreated) are referred to as 100%. Error bars, s.e.m. (*p < 0.05; **p < 0.01; ***p < 0.001; Students t-test).

wild-type human SOD1 as well as their mutant forms are released in association with exosomes [47,48], which may be implicated in ALS pathogenesis.

(c) Activation of signalling pathways by oligodendroglial exosomes

Oligodendroglial exosomes contribute to neuronal survival under distinct stress conditions. However, the molecular determinants of enhanced survival are still unknown. Here, we identify several kinases that are activated in the target neurons upon treatment with oligodendroglial exosomes under normal as well as stress conditions. Cellular phosphorylation levels of Akt and Erk1/2 kinases are enhanced within minutes in response to oligodendroglial exosomes. Erk exhibited faster activation kinetics compared with Akt, indicating that it may act more upstream in the signalling cascade. Both kinases have been implicated in promoting survival [28–31]. In addition to Akt and Erk1/2, we identified CREB, GSK-3α/β, GSK-3β and JNK as phosphorylated in response to oligodendroglial exosomes. CREB is present in all cells of the CNS and functions as a transcription factor promoting cell survival and, furthermore, has a well-documented role in neuronal plasticity and the transcriptional control of memory formation [49–51]. Intriguingly, the Akt pathway activates CREB and downstream targets of CREB include the transcripts of BDNF and VGF [52–55], which we identified to be significantly regulated in neurons exposed to oligodendroglial exosomes. Additionally, phosphorylation of GSK-3α/β and GSK-3β, further downstream targets of Akt [56], may contribute to the neuroprotective function of oligodendroglial exosomes [57–59]. JNKs (c-Jun N-terminal kinases) are stress-induced protein kinases with various functions [60,61]. Recently, it has been reported that activated JNK is required for axon formation [62]. The transcription factor ATF-2, a member of the ATF/CREB family, is a target of JNK and was suggested to play a role in neuronal survival [62–64]. Taken together, these results reveal a variety of pathways potentially involved in mediating the neuroprotective effect of oligodendroglial exosomes. It is open at present whether signalling pathway activation requires exosome endocytosis or whether cell surface binding of exosomes is sufficient to elicit signalling.

(d) Influence of exosomes on neuronal gene expression

Initiation of signalling pathways in recipient neurons may result in differentially expressed genes. In addition, exosomal mRNAs and miRNAs or transcription factors are likely to modify the neuronal transcriptome. However, the ability to modify neuronal gene expression by the release of exosomes enables oligodendrocytes to broadly modulate the phenotype of surrounding neurons.

The most significantly regulated genes are Plp, Ier3, Vgf and Bdnf. Since PLP mRNA can be found in oligodendroglial exosomes, the upregulation of Plp may be a consequence of exosomal transfer of Plp mRNA. It has been reported previously that mRNA is transferred between mast cells by the means of exosomes and translated into functional proteins within the target cells [6]. Knockdown of Ier3, Vgf and Bdnf may be a result of transferred miRNAs. The transfer of miRNAs and the subsequent knockdown of their target genes have been shown before [7]. Ier3 belongs to the immediate early genes that are induced by a variety of stimuli such as growth factors, cytokines, ionizing radiation, viral infection and other kinds of cellular stress [65]. IER3 plays a complex and contradictory role in apoptosis and cell cycle control depending on the cellular context and growth conditions [65]. Both VGF and BDNF belong to the class of growth factors and are essential mediators of neuronal development, survival, axonal outgrowth and synaptic plasticity. BDNF plays a role in hippocampal-dependent associative memory [66], neurogenesis of hippocampal neurons [67] and during the pathology of depression [68]. BDNF induces the expression of Vgf via the transcription factor CREB [69]. Thus, reduced Vgf levels may be a consequence of decreased Bdnf expression. VGF is further processed into distinct polypeptides, which are subsequently released and exhibit a variety of functions, for instance the regulation of hippocampal synaptic plasticity and depressive behaviour in rodents [70,71]. However, the results indicate that oligodendroglial exosomes have the potential to influence neuronal physiology on a broad spectrum by regulating neuronal gene expression either via induction of distinct signalling pathways or by the selective transfer of mRNAs and miRNAs.

