NR4A orphan nuclear receptors as mediators of CREB-dependent neuroprotection

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Induced expression of neuroprotective genes is essential for maintaining neuronal integrity after stressful insults to the brain. Here we show that NR4A nuclear orphan receptors are induced after excitotoxic and oxidative stress in neurons, up-regulate neuroprotective genes, and increase neuronal survival. Moreover, we show that NR4A proteins are induced by cAMP response element binding protein (CREB) in neurons exposed to stressful insults and that they function as mediators of CREB-induced neuronal survival. Animals with null mutations in three of six *NR4A* alleles show increased oxidative damage, blunted induction of neuroprotective genes, and increased vulnerability in the hippocampus after treatment with kainic acid. We also demonstrate that NR4A and the transcriptional coactivator PGC-1α independently regulate distinct CREB-dependent neuroprotective gene programs. These data identify NR4A nuclear orphan receptors as essential mediators of neuroprotection after exposure to neuropathological stress.

excitotoxicity | kainic acid | oxidative stress

Neuropathological conditions including stroke, Alzheimer's disease, and Parkinson's disease are associated with excitotoxic and oxidative stress. Transcriptional increases of neuroprotective genes, including antiapoptotic factors and scavengers of reactive oxygen species (ROS), are an important strategy for neuroprotection. Thus, understanding how neuroprotective gene programs are controlled at the transcriptional level is of considerable importance and may contribute to the identification of therapeutic strategies of disorders associated with neurodegeneration.

cAMP response element binding protein (CREB) is a transcription factor that is activated in response to stressful stimuli such as hypoxia, oxidative stress, excitotoxicity, and ischemia (1). Evidence from loss-of-function and other types of experiments shows that CREB plays an important role in neuronal survival (2–5) and neuroprotection (6). It is also well established that CREB is required for acquisition of ischemic tolerance, an endogenous neuroprotective mechanism whereby prior exposure to brief ischemia produces resilience to subsequent normally injurious ischemia (7, 8).

Despite the well-documented neuroprotective effect of CREB, only little is known of how CREB mediates this activity and only few directly regulated neuroprotective target genes have been identified (9–13). In addition to target genes that are directly neuroprotective, CREB-induced transcription factors or cofactors may also contribute to neuron survival by regulating downstream gene batteries controlled by elevated cAMP levels in a transcription factor cascade initiated by activated CREB. Indeed, CREB induces the expression of peroxisome proliferator-activated receptor gamma coactivator-1a (PGC-1α), an important regulator of ROS-detoxifying enzyme gene expression (14). However, how CREB mediates neuroprotective gene cascades via the induction of additional transcriptional regulators remains unexplored.

The NR4A orphan nuclear receptor (NR) family of transcription factors is rapidly and strongly up-regulated after stressful insults to the CNS (15–18). NR4A proteins consist of NR4A1 (NGFI-B), NR4A2 (Nurr1), and NR4A3 (Nor-1). Structural studies of the NR4A2 ligand-binding domain have shown that these receptors lack a ligand-binding pocket and appear to function as ligand-independent transcription factors (19). However, NR4A1 and NR4A2 can also form heterodimers with the retinoid X receptor (RXR) and they can activate gene expression in response to RXR ligands including 9-*cis* retinoic acid (20) and docosahexaenoic acid (21).

Because these proteins do not bind ligands, NR4A-regulated genes may be regulated by posttranslational modification of the NR4A proteins or altered *NR4A* gene expression. Indeed, the expression of *NR4A* genes can be induced by a wide range of physiological signals such as membrane depolarization and growth factors (22). Notably, NR4A receptors are robustly induced in the CNS by pathological stimuli such as ischemia (17, 18), seizures (16), and focal brain injury (15). These stimuli are also associated with CREB activation. Recently, using genome-wide expression profiling in mice lacking CREB in the forebrain, NR4A1 and NR4A2 were identified as two of the few genes that require CREB for their induction in the hippocampus after administration of the glutamate analog kainic acid (23). However, the functional consequences of stress-induced NR4A expression in neurons have remained unknown.

Considering their rapid induction by stimuli converging on CREB activation in neurons, we wished to investigate if NR4A receptors might be downstream mediators of the neuroprotective effects of CREB. We used experiments in cultured neurons to show that NR4A proteins are neuroprotective when cells are exposed to either excitotoxic or oxidative stress. Moreover, we have shown that NR4A receptors regulate neuroprotective genes and we investigated the response to excitotoxicity in vivo in *NR4A* gene-deficient mice. Collectively, our results identify NR4A nuclear receptors as essential transcription factors in a neuron survival program downstream of CREB and indicate that they may be suitable targets for intervention in neurodegenerative disease.

