Dexras1: A G Protein Specifically Coupled to Neuronal Nitric Oxide Synthase via CAPON

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Summary

Because nitric oxide (NO) is a highly reactive signaling molecule, chemical inactivation by reaction with oxygen, superoxide, and glutathione competes with specific interactions with target proteins. NO signaling may be enhanced by adaptor proteins that couple neuronal NO synthase (nNOS) to specific target proteins. Here we identify a selective interaction of the nNOS adaptor protein CAPON with Dexras1, a brain-enriched member of the Ras family of small monomeric G proteins. We find that Dexras1 is activated by NO donors as well as by NMDA receptor–stimulated NO synthesis in cortical neurons. The importance of Dexras1 as a physiologic target of nNOS is established by the selective decrease of Dexras1 activation, but not H-Ras or four other Ras family members, in the brains of mice harboring a targeted genomic deletion of $nNOS$ ($nNOS^{-/-}$). We also find that nNOS, CAPON, and Dexras1 form a ternary complex that enhances the ability of nNOS to activate Dexras1. These findings identify Dexras1 as a novel physiologic NO effector and suggest that anchoring of nNOS to specific targets is a mechanism by which NO signaling is enhanced.

Introduction

NO serves multiple roles in biology, acting as the endothelial-derived relaxing factor in regulating blood vessel relaxation (Furchgott and Zawadzki, 1980; Ignarro et al., 1981, 1987; Palmer et al., 1987), enabling activated macrophages to kill tumor cells and bacteria (Hibbs et al., 1987; Stuehr et al., 1989), and serving as a neurotransmitter both in the brain and the peripheral nervous system (Garthwaite et al., 1988; Bredt and Snyder, 1989; Knowles et al., 1989). NO is formed by the enzyme NO synthase (NOS), which oxidizes a guanidino nitrogen of arginine to NO with the stoichiometric formation of citrulline. Three distinct forms of NOS exist, derived from separate genes and designated endothelial NOS (eNOS), inducible or macrophage NOS (iNOS), and neuronal NOS (nNOS) (Griffith and Stuehr, 1995). nNOS is larger than eNOS and iNOS because of an N-terminal extension that contains a PDZ domain (Cho et al., 1992; Ponting and Phillips, 1995) that interacts with a variety of other proteins including PSD95/93 (Brenman et al., 1996) and CAPON (Jaffrey et al., 1998). These interactions are thought to facilitate the targeting of nNOS to distinct intracellular sites.

NO signals within cells through posttranslational covalent modification of proteins. The most studied example is the binding of NO to heme in soluble guanylyl cyclase, leading to activation of the enzyme and increased formation of cGMP (Denninger and Marletta, 1999). NO can also directly modify cysteine residues in proteins, leading to the formation of a nitrosothiol adduct (Stamler et al., 1997). The ability of endogenous NO to diffuse to various targets within the cell may be compromised by the high concentrations of glutathione and other molecules that can degrade NO. Accordingly, one might anticipate that physiologic targets of NO should be closely juxtaposed to NOS, with the juxtaposition established by appropriate targeting proteins. In a search for such targeting proteins, we identified CAPON, a 55 kDa protein that contains a C-terminal domain that binds to the PDZ domain of nNOS, as well as an N-terminal phosphotyrosine binding (PTB) domain (Jaffrey et al., 1998).

We sought to determine whether CAPON targets nNOS within the cell. In the present study, we identify a selective interaction of CAPON with Dexras1, a brain-enriched member of the Ras family of small monomeric G proteins that was recently identified in a screen for genes induced by dexamethasone (Kemppainen and Behrend, 1998). We find that Dexras1, like H-Ras, is S-nitrosylated by NO donors and is activated by NO donors and by coexpression with nNOS. The importance of Dexras1 as a physiologic target of nNOS is established by its activation by NMDA receptor–stimulated nNOS activity and by the finding that Dexras1 activation is diminished in the brains of mice harboring a targeted genomic deletion of $nNOS$ ($nNOS^{-/-}$). We also find that nNOS, CAPON, and Dexras1 form a ternary complex that enhances the ability of nNOS to activate Dexras1. These data identify a novel physiologic effector of NO in the brain and suggest that juxtaposition of nNOS and its targets may be a mechanism by which NO signaling is enhanced.

Results

Identification and Cloning of Dexras1
To identify proteins that are associated with the PTB domain of CAPON, we conducted a yeast two-hybrid screen. A rat hippocampal/cortical cDNA library was screened against the CAPON PTB domain (amino acids 20–180), and a single transformant expressing residues 235–280 of rat Dexras1 was isolated (see Experimental Procedures). A full-length cDNA of $Dexras1$ was cloned from a rat brain cDNA library whose deduced protein sequence is identical to that of mouse, except that glycine 267 is replaced by a serine in the rat sequence. Dexras1 was previously identified as a member of the Ras family within the Ras superfamily of small GTPases,

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Table 1. Binding of CAPON to Dexras1 in the Yeast Two-Hybrid System

Bait	Trap	Histidine Prototrophy	LacZ Activity
CAPON (aa 20–180)	Dexras1 (aa 235–280)	+	+
Dexras1 (aa 235–280)	CAPON (aa 20–180)	+	+
CAPON (aa 20–180)	Dexras1 (aa 1–232)	−	−
CAPON (aa 379–503)	Dexras1 (aa 235–280)	−	−
Cain	Dexras1 (aa 235–280)	−	−
FKBP12	Dexras1 (aa 235–280)	−	−

with 26% sequence identity with H-Ras. Dexras1 is a member of the Rap subfamily (Rap1, Rap1B, Rap2A, and Rap2B) of the Ras family, with 34% identity with Rap1B and 36% identity with Rap2B (Kemppainen and Behrend, 1998).

CAPON and Dexras1 Interact In Vitro and In Vivo
To establish the specificity of interactions between CAPON and Dexras1 in the two-hybrid screen, we examined several Dexras1 constructs (Table 1). The C terminus of Dexras1 interacts with the N-terminal PTB domain of CAPON but not with the C-terminal domain of CAPON (amino acids 379–503) or with two control proteins, CAIN and FKBP-12. Additionally, the N terminus of Dexras1 (amino acids 1–232) does not interact with the CAPON PTB domain. These data establish the specificity in yeast of the interaction between the CAPON PTB domain and the C terminus of Dexras1.

We confirmed the specificity of the interaction of CAPON and Dexras1 in binding studies utilizing immobilized GST-Dexras1 incubated with transfected HEK293 cell lysates containing various hemagglutinin (HA)-tagged CAPON fusions. GST-Dexras1 binds specifically to both full-length CAPON and its PTB domain (Figure 1A). GST-Dexras1 fails to interact with the C-terminal nNOS binding domain of CAPON or with HA-FKBP-12 (Figure 1A). We next compared the binding of HA-CAPON to GST-Dexras1 and GST-H-Ras. HA-CAPON binds to GST-Dexras1 but not to GST-H-Ras (Figure 1B). Additionally, we find that GST-Dexras1 is unable to bind to nNOS (Figure 1B). These results further support the notion that CAPON interacts specifically with Dexras1.

To determine if CAPON and Dexras1 interact in intact cells, we conducted immunoprecipitation studies in brain lysates, utilizing an antibody to Dexras1 (Figure 1C). Immunoprecipitation of Dexras1 results in the coprecipitation of CAPON, whereas both preimmune serum and an antibody to H-ras do not coprecipitate CAPON. These studies demonstrate that CAPON and Dexras1 form a physiologic complex in vivo.

