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SPECIES, STRAIN AND DEVELOPMENTAL VARIATIONS IN HIPPOCAMPAL NEURONAL AND ENDOTHELIAL NITRIC OXIDE SYNTHASE CLARIFY DISCREPANCIES IN NITRIC OXIDE-DEPENDENT SYNAPTIC PLASTICITY

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Abstract—Nitric oxide (NO) has been implicated in long-term potentiation (LTP) in pyramidal neurons in cellular area 1 (CA1) of the hippocampus. However, considerable confusion exists about the exact role of NO, and the contribution of the endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) isoforms of NO synthase to NO-dependent LTP (NO-LTP), with results often varying, depending on the organism and experimental paradigm used. Using immunohistochemistry and *in situ* hybridization, we contrast NO synthase expression and activity in rat, mouse, and human hippocampus. nNOS is prominently expressed in all CA1 pyramidal cells of C57B6 mice and humans, while in rats and SV129 mice, its levels are much lower and restricted to the caudal hippocampus. By contrast, eNOS is restricted to endothelial cells. We observe *N*-methyl-D-aspartate-dependent citrulline production in pyramidal cells of mouse hippocampus, which is absent in nNOS$^{\Delta/\Delta}$ animals. Finally, we observe robust nNOS expression in human CA1 pyramidal cells.

The considerable axial, developmental, strain and species-dependent variations in nNOS expression in CA1 pyramidal neurons can explain much of the variation observed in reports of NO-dependent LTP. Moreover, our data suggest that NO produced by eNOS in endothelial cells may play a paracrine role in modulating LTP.

Key words: long-term potentiation, pyramidal cell, species differences, rostro-caudal, *in situ* hybridization, citrulline.

In studies of long-term potentiation (LTP) numerous investigators have concluded that hippocampal LTP depends on release of a retrograde messenger from pyramidal cells (Schuman and Madison, 1994; Garthwaite and Boulton, 1995). The discovery that nitric oxide (NO) is a widely diffusible neurotransmitter (Bredt and Snyder, 1994; Garthwaite et al., 1995; Haley et al., 1996) suggested that it may function as a retrograde messenger for LTP. The first direct evidence for this came from studies showing that inhibitors of NO synthase (NOS) block hippocampal LTP in slices (Schuman and Madison, 1991; Nowicky and Bindman, 1993; Kantor et al., 1996), while exogenous NO donors facilitate the development of LTP (O'Dell et al., 1991; Haley et al., 1993; Kato and Zorumski, 1993; Zhuo et al., 1993, 1994; Arancio et al., 1996; Doyle et al., 1996). However, numerous researchers have failed to replicate some of the initial findings (Chetkovich et al., 1993; Bannerman et al., 1994a,b; Murphy et al., 1994; Ko and Kelly, 1999). Certain consistent themes have emerged in efforts to demonstrate a role of NO in LTP. NO-dependent LTP (NO-LTP) is much more readily elicited in mice than rats (Lu et al., 1999; Wilson et al., 1999; Doreulee et al., 2001). In rats NO-LTP is more robust in immature than adult animals, and is most consistently observed in hippocampal cultures obtained from late embryonic or neonatal rats (Williams et al., 1993; Arancio et al., 1996). Furthermore, NO-LTP is also more prominent in the stratum radiatum than the stratum oriens (Haley et al., 1996; Son et al., 1996).

Mice with targeted deletion of various forms of NOS have helped to clarify NO-LTP. Deletion of either neuronal NOS (nNOS) or endothelial NOS (eNOS) alone produces only minimal decrements in NO-LTP (O'Dell et al., 1994; Wilson et al., 1999; Doreulee et al., 2001). By contrast, mice with deletion of both nNOS and eNOS manifest reproducible declines in certain forms of hippocampal LTP (Son et al., 1996). Such findings raise questions about the localization of nNOS and eNOS. In

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Abbreviations: aCSF, artificial cerebrospinal fluid; αCSF, anti-granulocyte-monocyte colony stimulating factor; CA1, cellular area 1 of hippocampus; CA2, cellular area 2 of hippocampus; CA3, cellular area 3 of hippocampus; CA4, cellular area 4 of hippocampus; DG, dentate gyrus of the hippocampus; eNOS, endothelial form of nitric oxide synthase (NOS III); iNOS, inducible form of nitric oxide synthase (NOS II); LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NO-LTP, nitric oxide-dependent long-term potentiation; NOS, nitric oxide synthase; nNOS, neuronal from of nitric oxide synthase (NOS I); PCL, pyramidal cell layer; SO, stratum oriens of CA1; SR, stratum radiatum of CA1.

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the adult rat most studies reveal nNOS largely confined to GABA-containing interneurons in cellular area 1 (CA1) of the hippocampus and not in pyramidal cells (Bredt et al., 1991; Valtschanoff et al., 1993; Nomura et al., 1997; Lin and Totterdell, 1998; Lumme et al., 2000). Some reports, however, describe limited nNOS staining in CA1 pyramidal cells of adult rat hippocampus (Endoh et al., 1994; Wendland et al., 1994; Black et al., 1995; Lopez-Figueroa et al., 1998). Though some studies report eNOS in pyramidal cells of adult rat and mouse hippocampus (Dinerman et al., 1994; O'Dell et al., 1994; Kantor et al., 1996; Doyle and Slater, 1997; Teichert et al., 2000), others find eNOS restricted to blood vessels in the brain (Seidel et al., 1997; Stanarius et al., 1997; Topel et al., 1998). Variable results in linking NO to hippocampal LTP have been largely attributed to differences between laboratories in sample preparation or stimulus paradigms (Holscher, 1997). In the present study, we have explored the possibility that many of these differences can be attributed to variations in expression of nNOS in different species, strains and developmental stages.

EXPERIMENTAL PROCEDURES

Material

C57/B6 and SV129 mice were obtained from the Jackson Laboratory. Sprague–Dawley rats were obtained from Charles River Laboratories. $nNOS^{\Delta/\Delta}$ and $eNOS^{\Delta/\Delta}$ mice were derived from animals generated in the laboratory of Mark Fishman (Massachusetts General Hospital and Harvard Medical School). $nNOS^{\Delta/\Delta}$:$eNOS^{\Delta/\Delta}$ were generated in our laboratory and genotyped by PCR. All animals were housed at the Johns Hopkins Animal Care Facility on a 12-h light/dark cycle, and only males were used. All efforts were made to minimize the number of animals used and their suffering, and experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Human tissue (4–8 h postmortem) was obtained from Tokyo Metropolitan Matsuzawa Hospital, Japan. Three male individuals aged 37–52 at death, none of whom had any reported neurological abnormalities, were tested and showed substantially similar nNOS and eNOS expression.

