MICRO REPORT

A Jacob/nsmf gene knockout does not protect against acute hypoxia- and NMDA-induced excitotoxic cell death

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Abstract

Jacob is a synapto-nuclear messenger protein that encodes and transduces the origin of synaptic and extrasynaptic NMDA receptor signals to the nucleus. The protein assembles a signalosome that differs in case of synaptic or extrasynaptic NMDAR activation. Following nuclear import Jacob docks these signalosomes to the transcription factor CREB. We have recently shown that amyloid-β and extrasynaptic NMDAR activation triggers the translocation of a Jacob signalosome that results in inactivation of the transcription factor CREB, a phenomenon termed Jacob-induced CREB shut-off (JaCS). JaCS contributes to early Alzheimer's disease pathology and the absence of Jacob protects against amyloid pathology. Given that extrasynaptic activity is also involved in acute excitotoxicity, like in stroke, we asked whether nsmf gene knockout will also protect against acute insults, like oxygen and glucose deprivation and excitotoxic NMDA stimulation. nsmf is the gene that encodes for the Jacob protein. Here we show that organotypic hippocampal slices from wild-type and nsmf$^{-/-}$ mice display similar degrees of degeneration when exposed to either oxygen glucose deprivation or 50 μM NMDA to induce excitotoxicity. This lack of neuroprotection indicates that JaCS is mainly relevant in conditions of low level chronic extrasynaptic NMDAR activation that results in cellular degeneration induced by alterations in gene transcription.

Keywords Jacob, Nsmf, Oxygen–glucose deprivation, Cell death, Excitoxicity, Extrasynaptic N-methyl-D-aspartate receptor, Stroke

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Disruption of cAMP-responsive element-binding protein (CREB) transcriptional activity, a master regulator of cell survival and plasticity-related gene expression, is a hallmark of neurodegeneration [1]. Long-lasting dephosphorylation of CREB at serine 133, termed CREB shut-off, results in early synaptic dysfunction, contributes to pathology and eventually neuronal cell death. It is elicited by sustained activation of extrasynaptic N-methyl-D-aspartate-receptors (NMDAR). Glutamate spillover to peri- and extrasynaptic sites causes in conjunction with binding of amyloid-β (Aβ) detrimental activation of extrasynaptic NMDAR at early stages of Alzheimer's disease (AD). In previous work we found that the messenger protein Jacob encodes and transduces the synaptic or extrasynaptic origin of NMDAR signals to the nucleus

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[2]. In response to cell survival and plasticity-related synaptic NMDAR stimulation, macromolecular transport of Jacob from synapses to the nucleus docks the extracellular signaling-regulated kinase (ERK) to the CREB complex which results in sustained CREB phosphorylation at serine 133 [2]. Following disease-related activation of extrasynaptic NMDARs, Jacob associates with protein phosphatase-1γ (PP1γ) and induces dephosphorylation and transcriptional inactivation of CREB (Jacob-induced CREB shut-off (JaCS) [3]). Binding of the adaptor protein LIM domain only 4 (LMO4) distinguishes extrasynaptic from synaptic NMDAR signaling and determines the affinity for the association with PP1γ [3]. This mechanism contributes to transcriptional inactivation of CREB in the context of early synaptic dysfunction in AD [3]. Accordingly, Jacob protein knockdown attenuates Aβ-induced CREB shut-off induced via activation of extrasynaptic NMDARs and nsmf gene knockout is neuroprotective in a transgenic mouse model of AD [3]. Collectively the data suggest that long-distance protein transport from extrasynaptic NMDAR to the nucleus is part of early AD pathology and that Jacob docks a signalosome to CREB that is instrumental for CREB shut-off.

We now asked whether this mechanism is also relevant in cell death induced by acute excitotoxic insults, like those resulting from traumatic brain injury and stroke [4]. While the molecular underpinnings that drive cell death might differ between acute and chronic neurodegenerative insults, activation of extrasynaptic NMDAR appears to be fundamental in both conditions. To tackle this question, we employed organotypic hippocampal slice cultures (OHSC) of wild-type (wt) and nsmf knockout mice [5], and submitted them to two well-established protocols to study stroke-like excitotoxic insults. We predicted that the nsmf gene knockout would have a neuroprotective effect on OHSC exposed to either oxygen and glucose deprivation (OGD) or bath application of high doses of NMDA since both conditions are known to induce CREB shut-off [6].