**Dexras1, CAPON, and nNOS Form
a Ternary Complex**
Because nNOS interacts with the C terminus of CAPON and Dexras1 interacts with its N terminus, we suspected that CAPON may serve as an adaptor protein, linking nNOS to Dexras1. To detect these ternary complexes, we incubated the NOS affinity resin 2′, 5′ ADP sepharose (Bredt and Snyder, 1990) with brain lysates derived from wild-type and $nNOS^{-/-}$ mice. nNOS was eluted from the resin with 10 mM NADPH as described previously (Bredt

and Snyder, 1990). Dexras1 is detected in the eluates derived from wild-type mice but not from $nNOS^{-/-}$ mice (Figure 1D), demonstrating that nNOS is associated with Dexras1 in vivo. We explored the requirement of CAPON in this interaction using GST pulldown studies in transfected HEK293 cell lysates. In cells expressing GST-Dexras1, CAPON, and nNOS, precipitation of Dexras1 with glutathione agarose leads to the coprecipitation of nNOS and CAPON (Figure 1E). This complex requires CAPON, as precipitation of GST-Dexras1 fails to coprecipitate nNOS in lysates derived from cells that were not transfected with CAPON. Cells transfected with only CAPON and nNOS show no precipitation of CAPON or nNOS utilizing glutathione beads. Thus, CAPON functions as an adaptor linking nNOS to Dexras1.

Distribution of Dexras1
Northern blot analysis in rat tissues shows prominent expression of *Dexras1* mRNA in the brain, with somewhat lesser levels in the testes and still lower levels in the lung (Figure 2A). Negligible expression is evident in heart, spleen, liver, skeleton muscle, and kidney. Western blot analysis similarly reveals major enrichment in the brain (Figure 2B). A previous study of *Dexras1* mRNA expression in mouse showed more prominent expression in kidney and heart, which may reflect rodent-specific differences in *Dexras1* mRNA expression (Kemppainen and Behrend, 1998). Additionally, in the Northern blot analysis in mice (Kemppainen and Behrend, 1998), *GAPDH* mRNA levels appeared to be much less in brain samples than in heart and kidney, suggesting that, following correction for RNA loading, levels of *Dexras1* mRNA would be enriched in brain in mice as well.

Dexras1 contains a CAAX box, suggesting that it may be prenylated. We examined the membrane association of Dexras1 and five other proteins in the Ras superfamily (Figure 2C). Dexras1 is principally soluble, though a small fraction is detectable in membrane fractions. H-Ras, R-Ras, Rap2, and Rac1 are almost exclusively membrane associated, while Rab6 occurs both in soluble and membrane fractions. As CAPON, like Dexras1, is largely soluble (Jaffrey et al., 1998), CAPON/Dexras1 complexes are likely to be cytoplasmic. In other experiments, we assessed the subcellular localizations of CAPON and Dexras1 in $nNOS^{-/-}$ mice. We have found that these proteins exhibit similar localizations in $nNOS^{-/-}$ and wild-type mice, indicating that nNOS is not the primary determinant of the localization of these proteins (M. F., S. R. J., and S. H. S., unpublished data).

To identify neuronal pathways that might be regulated by Dexras1, we mapped the cellular distribution of *Dexras1* mRNA in the brain. In situ hybridization re-

A

Blot: anti-HA   Blot: anti-HA

B

Blot: anti-HA   Blot: anti-HA
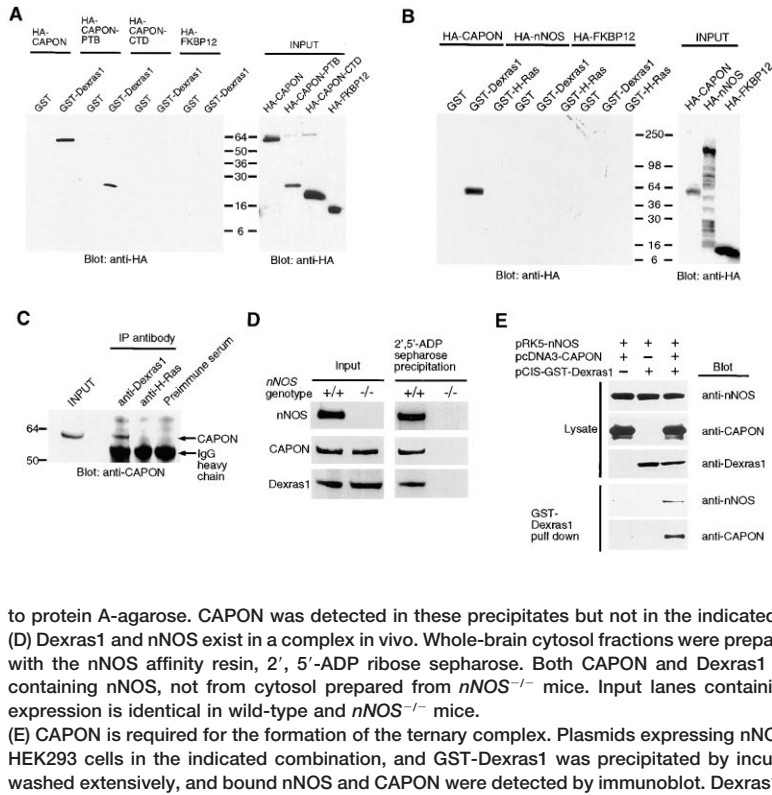
C

Blot: anti-CAPON

D

E

Figure 1. Dexras1 and CAPON Form a Complex In Vitro and In Vivo

(A) In vitro binding of GST-Dexras1 and CAPON. HEK293 cells were transfected with plasmids expressing the indicated HA-tagged CAPON constructs. Cell lysates were incubated with the indicated immobilized GST fusion protein, and specifically bound proteins were determined by washing the resin five times followed by elution in SDS-PAGE sample buffer and detection of HA fusion proteins by Western blotting with an anti-HA antibody. Input (5% of the material applied to resins) are included to demonstrate the size of the different tagged proteins.

(B) CAPON binds to GST-Dexras1 but not GST-H-Ras. As in Figure 1A, HA-CAPON, HA-NOS, and HA-FKBP12 were tested for their ability to interact with the indicated GST fusions.

(C) CAPON and Dexras1 exist as a physiologic complex in rat brain lysates. Dexras1 was immunoprecipitated from rat brain cytosol with a rabbit anti-Dexras1 antibody bound

to protein A-agarose. CAPON was detected in these precipitates but not in the indicated control immunoprecipitates.

(D) Dexras1 and nNOS exist in a complex in vivo. Whole-brain cytosol fractions were prepared from wild-type and $nNOS^{-/-}$ mice and incubated with the nNOS affinity resin, 2′, 5′-ADP ribose sepharose. Both CAPON and Dexras1 were precipitated by the resin but only in cytosol containing nNOS, not from cytosol prepared from $nNOS^{-/-}$ mice. Input lanes containing 5% of the starting material show that Dexras1 expression is identical in wild-type and $nNOS^{-/-}$ mice.

