

# Dexas1, a Small GTPase, Is Required for Glutamate-NMDA Neurotoxicity

Yong Chen,<sup>1</sup> Reas S. Khan,<sup>3</sup> Alyssa Cwanger,<sup>3</sup> Ying Song,<sup>3</sup> Catherine Steenstra,<sup>1</sup> Sookhee Bang,<sup>1</sup> Jaime H. Cheah,<sup>4</sup> Joshua Dunaief,<sup>3</sup> Kenneth S. Shindler,<sup>3</sup> Solomon H. Snyder,<sup>4,5,6</sup> and Sangwon F. Kim<sup>1,2</sup>

Departments of <sup>1</sup>Psychiatry and <sup>2</sup>Pharmacology, Center for Neurobiology and Behavior, and <sup>3</sup>F. M. Kirby Center for Molecular Ophthalmology, Scheie Eye Institute, Department of Ophthalmology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, <sup>4</sup>Solomon H. Snyder Department of Neuroscience, and the Departments of <sup>5</sup>Pharmacology and <sup>6</sup>Psychiatry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Dexas1, a small G-protein localized predominantly to the brain, is transcriptionally upregulated by the synthetic glucocorticoid dexamethasone. It has close homology to the Ras subfamily but differs in that Dexas1 contains an extended 7 kDa C-terminal tail. Previous studies in our laboratory showed that NMDA receptor activation, via NO and Dexas1, physiologically stimulates DMT1, the major iron importer. A membrane-permeable iron chelator substantially reduces NMDA excitotoxicity, suggesting that Dexas1-mediated iron influx plays a crucial role in NMDA/NO-mediated cell death. We here report that iron influx is elicited by nitric oxide but not by other proapoptotic stimuli, such as H<sub>2</sub>O<sub>2</sub> or staurosporine. Deletion of Dexas1 in mice attenuates NO-mediated cell death in dissociated primary cortical neurons and retinal ganglion cells *in vivo*. Thus, Dexas1 appears to mediate NMDA-elicited neurotoxicity via NO and iron influx.

## Introduction

The neurotransmitter glutamate acting via NMDA receptors elicits a variety of cellular alterations that are mediated by nitric oxide (NO). NO, in turn, can signal by activating guanylyl cyclase. Additionally, S-nitrosylation of cysteines in diverse proteins is increasingly appreciated as a major vehicle for NO actions (Kim et al., 2005; Hara and Snyder, 2007; Foster et al., 2009). One mode whereby NO is conveyed to its targets involves the binding of neuronal NO synthase (nNOS) to CAPON, a 55 kDa scaffold protein with a C-terminal domain that binds to the PDZ domain of nNOS (Jaffrey et al., 1998). CAPON then binds to Dexas1, a small GTPase that is a member of the Ras family and was discovered on the basis of selective induction by dexamethasone (Kemppainen and Behrend, 1998; Fang et al., 2000).

Dexas1 displays ~35% homology with the Ras family of proteins but differs in incorporating a 7 kDa C-terminal extension,

which it shares with Rhes (Ras Homolog Enriched in Striatum), a G-protein highly enriched in the corpus striatum and involved in the neurotoxicity associated with Huntington's disease (Blumer et al., 2005; Subramaniam et al., 2009). Dexas1 plays a role in synchronizing circadian rhythms, as its deletion impairs circadian entrainment to light cycles and alters phase shifts to light (Cheng et al., 2004). A variety of influences upon adenylyl cyclase and G-protein-linked neurotransmitter influences have been reported for Dexas1. Also, Dexas1 can interact with FE65, an adaptor protein that occurs in a complex with the intracellular domain of the amyloid precursor protein (Cismowski et al., 2000; Nguyen and Watts, 2005; Lau et al., 2008).

NMDA receptor-mediated neurotransmission, via stimulation of nNOS, enhances Dexas1 activity. Thus, NMDA transmission leads to the binding of nNOS to CAPON, which in turn binds to Dexas1 with the ternary complex of proteins facilitating the S-nitrosylation of Dexas1 to activate its GTP binding activity (Fang et al., 2000).