In the first set of experiments, OHSC from wt and nsmf$^{-/-}$ mice were submitted to OGD for 30 min and cell death was assessed via monitoring propidium iodide (PI) uptake at different intervals after the insult (3 h, 8 h, 12 h and 24 h; for detailed methods see Additional file 1). PI is a red-fluorescent nuclear counterstain not permeant to living cells, thus the increase in fluorescence provides a read out of cell death. Statistical analysis revealed that exposure of OHSC to 30 min OGD induces strong cell death in the CA1 and CA3 subregions of the hippocampus of wt slices, as early as 3 h after the insult (Fig. 1A, C–E, Two-way repeated measures ANOVA, time x OGD CA1 $F_{(12,140)}=25.43$, $p < 0.0001$; CA3 $F_{(12,140)}=10$,

$p < 0.0001$; DG $F_{(12,140)}=11.42$, $p < 0.0001$). Cell death was also detected in the dentate gyrus (DG), although to a lower degree when compared to the other subregions, which is in line with several studies applying OGD [7, 8]. Surprisingly, regardless of the subregion analyzed, no difference in cell death between wild-type and nsmf$^{-/-}$ slices was observed at all time points and in all subregions analyzed (Fig. 1A, C–E, Mixed-effects model analysis CA1 nsmf$^{+/+}$ x nsmf$^{-/-}$ $F_{(1,19)}=0.003$, $p=0.9057$; CA3 $F_{(1,19)}=0.3232$, $p=0.5763$; DG $F_{(1,19)}=0.1593$, $p=0.6942$).

In the next set of experiments, we assessed whether nsmf gene knockout confers protective effects on OHSC incubated with an excitotoxic dose of 50 μM NMDA. Statistical analysis revealed that 30 min bath application of NMDA induces strong cell death in wild-type slices over time, as indicated by the increase in PI uptake in all subregions as early as 3 h (Fig. 1F–I, Two-way repeated measures ANOVA, time x OGD CA1 $F_{(12,140)}=47.07$, $p < 0.0001$; CA3 $F_{(12,140)}=15.68$, $p < 0.0001$; DG $F_{(12,132)}=54.57$, $p < 0.0001$). NMDA-induced cell death reached a plateau in both CA1 and CA3 subregions 8 h after NMDA bath application (Fig. 1G, H), while cell death reached its peak at 24 h in DG during the examined time period (Fig. 1I). Similar to the OGD experiments, no difference in cell death between wild-type and nsmf$^{-/-}$ slices was observed at all time points and in all subregions analyzed (Fig. 1F–I, Mixed-effects model analysis CA1 nsmf$^{+/+}$ x nsmf$^{-/-}$ $F_{(1,23)}=1.097$, $p=0.3058$; CA3 $F_{(1,23)}=1.602$, $p=0.2182$; DG $F_{(1,23)}=0.2236$, $p=0.6408$). Lastly, as a control experiment, we co-applied the NMDAR antagonist MK-801 to OHSC in order to confirm that NMDA-induced cell death occurs via activation of NMDA receptors. Statistical analysis revealed that co-application of MK-801 with NMDA completely abolished PI uptake, at all time-points (Fig. 1J, K, Two-way repeated measures ANOVA groups x time $F_{(1,6)}=0.3347$, $p=0.5840$).