(E) CAPON is required for the formation of the ternary complex. Plasmids expressing nNOS, CAPON, and GST-Dexras1 were transfected into HEK293 cells in the indicated combination, and GST-Dexras1 was precipitated by incubation of the cell lysates with glutathione agarose, washed extensively, and bound nNOS and CAPON were detected by immunoblot. Dexras1 precipitated nNOS only in the presence of CAPON.

veals that Dexras1 is localized to discrete neuronal populations, with highest densities in the cortex as well as the accessory olfactory nucleus, supraoptic nucleus, and the Purkinje cell layer of the cerebellum (Figure 2D). *Dexras1* is also expressed in the dentate gyrus and CA1 and CA2 regions of the hippocampus, with lesser label-
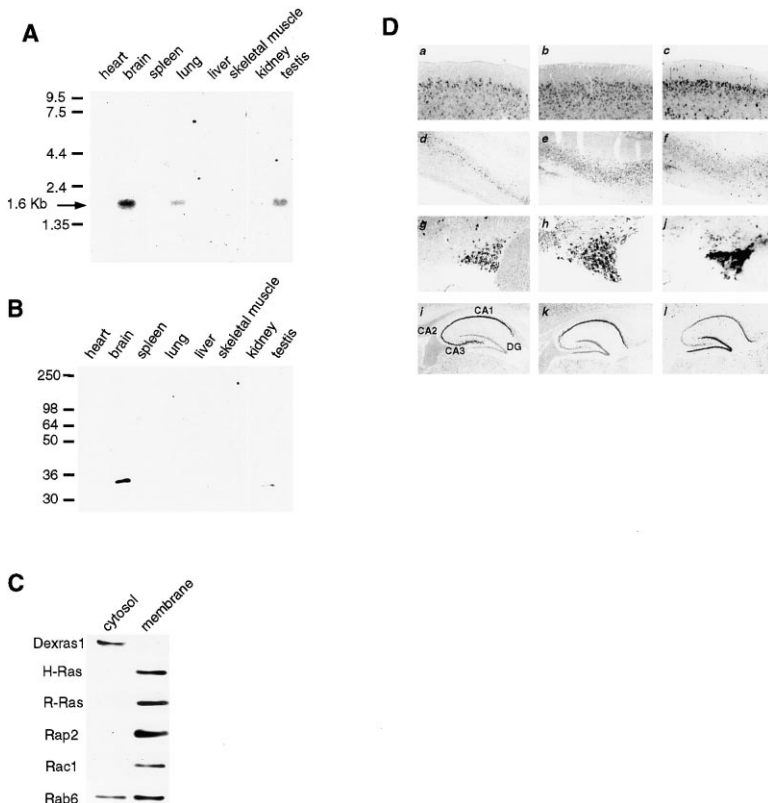
A

B

C

D

Figure 2. Tissue and Subcellular Distribution of Dexras1

(A) *Dexras1* Northern blot. Dexras1 mRNA was localized by Northern blot using a cDNA probe corresponding to amino acids 235–280 of Dexras1.

(B) Dexras1 is enriched in brain. The indicated tissue (50 µg) was subjected to SDS-PAGE and immunoblotted for Dexras1.

(C) Dexras1 is a predominantly cytoplasmic protein. Cytosol and membrane fractions were prepared (see Experimental Procedures). Each sample (50 µg) was separated by SDS-PAGE and immunoblotted with Dexras1 and the indicated G protein–specific antibodies.

(D) *Dexras1*, *CAPON*, and *nNOS* mRNA colocalize in several neuronal populations. *Dexras1*, *CAPON*, and *nNOS* mRNA were localized as described in Experimental Procedures. *Dexras1*, *CAPON*, and *nNOS* are detected in the cortex ([a], [b], and [c], respectively), ventral accessory olfactory nucleus ([d], [e], and [f], respectively), supraoptic nucleus ([g], [h], and [i], respectively), and hippocampus ([j], [k], and [l], respectively). The dentate gyrus (DG) as well as the CA1, CA2, and CA3 portions of the hippocampus are indicated. Magnification: (a–f), 5×; (g-i), 10×; (j-l), 2.5×.

ing of the CA3 region (Figure 2D). Lower levels of *Dexras1* are detected in the thalamus, diagonal band of Broca, and the substantia nigra, pars compacta. *Dexras1* also occurs in the pontine nucleus, the trapezoid body, the pedunculopontine tegmental nucleus, and the nucleus solitarius (data not shown).

We next compared the distribution of *Dexras1*, *CAPON*, and *nNOS* mRNAs. We find these mRNAs expressed in many similar neuronal populations. The three mRNAs are coexpressed in the cortex, although in scattered cortical medium aspiny neurons *nNOS* is expressed at an unusually high level, which is not the case for either *Dexras1* or *CAPON*. The three mRNAs are also coexpressed in the accessory olfactory nucleus and the supraoptic nucleus (Figure 2D). All three mRNAs are expressed in the hippocampus, with higher levels of mRNA in the CA1 and CA2 and with lower levels in CA3 (Figure 2D). In the dentate gyrus, *nNOS* mRNA is more prominent than either *CAPON* or *Dexras1*. Several other regions, including the pontine nucleus, trapezoid body, the pedunculopontine nucleus, and the nucleus solitarius, also express all three mRNAs, albeit at a lower level.

Dexras1 Is S-Nitrosylated by NO Donors
Ras-like G proteins alternate between an "inactive" GDP-bound state and an "active" GTP-bound state through the action of guanine-nucleotide exchange factors (GEFs), which enable GTP to bind by triggering the dissociation of GDP, and by GTPase-activating proteins, which increase the hydrolysis of GTP to GDP. In addition to these mechanisms, H-Ras can also be activated by S-nitrosylation, which leads to enhanced guanine-nucleotide exchange activity and a resulting increase in the concentration of active GTP-bound H-Ras (Lander et al., 1995). This effect is mediated by the nitrosothiol-modified protein alone and does not require a separate GEF protein.

The 26% sequence identity between H-ras and Dexras1 raised the possibility that Dexras1 may also be S-nitrosylated by NO. To determine if Dexras1 was susceptible to S-nitrosylation, we incubated purified GST-Dexras1 with the NO donor SNAP or the control compound NAP for 30 min and then isolated the protein by spin column chromatography. Dexras1 in the eluate was examined for the presence of nitrosothiol adducts using an S-nitrosylation assay in which S-nitrosocysteine residues are biotinylated and the biotinylated proteins are subsequently detected by immunoblotting with an anti-biotin antibody (Jaffrey et al., 2000). Preincubation of GST-Dexras1 with SNAP leads to S-nitrosylation of the protein (Figure 3A). The absence of a signal in GST-Dexras1 preparations treated with NAP indicates that the signal is dependent on the NO moiety of SNAP.

To determine if NO donors preferentially S-nitrosylate Dexras1 relative to other G proteins, we incubated tissue lysates with glutathione (GSH) or S-nitrosoglutathione (GSNO). The latter compound is present in the brain at micromolar concentrations and has been proposed to be a physiologic carrier of NO for S-nitrosylation reactions in the cytosol (Gaston et al., 1993; Kluge et al., 1997). Tissue lysates treated with GSNO or GSH were subjected to the S-nitrosylation assay (Jaffrey et al.,
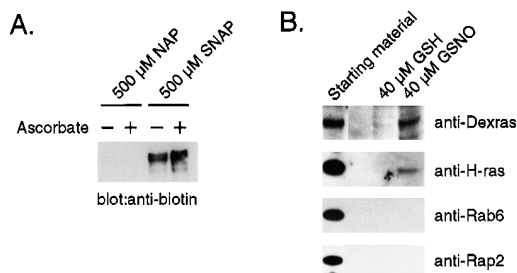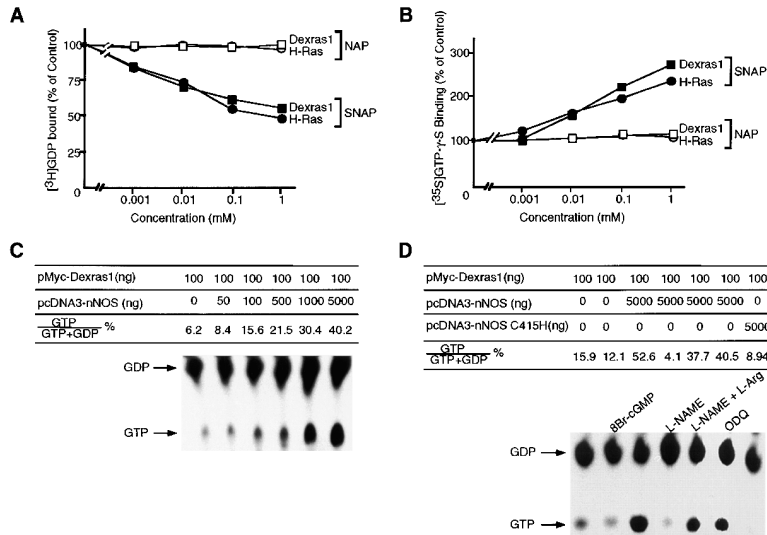