Recently, we discovered a signaling cascade wherein Dexas1 binds to the peripheral benzodiazepine receptor-associated protein (PAP7), which in turn binds to the divalent metal transporter (DMT1), an iron import channel (Cheah et al., 2006). Stimulation of NMDA receptors activates nNOS, leading to nitrosylation and activation of Dexas1, which, through linkage to PAP7 and DMT1, physiologically enhances iron uptake. As iron is a potentially toxic substance, we wondered whether, following cell stress, Dexas1 might mediate neurotoxicity via an excitotoxic pathway elicited by NMDA neurotransmission and iron entry. In the present study, we have developed mice with targeted deletion of the gene for Dexas1. We demonstrate that deletion of Dexas1 markedly impairs iron uptake elicited by neurotoxic concentra-

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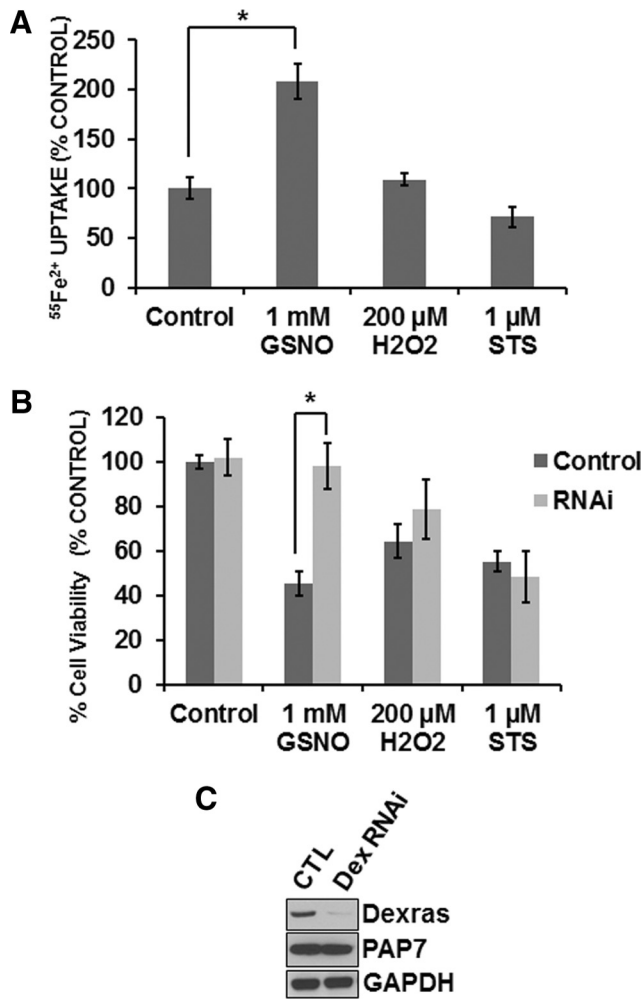
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Correspondence should be addressed to either of the following: Dr. Sangwon F. Kim, Departments of Psychiatry and Pharmacology, Center for Neurobiology and Behavior, Perelman School of Medicine University of Pennsylvania, 125 S 31st Street, TRL Rm 2207, Philadelphia, PA 19104, E-mail: sangwonk@mail.med.upenn.edu; or Dr. Solomon H. Snyder, Solomon H. Snyder Department of Neuroscience, Pharmacology, and Psychiatry, Johns Hopkins University School of Medicine, 725 N Wolfe Street, WBSB Rm 803, Baltimore, MD 21205, E-mail: ssnyder@jhmi.edu.

J. H. Cheah's present address is Eli and Edythe L. Broad Institute of Harvard and Massachusetts Institute of Technology, Chemical Biology Program, 7 Cambridge Center, Cambridge, MA 02142.

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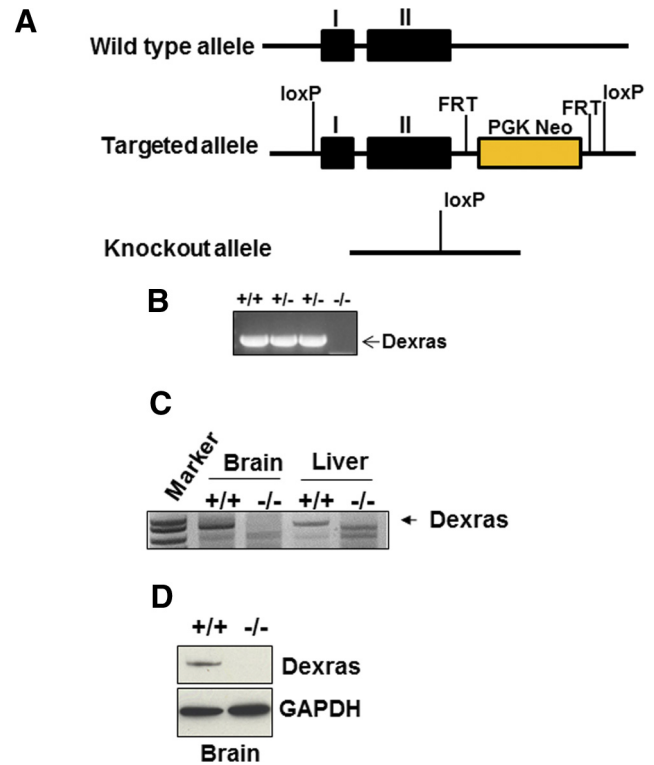
**Figure 1.** Dexras selectively mediates NO-associated toxicity. **A**, PC12 cells were treated with the indicated reagents for 2 h, and nontransferrin iron uptake was measured. \* $p < 0.05$ . **B**, PC12 cells were transfected with either scrambled control RNAi or Dexras1 RNAi plasmids and treated with indicated reagent for 24 h. Cell viability was measured by MTT assay. \* $p < 0.05$ . **C**, Western blotting was performed with Dexras antibody after Dexras RNAi transfection. Experiments were repeated three times, each sample in triplicate.