In situ hybridization

Fresh-frozen tissue was used for all *in situ* hybridization studies. For all species and conditions, at least three individuals were tested. Sections (20 μm) were stored at −80°, and fixed for 10 min in 4% paraformaldehyde. Nonradioactive *in situ* hybridization was carried out as previously described (Blackshaw and Snyder, 1997; Eliasson et al., 1997; Demas et al., 1999). Radioactive *in situ* hybridization was carried out as described elsewhere (Vassar et al., 1993). Probes were used at a dilution of 1,000,000 cpm/ml hybridization solution. Cellular quantification of mRNA levels was achieved by coating slides hybridized with radioactive cRNA probes with photoemulsion as previously described (Eliasson et al., 1997) and counting the number of silver grains per cell.

cRNA probes for both radioactive and nonradioactive *in situ* hybridization were generated as follows: (1) rat C-terminus of nNOS: residues 4196–5057 of coding sequence; (2) mouse N-terminus of nNOS: residues 100–786 of coding sequence; (3) mouse eNOS: residues 2282–3499 of coding sequence; (4) human nNOS: residues 2686–3491 of coding sequence; and (5) human eNOS: residues 3007–3612 of coding sequence.

The cDNA corresponding to the C-terminus of the rat nNOS was generated by subcloning a *Sal*I-*Eco*RI fragment into pBluescript. All other probes were generated by PCR, followed by subcloning into pBluescript. Sense and antisense cRNA probes were generated by T3 and T7 RNA polymerases.

Immunohistochemistry

Immunohistochemistry for nNOS and citrulline were done as previously described (Eliasson et al., 1997, 1999). The anticitrulline antiserum was used at a dilution of 1:12,000. The anti-nNOS antibody (which corresponded to the N-terminal region of human nNOS and was a kind gift of Jeffery Spangenberg of Incstar, Stillwater, MN, USA) was used at a 1:2000 dilution of purified antibody. Antiserum to the inducible form of NOS (iNOS; 1 mg/ml to 10 μg/ml; Transduction Laboratories, Lexington, KY, USA) did not show any specific immunoreactivity on brain sections. Specificity of iNOS antibody was examined by omission of the primary antibody. Antiserum to granulocyte macrophage-colony stimulating factor was used at 1:500 dilution (Research Diagnostics, Flanders, NJ, USA).

Animals were perfused through the left ventricle with 37° oxygenated Krebs-Henseleit buffer, followed by 250 ml of 37° 5% glutaraldehyde-0.5% paraformaldehyde containing $Na_2S_2O_5$ in 0.1 M sodium phosphate, pH 7.4. Brains were postfixed in the same buffer for 2 h at room temperature and cryoprotected for 2 days at 4 ° in 50 mM sodium phosphate, pH 7.4, 0.1 M NaCl, 20% (vol/vol) glycerol. Brain sections (40 μm) were cut on a sliding microtome. Free-floating brain sections were reduced for 20 min with 0.5% $NaBH_4$ and 0.2% $Na_2S_2O_5$ in PBS (10 mM, pH 7.4, and 0.19 M NaCl) to reduce background staining caused by the glutaraldehyde fixation. After washing for 45 min at room temperature in PBS containing $Na_2S_2O_5$, sections were blocked with 4% normal goat serum for 1 h in the presence of 0.2% Triton X-100. Immunoreactivity was visualized with the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) using 3,3'-diaminobenzidine-HCl as chromogen. The specificity of the antisera and detection assay were tested by preabsorption with the antigen and omission of the primary antibody, respectively.

N-methyl-D-aspartate (NMDA) application

NMDA was administered by intrahippocampal perfusion. Mice (20–27 g) were anesthetized with 1.5% halothane in 2:1 air and oxygen. Mice were placed in a custom-designed stereotactic frame (David Kopf, Tujunga, CA, USA) and microdialysis probes (CMA10: 1 mm membrane length) were placed into the cerebral cortex, and dorsorostral hippocampus was injected. Microdialysis probes were perfused with artificial cerebrospinal fluid (aCSF) [containing (mM): NaCl 125, KCl 2.5, $CaCl_2$ 1.2, NaH_2PO4 H_2O 0.5, Na_2HPO_4 5, $MgCl_2$ $6H_2O$ 1, and ascorbic acid 0.2, pH 7.4] at a flow rate of 2 μl/min microinfusion pump. After implantation, stabilization of the tissue was allowed by waiting for 1 h, during which aCSF was infused. A 30-min baseline control was obtained with aCSF superfusion, followed by 10 min of 1 mM NMDA superfusion. Six minutes later, the animals were perfused for immunohistochemistry.

Two mice were used for wild type and $eNOS^{\Delta/\Delta}$, while one mouse was used for $nNOS^{\Delta/\Delta}$.

RESULTS

Pronounced species, strain, developmental and spatial differences in nNOS expression

In the adult mouse, *in situ* hybridization at low power reveals prominent expression of nNOS mRNA in pyramidal layers of CA1 and CA3 in hippocampus (Fig. 1). High-