(e) Transfer of exosomes decreases neuronal doublecortin expression

Uptake of exosomes derived from the oligodendroglial precursor cell line Oli-*neu* but not from mature oligodendrocytes leads to a decreased DCX expression in recipient neurons, possibly by the transfer of glial miRNAs. We found the miRNAs miR-9 and miR-19a with predicted binding to DCX in oligodendroglial exosomes. It has been shown before that expression of both miRNAs is downregulated during oligodendrocyte maturation [35]. This is consistent with the finding that transfer of exosomes derived from the oligodendroglial precursor cell line Oli-*neu* but not from mature oligodendrocytes decreases neuronal DCX expression. DCX is a microtubule-stabilizing protein, which is mainly expressed by neuronal precursor cells and immature neurons [36]. Decreased DCX expression is associated with neuronal maturation and differentiation. The exosome-mediated downregulation of DCX may be important during development, where oligodendroglial exosomes could provide a differentiation signal to neurons, either initiating differentiation of neuronal precursor cells or keeping mature neurons in a differentiated state. Alternatively, neuronal precursors that migrate from the subventricular zone to the olfactory bulb along the rostral migratory stream could start to differentiate once they have come into contact with exosomes. And finally, oligodendroglial precursors accumulate at CNS injuries [72] and may be involved in on-going repair processes of the adult brain by stimulating axonal regeneration via the secretion of exosomes.

5. Conclusion

In this study, we provide evidence that oligodendroglial exosomes exhibit versatile effects on neuronal physiology. We identify exosomes as signalling entity in the brain able to influence neurons in multiple ways. However, we are only beginning to understand these effects, and future studies are needed to unravel the detailed mechanisms. Elucidating these mechanisms will enhance our understanding of brain performance in health and disease.

References

1. Raposo G, Stoorvogel W. 2013 Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* **200**, 373–383. (doi:10.1083/jcb.201211138)

2. Johnstone RM, Mathew A, Mason AB, Teng K. 1991 Exosome formation during maturation of mammalian and avian reticulocytes: evidence that exosome release is a major route for externalization of obsolete membrane proteins. *J. Cell Physiol.* **147**, 27–36. (doi:10.1002/jcp.1041470105)

3. Ludwig AK, Giebel B. 2012 Exosomes: small vesicles participating in intercellular communication.

Int. J. Biochem. Cell Biol. **44**, 11–15. (doi:10.1016/j.biocel.2011.10.005)

4. Bellingham SA, Guo BB, Coleman BM, Hill AF. 2012 Exosomes: vehicles for the transfer of toxic proteins associated with neurodegenerative diseases? *Front. Physiol.* **3**, 124. (doi:10.3389/fphys.2012.00124)

5. Thery C, Ostrowski M, Segura E. 2009 Membrane vesicles as conveyors of immune responses. *Nat. Rev. Immunol.* **9**, 581–593. (doi:10.1038/nri2567)

6. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. 2007 Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of

genetic exchange between cells. *Nat. Cell Biol.* **9**, 654–659. (doi:10.1038/ncb1596)

7. Pegtel DM et al. 2010 Functional delivery of viral miRNAs via exosomes. *Proc. Natl Acad. Sci. USA* **107**, 6328–6333. (doi:10.1073/pnas.0914843107)

8. Skog J, Würdinger T, van Rijn S, Meijer DH, Gainche L, Curry WT, Carter BS, Krichevsky AM, Breakefield XO. 2008 Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* **10**, 1470–1476. (doi:10.1038/ncb1800)

9. Zhang Y et al. 2010 Secreted monocytic miR-150 enhances targeted endothelial cell migration. Mol. Cell 39, 133–144. (doi:10.1016/j.molcel.2010.06.010)