tions of NMDA and virtually abolishes NMDA neurotoxicity in cortical cultures. In intact mice, NMDA destruction of retinal ganglion cells is abolished in Dexras1 knock-out (KO) mice.

### Materials and Methods

**Cells and reagents.** HEK 293T cells were maintained in DMEM with 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin-streptomycin at 37°C with 5% CO<sub>2</sub> atmosphere in a humidified incubator. PC12 cells were maintained in DMEM with 10% FBS, 5% horse serum, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin in the same environment. All chemicals were purchased from Sigma, unless otherwise indicated.

**Generation and maintenance of Dexras1/RASD1 KO mice.** The gene-encoding mouse Dexras1, *Rasd1*, is located on chromosome 17 and consists of two exons. *Rasd1*<sup>+/-</sup> mice were generated at Ozgene. The targeting construct was based on the sequence of the C57BL/6 strain *Rasd1* gene (GenBank accession number AF239157). The PGK-neo selection cassette was inserted downstream of exon 2. The PGK-neo cassette was flanked by flippase recognition target sites and can be deleted with enhanced flippase recombinase. All the exons were flanked by loxP sites and can be deleted with Cre recombinase. All mice were maintained on a C57BL/6 background. Mice were housed in a 12 h light/dark cycle at an ambient temperature of 22°C and fed standard rodent chow. Animal



**Figure 2.** Generation and characterization of Dexras1 KO mice. **A**, Schematic representation of the mouse Dexras1 gene (*rasd1*) with coding regions shaded black and the exon number indicated on top. The KO allele loses the whole coding regions. **B**, Genotyping. **C**, RT-PCR analysis from brain and liver tissues. **D**, Western blotting from the brain tissue was performed.

protocols, approved by the Institutional Animal Care and Use Committee of University of Pennsylvania, were used in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*.

**Genotyping and RT-PCR analysis.** Mice were genotyped by PCR analysis of genomic DNA from tail biopsies. Primer sets P1 (CGATCCGCG-GCGAAGTCTAC) and P2 (GCGGTGCAAGTCGGGGCTCATCT) yielded a 579 bp product from the wild-type (*RASD1*<sup>+</sup>) allele. RT-PCR analysis was used to assess whether the *RASD1* transcript was missing in the KO mice. RNA was prepared from brain tissues obtained from *RASD1* KO mice and their wild-type littermates by using TRIZOL reagent (Invitrogen). cDNA was prepared by using Oligo(dT) primers (Invitrogen) and the Omniscript reverse transcriptase kit (QIAGEN). PCR was performed by using cDNA templates and primers P1 and P2 as above.

**Quantification of mRNA and iron in retina.** qPCR was performed as we have published previously (Hadziachmetovic et al., 2011), as was retinal iron quantification using bathophenanthroline sulfate.

**Iron uptake assay.** Primary cortical neurons were dissected out of E16–E18 wild-type or Dexras KO mice and plated in 6-well plates at 3 × 10<sup>6</sup> cells per well. Cells were maintained in Primary Neuron Media (Neurobasal media supplemented with B27 serum, 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin) at 37°C with 5% CO<sub>2</sub> atmosphere in a humidified incubator. Neurons were aged 14–20 d after plating before being used for iron uptake assays. Cells were treated with various concentration of NMDA for 10 min. Cells were then washed once with warm PBS. Iron uptake was measured as described previously (Cheah et al., 2006).

**Measurement of cell death.** A 5 mg/ml stock of MTT (thiazolyl blue tetrazolium bromide; Sigma) was diluted to a final concentration of 0.25 mg/ml in HBSS buffer and added to cells after various treatments. Cells were incubated at 37°C for 2–4 h; then the MTT reagent was removed and the cells were washed one time in HBSS. The samples were read in a spectrophotometer at OD 580 nm and OD 630 nm. The OD 580 nm/OD

630 nm reading was normalized to control and expressed as a percentage of cell viability.