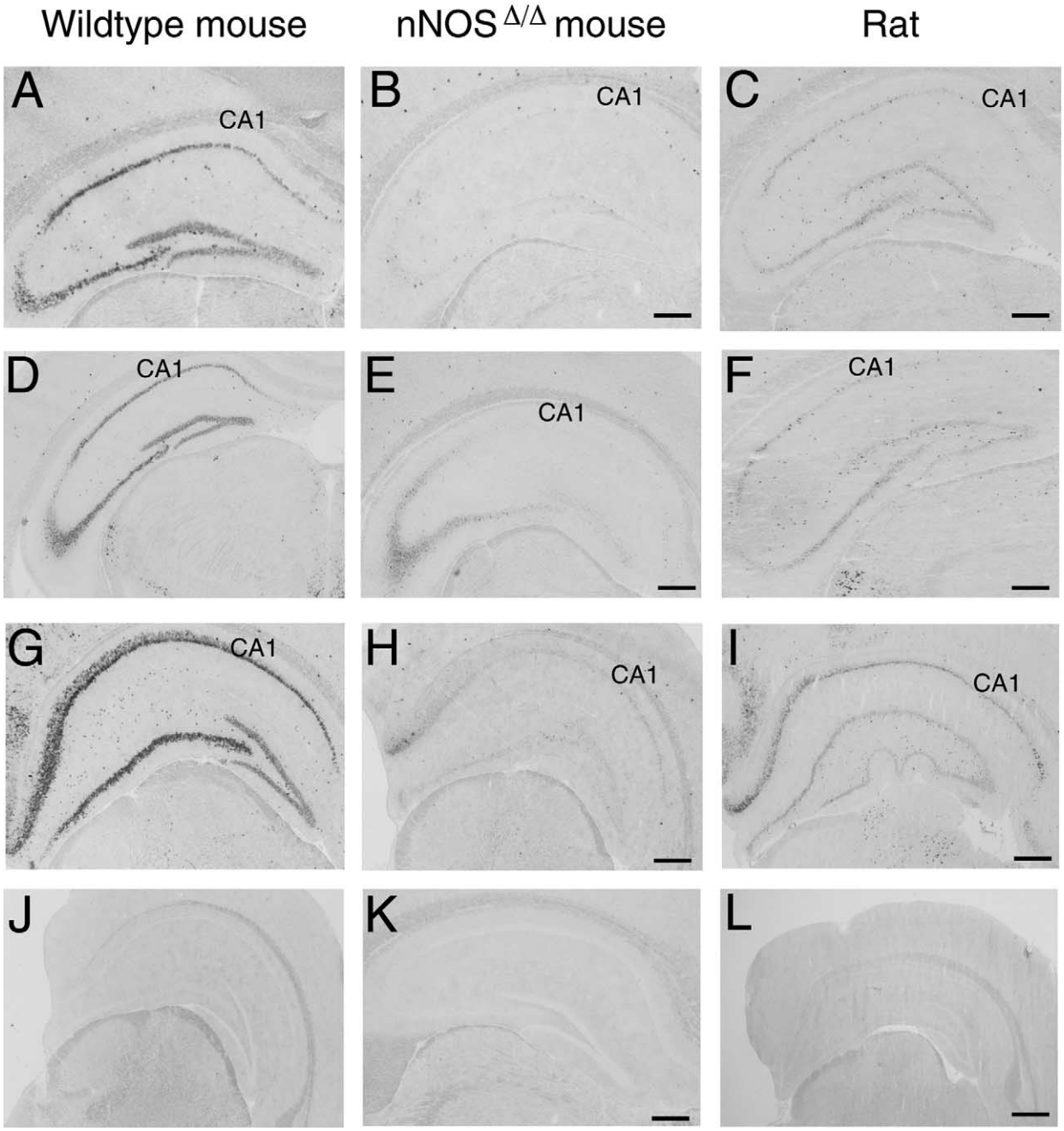
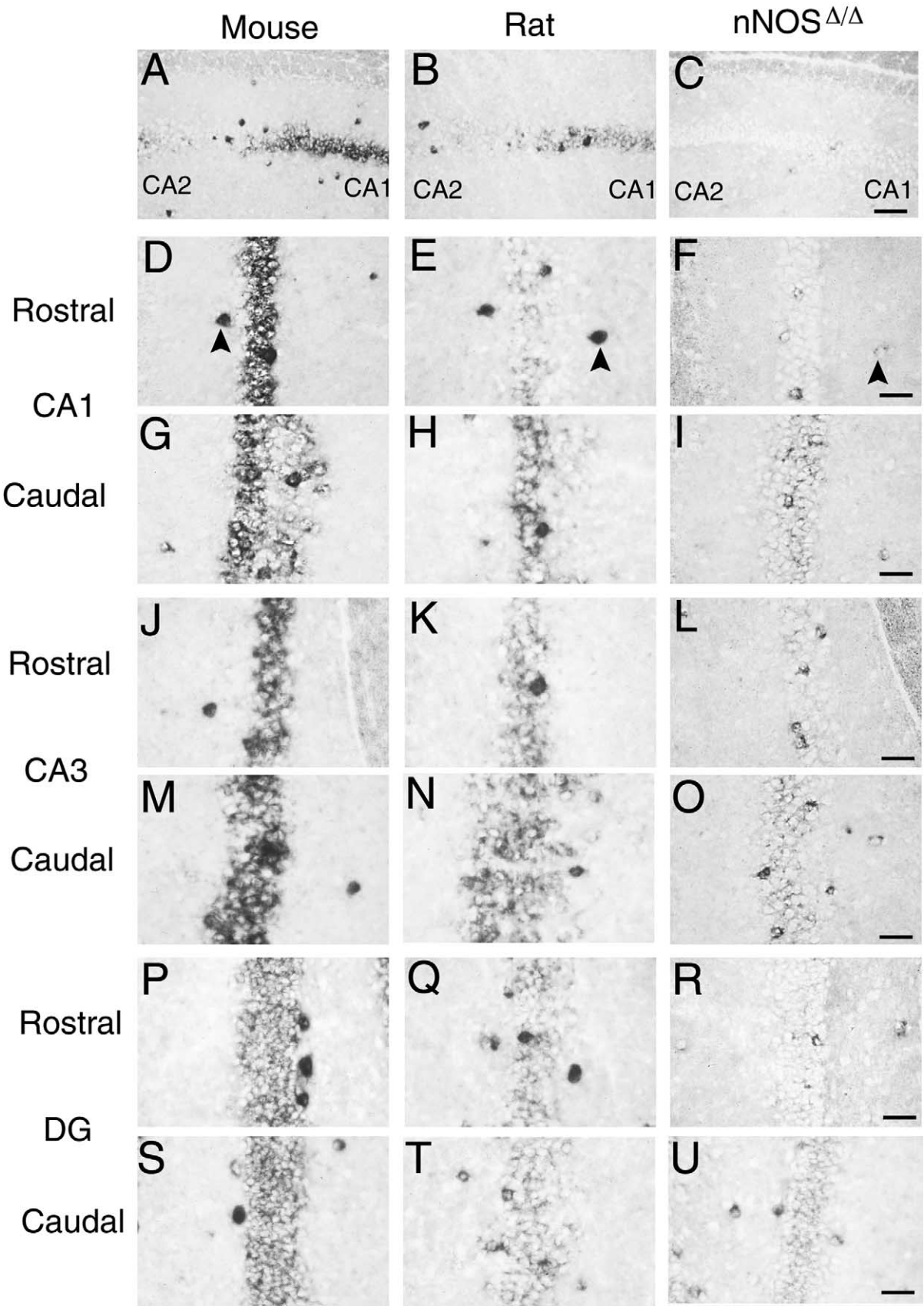


Fig. 1. Hippocampal nNOS mRNA levels substantially greater in mouse than rat. A pronounced rostro-caudal gradient is observed in CA1 and CA3 pyramidal cells, but not in dentate gyrus. Coronal sections are shown. A probe matching the C-terminus of rat nNOS is used, and detects nNOSβγ in nNOS$^{\Delta/\Delta}$ mice. nNOS expression in rostral hippocampus is shown for C57B/6 (A), nNOS$^{\Delta/\Delta}$ mice (B), and S.D. rats (C). Medial hippocampus is shown for C57B/6 (D), nNOS$^{\Delta/\Delta}$ (E), rat (F), while caudal hippocampus is shown for C57B/6 (G), nNOS$^{\Delta/\Delta}$ mice (H), and S.D. rats (I). Sense controls reveal no signal in either C57B/6 (J), nNOS$^{\Delta/\Delta}$ (K), or S.D. rats (L). Scale bars=250 μm for 1A, B, D, E, G, H, K, and 500 μm for 1C, F, I, L.

power magnification shows nNOS mRNA localized to perikarya of pyramidal cells in CA1 and CA3 layers (Fig. 2D, G, J, M).

In comparing mouse strains routinely employed for gene-knockout studies, pronounced differences are evident in expression of nNOS mRNA in CA1 pyramidal cells, with much higher expression in C57/B6 than in 129/SV mice (Fig. 3G, H). Interestingly, nNOS expression in eNOS$^{\Delta/\Delta}$ hippocampus, which is a mixture of the two strains, is intermediate between levels of wild type C57/B6 and 129/SV (data not shown). Immunohistochemical investigation shows similar patterns for nNOS protein with higher expression in C57/B6 than SV/129 mice (Fig. 3D, E). In CA3 and the dentate gyrus as well as the rest of the brain nNOS protein and mRNA expression is essentially the same in the two mouse strains.


Mouse
Rat
nNOS Δ/Δ
A
B
C
CA2
CA1
CA2
CA1
CA2
CA1
Rostral
CA1
D
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F
Caudal
G
H
I
Rostral
CA3
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Fig. 2.