Results

Rolipram-Induced CREB-Dependent Neuroprotection by NR4A Receptors. We studied neuronal survival after neurotoxic stress in mouse embryonic stem (ES) cell-derived neurons (24). By this protocol GABAergic and glutamatergic neurons expressing forebrain markers are generated (*SI Appendix*, Fig. S1). Neurons were enriched by magnetic cell sorting (MACS) (*SI Appendix*, Fig. S1). To study CREB-dependent neuroprotection, neuronal survival was assessed after pharmacological up-regulation of CREB by pretreatment

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The authors declare no conflict of interest.

Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE20392).

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with rolipram, a type IV phosphodiesterase that induces CREB phosphorylation (p-CREB) and confers neuroprotection from various types of stress (25, 26) (Fig. 1*A*). CREB function was analyzed by a decoy strategy whereby cultured neurons were transfected with DNA oligos containing the CREB DNA responsive element (CRE) (*SI Appendix,* Fig. S2). The CRE decoy titrates CREB away from its natural promoter targets and shows if an observed effect is CREB dependent. As expected, rolipram preconditioning resulted in CREB-dependent resistance to hydrogen peroxide-, ionomycin-, and glutamate-induced toxicity (Fig. 1*B*).

NR4A receptors are induced as downstream CREB targets in rolipram-treated neurons as shown in *SI Appendix,* Fig. S3 *A* and *B*. In addition, neuropathological stress (ionomycin and hydrogen peroxide) known to induce CREB also induced CREB-dependent NR4A mRNA and protein (*SI Appendix,* Fig. S3*D*). Moreover, we confirmed the ability of CREB to directly regulate NR4A2 mRNA expression in neural cells by direct promoter binding and activation in transfected neural c17.2 cells (*SI Appendix,* Fig. S4). In conclusion, NR4A receptors are directly and robustly regulated in neurons in which CREB has been activated by neuronal stress (2- to 8-fold) or after pharmacological treatments that increase intracellular levels of cAMP (4- to 11-fold).

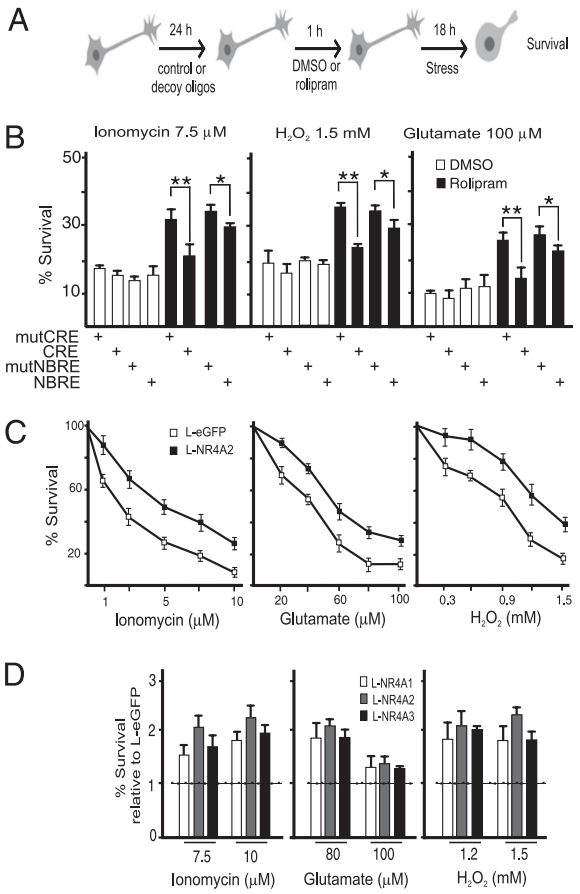

Fig. 1. NR4A-mediated neuroprotection. (*A* and *B*) MACS-sorted neurons were transfected with the indicated oligos for 5 h, received fresh medium for 19 h, and were then treated with rolipram or DMSO for 1 h and subsequently stressed with the indicated concentrations of ionomycin, H_2O_2, or glutamate for 18 h. Cell survival was assayed by adding cell titer reagent and measuring the absorbance at 490 nm. Error bars indicate SD, $n = 9$; *significant at $P < 0.05$; **significant at $P < 0.01$. (*C*) MACS-sorted neurons infected with eGFP or NR4A2 lentivirus were treated for 18 h with increasing concentrations of ionomycin, glutamate, or H_2O_2. Cell survival was assayed by adding cell titer reagent and measuring the absorbance at 490 nm. Error bars indicate SD, $n = 5$. (*D*) MACS-sorted neurons infected with eGFP or the indicated NR4A lentiviruses were treated for 18 h with the indicated concentrations of ionomycin, glutamate, or H_2O_2. Cell survival was assayed by adding cell titer reagent and measuring the absorbance at 490 nm. The values obtained with the different NR4A lentiviruses were normalized to the eGFP lentivirus values. Error bars indicate SD, $n = 5$.