**Propidium iodide (PI) staining.** The media was removed and the cells were rinsed once with PBS. PI was diluted into full media at a final concentration of 1  $\mu\text{g}/\text{ml}$  and then incubated with cells at 37°C for 10 min. Cells were washed once with PBS and then fixed with 4% paraformaldehyde in PBS for 30 min. To identify cells, the nuclei were stained with Hoechst stain at 1:15,000 for 5 min. PI selectively stains dying cells nuclei (red), whereas Hoechst stains all cells (blue).

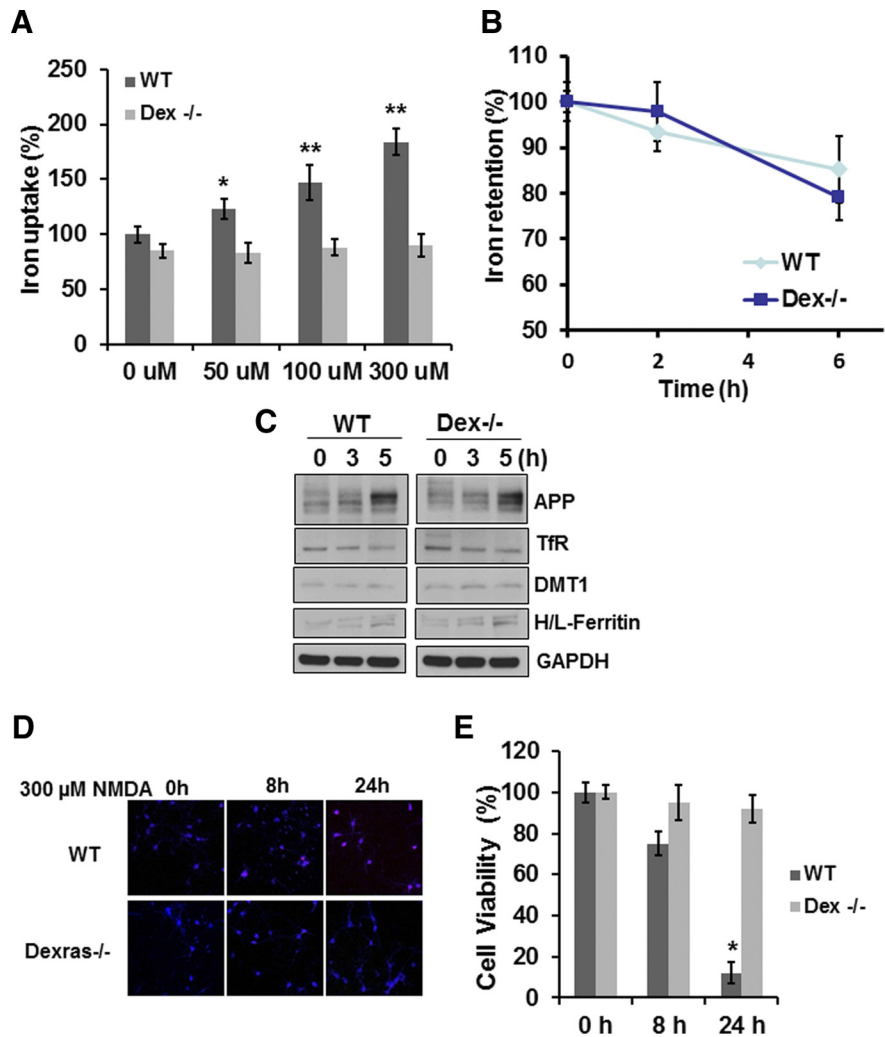
**Retinal ganglion cell (RGC) labeling.** Retrograde labeling of RGCs was performed as described previously (Shindler et al., 2006). Briefly, Dexas KO male mice and wild-type male mice were anesthetized by intraperitoneal injection with 2 mg of ketamine (Sigma) and 0.2 mg of xylazine (Sigma). Holes were drilled through the skull above each superior colliculus through a midsagittal skin incision; 2.5  $\mu\text{l}$  of 1.25% hydroxystilbamidine (Fluorogold; Invitrogen) in sterile water was injected stereotactically into each superior colliculus 1 week before NMDA injection.