Here we showed that both OGD and NMDA protocols induce cell death in wild-type and nsmf$^{-/-}$ OHSC slices to the same extent, suggesting that JaCS is not involved in acute excitotoxic insults. Excessive entry of Ca^{2+} via NMDARs causes disruption of mitochondrial calcium homeostasis, leading to neuronal cell death by apoptosis [9]. In the face of an acute excitotoxic insult, production of reactive oxygen species and breakdown of the mitochondrial membrane potential are the probable culprits for neurodegeneration [6]. In conclusion, JaCS appear to be relevant in scenarios where activation of extrasynaptic NMDARs builds up slowly, is chronic and results in cellular degeneration due to alterations in gene transcription.
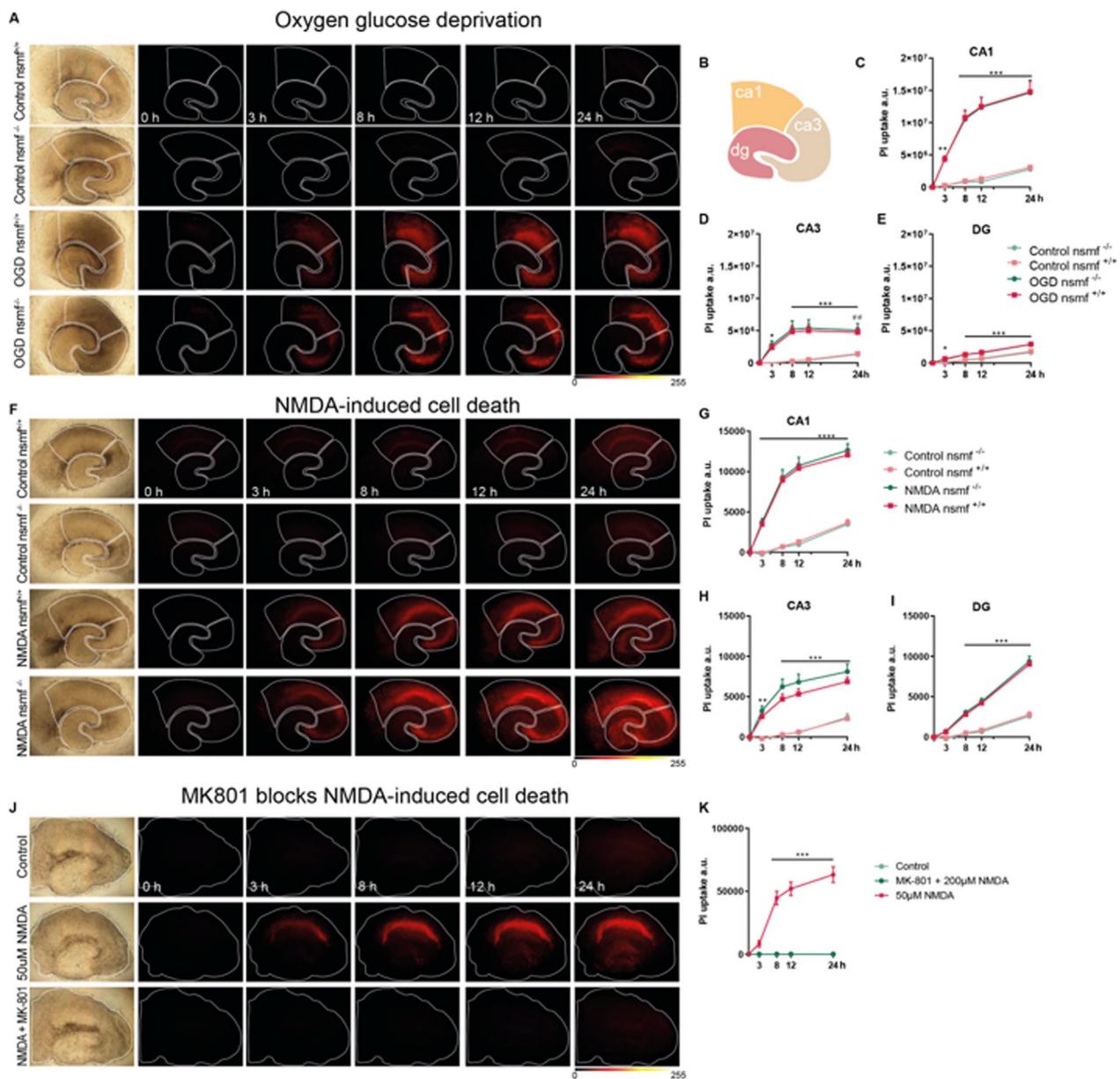
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Fig. 1 Jacob/nsmf gene knockout does not protect against OGD- and NMDA-induced cell death. **A–E** Oxygen glucose deprivation (OGD) induces cell death in organotypic hippocampal slice culture irrespective of mice genotype. **A** Bright field and fluorescent images of propidium iodide (PI) signal in organotypic hippocampal slices from wild-type (nsmf$^{+/+}$) and Jacob/nsmf constitutive knock-out animals (snmf$^{-/-}$) 0, 3, 8, 12, and 24 h after 30 min OGD and control. **B** Scheme representing CA1, CA3, and DG areas. **C–E** Graphs representing the degree of PI uptake in arbitrary units (A.U.) over time (h) after OGD insult. The OGD protocol induced cell death to the same degree in CA1 (**C**), CA3 (**D**), and DG (**E**) irrespective of genotype. N = Control nsmf$^{+/+}$: 11; OGD nsmf$^{+/+}$: 13; Control nsmf$^{-/-}$: 7; OGD nsmf$^{-/-}$: 8 slices per group. **$p < 0.01$, ***$p < 0.001$ OGD Jac$^{+/+}$ x control Jac$^{+/+}$, $^{\#\#}p < 0.01$ OGD Jac$^{-/-}$ x control Jac$^{-/-}$ by repeated measures (RM) two-way ANOVA followed by Bonferroni's multiple comparisons test. Data represented as mean ± SEM. **F–I** Acute NMDA (50 μM) treatment induces cell death in OHSC irrespective of genotype. **F** Brightfield and PI signal in organotypic hippocampal slices from Jac$^{+/+}$ Jac$^{-/-}$ animals after 0, 3, 8, 12, and 24 h post treatment with 50 μM NMDA or control. **G, H, I** Graphs representing the degree of PI uptake (A.U.) over time (h) after treatment with 50 μM NMDA. 50 μM NMDA induced cell death to the same degree in CA1, CA3, and DG irrespective of genotype. N = Control nsmf$^{+/+}$: 8; NMDA nsmf$^{+/+}$: 15; Control nsmf$^{-/-}$: 6; NMDA nsmf$^{-/-}$: 10 slices per group. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ NMDA nsmf$^{+/+}$ x control nsmf$^{+/+}$ by RM two-way ANOVA followed by Bonferroni's multiple comparisons test. Data represented as mean ± SEM. **J, K** MK-801 blocks NMDA-induced cell death in OHSC from C57BL6/J mice. **J** Brightfield and PI signal from OHSC 0, 3, 8, 12 and 24 h after treatment with 50 μM NMDA, co-application of MK-801 + 200 μM NMDA, or control. **K** The co-application of MK-801 completely abolished the effects of NMDA treatment. N = Control: 4; NMDA: 8; NMDA + MK-801: 4 slices per group. ***$p < 0.001$ vs. control by RM two-way ANOVA followed by Bonferroni's multiple comparisons test. Data represented as mean ± SEM. Lookup table indicates the pixel intensities from 0 to 255

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Abbreviations

NMDAR	N-methyl-D-aspartate receptor
AD	Alzheimer's disease
ERK	Extracellular signaling-regulated kinase
nsmf	NMDAR synaptonuclear signaling and neuronal migration factor
PP1γ	Protein phosphatase-1γ
LMO4	LIM domain only 4
Aβ	Amyloid-beta peptide
OHSC	Organotypic hippocampal slice cultures
WT	Wild-type
CREB	CAMP response element-binding protein
NMDA	N-methyl-D-aspartate
OGD	Oxygen and glucose deprivation
DG	Dentate gyrus
CA1	Cornus ammonis 1
CA3	Cornus ammonis 3
ANOVA	Analysis of variance
PI	Propidium Iodide

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13041-023-01012-2.

> **Additional file 1.** Extended materials and methods, detailed information on statistics.

Availability of data and materials

Please contact the corresponding author for data requests.

Declarations

Ethics approval and consent to participate

Experiments were conducted following ethical animal research standards defined by the German Law/European directive and approved by the Landesverwaltungsamt Saxony-Anhalt (Referat 203, Verbraucherschutz und Veterinärangelegenheiten). The competent authority follows the advice of an official animal welfare committee of the Federal State of Saxony-Anhalt, Germany.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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