We next tested if NR4A receptors mediate CREB-induced neuroprotective effects. An oligo containing NR4A DNA binding sites (referred to as NGFI-B response element, NBRE) was used as a decoy to block NR4A activity (*SI Appendix,* Fig. S2). Notably, rolipram pretreatment resulted in enhanced survival after exposure to all three stressors, but the neuroprotective effect was significantly reduced when neurons were transfected with NBRE decoy (Fig. 1*B*). The extent of inhibition was about half of that seen when cells were pretreated with CRE decoy (Fig. 1*B*). This result suggests that the NR4A receptors mediate a significant portion of the rolipram-induced neuroprotection. Lentiviral vectors were used to further investigate the involvement of NR4A receptors in neuroprotection. Lenti-NR4A2 (L-NR4A2), but not the Lenti-eGFP (L-eGFP)-transduced neurons, showed increased survival from ionomycin-, glutamate-, and hydrogen peroxide-induced toxicity (Fig. 1*C*). Moreover, lentiviral vectors for the expression of closely related NR4A1 and NR4A3 proteins also conferred neuroprotection (Fig. 1*D*). Thus, loss-of-function (by decoy blocking) and lentiviral-mediated gain-of-function show that NR4A receptors contribute to the neuroprotection observed in CREB-activated neurons.

NR4A2 Activates Neuroprotective Gene Expression. To gain further insight into how NR4A proteins promote survival after neuropathological stress, changes in gene expression resulting from increased NR4A expression were assessed. Sorted neurons were transduced with either L-NR4A2 or L-eGFP (*SI Appendix,* Fig. S5) and mRNA was subjected to microarray analysis. One hundred forty-nine genes were found to be up-regulated in L-NR4A2 samples at a statistically significant level (>1.6-fold). Thirty-nine genes were down-regulated (>1.6-fold). Interestingly, several of the induced genes have previously been shown to promote neuronal survival after stressful insults (Fig. 2*A* and *SI Appendix,* Table S1) Moreover, a gene list enrichment analysis showed that gene sets associated with additional functions or pathways linked to neuroprotection are up-regulated in mRNA samples from L-NR4A2–transduced neurons (*SI Appendix,* Table S2). qRT-PCR confirmed up-regulation of neuroprotective genes in mRNA samples derived from L-NR4A2-transduced neurons. Moreover, we wished to test if NR4A-regulated genes would also be induced in neurons treated with 8CPT-cAMP, a treatment that activates CREB. Indeed, all analyzed mRNAs were up-regulated ~2-fold in neurons exposed to 8CPT-cAMP, and CRE decoy transfection lowered induced mRNA levels (Fig. 2*B*). Importantly, 8CPT-cAMP-treated cells that were transfected with NBRE decoy showed diminished expression of neuroprotective genes by at least 50%, demonstrating that NR4A proteins are clearly important for CREB-mediated gene activation of these protective gene products (Fig. 2*C*).

CREB-Induced PGC-1α-Mediated Neuroprotection. The coactivator PGC-1α regulates the expression of a broad set of ROS defense genes and its promoter has been shown to be regulated by CREB under oxidative stress (14). We wished to investigate the relationship between PGC-1α and NR4A-induced neuroprotection. We first confirmed that both rolipram and 8CPT-cAMP treatment of neurons resulted in induction of PGC-1α mRNA in a CREB-dependent way (Fig. 3 *A* and *B*). We next used a Lenti-PGC-1α shRNA expression vector (*SI Appendix,* Fig. S6) to confirm that PGC-1α mediated neuroprotection was downstream of CREB in cultured neurons treated with rolipram. Results confirmed that PGC-1α could protect against oxidative stress, but we also found that PGC-1α did not promote protection against excitotoxic stress