**Intravitreal injections.** The method for intravitreal injections was adapted from previous studies (Liang et al., 2001). Dexas KO and wild-type mice were anesthetized with ketamine/xylazine, and eyes were visualized under a dissecting microscope. The conjunctiva was lifted with forceps and cut from the sclera with Vannas scissors along the corneal limbus. Sclera was penetrated with a 30-gauge needle passed into the vitreous just posterior to the lens. NMDA (0.8  $\mu\text{l}$ , 1.2  $\mu\text{M}$ ; Sigma) in PBS was then injected into the vitreous using a 10  $\mu\text{l}$  Hamilton syringe with a 32-gauge blunt tip needle. PBS alone was injected in the contralateral eye of each mouse. After injection, antibiotic ointment (Polysporin; Pfizer) was applied to each eye. The final NMDA concentration in the eye is estimated to be 200 nM, one sixth the concentration of the solution injected based on the volume injected and the average size of the vitreal space.

**Quantification of RGC numbers.** RGC numbers were counted as described previously (Shindler et al., 2006). Briefly, after death, each eye was removed and fixed in 4% paraformaldehyde. Dissected retinas were flat mounted on glass slides, viewed by fluorescence microscopy (Eclipse 80i; Nikon), and photographed at 20 $\times$  magnification in 12 standard fields: 1/6, 3/6, and 5/6 of the retinal radius from the center of the retina in each quadrant. RGC numbers shown in each experiment represent the total number of RGCs counted in 12 fields per eye. RGCs were counted by a blinded investigator using image analysis software (Image-Pro Plus 5.0; Media Cybernetics). Statistical comparisons of RGC numbers were performed by ANOVA.

## Results

PC12 cells display many neuronal properties and possess endogenous Dexas1 as well as PAP7 and DMT1 (Cheah et al., 2006). Accordingly, we selected these cells for studies of a possible role of Dexas1 in mediating the effects of neurotoxic levels of NO upon iron uptake and cell viability (Fig. 1). We exposed cells to the NO donor GSNO (1 mM), H<sub>2</sub>O<sub>2</sub> (200  $\mu\text{M}$ ) or staurosporine (1  $\mu\text{M}$ ). The concentrations at which we administered these agents are cytotoxic with 50% cell death 24 h after treatment (data not