Figure 3. Dexras1 Is S-Nitrosylated by NO Donors

(A) GST-Dexras1 is S-nitrosylated by SNAP. GST-Dexras1 was pre-incubated with 0.5 mM SNAP or NAP, desalted, and then subjected to the S-nitrosylation assay. After the S-nitrosylation assay, the samples were resolved by SDS-PAGE and immunoblotted with an anti-biotin antibody. Only Dexras1 incubated with the NO donor was capable of eliciting a signal in this assay.

(B) Dexras1 and H-ras are preferentially S-nitrosylated in GSNO-treated brain lysates. Brain lysates were incubated with 40 μM GSNO or GSH, and S-nitrosylated proteins were biotinylated using the S-nitrosylation assay and then purified on neutravidin-agarose by 2-ME elution. 2-ME eluates were blotted with the indicated G protein antibodies. Only Dexras1 and H-Ras were detected in 2-ME eluates treated with NO donors. Input represents 0.2% of the material applied to the neutravidin agarose.

2000). The biotinylated proteins were bound to neutravidin agarose and then purified by cleavage of the disulfide cross-linked biotin with 2-mercaptoethanol (2-ME). The 2-ME eluates derived from lysates treated with GSH exhibit no detectable G protein, while 2-ME eluates from lysates treated with GSNO contain detectable amounts of H-Ras and Dexras1 but not two other control G proteins, Rab6 and Rap2 (Figure 3B). These findings indicate that Dexras1 reacts with NO donors to form nitrosothiol adducts and that incubation of tissue lysates with NO donors leads to preferential nitrosylation of Dexras1 and H-Ras.

Dexras1 Guanine-Nucleotide Exchange Activity Is Activated by NO Donors
S-nitrosylation of H-ras by NO donors is associated with enhanced guanine-nucleotide exchange activity. We compared the ability of an NO donor to activate Dexras1 and H-Ras (Figures 4A and 4B). S-nitroso-acetylpenicil-lamine (SNAP) increases the dissociation of [^{3}H]GDP from Dexras1 and H-Ras to a similar extent, with a significant effect evident at 1 μM and further increases at higher concentrations of SNAP (Figure 4A). By contrast, N-acetylpenicillamine (NAP) has no effect at concentrations as high as 1 mM, establishing that the effects of SNAP are due to its NO moiety. In other experiments, Dexras1 and H-Ras were first treated with unlabeled GDP, followed by [^{35}S]GTP-γ-S to facilitate detection of NO-stimulated guanine-nucleotide exchange. SNAP augments [^{35}S]GTP-γ-S binding to Dexras1 and H-Ras with similar concentration–response characteristics (Figure 4B). We find a similar dose–response effect on Dexras1 guanine-nucleotide exchange activity in both these assays, using sodium nitroprusside as the NO donor and potassium ferricyanide as the inactive control (data not shown). These experiments show that Dexras1, like H-Ras (Lander et al., 1995), can be activated by

A

B

C

pMyc-Dexras1 (ng)	100	100	100	100	100	100
pcDNA3-nNOS (ng)	0	50	100	500	1000	5000
$\frac{GTP}{GTP+GDP}$ %	6.2	8.4	15.6	21.5	30.4	40.2

D

pMyc-Dexras1 (ng)	100	100	100	100	100	100	100
pcDNA3-nNOS (ng)	0	0	5000	5000	5000	5000	0
pcDNA3-nNOS C415H (ng)	0	0	0	0	0	0	5000
$\frac{GTP}{GTP+GDP}$ %	15.9	12.1	52.6	4.1	37.7	40.5	8.94

Figure 4. Dexras1 Is Activated by NO Donors and Enzymatically Generated NO

(A) A NO donor stimulates GDP release from Dexras1 and H-Ras to a similar extent. GST-Dexras1 and GST-Ras, preloaded with [^{3}H]GDP, were treated with various amounts of the NO donor SNAP or the control compound NAP for 30 min at 30°C, followed by determination of bound [^{3}H]GDP. The control compound (NAP) did not induce [^{3}H]GDP release. Experiments shown are the average of three separate trials with standard error less than 5% in each.

(B) A NO donor stimulates Dexras1 and H-Ras activation as measured by [^{35}S]GTP-γ-S binding. In these assays, GST fusion proteins, preloaded with 2 mM unlabeled GDP, were incubated with the indicated concentration of SNAP or NAP and [^{35}S]GTP-γ-S for 30 min, followed by assay of bound [^{35}S]GTP-γ-S. Experiments shown are the average of three separate trials with standard error less than 5% in each.

(C) Transfection of nNOS leads to activation of Dexras1 in HEK293 cells. Cells were transfected with the indicated amounts of pcDNA3-nNOS and 100 ng of pMyc-Dexras1 and then metabolically labeled with [^{32}P]orthophosphate to label intracellular nucleotide pools. Myc-Dexras1 was immunoprecipitated, and bound GDP and GTP were detected by TLC. Transfection of nNOS leads to an increased amount of GTP bound to Dexras1.

(D) Dexras1 activation by nNOS is NO dependent and cGMP independent. HEK293 cells were transfected with pMyc-Dexras1 and pcDNA3-nNOS, as indicated. 8-Bromo-cGMP (5 mM) failed to mimic the effect of nNOS, and the guanylyl cyclase inhibitor ODQ (10 μM) failed to inhibit nNOS-dependent activation of Dexras1. nNOS activation of Dexras1 was inhibited by 500 μM L-NAME, which could be reversed by adding 5 mM L-arginine.

different classes of NO donors and, like H-ras, this activation involves increased guanine-nucleotide exchange.

nNOS Activates Dexras1 in a cGMP-Independent Manner

To determine whether NO generated from nNOS can activate Dexras1, we transfected HEK293 cells, which do not contain endogenous Dexras1 (data not shown), with a Dexras1 plasmid and with varying amounts of an nNOS-expressing plasmid. Cells were metabolically labeled with [^{32}P]orthophosphate to label nucleotide pools, and Dexras1 was immunoprecipitated. Bound nucleotides were resolved by TLC to measure the GTP:GDP ratio, as an increased ratio reflects activation. nNOS transfection leads to activation of Dexras1 in a dose-dependent fashion (Figure 4C). nNOS transfection has no effect in cells that are not cotransfected with the Myc-Dexras-expressing plasmid (data not shown). NO generation mediates the effect of nNOS, as the NOS inhibitor L-nitroarginine methyl ester (L-NAME) abolishes Dexras1 activation, while L-arginine reverses the effects of L-NAME (Figure 4D). A nNOS C415H mutant, which lacks catalytic activity (Richards et al., 1996), does not activate Dexras1. To determine if Dexras1 activation stems from the augmentation of cyclic GMP formation by NO, we treated cells with 5 mM 8-bromo-cyclic GMP, a nonhydrolyzable cGMP analog that fails to activate Dexras1. Moreover, 10 μM ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), a guanylyl cyclase inhibitor (Garthwaite et al., 1995), does not affect the activation of Dexras1 by nNOS (Figure 4D). Thus, Dexras1 is activated by enzymatically generated NO in a cGMP-independent manner.