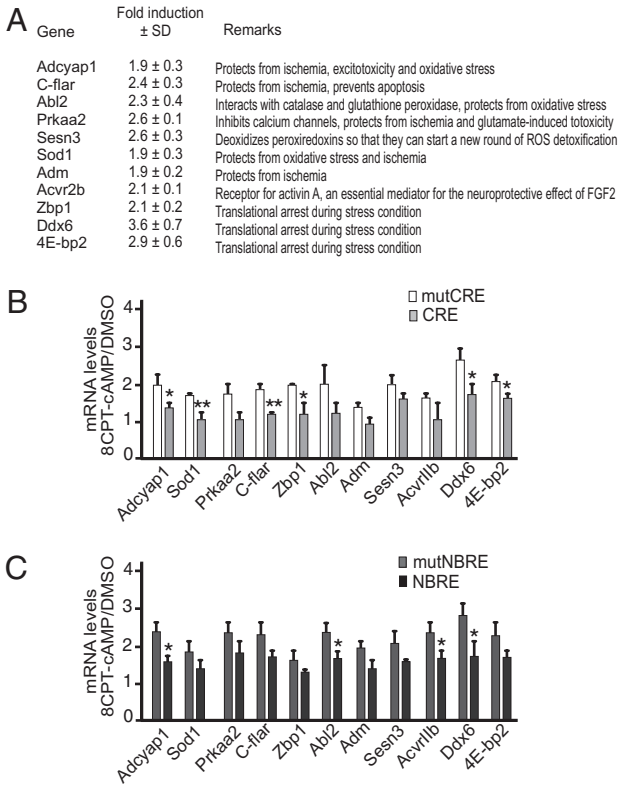

Fig. 2. NR4A2 activates neuroprotective gene expression. (*A*) Neuroprotective genes up-regulated in MACS-sorted neurons infected with L-NR4A2, $n = 3$. (*B* and *C*) MACS-sorted neurons were pretransfected with CRE or mutCRE oligo (*B*) or NBRE or mutNBRE oligo (*C*) and treated with 8CPT-cAMP or DMSO. Total RNA was extracted after 6 h and the expression levels of the indicated genes were determined by real-time PCR. Error bars indicate SD, $n = 4$; $*P < 0.05$; $**P < 0.01$. All genes as a group were significantly down-regulated by both CRE ($**P < 0.01$) and NBRE ($*P < 0.05$) decoys.

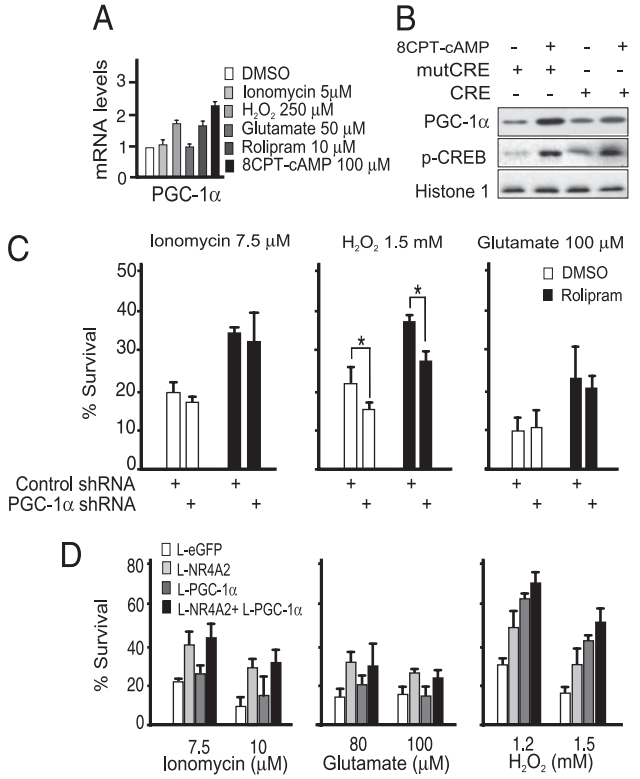

Fig. 3. CREB-induced PGC-1α-mediated neuroprotection. (*A*) MACS-sorted neurons were treated with the indicated chemicals. Total RNA was extracted after 2 h and the expression levels of PGC-1α were determined by real-time PCR. Error bars indicate SD, $n = 4$. (*B*) MACS-sorted neurons were pretransfected with CRE or mutCRE oligo and treated for 2 h with 8CPT-cAMP or DMSO. Nuclear extracts were prepared, resolved on SDS/PAGE, and probed with the indicated antibodies. Expression of Histone 1 was used as a loading control. (*C*) MACS-sorted neurons were infected with control or PGC-1α shRNA lentivirus for 24 h, received fresh medium for another 24 h, and were then treated with rolipram or DMSO for 1 h and subsequently stressed with the indicated concentrations of ionomycin, H_2O_2, or glutamate for 18 h. Cell survival was assayed by adding cell titer reagent and measuring the absorbance at 490 nm. Error bars indicate SD, $n = 9$; *significant at $P < 0.05$. (*D*) MACS-sorted neurons infected with the indicated lentiviruses were treated for 18 h with the indicated concentrations of ionomycin, glutamate, or H_2O_2. Cell survival was assayed by adding cell titer reagent and measuring the absorbance at 490 nm. Error bars indicate SD, $n = 5$.