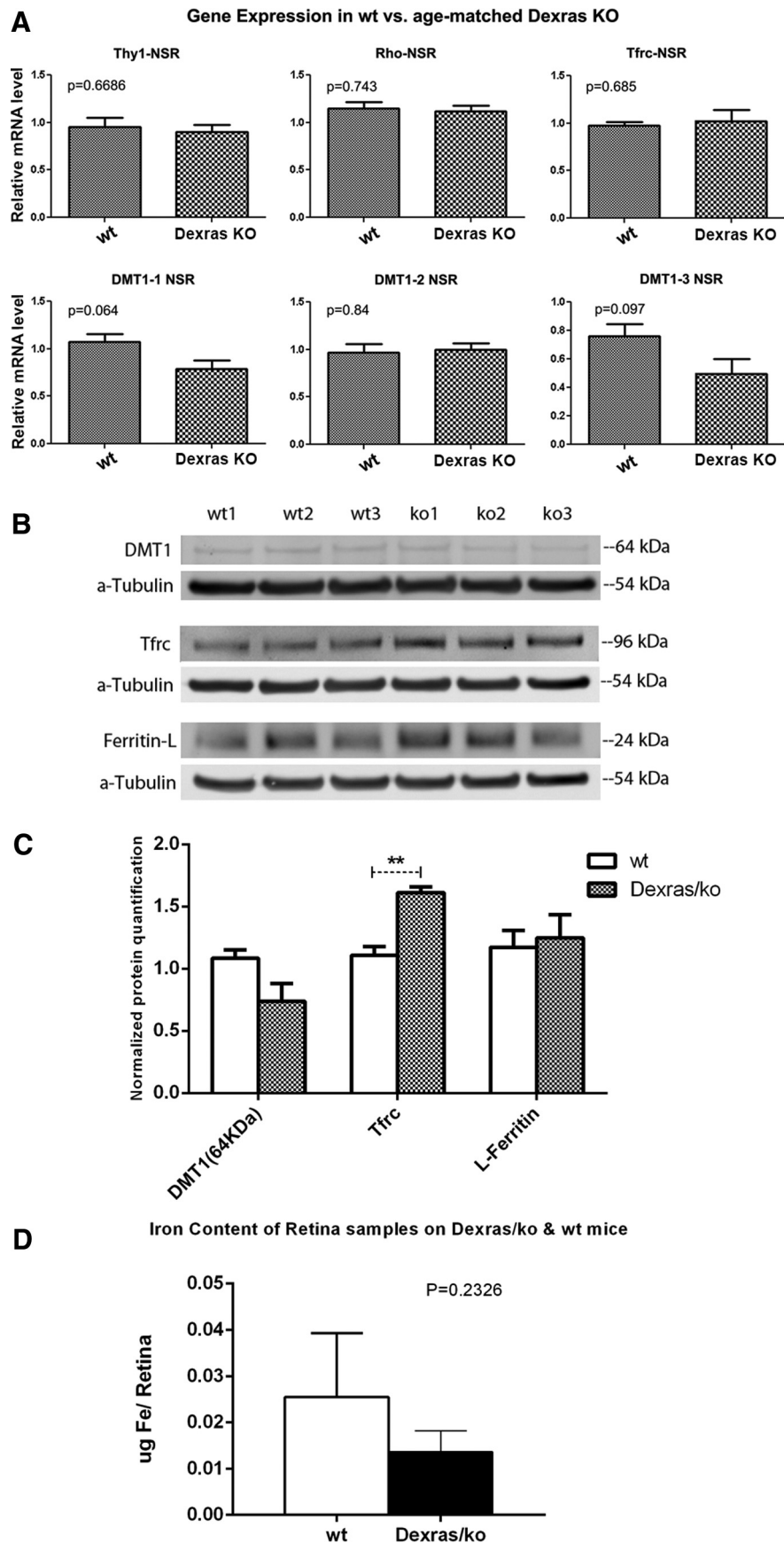


**Figure 3.** Iron uptake and neurotoxicity mediated by glutamate–NMDA neurotransmission are abolished in Dexas KO mice. **A**, NMDA stimulation increases NTBI uptake (15 min) in primary cortical neurons in a concentration-dependent manner. No increase is seen in neurons from Dexas KO mice. All NTBI uptake experiments were repeated three times, each sample in triplicate. Error bars indicate SEM.  $*p < 0.05$ ,  $**p < 0.005$  (two-tailed Student's *t* test). **B**, Primary cortical neurons were treated with NTBI as performed in **A**. Cells were washed with PBS and incubated normal growth media for different time periods, and then the amount of iron retained in the cells was measured. **C**, Primary cortical neurons from either WT or Dexas<sup>-/-</sup> mice were treated with 300  $\mu\text{M}$  NMDA for 10 min and harvested at the different time points. Western blotting was performed. **D**, Primary cortical neurons were treated with 300  $\mu\text{M}$  NMDA for 10 min, and neuronal cell death was examined at the indicated time points using PI staining (red) with DAPI (nucleus). A representative image is shown. **E**, Quantification of three independent experiments of neurotoxicity assays.  $*p < 0.005$ .

shown). At 2 h, when cell viability is normal, GSNO treatment doubles iron uptake, whereas H<sub>2</sub>O<sub>2</sub> and staurosporine did not change uptake (Fig. 1A). To assess a role for Dexas1 in the neurotoxic actions of these substances, we developed an shRNA construct that provides almost complete elimination of Dexas1 protein in PC12 cells (Fig. 1B,C). Depletion of Dexas1 abolishes the cytotoxic actions of GSNO. Strikingly, loss of Dexas1 does not impair the cytotoxic actions of staurosporine or H<sub>2</sub>O<sub>2</sub>.

To evaluate the impact of Dexas1 on neurotoxicity in intact rodents, we developed mice with targeted deletion of Dexas1 (Fig. 2). We used a targeting vector to delete the complete open reading frame of Dexas1 (Fig. 2A). PCR genotyping and RT-PCR confirm the complete genomic deletion of Dexas and the absence of Dexas mRNA in brain and liver (Fig. 2B,C). The mutant mice appear grossly normal. There was no difference from wild-type in body size, weight, or locomotor activity at the age of 8 weeks. Mice lived up to 16–18 months, and gross ana-





**Figure 4.** Effect of Dexas KO on mRNAs and proteins involved in iron homeostasis or iron levels in the retina. **A**, RT-PCR of various genes involved in iron homeostasis and retina cell markers. **B**, **C**, Western analysis was performed to detect DMT1, Tfrc (transferrin receptor), and ferritin-L. \*\**p* < 0.01. **D**, Retinal nonheme iron content.

tomically dissected 16-month-old mice reveals no apparent abnormalities in organs of the adult mutant mice. The absence of major aberrations in the mice corresponds to results from another group that also developed Dexas1 KOs (Cheng et al., 2004).