Dexras1 Is Regulated by NO and NMDA Receptor Activation in Cortical Neurons

To identify mechanisms by which Dexras1 activity may be regulated in neurons, we examined the GTP:GDP ratio in Dexras1 immunoprecipitates derived from metabolically labeled cortical neurons. Dexras1 immunoprecipitates derived from cortical neurons stimulated with 100 μM NMDA exhibit increased GTP loading, indicating that Dexras1, like H-ras, is activated by NMDA receptor stimulation (Yun et al., 1998). Pretreatment of neurons with 10 μM MK-801 prevented NMDA-induced Dexras1 activation, indicating that the stimulatory effect of NMDA on Dexras activity requires calcium flux through the NMDA receptor. As NMDA receptor stimulation is coupled to nNOS activation (Bredt and Snyder, 1989), we tested whether the effect of NMDA was mediated by nNOS using L-NAME. Pretreatment of neurons with 500 μM L-NAME blocks NMDA-stimulated Dexras1 activation, indicating that the stimulatory effect of NMDA on Dexras1 function requires NO synthesis. Thus, Dexras1 activation is coupled to NMDA receptor–dependent NO synthesis.

Dexras1 Guanine-Nucleotide Exchange Activity Is Decreased in *nNOS*$^{-/-}$ Mice

To determine whether Dexras1 is regulated by nNOS in the intact organism, we utilized *nNOS*$^{-/-}$ mice. We immunoprecipitated Dexras1, as well as five other members of the Ras superfamily, from the brains of wild-type and *nNOS*$^{-/-}$ mice and monitored their guanine-nucleotide exchange activity (Figure 5A). In these experiments, we assessed the guanine-nucleotide exchange activity of the Ras proteins by measuring [^{35}S]GTP-γ-S binding to proteins that had been preloaded with un-
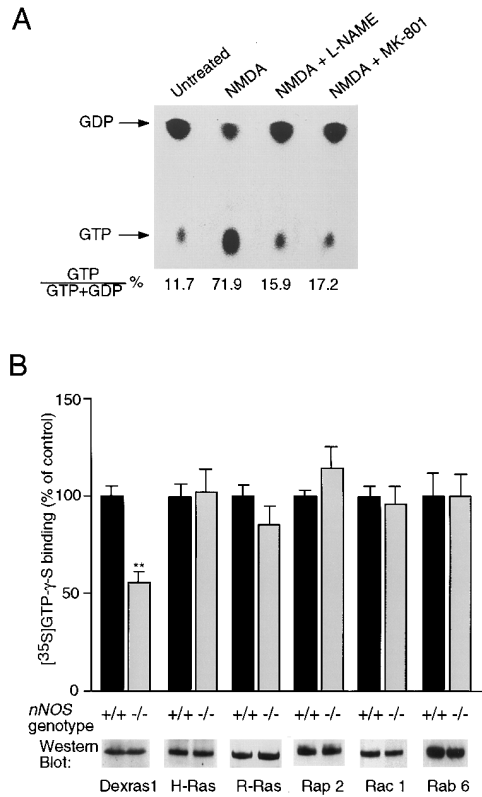
A

Lanes: Untreated, NMDA, NMDA + L-NAME, NMDA + MK-801

GDP →

GTP →

$\frac{GTP}{GTP+GDP}$ %   11.7   71.9   15.9   17.2

B

$[^{35}S]GTP\text{-}\gamma\text{-}S$ binding (% of control)

nNOS genotype: +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/-

Western Blot:

Dexras1   H-Ras   R-Ras   Rap 2   Rac 1   Rab 6

Figure 5. Regulation of Dexras1 by nNOS in Cortical Neurons and in Intact Animals

(A) Dexras1 is activated in a nNOS- and NMDA-dependent manner in cortical neurons. Cortical neurons were metabolically labeled with [^{32}P]orthophosphate to label intracellular nucleotide pools and treated as indicated. Endogenous Dexras1 was immunoprecipitated, and bound GDP and GTP were detected by TLC. Treatment of neurons with 100 μM NMDA leads to increased Dexras1 guanine-nucleotide exchange activity. Pretreatment of cortical neurons with the NMDA receptor antagonist MK801 (10 μM) blocked this effect, as did 500 μM L-NAME, indicating that NMDA receptor–dependent activation of nNOS is required for the activation of Dexras1. Immunoprecipitates using preimmune serum failed to precipitate a detectable amount of radiolabeled nucleotides (data not shown).

(B) Dexras1 is regulated by nNOS in intact animals. To detect G protein activation, a [^{35}S]GTP-γ-S binding assay was used. The indicated G proteins were immunoprecipitated with specific antibodies and preloaded with unlabeled GDP. Extra GDP was then removed, and the immunoprecipitated proteins were incubated with [^{35}S]GTP-γ-S for 30 min at 30°C to detect guanine-nucleotide exchange. Dexras1 immunoprecipitated from wild-type mice exhibited nearly twice as much guanine-nucleotide exchange as Dexras1 immunoprecipitated from nNOS$^{-/-}$ mice. H-Ras guanine-nucleotide exchange activity was identical in both wild-type and nNOS$^{-/-}$ mice, as were the guanine-nucleotide exchange activities of several other control G proteins. The results shown are the average of at least four separate trials. Statistical significance was determined by t test (p < 0.005).

labeled GDP and normalized the values to those found in immunoprecipitates derived from wild-type mice. Dexras1 activation is reduced about 50% in nNOS$^{-/-}$ brains (Figure 5A), while the activation of H-Ras, R-Ras, Rap2, Rac1, and Rab6 is unaffected by nNOS deletion. Protein levels of the six Ras superfamily members tested are the same in nNOS$^{-/-}$ and wild-type mouse brains (Figure 5). We also confirmed that the same amount of
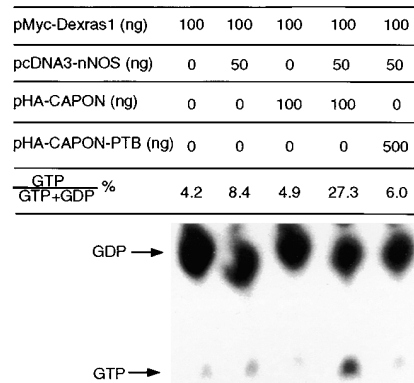
pMyc-Dexras1 (ng)	100	100	100	100	100
pcDNA3-nNOS (ng)	0	50	0	50	50
pHA-CAPON (ng)	0	0	100	100	0
pHA-CAPON-PTB (ng)	0	0	0	0	500
$\frac{GTP}{GTP+GDP}$ %	4.2	8.4	4.9	27.3	6.0

GDP →

GTP →

Figure 6. CAPON Enhances nNOS-Dependent Activation of Dexras1

pMyc-Dexras1 was cotransfected with a subthreshold quantity of pcDNA3-nNOS as well as pHA-CAPON or with pHA-CAPON-PTB. A total of 650 ng of DNA was transfected into each plate, using the parent vector pRK5 as the balance. Cells were metabolically labeled with [^{32}P]orthophosphate, as in Figure 5C and 5D. Myc-Dexras1 was immunoprecipitated, and GTP:GDP ratios were determined by phosphorimaging quantitation of TLC-separated bound nucleotides. nNOS minimally activates Dexras1 alone, while the addition of CAPON leads to a significant increase of nNOS-mediated Dexras1 activation. CAPON alone fails to activate Dexras1. Expression of the CAPON PTB domain, which is capable of binding to Dexras1 but not nNOS, fails to alter nNOS-mediated activation of Dexras1. Experiments shown were replicated three times with essentially identical results.