10. Frühbeis C, Fröhlich D, Kuo WP, Krämer-Albers E-M. 2013 Extracellular vesicles as mediators of neuron-glia communication. Front. Cell Neurosci. 7, 182. (doi:10.3389/fncel.2013.00182)

11. Lachenal G et al. 2011 Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. Mol. Cell Neurosci. 46, 409–418. (doi:10.1016/j.mcn.2010.11.004)

12. Chivet M, Hemming F, Pernet-Gallay K, Fraboulet S, Sadoul R. 2012 Emerging role of neuronal exosomes in the central nervous system. Front. Physiol. 3, 145. (doi:10.3389/fphys.2012.00145)

13. Faure J et al. 2006 Exosomes are released by cultured cortical neurones. Mol. Cell Neurosci. 31, 642–648. (doi:10.1016/j.mcn.2005.12.003)

14. Frühbeis C et al. 2013 Neurotransmitter-triggered transfer of exosomes mediates oligodendrocyte-neuron communication. PLoS Biol. 11, e1001604. (doi:10.1371/journal.pbio.1001604)

15. Bakhti M, Winter C, Simons M. 2011 Inhibition of myelin membrane sheath formation by oligodendrocyte-derived exosome-like vesicles. J. Biol. Chem. 286, 787–796. (doi:10.1074/jbc.M110.190009)

16. Fitzner D, Schnaars M, van Rossum D, Krishnamoorthy G, Dibaj P, Bakhti M, Regen T, Hanisch U-K, Simons M. 2011 Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. J. Cell Sci. 124, 447–458. (doi:10.1242/jcs.074088)

17. Lopez-Verrilli MA, Picou F, Court FA. 2013 Schwann cell-derived exosomes enhance axonal regeneration in the peripheral nervous system. Glia 61, 1795–1806. (doi:10.1002/glia.22558)

18. Krämer EM et al. 1997 Oligodendrocytes direct glycosyl phosphatidylinositol-anchored proteins to the myelin sheath in glycosphingolipid-rich complexes. J. Biol. Chem. 272, 8937–8945. (doi:10.1074/jbc.272.16.10558)

19. Feldmann A et al. 2011 Transport of the major myelin proteolipid protein is directed by VAMP3 and VAMP7. J. Neurosci. 31, 5659–5672. (doi:10.1523/JNEUROSCI.6638-10.2011)

20. Gould SJ, Raposo G. 2013 As we wait: coping with an imperfect nomenclature for extracellular vesicles. J. Extracell. Vesicles 2, 20389. (doi:10.3402/jev.v2i0.20389)

21. Witwer KW et al. 2013 Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J. Extracell. Vesicles 2, 20360. (doi:10.3402/jev.v2i0.20360)

22. Gentleman RC et al. 2004 Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80. (doi:10.1186/gb-2004-5-10-r80)

23. Nimmervoll B, White R, Yang J-W, An S, Henn C, Sun J-J, Luhmann HJ. 2013 LPS-induced microglial secretion of TNFalpha increases activity-dependent neuronal apoptosis in the neonatal cerebral cortex.

24. Sun JJ, Kilb W, Luhmann HJ. 2010 Self-organization of repetitive spike patterns in developing neuronal networks in vitro. Eur. J. Neurosci. 32, 1289–1299. (doi:10.1111/j.1460-9568.2010.07383.x)

25. Antonucci F et al. 2012 Microvesicles released from microglia stimulate synaptic activity via enhanced sphingolipid metabolism. EMBO J. 31, 1231–1240. (doi:10.1038/emboj.2011.489)

26. Newcomb-Fernandez JK, Zhao X, Pike BR, Wang KKW, Kampfl A, Beer R, DeFord SM, Hayes RL. 2001 Concurrent assessment of calpain and caspase-3 activation after oxygen–glucose deprivation in primary septo-hippocampal cultures. J. Cereb. Blood Flow Metab. 21, 1281–1294. (doi:10.1097/00004647-200111000-00004)

27. Abramov AY, Scorziello A, Duchen MR. 2007 Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. J. Neurosci. 27, 1129–1138. (doi:10.1523/JNEUROSCI.4468-06.2007)

28. Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME. 1997 Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275, 661–665. (doi:10.1126/science.275.5300.661)

29. Kennedy SG, Wagner AJ, Conzen SD, Jordan J, Bellacosa A, Tsichlis PN, Hay N. 1997 The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev. 11, 701–713. (doi:10.1101/gad.11.6.701)

30. Atwal JK, Massie B, Miller FD, Kaplan DR. 2000 The TrkB-Shc site signals neuronal survival and local axon growth via MEK and PI3-kinase. Neuron 27, 265–277. (doi:10.1016/S0896-6273(00)00035-0)

31. Jin K, Mao XO, Zhu Y, Greenberg DA. 2002 MEK and ERK protect hypoxic cortical neurons via phosphorylation of Bad. J. Neurochem. 80, 119–125. (doi:10.1046/j.0022-3042.2001.00678.x)

32. Barco A, Marie H. 2011 Genetic approaches to investigate the role of CREB in neuronal plasticity and memory. Mol. Neurobiol. 44, 330–349. (doi:10.1007/s12035-011-8209-x)

33. Sassone-Corsi P. 1998 Coupling gene expression to cAMP signalling: role of CREB and CREM. Int. J. Biochem. Cell Biol. 30, 27–38. (doi:10.1016/S1357-2725(97)00093-9)

34. Smyth GK. 2005 Limma: linear models for microarray data. In Bioinformatics and computational biology solutions using R and bioconductor (eds R Gentleman, VJ Carey, W Huber, RA Irizarry, S Dudoit), pp. 397–420. Berlin, Germany: Springer.

35. Lau P, Verrier JD, Nielsen JA, Johnson KR, Notterpek L, Hudson LD. 2008 Identification of dynamically regulated microRNA and mRNA networks in developing oligodendrocytes. J. Neurosci. 28, 11 720–11 730. (doi:10.1523/JNEUROSCI.1932-08.2008)

36. Horesh D, Sapir T, Francis F, Grayer Wolf S, Caspi M, Elbaum M, Chelly J, Reiner O. 1999 Doublecortin, a stabilizer of microtubules. Hum. Mol. Genet. 8, 1599–1610. (doi:10.1093/hmg/8.9.1599)

37. Eldh M, Ekström K, Valadi H, Sjöstrand M, Olsson B, Jernås M, Lötvall J, Tailleux L. 2010 Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. PLoS ONE 5, e15353. (doi:10.1371/journal.pone.0015353)

38. Taylor AR, Robinson MB, Gifondorwa DJ, Tytell M, Milligan CE. 2007 Regulation of heat shock protein 70 release in astrocytes: role of signaling kinases. Dev. Neurobiol. 67, 1815–1829. (doi:10.1002/dneu.20559)

39. Schneider A, Simons M. 2013 Exosomes: vesicular carriers for intercellular communication in neurodegenerative disorders. Cell Tissue Res. 352, 33–47. (doi:10.1007/s00441-012-1428-2)

40. Goldberg MP, Choi DW. 1993 Combined oxygen and glucose deprivation in cortical cell culture: calcium-dependent and calcium-independent mechanisms of neuronal injury. J. Neurosci. 13, 3510–3524.

41. De Cristobal J, Cardenas A, Lizasoain I, Leza JC, Fernandez-Tome P, Lorenzo P, Moro MA. 2002 Inhibition of glutamate release via recovery of ATP levels accounts for a neuroprotective effect of aspirin in rat cortical neurons exposed to oxygen–glucose deprivation. Stroke 33, 261–267. (doi:10.1161/hs0102.101299)

42. Brown IR. 2007 Heat shock proteins and protection of the nervous system. Ann. N Y Acad. Sci. 1113, 147–158. (doi:10.1196/annals.1391.032)

43. Tytell M. 2005 Release of heat shock proteins (Hsps) and the effects of extracellular Hsps on neural cells and tissues. Int. J. Hyperthermia 21, 445–455. (doi:10.1080/02656730500041921)