To determine whether Dexas1 influences iron uptake associated with neurotoxicity, we exposed cortical cultures to various concentrations of NMDA, including cytotoxic levels (100–300  $\mu$ M) for 10 min and measured iron uptake. At the cytotoxic concentrations of NMDA, we observe substantial increases in iron uptake with 300  $\mu$ M NMDA eliciting a doubling of iron uptake (Fig. 3A). No increase of iron uptake is apparent in Dexas1-deleted brain cultures. On the other hand, iron efflux was not affected in primary neurons from Dexas<sup>-/-</sup> mice (Fig. 3B). Moreover, we found that the expression levels of proteins involved in iron homeostasis, such as amyloid precursor protein, TfR, DMT1, and ferritin, are similar in neuronal cultures between WT and Dexas<sup>-/-</sup> mice (Fig. 3C). Thus, these findings establish a role for Dexas1 in mediating iron uptake in NMDA-mediated neurotoxic insults. These findings confirm and extend our previous observations in PC12 cells that the stimulation of iron uptake by GSNO (100  $\mu$ M) is prevented by depletion of Dexas1 using RNA interference (Cheah et al., 2006).

We also examined cytotoxic actions of NMDA in the same cortical cultures (Fig. 3D,E). We used the well-characterized regimen of NMDA treatment that elicits delayed neurotoxicity, thought to mimic events in vascular strokes (Koh and Choi, 1988; Dawson et al., 1991). Cortical cultures were exposed to 300  $\mu$ M NMDA for 10 min and then examined after 8 and 24 h. As has been reported by numerous investigators, no loss of cell viability is evident at 8 h, whereas at 24 h viability is decreased by >90%. Dexas1 deletion provides dramatic protection against this toxicity, with no evident loss of viability.

To extend these findings to intact animals, we evaluated retinal toxicity elicited by NMDA injection into the eyes of mice, a procedure known to selectively destroy retinal ganglion cells (Sucher et al., 1997). First, we examined various genes involved in iron trafficking as well as markers for retinal cells. The qPCR cycle threshold for Dexas1 in wild-type neural retinas was 29 (mid-range, of 40 cycles) but was undetectable in Dexas1 KOs. There were no significant differences for wild-type versus KO for transferrin receptor, rhodop-

sin (rod photoreceptor-specific), Thyl (ganglion cell-specific), or any of the DMT1 isoforms. These results are further confirmed by Western blotting examining the levels of protein expression for ferritin and DMT1 (Fig. 4B,C). Interestingly, the protein level of TfR is slightly higher in *Dexas1*<sup>-/-</sup>, and it may be a reflection of slightly lower levels of iron. We measured retinal iron levels directly, but the levels were variable and there was no statistically significant (Fig. 4D) difference between the genotypes. Thus, *Dexas1* KO does not notably affect baseline iron levels or ganglion cell or rod photoreceptor numbers in the normal retina.

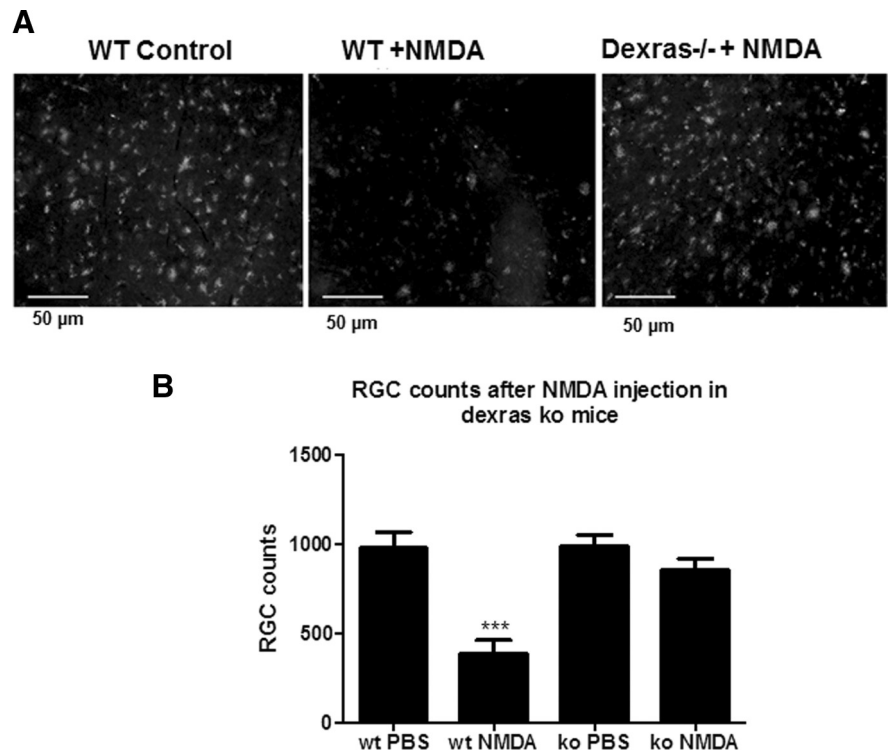
Five days after NMDA administration, we observed a 60% reduction in numbers of retinal ganglion cells (Fig. 5A,B). The *Dexas1*-deleted mice are completely protected from this neurotoxicity. Detailed histologic examination reveals no difference in the morphology of retinal ganglion cells or any other cell type of the retina between wild-type mice and *Dexas1*-deleted mice in the absence of NMDA administration (data not shown).