G protein was immunoprecipitated from wild-type and nNOS$^{-/-}$ animals by loading immunoprecipitates with [^{35}S]GTP-γ-S. Levels of G proteins in the immunoprecipitates were quantitated by scintillation counting and varied by less than 5% between wild-type and nNOS$^{-/-}$ animals. The selective reduction in guanine-nucleotide exchange activity of Dexras1 derived from nNOS$^{-/-}$ mice demonstrates that Dexras1 activation is physiologically determined by nNOS.

CAPON Enhances nNOS Activation of Dexras1

Dexras1 is noteworthy in that it is both activated by nNOS and physically coupled to nNOS. To determine whether the ternary complex of nNOS, CAPON, and Dexras1 plays a role in Dexras1 activation, we cotransfected various combinations of Dexras1, nNOS, and CAPON into HEK293 cells labeled with [^{32}P]orthophosphate and monitored Dexras1 activation by quantitation of bound guanine nucleotides in immunoprecipitates (Figure 6). Our experiments transfecting different amounts of nNOS had established that detectable activation of Dexras1 can occur following transfection with 100 ng of the nNOS expression vector (Figure 4C). Cotransfection of 50 ng of the nNOS plasmid and pMyc-Dexras1 resulted in only a small amount of Dexras1 activation. However, the combination of nNOS, CAPON, and Dexras1 reproducibly activates Dexras1 to levels higher than those seen with cotransfection of 500 ng of the nNOS-expressing plasmid with pMyc-Dexras1. CAPON transfection by itself does not lead to activation, ruling out the possibility that CAPON is a Dexras1-specific GEF (Figure 6). Also, we cotransfected nNOS, Dexras1,

and the PTB domain of CAPON, which can bind Dexras1 but not nNOS. This CAPON construct fails to activate Dexras1 beyond the level seen with nNOS alone. Thus, CAPON enhances nNOS-mediated activation of Dexras1 through the ternary complex whereby CAPON links nNOS to Dexras1.

Discussion

In this study, we identify Dexras1 as a novel G protein effector of nNOS. Dexras1 is S-nitrosylated by NO donors, and Dexras1 is regulated by NMDA-dependent NO production in cortical neurons. Dexras1 activity is regulated by nNOS under basal physiologic conditions, as Dexras1 activation is markedly diminished in $nNOS^{-/-}$ mice. This evidence that Dexras1 is a physiologic target of basally generated NO is notable. Since NO is a reactive free radical, its application affects a wide range of proteins, such as H-ras, that have been proposed as physiologic targets. However, there are few examples of proteins whose activity differs in wild-type animals and $nNOS^{-/-}$ mice. A role for the physiologic linkage by CAPON of nNOS to Dexras1 is suggested by our finding that NO-mediated activation of Dexras1 is markedly enhanced by CAPON. This implies that NO signaling is regulated by protein–protein interactions that juxtapose nNOS with its targets.

One of the remarkable aspects of Dexras1 activation is the selectivity of its regulation by nNOS, as five other members of the Ras superfamily exhibited identical guanine-nucleotide exchange activity in both wild-type and $nNOS^{-/-}$ mice. Importantly, H-ras, which can be regulated by iNOS in HUVEC cells (Lander et al., 1995) and by nNOS in PC12 cells (Teng et al., 1999) and primary cortical neurons (Yun et al., 1998), exhibited identical activity in both wild-type and knockout mice. The selectivity of nNOS-mediated activation of Dexras1 appears to derive from Dexras1's unique ability to interact with CAPON/nNOS complexes through its extended C terminus. This C-terminal 7 kDa extension results in a 31 kDa G protein that is substantially larger than most other Ras superfamily members, which are typically 21–24 kDa. No other G protein has a C-terminal domain with sequence similarity to that found in Dexras1, suggesting that Dexras1 is the only Ras-like G protein capable of interacting with the CAPON PTB domain.

Dexras1 binds to the PTB domain located at the N terminus of CAPON. The PTB domain of CAPON is most similar to the PTB domain of mouse Numb, which, like CAPON, lacks certain residues that are expected to contact the phosphotyrosine, based on extrapolation from the crystal structure of the Shc PTB domain (Zhou et al., 1995). In the case of Numb, its protein targets lack phosphotyrosine, as exemplified by Numb-associated kinase (Chien et al., 1998) and LNX (Dho et al., 1998). This also appears to be the case for the interaction of Dexras1 and CAPON, as we found that Dexras1 expressed in bacteria, which is not phosphorylated, robustly binds CAPON, indicating that this interaction does not require phosphotyrosine.

Several studies have shown that H-ras is regulated by iNOS in HUVEC cells (Lander et al., 1995) and by nNOS in PC12 cells (Teng et al., 1999) and primary corti-
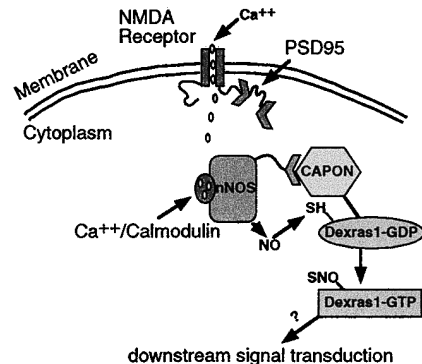

Figure 7. Model of Dexras1 Activation by nNOS

Dexras1, CAPON, and nNOS can form a complex that places Dexras1 in proximity to CAPON. The most likely mechanism of Dexras1 activation is S-nitrosylation, which is likely to be highly efficient due to the juxtaposition of nNOS and Dexras1. nNOS is a dimer (not shown), and, conceivably, one monomer may bind to PSD95 and the other to CAPON/Dexras, thus allowing Dexras1 to be activated by NMDA receptor–gated calcium.

cal neurons (Yun et al., 1998). Although comparatively large amounts of NO do activate H-Ras in transfected HEK293 cells (M. F., S. R. J., and S. H. S., unpublished data) as well as in other cell types (Lander et al., 1995), the failure to see differences in H-ras activation between wild-type and $nNOS^{-/-}$ mice may derive from the higher levels of NO generated in those studies. It is possible that substantial NO release, as occurs during vascular stroke, might produce sufficient NO to activate both H-Ras and Dexras1 and their respective signaling pathways.

There has been little characterization of Dexras1, and its downstream targets are not definitively established. Members of the Rap subfamily of Ras-like G proteins transmit growth factor signals to MAP kinase, signaling cascades through B-Raf preferentially over A-Raf (Ohtsuka et al., 1996; York et al., 1998; Dugan et al., 1999). Dexras1 may have similar effectors as other Rap subfamily members, since its effector loop (residues 53–61) is 78% identical to the effector loop in Rap2b (residues 32–40). Indeed, in preliminary experiments, we have transfected Dexras1 into HEK293 cells and detected activation of MAP kinase activity, monitored by the phosphorylation of myelin basic protein (data not shown). Conceivably, Dexras1-specific effectors may exist that mediate the effects of NO in the central nervous system.