(Fig. 3*C*). In accordance with these results, infection of neurons with a PGC-1α lentivirus (L-PGC-1α) conferred neuroprotection, but only against hydrogen peroxide-induced oxidative stress (Fig. 3*D*). Virus cotransduction using lentivirus vectors for both NR4A2 and PGC-1α resulted in a very high survival (70%) after exposure to hydrogen peroxide (Fig. 3*D*). However, the effect was additive rather than synergistic.

Because both NR4A2 and PGC-1α can mediate neuroprotection from hydrogen peroxide, we determined whether they regulate similar or distinct target genes. qRT-PCR was used to confirm that a number of previously identified PGC-1α target genes implicated in protection from oxidative stress (14) are up-regulated after transduction of L-PGC-1α. As seen in Fig. 4*A*, all analyzed target genes were robustly activated by forced expression of PGC-1α (2- to 7-fold); however, L-NR4A2 virus transduction resulted in only marginal up-regulation (2-fold) of one PGC-1α target (Prdx5). Reciprocally, only one of the analyzed neuroprotective NR4A2 target genes (Sod1) was significantly up-regulated (2-fold) by forced expression of PGC-1α (Fig. 4*B*). Thus, NR4A and PGC-1α appear to mediate distinct and complementary neuroprotective pathways because they induce distinct sets of neuroprotective gene targets.

NR4A Nuclear Receptors Protect Hippocampus Neurons Against Kainic Acid Toxicity. Finally, the significance of NR4A proteins in neuroprotection was evaluated in vivo by analyzing if NR4A proteins can confer neuroprotection to kainic acid (KA), a glutamate analogue that is associated with excitotoxicity and oxidative stress within the hippocampus. KA induces CREB-dependent expression of NR4A receptors in the cortex and the hippocampus in mice (23). In our experiments, wild-type mice or mice lacking three of six *NR4A* alleles (*NR4A2*$^{+/-}$; *NR4A3*$^{-/-}$; here referred to as *NR4Amut*) were injected with 25 mg/kg KA or saline. Three days following status epilepticus the histology of mice was analyzed within the hippocampus. As seen in Fig. 5*A*, saline-treated *NR4Amut* mice showed decreased cell density within the CA1 hippocampal field, consistent with the previous finding that NR4A3 knockout mice show a developmental decrease in neuron density within the hippocampus (27). Importantly, in contrast to wild type, *NR4mut* mice showed a significant decrease in the number of remaining cells within the CA1 region of the hippocampus following KA treatment (Fig. 5*A*). Moreover, in KA-treated *NR4mut* mice, but not in wild-type controls, an increase in oxidative stress was evident from an increased number of cells staining positive using an antibody detecting nitrosylated tyrosine species and an increased number of degenerating neurons were detected by Fluoro-Jade staining within the CA3 region (Fig. 5*B*).

Tissue from saline- and KA-treated mice was collected, and mRNA was extracted and subjected to qRT-PCR analysis. As

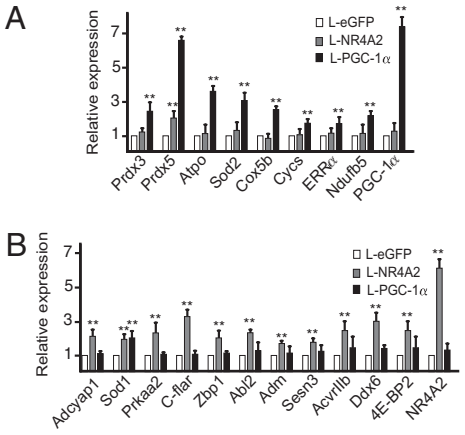

Fig. 4. NR4A2 and PGC-1α have distinct neuroprotective targets. (*A* and *B*) MACS-sorted neurons were infected with the indicated lentiviruses. Total RNA was extracted after 18 h and the expression levels of the indicated genes were determined by real-time PCR. Error bars indicate SD, $n = 4$; $^{**}P < 0.01$.

expected, all three NR4A genes were up-regulated by KA in hippocampus of wild-type mice, but several NR4A target genes that were induced by KA in wild-type controls were diminished in *NR4Amut* mice (Fig. 5*C*). PGC-1α was also induced in the hippocampus 3 d after KA injection and genetic deletion of NR4A alleles did not affect its induction. Taken together, these results show that NR4A-deficient mice are more sensitive to neurodegeneration, presumably as a result of decreased induction of an NR4A-dependent neuroprotective gene program.