## Discussion

In the present study, we have established a major role for *Dexas1* in mediating both iron uptake and cell viability under NMDA-excitotoxic conditions. The linkage between cell viability and iron uptake is selective for NO, as cytotoxic concentrations of GSNO increase iron uptake, whereas comparably toxic levels of H<sub>2</sub>O<sub>2</sub> and staurosporine fail to do so. Moreover, the neurotoxic actions of GSNO, but not those of other agents, are prevented by *Dexas1* depletion. Experiments using *Dexas1* KO mice provide compelling evidence for its importance in iron uptake and neurotoxicity. In cortical cultures of *Dexas1* KOs, stimulation by NMDA both of iron uptake and neurotoxicity is abolished. In intact mice, the marked loss of retinal ganglion cells elicited by NMDA is completely prevented in the *Dexas1* KOs. Our findings suggest that *Dexas1* mediates NMDA neurotoxicity via its enhancement of iron uptake, as *Dexas1* deletion prevents both processes. Moreover, previously we observed that NMDA neurotoxicity in cortical cultures is prevented by iron chelator treatment (Cheah et al., 2006).

The use of *Dexas1* mutant mice substantially strengthens evidence for a signaling cascade wherein glutamate, acting via NMDA receptors, activates nNOS to form NO, which nitrosylates and activates *Dexas1*, which, through a link to PAP7, increases iron uptake via DMT1. As reported previously (Cheah et al., 2006), *Dexas1* stimulation of iron uptake stems from its GTPase activity, as constitutively active *Dexas1* is associated with enhanced stimulation of iron uptake.

Rhes (*Dexas2*) is the only Ras homolog that closely resembles *Dexas1*, with ~62% amino acid homology (Falk et al., 1999). Whereas *Dexas1* is induced by glucocorticoids, Rhes is selectively stimulated by thyroid hormone (Vargiu et al., 2001). Most strikingly, Rhes is uniquely concentrated in the corpus striatum, where its binding to mutant huntingtin is thought to underlie the selective damage to the corpus striatum in



**Figure 5.** Genetic deletion of *Dexas1* blocks NMDA toxicity *in vivo*. **A**, NMDA (200 nM) was injected in the left eye of 6 wild-type mice and 6 *Dexas1* KO mice. An equal volume of PBS was injected in the right eye of each mouse as control. Mice were killed 5 d later. One representative field/retina is shown. **B**, The number of RGCs was counted. Average numbers of RGCs/eye are significantly decreased in NMDA-injected eyes compared with wild-type mice (\*\*\*) ( $p < 0.0001$ ), but little or no RGC loss occurs in *Dexas1* KO mouse eyes injected with NMDA.

Huntington's disease (Subramaniam et al., 2009). Like *Dexas1*, Rhes does bind to PAP7 (Cheah et al., 2006) and so might regulate striatal iron deposition, which might participate in Huntington's disease pathophysiology. Consistent with this possibility, iron levels in the striatum are increased in Huntington's disease patients (Dexter et al., 1991).

In our earlier study, we addressed physiologic regulation of iron transport by *Dexas1* in response to NMDA-NO activation. Our present study focuses on the pathogenic actions of iron. Iron is well known to be toxic in excess; and in the brain, iron accumulation has been linked to numerous neurodegenerative diseases (McCord, 1998; Thomas and Jankovic, 2004). In particular, Salazar et al. (2008) showed that DMT1, which is modulated by *Dexas1*, plays a critical role in iron-mediated neurodegeneration of Parkinson's disease (see also Snyder and Connor, 2009).

The dramatic protection from NMDA neurotoxicity elicited by deletion of *Dexas1* may have therapeutic implications. Glutamate excitotoxicity has been implicated in the retinal ganglion cell loss of numerous optic neuropathies, including glaucoma (Dreyer, 1998; McCord, 1998) and dominant optic atrophy (Nguyen et al., 2011), as well as neuronal loss in multiple sclerosis models (Pitt et al., 2000), where significant ganglion cell loss occurs secondary to optic neuritis (Shindler et al., 2008; Quinn et al., 2011). Conceivably, drugs that selectively block *Dexas1* may be neuroprotective in these optic neuropathies, vascular stroke, and other neurodegenerative diseases. Because *Dexas1* differs markedly in structure from other members of the Ras family and other small G-proteins, it may be feasible to develop highly selective and safe inhibitors of *Dexas1* function with therapeutic potential.

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