Ras family members have been implicated in growth and differentiation of neurons (Curtis and Finkbeiner, 1999). nNOS exhibits a dynamic cell-type specific pattern of expression during development of the embryonic brain (Bredt and Snyder, 1994), and neurons in $nNOS^{-/-}$ mice have impaired dendritic arborization (Inglis et al., 1998). A role for NO in the differentiation of PC12 cells has also been established (Peunova and Enikolopov, 1995). It will be important to determine which of these NO-dependent functions are mediated by Dexras1.

S-nitrosylation is the most likely mechanism for NO-dependent activation of Dexras1. This is likely as NO donors directly S-nitrosylate Dexras1, and Dexras1 is

preferentially S-nitrosylated over other similar G proteins. Secondly, S-nitrosylation is also associated with the activation of H-ras (Lander et al., 1995, 1996; Mott et al., 1997), and mutagenesis of cysteine 118, the S-nitrosylated residue, in H-ras abolishes its sensitivity to NO (Lander et al., 1996). Although there is no cysteine in Dexras1 that corresponds to cysteine 118 in H-Ras, several candidate cysteines can be identified by the presence of adjacent polar amino acids that correspond to a proposed consensus site for S-nitrosylation (Stamler et al., 1997).

Although nNOS directly S-nitrosylates peptides (Mayer et al., 1998) and proteins (Stamler et al., 1992), there are conceptual difficulties in invoking S-nitrosylation as a physiologic signaling mechanism for NO. One problem is the abundance of iron-containing proteins and cofactors that might interact nonselectively with NO. Moreover, most cells contain very high levels of glutathione, about 5 mM, which would be expected to compete with thiol groups in proteins for binding NO. Juxtaposition of Dexras1 and nNOS should increase the effective concentration of NO in the vicinity of Dexras1, making it a preferred target for S-nitrosylation. The ternary complex of nNOS, CAPON, and Dexras1 provides a system that is uniquely suited for targeting NO to protein targets for S-nitrosylation (Figure 7).

By analogy with kinase signaling, where anchoring proteins such as AKAPs (A-kinase anchoring proteins) contribute substantially to the specificity and rate of kinase signaling reactions (Colledge and Scott, 1999), we speculate that nNOS may rely on CAPON and other nNOS-associated proteins, such as PSD95/93 (Brenman et al., 1996), to facilitate reactions of NO with its targets. Interestingly, the NMDA receptor, whose activity can be modulated by NO donors (Choi et al., 2000), is coupled to nNOS through a mutual interaction with PSD95 (Sattler et al., 1999). Conceivably, this anchoring of nNOS may be required for the regulation of NMDA receptors by NO in vivo.

Experimental Procedures

General Methods and Materials
Molecular biology reagents were from New England Biolabs (Beverly, MA). G protein antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), SNAP was from RBI (Natick, MA), and NAP was from Aldrich Chem. Co. (Milwaukee, WI). All other reagents were from Sigma (St. Louis, MO), except as indicated.

cDNA Cloning and Localization of Rat *Dexras1*
The two-hybrid screens were done as described (Jaffrey et al., 1998). The PTB domain of rat CAPON, comprising amino acids 20–180, was constructed by PCR and subcloned into the SalI and NotI sites of pPC97, encoding a GAL4 BD-CAPON-PTB fusion protein. Other control plasmids were also prepared by PCR and subcloned into SalI and NotI sites of either pPC86 or pPC97, as indicated. A total of 2×10^6 independent clones were screened, and a positive clone carrying 330 bp cDNA was identified by selecting for histidine prototrophy and the presence of β-galactosidase activity, as described previously (Jaffrey et al., 1998). The two-hybrid 330 bp *Dexras1* fragment was used to screen a rat brain lamdaZAP II cDNA library (Stratagene). A 1.6 kb full-length Dexras1 cDNA was isolated and subcloned into pCMV for eukaryotic expression.

A commercial rat multiple tissue Northern blot (Clontech) was hybridized in ExpressHyb solution (Clontech) at 68°C for 1 hr with the 330 bp *Dexras1* DNA probe labeled with [^{32}P]CTP in a nick translation system (Boehringer Mannheim). The blots were washed sequentially in 2× SSC with 0.05% SDS four times at room temperature for 30 min and in 0.1× SSC with 0.1% SDS twice at 40°C. The blot was apposed to film using indirect autoradiography for 24 hr at −70°C.

Tissues from an adult male rat were homogenized in ice-cold lysis buffer A (50 mM Tris-HCl [pH 7.7], 100 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A). Cellular debris was removed by centrifugation at 800 × g for 10 min at 4°C, the supernatant was centrifuged at 100,000 × g for 20 min at 4°C, and the supernatant was saved as "cytosol" fraction. Immunoblotting was performed using a rabbit anti-Dexras1 antibody generated against the unique C-terminal Dexras1 fragment isolated in the yeast two-hybrid screen and purified on a resin consisting of cross-linked GST-Dexras1 prepared with dimethylpimelimidate, as described for the anti-CAPON antibody (Jaffrey et al., 1998). To obtain the "membrane" fraction of brain, the pellet from the above centrifugation was sonicated in lysis buffer B (lysis buffer A adjusted to 0.1% Triton X-100), followed by centrifugation at 100,000 × g for 20 min at 4°C.

In Situ Hybridization
In situ hybridization was performed essentially as described previously (Borjigin et al., 1999), with minor modifications. In brief, fresh-frozen rat brain sections were fixed with 4% paraformaldehyde/PBS and permeabilized, prehybridized, and hybridized in 50% formamide, 5× SSC with 100 ng/ml unhydrolyzed digoxygenin-labeled probe overnight at 55°C (nNOS, CAPON) or 65°C (Dexras). Sections were washed, blocked, and incubated overnight at 4°C in 4% normal goat serum in TBS with antidigoxygenin-AP antibody (Boehringer) at 1:5000. After washing in TBS, slides were developed with 1 ml of color development solution, containing 3.375 mg/ml nitroblue tetrazolium, 3.5 mg/ml BCIP, and 0.24 mg/ml levamisole, in the dark. The color reaction was allowed to run 4–8 hr at room temperature. The reaction was stopped in ddH2O, and the slides were sealed in Aquapolymount. Unique probes to *CAPON*, *nNOS*, and *Dexras1* were generated from cDNA corresponding to amino acids 20–180 of CAPON, the full open reading frame (ORF) sequences of *nNOS*, and the 3′UTR sequences of *Dexras1*, respectively. Probes generated against the *Dexras1* ORF sequence and the *Dexras1* 3′UTR generated identical results. Templates were generated by PCR, subcloned into pBS (Stratagene), and antisense and sense cRNA probes were generated by T7 and T3 RNA polymerases. Sense control probes used at equal concentration generated no specific signal. CAPON was localized to the supraoptic nucleus by immunohistochemistry (Jaffrey et al., 1998).

Protein Binding Assays
GST fusion proteins were prepared as described previously (Jaffrey et al., 1998). Transfections were performed with 10 μg or the indicated quantity of each plasmid, using the calcium phosphate method. At 48 hr after transfection, cells were lysed in 1 ml lysis buffer C (50 mM Tris-HCl [pH 7.4], 40 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1.5 mM NaVO$_4$, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 1 mM PMSF, 5 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A) and cleared by centrifugation. This cellular lysate was incubated with GST fusion protein immobilized on glutathione agarose for 1 hr at 4°C and washed four times with washing buffer (lysis buffer C plus 300 mM NaCl). In GST pulldown assays, HEK293 cells transfected with pCIS-GST-Dexras1 as well as the indicated plasmids were lysed in 0.5 ml lysis buffer C. GST agarose was incubated with clarified cell lysates for 1 hr. After incubation, the resin was washed five times with lysis buffer C adjusted to 250 mM NaCl, and bound proteins were eluted by boiling in 1× SDS-PAGE sample buffer.