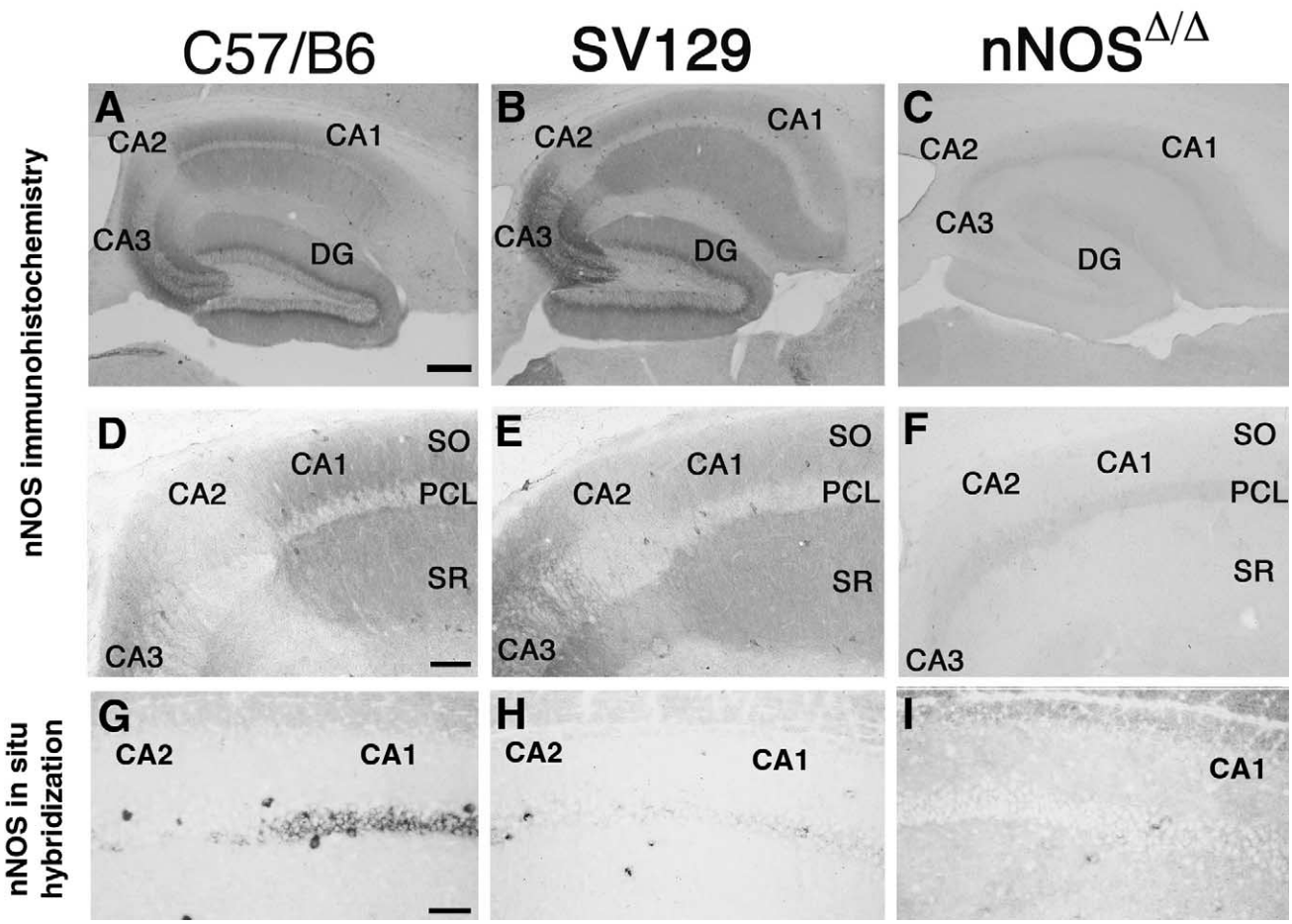

Fig. 3. nNOS protein expression is greater in CA1 pyramidal cells of C57/B6 than SV129 mice. nNOS immunoreactivity is found in dendrites of CA1 pyramidal cells in both C57/B6 (A, D) and SV129 (B, E) mice. nNOS protein is expressed at higher levels in C57/B6 than in SV129, and is seen in both stratum oriens (SO) and stratum radiatum (SR). Since the epitope used to generate the nNOS antibody is in exon 2, which is missing in nNOS$\beta\gamma$, no protein is observed in nNOS$^{\Delta/\Delta}$ mice (C, F). *In situ* hybridization of rostral CA1–CA2 regions shows dramatically higher levels of nNOS mRNA in CA1 pyramidal cells of C57B/6 mice (G) than in SV129 mice (H). Low levels of mRNA corresponding to nNOS$\beta\gamma$ are found in nNOS$^{\Delta/\Delta}$ mice (I). Scale bars=200 μm for A–C, and 50 μm for D–I.

nNOS mRNA levels are much lower in adult rat than mouse hippocampus (Figs. 1 and 2), whereas in the rest of the brain (data not shown) we find no difference in nNOS mRNA expression between rat and mouse. We observe rostro-caudal gradients in expression of nNOS in CA1 and CA3 in rat with substantially higher expression in caudal than in rostral areas (Fig. 2G, H, K, N; Table 1). We also observed strong rostro-caudal gradients in SV129 mice (Table 1) and, to a much lesser extent, in C57B/6 mice (Fig. 2D, G, J, M; Table 1). Rostro-caudal gradients of expression are seen in DG, as well as CA1 and CA3, for nNOS$\beta\gamma$ isoforms in nNOS$^{\Delta/\Delta}$ animals (Fig. 2F, I, L, O, R, U; Table 1). High-power magnification reveals expression of nNOS in caudal, but not rostral, rat CAl pyramidal cells (Fig. 2E, H). In nNOS$^{\Delta/\Delta}$ mice expression of nNOS mRNA is greatly reduced, but there is some residual expression, especially in CA3 (Fig. 2L, O). Presumably, this reflects persistence of alternatively spliced forms of nNOS that are not deleted in nNOS$^{\Delta/\Delta}$ animals (Eliasson et al., 1997). Sense controls show negligible staining both in mice and rats (Fig. 1J, K, L), ensuring the specificity of the procedure. High-power magnification reveals the nNOS expression in rat CA1 to be primarily in small interneurons, as previously observed

Fig. 2. High-power view of the rostro-caudal gradient in nNOS expression. nNOS expression is very low in rostral CA1 pyramidal cells in the rat, but is much more pronounced in caudal CA1. Strong expression is seen in interneurons of both rat and mouse (arrows). Residual nNOS$\beta\gamma$ expression is also seen in caudal CA1 and CA3 of nNOSΔ/Δ mice, as well as in interneurons. nNOS expression at the CA1–CA2 boundary of rostral hippocampus is shown for C57B/6 (A), S.D. rats (B), and nNOSΔ/Δ mice (C). Higher-power pictures are shown of nNOS expression in rostral (D–F) and caudal (G–I) CA1, rostral (J–L) and caudal (M–O) CA3, and rostral (P–R) and caudal (S–U) dentate gyrus. Scale bars=80 μm for A–C, and 40 μm for D–U. The orientation of the sections in D–U has been rotated 90° relative to A–C.