44. Guzhova I, Kislyakova K, Moskaliova O, Fridlanskaya I, Tytell M, Cheetham M, Margulis B. 2001 In vitro studies show that Hsp70 can be released by glia and that exogenous Hsp70 can enhance neuronal stress tolerance. Brain Res. 914, 66–73. (doi:10.1016/S0006-8993(01)02774-3)

45. Tytell M, Greenberg SG, Lasek RJ. 1986 Heat shock-like protein is transferred from glia to axon. Brain Res. 363, 161–164. (doi:10.1016/0006-8993(86)90671-2)

46. Kassmann CM et al. 2007 Axonal loss and neuroinflammation caused by peroxisome-deficient oligodendrocytes. Nat. Genet. 39, 969–976. (doi:10.1038/ng2070)

47. Basso M et al. 2013 Mutant copper-zinc superoxide dismutase (SOD1) induces protein secretion pathway alterations and exosome release in astrocytes: implications for disease spreading and motor neuron pathology in amyotrophic lateral sclerosis. J. Biol. Chem. 288, 15 699–15 711. (doi:10.1074/jbc.M112.425066)

48. Gomes C, Keller S, Altevogt P, Costa J. 2007 Evidence for secretion of Cu,Zn superoxide dismutase via exosomes from a cell model of amyotrophic lateral sclerosis. Neurosci. Lett. 428, 43–46. (doi:10.1016/j.neulet.2007.09.024)

49. Kandel ER. 2012 The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. Mol. Brain 5, 14. (doi:10.1186/1756-6606-5-14)

50. Sakamoto K, Karelina K, Obrietan K. 2011 CREB: a multifaceted regulator of neuronal plasticity and

protection. *J. Neurochem.* **116**, 1–9. (doi:10.1111/j. 1471-4159.2010.07080.x)

51. Walton MR, Dragunow I. 2000 Is CREB a key to neuronal survival? *Trends Neurosci.* **23**, 48–53. (doi:10.1016/S0166-2236(99)01500-3)

52. Tao X, Finkbeiner S, Arnold DB, Shaywitz AJ, Greenberg ME. 1998 Ca^{2+} influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron* **20**, 709–726. (doi:10.1016/S0896-6273(00)81010-7)

53. Lonze BE, Ginty DD. 2002 Function and regulation of CREB family transcription factors in the nervous system. *Neuron* **35**, 605–623. (doi:10.1016/S0896-6273(02)00828-0)

54. Riccio A, Alvania RS, Lonze BE, Ramanan N, Kim T, Huang Y, Dawson TM, Snyder SH, Ginty DD. 2006 A nitric oxide signaling pathway controls CREB-mediated gene expression in neurons. *Mol. Cell* **21**, 283–294. (doi:10.1016/j.molcel.2005.12.006)

55. Du K, Montminy M. 1998 CREB is a regulatory target for the protein kinase Akt/PKB. *J. Biol. Chem.* **273**, 32 377–32 379. (doi:10.1074/jbc.273. 49.32377)

56. Beitner-Johnson D, Rust RT, Hsieh TC, Millhorn DE. 2001 Hypoxia activates Akt and induces phosphorylation of GSK-3 in PC12 cells. *Cell Signal.* **13**, 23–27. (doi:10.1016/S0898-6568(00)00128-5)

57. Doble BW, Woodgett JR. 2003 GSK-3: tricks of the trade for a multi-tasking kinase. *J. Cell Sci.* **116**, 1175–1186. (doi:10.1242/jcs.00384)

58. Rockenstein E, Torrance M, Adame A, Mante M, Bar-on P, Rose JB, Crews L, Masliah E. 2007 Neuroprotective effects of regulators of the glycogen synthase kinase-3beta signaling pathway in a transgenic model of Alzheimer's disease are associated with reduced amyloid precursor protein

phosphorylation. *J. Neurosci.* **27**, 1981–1991. (doi:10.1523/JNEUROSCI.4321-06.2007)