Immunoprecipitations were performed by homogenizing one rat brain in 3 ml lysis buffer B, followed by centrifugation at 100,000 × g for 30 min at 4°C. The supernatant (0.5 ml) was incubated with 40 μl of protein A-agarose (Calbiochem, La Jolla, CA) and 5 μg of the indicated antibody for 60 min at 4°C. The resins were then washed with IP wash buffer (50 mM Tris-HCl [pH 7.7], 200 mM NaCl, and 2 mM EDTA) three times and eluted in 30 μl of 1× SDS-PAGE sample buffer by boiling. Detection of ternary complexes was done by incubating brain supernatants prepared from wild-type and *nNOS*$^{-/-}$

mice as above and then incubating with 100 μl 2′, 5′ ADP sepharose 4B (Pharmacia) for 1 hr and then washing with IP wash buffer five times. Protein was eluted by incubation of the resin in 10 mM NADPH for 30 min.

S-Nitrosylation Assays

Purified GST-Dexras1 in HEN buffer (250 mM HEPES [pH 7.7], 1 mM EDTA, and 0.1 mM neocuproine) (1 ng/μl) was incubated with the indicated concentrations of SNAP or NAP for 30 min at 25°C in the dark, and the NO donors were removed by desalting through a MicroBioSpin6 column (Biorad). The S-nitrosylated protein was subjected to the S-nitrosylation assay as described elsewhere (Jaffrey et al., 2000). In brief, 75 μl of the flowthrough was blocked with methylmethanethiosulfonate (MMTS) blocking solution for 20 min at 50°C, and, following removal of MMTS by desalting, S-nitrosylated cysteines were detected with biotin-HPDP (Pierce) and detected by Western blotting with a biotin-specific antibody (Sigma). Detection of nitrosylated proteins in lysates incubated with GSNO was performed as described previously (Jaffrey et al., 2000). In brief, 750 mg of rat brain cortex was incubated with either 40 μM GSNO or GSH for 20 min in the dark and subjected to the S-nitrosylation assay. Following the S-nitrosylation assay, biotinylated proteins were purified on neutravidin-agarose (Pierce) and then eluted with 2-mercaptoethanol and blotted with the indicated G protein–specific antibody.

Quantitation of Dexras1-Associated Nucleotides

GTP loading assays were performed as previously described (Rosen et al., 1994). In brief, HEK293 cells were washed once with phosphate-free Dulbecco's modified Eagle's medium (GIBCO-BRL) before incubation in the same medium plus 0.25 mCi/ml [^{32}P]H$_3$PO$_4$ for 3 hr at 37°C. Cortical neurons were prepared as described previously (Dawson et al., 1993; Yun et al., 1998). After metabolic labeling, cells were treated with 500 μM L-NAME, 500 μM L-NAME plus 5 mM L-arginine, 10 μM MK-801, or 10 μM ODQ for 20 min or 5 mM 8-Br-cGMP for 5 min, if indicated. In studies with cortical neurons, drugs were removed by rinsing the cells once with media, and 100 μM NMDA was added for 5 min. Cells were then lysed in 0.5 ml of lysis buffer D (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM MgCl$_2$, 1% Triton X-100, and 1 μg anti-Myc antibody). Extracts were drawn ten times through a 0.22 gauge needle and mixed with 1:10 volume of PBS/1% bovine serum albumin/10% charcoal slurry and then rocked at 4°C for 30 min. After centrifugation for 5 min at 12,000 × g, the supernatant was mixed with 1 μg anti-Myc antibody or 1 μg anti-Dexras1 antibody and 30 μl protein G or protein A-agarose, respectively. Samples were incubated for 2 hr and washed three times with lysis buffer D and once with PBS. Immunoprecipitates from cortical neurons were washed four times. Pellets were resuspended in 30 μl of 1 M KH$_2$PO$_4$ (pH 3.4) and incubated at 85°C for 3 min. After centrifugation at 12,000 × g for 5 min, 3 μl (HEK293 cells) or 30 μl (cortical neurons) of the supernatant was spotted onto polyethyleneimine-cellulose TLC plates (EM Science), and guanine nucleotides were resolved in 1 M KH$_2$PO$_4$ (pH 3.4) for 2.5 hr. The TLC plate was exposed to film for 1 day at −70°C. Each GDP and GTP fractionation was quantitated using a Phosphorimager (Molecular Dynamics).

Guanine-Nucleotide Exchange Assays

GDP release assays were performed as described (Lander et al., 1995). Briefly, GST-Dexras1 (1 μg) or GST-H-Ras (1 μg) was incubated at 30°C for 30 min in 200 μl of GDP binding buffer (Tris-HCl [pH 7.4], 1 mM MgCl$_2$, 100 mM NaCl, and 0.1 mg/ml BSA) plus 100 pmol of [^{3}H]GDP (10 Ci/mmol). Then, 20 μl of 5 mM GTP was added with different concentrations of SNAP or NAP, as indicated in the figure. After incubation at 30°C for 30 min, 40 μl aliquots were removed, and bound [^{3}H]GDP was separated from free by washing with 10 ml of washing solution (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, and 25 mM MgCl$_2$) on nitrocellulose filters (Millipore, Bedford, MA) three times. The filters were dried and counted in a liquid scintillation counter.

[^{35}S]GTP-γ-S binding assays were performed as described (Lander et al., 1995), with slight modifications. GST-Dexras1 (1 μg) or GST-Ras (1 μg) coupled to glutathione-sepharose beads was preloaded with 2 mM GDP in 200 μl of GDP loading buffer (20 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 5 mM MgCl$_2$). After incubation at 30°C for 30 min, samples were washed three times with 500 μl of reaction buffer (150 mM HEPES [pH 8.0], 180 mM MgCl$_2$, 6 mM EDTA, and 600 mM NaCl). The agarose beads were then resuspended in 50 μl of reaction buffer plus 3 mM GTP-γ-S and 1 pmol of [^{35}S]GTP-γ-S (1000 Ci/mmol) in the presence of different concentrations of SNAP or NAP, as indicated in the figures. After incubation at 30°C for 30 min, samples were washed with 500 μl of the washing solution described above for three times. The agarose containing [^{35}S]GTP-γ-S bound to GST fusion proteins were counted in a liquid scintillation counter.

Immunoprecipitations for nucleotide exchange assays were performed by homogenizing two brains from either wild-type or $nNOS^{-/-}$ mice in 4 ml of lysis buffer D, followed by centrifugation at 100,000 × g for 20 min at 4°C. Care was taken to rapidly homogenize the tissue after decapitation to minimize postmortem NO production. The supernatant (0.5 ml) was incubated at 4°C for 2 hr in the dark with 1 μg of the indicated antibody and 30 μl of protein-A agarose and then washed three times with 0.5 ml of lysis buffer D and once with GDP loading buffer. Samples were preloaded with GDP and incubated with [^{35}S]GTP-γ-S, as described above, except that no SNAP or NAP was added. After samples were washed three times with 0.5 ml of washing solution, the bound [^{35}S]GTP-γ-S was counted as described above. Mice harboring a genomic deletion of nNOS have been described previously (Huang et al., 1993) and backcrossed a minimum of seven times against the C57/BL6 strain, which was the strain used for wild-type controls.

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GenBank Accession Number

The GenBank accession number for the rat *Dexras1* sequence has been deposited under the accession number AF239157.