Table 1. Quantification of nNOS mRNA levels in rodent hippocampal neurons

	CA1		CA3		DG	
	Rostral	Caudal	Rostral	Caudal	Rostral	Caudal
C57/B6	8.76±0.37	9.78±0.29	6.86±0.32	10.64±0.46	4.52±0.23	6.42±0.26
SV129	1.79±0.17	4.35±0.26	6.38±0.43	6.62±0.39	1.92±0.18	2.11±0.21
$nNOS^{\Delta/\Delta}$	0.98±0.10	1.30±0.10	0.89±0.09	1.96±0.16	0.81±0.09	1.13±0.14
$eNOS^{\Delta/\Delta}$	2.92±0.20	5.07±0.26	5.98±0.40	6.38±0.35	2.26±0.23	1.92±0.23
Adult rat	2.85±0.16	5.74±0.46	5.47±0.27	3.85±0.84	3.73±0.23	4.48±0.47
Neonatal rat	6.89±0.44	6.43±0.48	7.23±0.46	7.73±0.51	0.82±0.11	1.04±0.14
Mouse sense control	0.69±0.07	0.37±0.08	0.52±0.93	0.56±0.10	0.38±0.07	0.57±0.10

This shows the average number of silver grains per cell in pyramidal cells of CA1 and CA3, along with granule cells of the dentate gyrus. A probe matching the C-terminus of rat nNOS is used. The standard error of the mean is shown. Sections from between three to six different individual wildtype and transgenic animals are considered for each species, strain and region shown. Between six and ten sections per individual are considered.

both for nNOS mRNA and nNOS protein (Fig. 2E, H; Bredt et al., 1991; Lin and Totterdell, 1998; Lumme et al., 2000). Table 1 summarizes overall level of nNOS mRNA per cell in the pyramidal neurons of CA1 and CA3, and the granule cells of the dentate gyrus in both rats and mice. No obvious inter-individual variation in hippocampal nNOS expression was observed.

In contrast to the negligible expression of nNOS in rostral hippocampal pyramidal cells of adult rat, neonatal rat displays substantial levels of nNOS mRNA which are most prominent in the CA1 pyramidal layer, with lesser levels in CA3 and very low levels in the dentate gyrus (Fig. 4). Close examination reveals the nNOS of neonatal hippocampus localized to pyramidal cells in marked contrast to the adult rat where it is exclusively in interneurons. In the rostral hippocampus of adult rats nNOS is exclusively in interneurons, while in the caudal hippocampus a notable minority of pyramidal cells also displays nNOS.

All of the above studies have employed a digoxigenin riboprobe corresponding to the C-terminal portion of rat nNOS, which is about 95% identical in sequence to mouse nNOS. However, to ensure specificity of the procedure, we have conducted some studies with a mouse riboprobe of similar length and corresponding to the N-terminus of nNOS. We observe essentially the same patterns both in mouse and rat as were evident with the rat probe (data not shown).

eNOS is localized to blood vessels of rat and mouse brain

Several studies have implicated eNOS in hippocampal LTP suggesting that eNOS ought to be localized to neurons, consistent with early reports of eNOS in hippocampal pyramidal cells though other studies revealed eNOS only in brain blood vessels. To clarify these questions, we have conducted high-stringency *in situ* hybridization of eNOS in mouse and rat brain (Fig. 5). The distribution and levels of eNOS are similar in rat and mouse with punctate patterns that at high power exclusively reflect blood vessels. There is no reduction of eNOS mRNA in $nNOS^{\Delta/\Delta}$ mouse brain, while expression is virtually abolished in $eNOS^{\Delta/\Delta}$ brains. eNOS expression is greater in the stratum radiatum than the stratum oriens both in rat and mouse (Fig. 5D, E). This presumably reflects a substantially higher density of blood vessels in stratum radiatum than stratum oriens. The absence of staining in sense controls ensures the specificity of the procedure.

NMDA3 stimulation activates hippocampal NOS

Much of the theoretical underpinning for a retrograde messenger role for NO in hippocampal pyramidal cells depends on the notion that NMDA-dependent LTP stimulates the formation of NO in pyramidal cells. Heretofore, there has been no direct demonstration that NO is formed by pyramidal cells, especially in response to NMDA receptor activation. Accordingly, we microinjected NMDA into the pyramidal cell layer of CA1 of mouse hippocampus (Fig. 6). We monitored NOS activity by immunohistochemical staining for citrulline, which is formed by NOS stoichiometrically with NO. Under basal conditions with saline injection, negligible staining of citrulline is evident (Fig. 6E). NMDA administration elicits pronounced staining of citrulline with the greatest intensity in the pyramidal cells extending into the apical dendrites of the stratum radiatum as well as the basal dendrites of the stratum oriens (Fig. 6A, B).

To ascertain which type of NOS is activated by NMDA, we employed knockout mice. Citrulline staining is abolished in $nNOS^{\Delta/\Delta}$ animals treated with NMDA (Fig. 6C), while it persists in $eNOS^{\Delta/\Delta}$ animals (Fig. 6D). Though low levels of nNOS$\beta\gamma$ mRNA persist in $nNOS^{\Delta/\Delta}$ hippocampus (Figs. 1B, E, H; 2F, I, L, O, R, U) these isoforms lack a PDZ domain and are not activated in response to NMDA receptor stimulation (Brenman et al., 1996).

NO can also be formed by iNOS. We have failed to detect iNOS expression by *in situ* hybridization either in wild type animals or after extensive stimulation by various perturbations including maximal electroconvulsive shock (data not shown). Also, we observe no expression of iNOS mRNA at various time points following focal ischemia induced by middle cerebral artery ligation. iNOS is formed by microglia in the brain. To ascertain whether microglia are activated by the NMDA injection, we stained for granulocyte macrophage-colony stimulating factor but did not detect any notable activation compared with uninjected controls (data not shown; Fig. 6F).

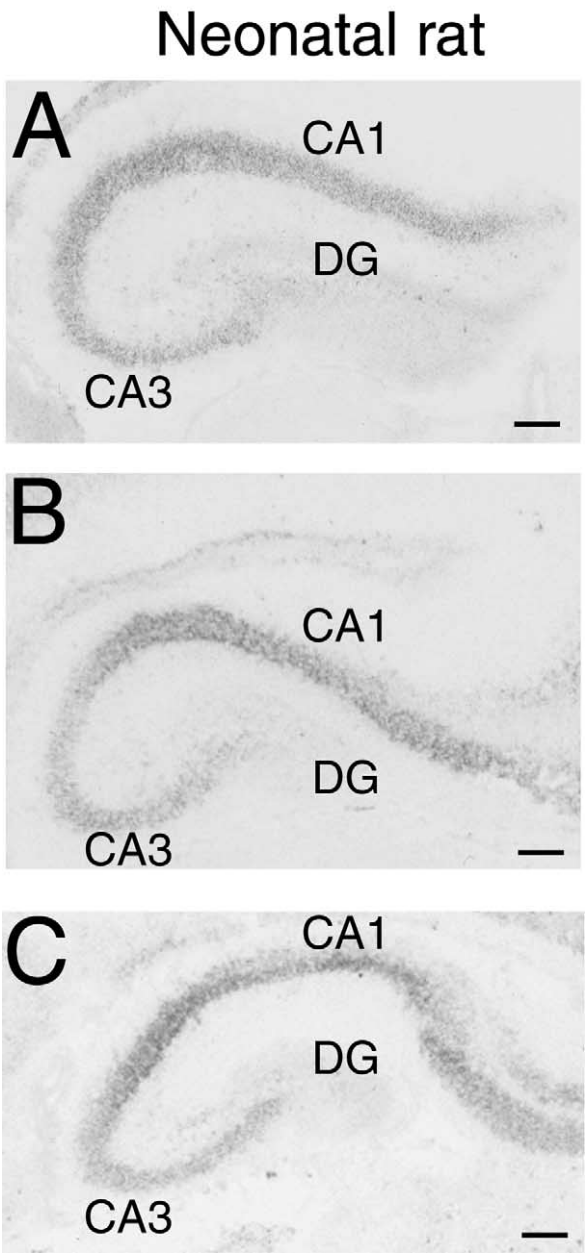

Fig. 4. nNOS expression in neonatal rat hippocampus. Low-power views of postnatal day 2 hippocampus are shown. Rostral (A), medial (B), and caudal (C) coronal sections of hippocampus are shown. The rostro-caudal gradient of nNOS mRNA seen in the adult CA1 and CA3 is not observed here. Scale bars=90 μm.