Discussion

The central role of CREB in neuroprotection emphasizes the importance of understanding how it maintains neuronal integrity. The rolipram paradigm used here to increase CREB activity and thereby promote neuronal protection has been used before to demonstrate how pharmacological activation of CREB before neurological insult will alleviate later neuronal damage both in vivo and in vitro. However, understanding how CREB regulates neuroprotection has remained largely unknown. Our results point to a role played by NR4A proteins as key downstream mediators of CREB-induced neuroprotection. Importantly, our results in vivo, showing that NR4A knockout mice are more vulnerable to kainic acid toxicity, most likely underestimate the importance of these proteins because only three of six *NR4A* alleles were targeted in these experiments.

Forced expression of NR4A2 in cultured neurons induced genes identified in gene arrays that were also confirmed targets of rolipram-activated CREB. A considerable proportion of NR4A2-activated genes have previously been associated with neuroprotection. Adcyap1 is a signaling peptide that via its receptor increases intracellular cAMP. Thus, its activation by NR4A can likely contribute to a positive feed-forward mechanism for increased and maintained CREB activation in exposed neurons. Moreover, it may provide a means whereby stressed neurons may provide protection by paracrine signaling in adjacent neuronal tissue (28, 29). Overexpression of Adm (30) and Prkaa2 (31) has been shown to confer neuroprotection in a variety of contexts but the precise downstream mechanisms are not well characterized. Ddx6 and zbp1 are involved in stress granule formation and 4E-bp2 in translational arrest. These two processes are of major importance for the protection of mRNA from decay during conditions of cellular stress and thus cell survival from stress (32). Abl2 (33), Sesn3 (34), and Sod1 (35) are genes involved in ROS detoxification, whereas c-flar is an apoptosis inhibitor that prevents the formation of active caspase 8 (36). Finally, the induction of Acvr2b, a receptor for activin A, which is essential

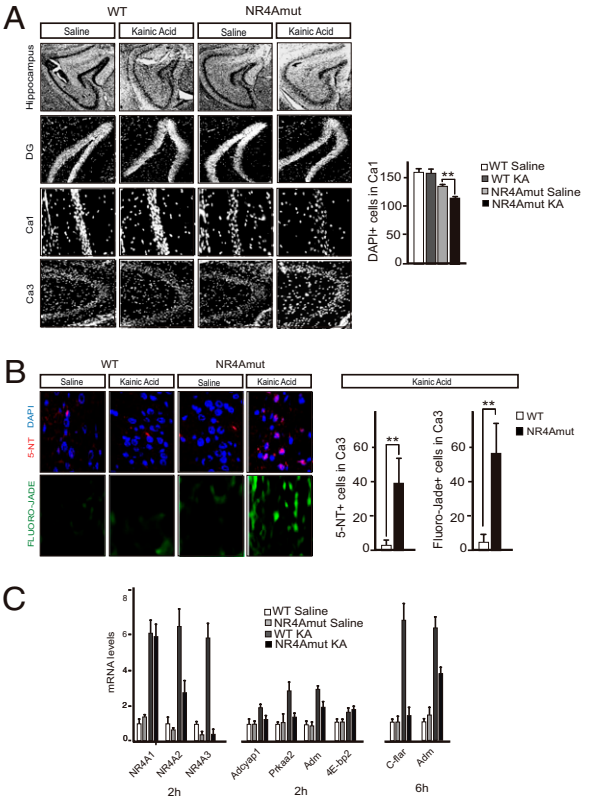

Fig. 5. In vivo evaluation of the neuroprotective role of NR4A receptors. (*A*) Cresyl violet and DAPI staining of hippocampal sections from WT C57/BL and NR4Amut mice injected with saline or 25 mg/kg kainic acid and killed 72 h postinjection. (*B*) DAPI, 5-nitro-tyrosine, and fluoro-jade staining of the CA3 hippocampal region of WT and NR4Amut mice injected with saline or 25 mg/kg kainic acid and killed 72 h postinjection. (*C*) Total RNA was extracted at the indicated time points from the hippocampus of WT and NR4Amut mice injected with saline or 25 mg/kg kainic acid. The expression levels of the indicated genes were determined by real-time PCR. Error bars indicate SD, $n = 3$.

for the in vivo neuroprotective effect of basic fibroblast growth factor (37), suggests a potential interplay between NR4A receptors and other neuroprotective pathways. Interestingly, a gene list enrichment analysis that identifies gene sets associated with common functions or pathways revealed that additional pathways linked to neuroprotection are up-regulated in mRNA samples from L-NR4A2-transduced neurons (*SI Appendix*, Table S2). Notably, mTOR (38), adrenergic (39), insulin receptor (40), and VEGF signaling pathways (41) have been linked to neuroprotection and are significantly enriched in NR4A2-expressing neurons. Thus, we speculate that NR4A promotes neuroprotection as a result of a combined effect by mutiple genes rather than any individual NR4A target gene.