59. Chin PC, Majdzadeh N, D'Mello SR. 2005 Inhibition of GSK3beta is a common event in neuroprotection by different survival factors. *Brain Res. Mol. Brain Res.* **137**, 193–201. (doi:10.1016/j.molbrainres. 2005.03.004)

60. Dhanasekaran DN, Reddy EP. 2008 JNK signaling in apoptosis. *Oncogene* **27**, 6245–6251. (doi:10.1038/ onc.2008.301)

61. Leppa S, Bohmann D. 1999 Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. *Oncogene* **18**, 6158–6162. (doi:10.1038/ sj.onc.1203173)

62. Oliva Jr AA, Atkins CM, Copenagle L, Banker GA. 2006 Activated c-Jun N-terminal kinase is required for axon formation. *J. Neurosci.* **26**, 9462–9470. (doi:10.1523/JNEUROSCI.2625-06.2006)

63. Kreutz MR, Bien A, Vorwerk CK, Böckers TM, Seidenbecher CI, Tischmeyer W, Sabel BA. 1999 Co-expression of c-Jun and ATF-2 characterizes the surviving retinal ganglion cells which maintain axonal connections after partial optic nerve injury. *Brain Res. Mol. Brain Res.* **69**, 232–241. (doi:10. 1016/S0169-328X(99)00113-8)

64. Robinson GA. 1996 Changes in the expression of transcription factors ATF-2 and Fra-2 after axotomy and during regeneration in rat retinal ganglion cells. *Brain Res. Mol. Brain Res.* **41**, 57–64. (doi:10.1016/ 0169-328X(96)00070-8)

65. Arlt A, Schafer H. 2011 Role of the immediate early response 3 (IER3) gene in cellular stress response, inflammation and tumorigenesis. *Eur. J. Cell Biol.* **90**, 545–552. (doi:10.1016/j.ejcb.2010.10.002)

66. Gorski JA, Balogh SA, Wehner JM, Jones KR. 2003 Learning deficits in forebrain-restricted brain-

derived neurotrophic factor mutant mice. *Neuroscience* **121**, 341–354. (doi:10.1016/S0306-4522(03)00426-3)

67. Scharfman H, Goodman J, Macleod A, Phani S, Antonelli C, Croll S. 2005 Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats. *Exp. Neurol.* **192**, 348–356. (doi:10.1016/j.expneurol.2004. 11.016)

68. Shimizu E et al. 2003 Alterations of serum levels of brain-derived neurotrophic factor (BDNF) in depressed patients with or without antidepressants. *Biol. Psychiatry* **54**, 70–75. (doi:10.1016/S0006-3223(03)00181-1)

69. Bozdagi O, Rich E, Tronel S, Sadahiro M, Patterson K, Shapiro ML, Alberini CM, Huntley GW, Salton SRJ. 2008 The neurotrophin-inducible gene Vgf regulates hippocampal function and behavior through a brain-derived neurotrophic factor-dependent mechanism. *J. Neurosci.* **28**, 9857–9869. (doi:10. 1523/JNEUROSCI.3145-08.2008)

70. Alder J et al. 2003 Brain-derived neurotrophic factor-induced gene expression reveals novel actions of VGF in hippocampal synaptic plasticity. *J. Neurosci.* **23**, 10 800–10 808.

71. Thakker-Varia S, Krol JJ, Nettleton J, Bilimoria PM, Bangasser DA, Shors TJ, Black IB, Alder J. 2007 The neuropeptide VGF produces antidepressant-like behavioral effects and enhances proliferation in the hippocampus. *J. Neurosci.* **27**, 12 156–12 167. (doi:10.1523/JNEUROSCI.1898-07.2007)

72. Hughes EG, Kang SH, Fukaya M, Bergles DE. 2013 Oligodendrocyte progenitors balance growth with self-repulsion to achieve homeostasis in the adult brain. *Nat. Neurosci.* **16**, 668–676. (doi:10.1038/ nn.3390)