nNOS is expressed in human hippocampus

While rodent LTP is presumed to model human learning and memory, because of the pronounced species variations in NOS expression, we have also examined adult human hippocampus (Fig. 7). We detect substantial expression of nNOS mRNA in CA1, CA3 and the dentate gyrus (Fig. 7A). The specificity of this hybridization is demonstrated by the absence of signal in sense probe controls (Fig. 7B). High-power magnification reveals nNOS of CA1 and CA3 predominantly in pyramidal cells but with expression also in a limited number of interneurons (Fig. 7D, E). In the dentate gyrus nNOS mRNA is restricted to granule cells (Fig. 7C). eNOS expression in human occurs exclusively in blood vessels in CA1, CA3, CA4 and the dentate gyrus (Fig. 7F–H). Examination of multiple sections fails to reveal any expression of eNOS in human hippocampal neurons.

DISCUSSION

A major finding of our study is that nNOS expression in CA1 pyramidal cells varies markedly in several dimensions: spatially, in different species, strains and as a function of development. The differences we have observed may explain many discrepant observations regarding NO-LTP (Son et al., 1996). For instance, NO-LTP is not reproducibly demonstrable in the adult rat, whereas robust NO-LTP is evident in adult mouse hippocampus. It has been suggested that variations in temperature, slice preparation and stimulus protocol account for variable results among different laboratories (Holscher, 1997). By contrast, in mice NO-LTP is reproducibly demonstrable (O'Dell et al., 1994; Son et al., 1996). We suggest that difficulties in demonstrating NO-LTP in adult rat result in part from its very low expression in adult rat CA1 pyramidal cells. Moreover, the higher level of nNOS in caudal than rostral areas and the pyramidal cell localization in caudal but not rostral areas suggest that greater success in observing NO-LTP might come with use of more caudal preparations.

It is striking that the pronounced differences between rat and mouse in hippocampal nNOS expression are restricted to the hippocampus. In an extensive evaluation of numerous other brain areas, we find no differences in the localization and intensity of expression of nNOS between mouse and rat.

Some groups (Williams et al., 1993; Son et al., 1996) found NO-LTP much more readily in neonatal rather than adult rat hippocampus. Our observations that nNOS is abundant in pyramidal cells of the neonatal but not adult rat hippocampus can account for these findings.

We observe substantially higher expression of nNOS in C57/B6 than SV129 mice in CA1 of the hippocampus. In the rest of the hippocampus and the rest of the brain, nNOS expression is similar in both mouse strains. These differences may have considerable bearing on interpretation of LTP alterations in NOS knockouts, as most gene knockouts are constructed from hybrids of C57/B6 and SV129 mice. Interestingly, we found nNOS expression in $eNOS^{\Delta/\Delta}$ animals to be substantially greater than in the SV129 parent strain. Had we not also examined the C57/B6 parent strain, we might have concluded that nNOS was up-regulated in $eNOS^{\Delta/\Delta}$ animals as some sort of compensatory mechanism.

Differences in learning and memory between C57/B6 and SV129 mice have been well documented; for instance, spatial memory seems to be less efficient in SV129 than C57/B6 animals though NO-LTP does not vary markedly between the strains (Son et al., 1996). Even though nNOS expression is less in SV129 than C57/B6 animals, there