CREB is a central intracellular regulator in several signaling pathways and influences a variety of neural processes. Pharmacological treatment by drugs such as rolipram confers diverse responses in treated neurons. Thus, therapeutic intervention designed to target factors acting downstream of CREB may be advantageous and confer higher precision in treatments aimed at blocking neurodegeneration. Treatments that can increase NR4A activity in neurons exposed to insult could be therefore be of significant clinical relevance. Several strategies whereby NR4A activity could be increased can be envisioned. For example, NR4A belongs to the nuclear receptor family whose members are commonly regulated by small lipophilic ligands. However, the ligand-binding domain of NR4A receptors is unconventional and lacks a ligand-binding pocket, but two NR4A members, NR4A1 and

NR4A2, form heterodimers with RXR. It is interesting to note that NR4A2 plays a key role in the development of dopamine neurons and ligands activating RXR can protect these cells in culture (19, 42). Thus, RXR may be a relevant target for ligand modulation of NR4A-regulated processes. Moreover, NR4A activity is possible to modulate, e.g., by the leukemia drug 6-mercaptopurine (43). Although 6-mercaptopurine mediates serious side effects, other compounds with higher specificity may be possible to identify.

CREB has also been found to induce PGC-1α expression (3, 14, 44–46). We show here that PGC-1α is expressed in neurons exposed to oxidative stress, as previously observed in fibroblasts (14), but it failed to be induced by excitotoxic insult induced by ionomycin and glutamate. Interestingly, the selective regulation by different stimuli correlated with the ability of PGC-1α to protect neurons against oxidative but not excitotoxic stress. Thus, distinct insults elicit selective CREB-dependent cellular responses adapted to the nature of the neuronal insult.

As both NR4A and PGC-1α protect against oxidative stress in response to activated CREB, an important question concerns the functional relationship between these two CREB-dependent transcriptional regulators. PGC-1α is a coactivator that exerts pleiotropic effects in the control of energy homeostasis by interacting with transcription factors. Notably, an important group of PGC-1α-interacting factors are members of the nuclear receptor family. However, PGC-1α is probably not a coactivator of NR4A receptors (47). In contrast, we favor that these transcriptional regulators act via independent pathways for the following reasons: First, NR4A and PGC-1α activate almost completely independent sets of target genes. Second, coexpression of PGC-1α together with NR4A2 did not enhance the expression of tested NR4A targets (*SI Appendix*, Fig. S7). Third, coexpression of both PGC-1α and NR4A2 resulted in additive rather than synergistic protection of neurons. Fourth, analogous to the pathway described here, NR4A and PGC-1α cooperate in the glucagon-cAMP-induced transcriptional regulation of hepatic glucose metabolism by independent regulation of distinct gene sets important in the control of hepatic glucose metabolism (47). Thus, similar to the glucagon-cAMP axis in the liver, NR4A2 and PGC-1α appear to represent two independent but complementary CREB-dependent regulatory "legs" in the adaptation to neuronal stress.

Oxidative stress is central to the pathology in many disorders associated with neurodegeneration, including Alzheimer's and Parkinson's diseases. Excitotoxicity induced by kainic acid induces oxidative stress as reflected in the appearance of nitrosylated protein species following kainic acid treatment. It is interesting to note that gene ablation of only three of six NR4A alleles reported here shows a severe increase in oxidative stress and neuronal death in animals treated with kainic acid. Thus, our results in in vitro cultured neurons and in vivo collectively demonstrate that NR4A receptors mediate a unique and essential downstream pathway of neuroprotection after insults leading to excitotoxic and oxidative stress.

Materials and Methods

Chemicals. DMSO, hydrogen peroxide, ionomycin, glutamate, rolipram, and 8CPT-cAMP were purchased from Sigma. The absolute volumes of DMSO added to the cells were minimal (<0.5%) and did not affect cell survival. The CellTiter 96 reagent to assay cell survival was purchased from Promega and used according to the manufacturer's instructions.

Plasmids. NR4A2 (NM_013613), NR4A3 (NM_015743), NR4A1 (NM_010444), and PGC-1α (NM_008904) cDNAs were cloned into the HIV-PGK-GFP vector (48) after removal of the GFP cDNA. Lenti-PGC-1α shRNA was made by cloning a shRNA against PGC-1α (49) into the pLKO.1 vector (Open Biosystems).