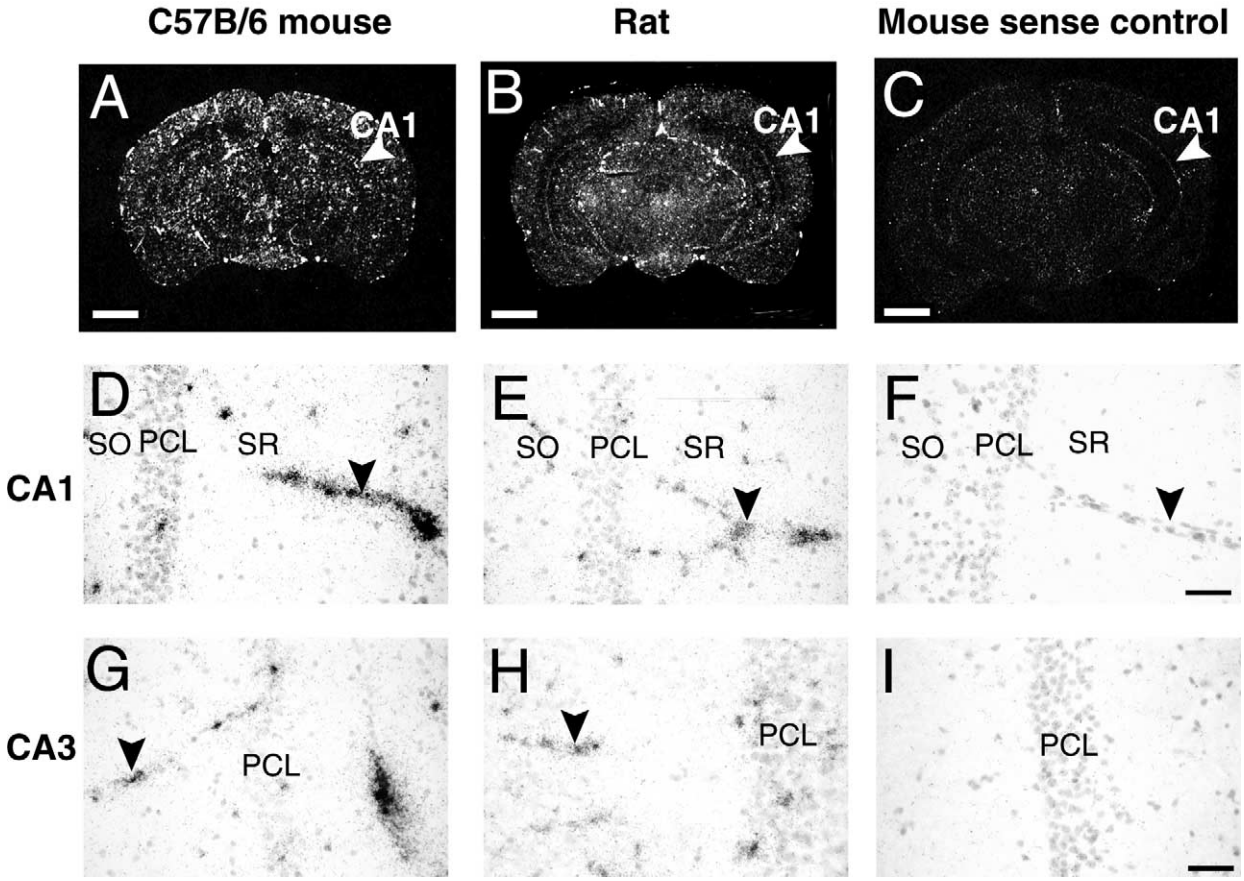

Fig. 5. eNOS mRNA expression is restricted to blood vessels. A probe derived from the mouse eNOS cDNA is used. Arrowheads indicate select cerebral blood vessels. A low-power, dark-field view of ^{33}P *in situ* hybridization is shown for C57B/6 mouse (A), or S.D. rat (B). Sense control probes (C) produce no specific signal. No neuronal expression of eNOS is observed in CA1 or anywhere else in any of the samples tested. High-power autoradiographic emulsion views of ^{33}P *in situ* hybridization show that eNOS mRNA is expressed only in endothelial cells of CA1 and CA3. Prominent endothelial signal is seen in CA1 of both mouse (D) and rat CA1 (E), and mouse (G) and rat (H) CA3. Sense control probes show no signal in either CA1 (F) or CA3 (I). SO, stratum oriens; SR, stratum radiatum; PCL, pyramidal cell layer. Scale bars=1 mm for A, C, 2 mm for B and 25 μm for D–I.

still is abundant NOS activity in SV129 hippocampus. Thus, in SV129 mice receiving NMDA injections into the hippocampus, we observe robust stimulation of staining.

Several studies have compared the relative contributions of nNOS and eNOS to LTP. The persistence of NO-LTP in nNOS$^{\Delta/\Delta}$ animals might be attributable to the persistence of alternatively spliced forms of nNOS. These alternatively spliced forms, nNOSβ and nNOSγ, continue to be expressed in a variety of brain regions (Eliasson et al., 1997). In the present study we have demonstrated the persistence of nNOS expression in pyramidal cells of the caudal hippocampus of nNOS$^{\Delta/\Delta}$ animals as well as in hippocampal interneurons.

The persistence of NO-LTP in nNOS$^{\Delta/\Delta}$ animals might also be attributable to activity of eNOS. For instance, Schumann and collaborators (Kantor et al., 1996) obtained evidence that eNOS is involved in NO-LTP, because a drug that inhibits myristoylation interferes with NO-LTP, and myristoylation is crucial for eNOS activity. Moreover, transfection with myristoylation-deficient mutants of eNOS interferes with NO-LTP. Kandel and associates (Son et al., 1996) found decrements in at least one form of LTP in double knockouts of eNOS and nNOS, whereas the individual gene knockout mice manifested normal LTP. It has been assumed that the NO which mediates LTP is formed in neurons, and there have been reports of eNOS protein identified by immunohistochemistry in hippocampal pyramidal cells (Dinerman et al., 1994; Doyle and Slater, 1997). However, subsequent reports have identified eNOS protein only in blood vessels in the brain (Seidel et al., 1997; Stanarius et al., 1997; Topel et al., 1998). Utilizing a highly sensitive ^{33}P *in situ* hybridization analysis, we also find eNOS mRNA localized exclusively to blood vessels in the hippocampus in all species examined. This suggests that NO formed by eNOS in hippocampal blood vessels diffuses into neuronal parenchyma to influence LTP. As vascular endothelium in the brain is abundantly innervated (Kobayashi et al., 1985; Wahl and Schilling, 1993), neuronal activity may well stimulate eNOS to generate neuronally active NO (Lovick et al., 1999). Evidence for neuronal functionality of eNOS comes from recent studies showing behavioral alterations and altered

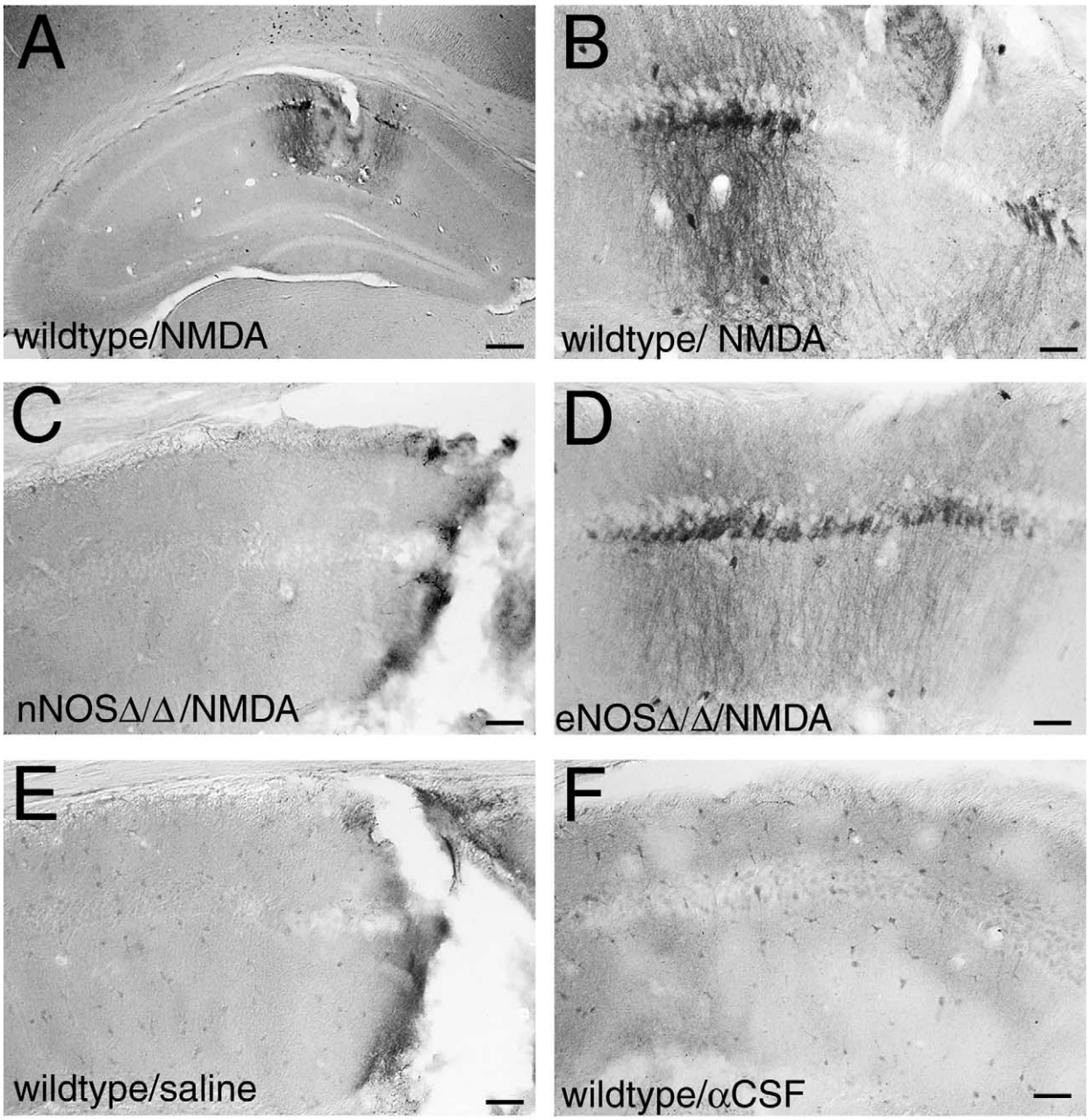

Fig. 6. NMDA-dependent activation of nNOS in dendrites of CA1 pyramidal neurons. Injection of NMDA into CA1 of C57/B6 mice produces high levels of citrulline, shown in (A) at low power and (B) at high power, indicative of NO production from arginine, in cell bodies and dendrites of CA1 pyramidal neurons. This is not observed in $nNOS^{\Delta/\Delta}$ mice (C) but is seen in $eNOS^{\Delta/\Delta}$ mice (D). Saline injection results in no citrulline production in CA1 (E). Low levels of granulocyte macrophage-colony stimulating factor immunoreactivity in hippocampal microglia are seen in wild type mice, but these levels do not increase substantially following saline injection, and do not differ in $nNOS^{\Delta/\Delta}$ or $eNOS^{\Delta/\Delta}$ mice (F). SO, stratum oriens; SR, stratum radiatum; PCL, pyramidal cell layer; DG, dentate gyrus. Scale bars = 180 μm for A and 60 μm for B–F.

LTP in $eNOS^{\Delta/\Delta}$ mice (Demas et al., 1999; Haul et al., 1999; Wilson et al., 1999; Frisch et al., 2000; Doreulee et al., 2001).

Many studies of NO-LTP have utilized adult rat hippocampus, which displays substantially lower CA1 pyramidal cell nNOS than mouse. Since the rationale for much of this research is clarification of learning and memory in humans, we explored nNOS expression in human hippocampus. The levels and localization of nNOS in the human more closely resemble those of mice than rat, suggesting that one can extrapolate from studies of NO-LTP in mouse to human and emphasizing the likely importance for human synaptic plasticity of hippocampal NO systems. Mapping of various protein markers is routinely

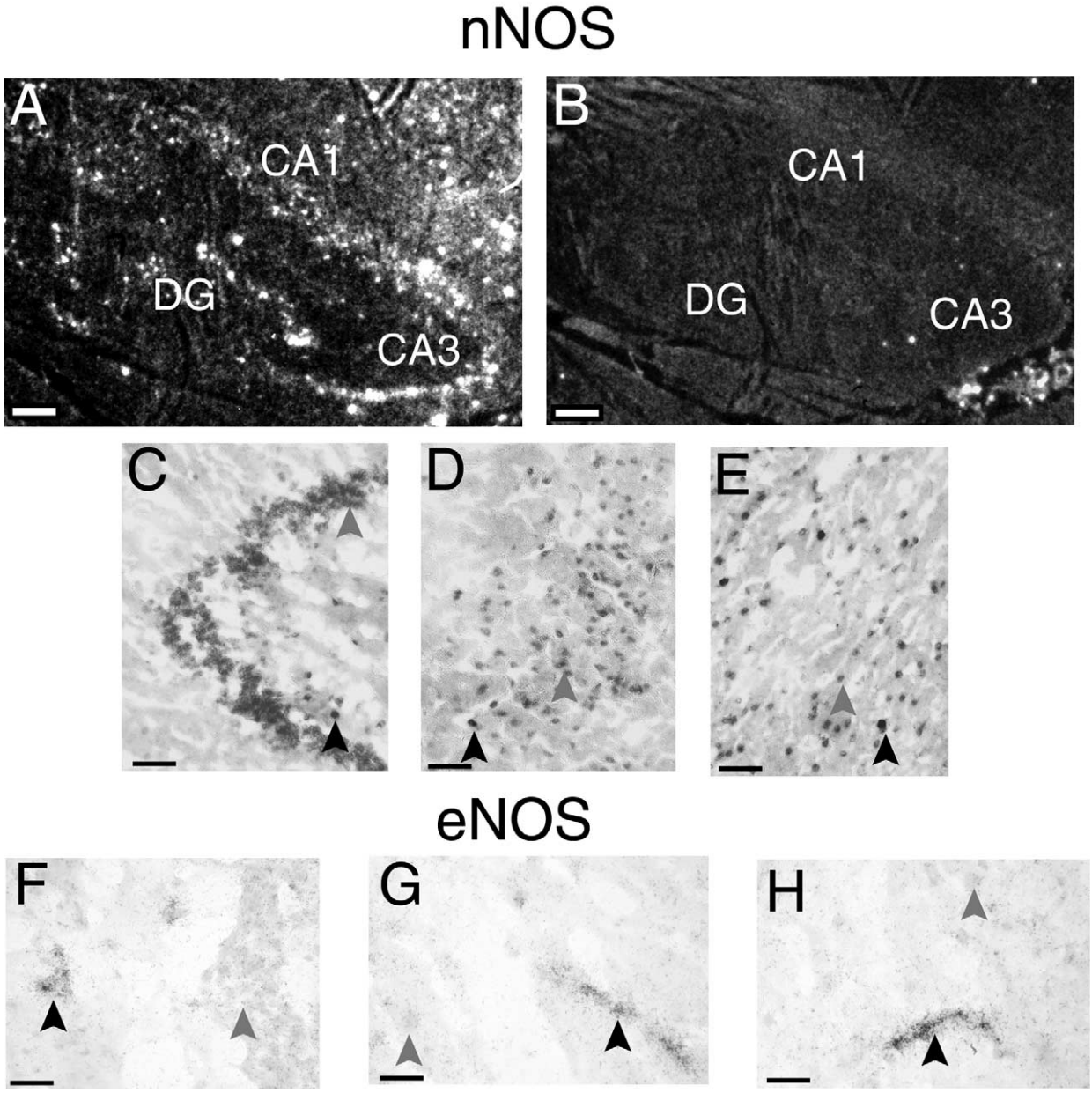

Fig. 7. nNOS and eNOS expression in human hippocampus. Low-power ^{33}P *in situ* hybridization for nNOS demonstrates signal in pyramidal cells of CA1 and CA3 and dentate gyrus (A). Sense control probe shows no signal (B). High-power digoxigenin *in situ* hybridization indicates expression in granule cells of the dentate gyrus (C) and pyramidal cells of CA1 (D) and CA3 (E). As in the rodent, high levels of expression are seen in hippocampal interneurons (black arrowheads). High-power emulsion ^{33}P *in situ* hybridization of eNOS indicates expression in endothelial cells (black arrowheads) but not in neurons (gray arrowheads) of DG/CA4 (F), CA3 (G) or CA1 (H). Scale bars=200 μm for A, B and 50 μm for C–F.

performed in the rodent brain, but our study illustrates the importance of closely examining species and strain variations that correspond to those employed for physiologic experiments.

In summary, our findings may clarify a number of controversies regarding the role of NO in hippocampal LTP. Differences in the abilities of various laboratories to reproduce these findings have raised much confusion about the nature and role of NO-LTP. Our findings imply that NO-LTP is a valid phenomenon whose expression correlates with the extent of nNOS expression.

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