Cell Culture. E14.1 mouse stem cells were propagated in Cellbind culture flasks (Corning) in DMEM (Invitrogen) supplemented with 2,000 units/mL LIF (Chemicon), 10% KSR (Invitrogen), 2% FCS (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), and 0.1 mM β2-mercaptoethanol (Sigma). For differentiation, stem cells were plated on gelatinized Cellbind T75 culture flasks and incubated in the above medium for 4 h. Thereafter, the cells were washed with PBS and grown in N2B27 differentiation medium (50) for 15 d. The medium was supplemented with 1 mM cyclopamine (Calbiochem) during the first week.

Western Blot Analysis. Nuclear extracts were performed using the protocol described in ref. 51. Equal amounts of extracts were loaded on 10% SDS-polyacrylamide gels, electrophoresed, and blotted onto polyvinylidene fluoride membranes (BioRad). For a list of antibodies used, see *SI Appendix, SI Text*.

Immunohistochemistry. Cells were fixed in 2% paraformaldehyde for 30 min at room temperature, washed with PBS, blocked in 0.3% Triton X-100 (Sigma), 1% FCS for 1 h at room temperature, and then incubated overnight at 4 °C with primary antibodies diluted in block solution. For a list of antibodies used, see *SI Appendix, SI Text*. Slides were then washed with PBS and incubated for 1 h at room temperature with secondary antibodies (Alexa; Molecular Probes) diluted 1:200 in PBS. Finally slides were washed with PBS and examined using a NICON inverted microscope.

Magnetic Sorting and Lentiviral Infections. Cells were washed with PBS, collected by trypsinization, and incubated in suspension with mouse anti-NCAM antibody (Chemicon) diluted 1:1,000 in MACS buffer (PBS, 0.5% BSA, 2 mM EDTA) for 15 min at 4 °C. The cells were collected by centrifugation, washed twice with MACS buffer, and incubated in suspension in rat anti-mouse IgM microbeads (Miltenyi Biotech) diluted 1:5 in MACS buffer for 15 min at 4 °C. Then, the cells were washed once with MACS buffer and sorted using MACS separation columns (Miltenyi Biotech). After sorting, cells were plated in N2B27 medium on 24-well plates (100,000 cells/well) coated with laminin (Sigma) and polyornithin (Sigma). Four days after sorting, cells were infected with lentivirus at a multiplicity of infection of 10. Media were changed after overnight infection.

Real-Time PCR Analysis. Total RNA was prepared using the RNAeasy mini kit (Qiagen), treated with DNase (Qiagen), and reverse transcribed using SuperScriptIII (Invitrogen). Real-time PCR analysis was performed on the Rotor-Gene RG-3000A (Corbett Research), using SYBR green PCR Mastermix (Applied Biosystems). For a list of primers used see *SI Appendix, SI Text*. All values were normalized against GAPDH and presented as relative increase of mRNA levels. Data are presented as the mean ± SEM of quadruplicate samples.

Treatment of Cells with Oligonucleotides. Single-stranded phosphorothioate oligonucleotides were synthesized by IBA, annealed by heating to 95 °C for 10 min followed by cooling to room temperature over 2 h, and added to the cells at a concentration of 600 nM in the presence of DOTAP (Boehringer Mannheim). After 5 h the medium was changed. For a list of oligonucleotides used see *SI Appendix, SI Text*.

Animal Experiments. NR4A3$^{-/-}$ mice (provided by Deltagen; *SI Appendix*, Fig. S8) were crossed with NR4A2$^{+/-}$ mice to generate NR4A3$^{+/-}$; NR4A2$^{+/-}$ mice, which were backcrossed with NR4A3$^{-/-}$ mice to finally generate NR4Amut mice (NR4A3$^{-/-}$; NR4A2$^{+/-}$). Eight-week-old mice were single caged, handled for 5 d, and then injected intraperitoneally with KA (Sigma) at 25 mg/kg of body weight or saline. The animals were killed at different time points after the injection by carbon monoxide inhalation and the whole brain was removed. Half of the brain was sliced into 14-mM-thick cryostat sections whereas the other half of the brain was used to dissect the hippocampus, which was placed in RNA later (Qiagen). Sections were stained with cresyl violet (Sigma-Aldrich), FluoroJade-B (Millipore), DAPI (Molecular Probes), or a rabbit antibody against 5-nitrotyrosine (Sigma) according to the manufacturer's recommendations. Three saline- and three KA-injected mice of each genotype were used for each of the different time points. All animal procedures were approved by the animal welfare office of the Stockholm district court.

Microarray Analysis. Microarray experiments were carried out using GeneChip 430A 2.0 arrays (Affymetrix) according to the manufacturer's instructions. See *SI Appendix, SI Text* for details.

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