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A role for Dimethylarginine Dimethylaminohydrolase 1 (DDAH1) in mammalian development

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ABSTRACT Nitric oxide has been linked to a number of embryonic processes, yet the role of nitric oxide signalling in development remains largely unknown. Dimethylarginine Dimethylaminohydrolase 1 and 2 (DDAH1/2) catalyse the breakdown of asymmetric dimethylarginine, an endogenous inhibitor of nitric oxide production, and may also have nitric oxide-independent functions. We have generated transgenic mice targeting the DDAH1 and DDAH2 loci. Here we report that homozygous DDAH1 null embryos are generated at low frequency, and do not progress through embryonic development. During normal development *DDAH1* RNA is expressed in the left ventricle, cardiac outflow tract and developing vasculature. In contrast, DDAH2 homozygous null mice are viable and fertile, with a normal lifespan. *DDAH2* expression is seen in the developing left ventricle and cardiac outflow tract, and additionally in the peripheral nervous system. Both DDAH1 and 2 are expressed in the developing limb buds in patterns overlapping areas with high nitric oxide synthase activity. These expression patterns implicate DDAH1 and DDAH2 in embryonic development, possibly through specific effects on nitric oxide pathways.

KEY WORDS: *DDAH, nitric oxide, embryonic development, mouse, transgenic*

Introduction

Dimethylarginine Dimethylaminohydrolase (DDAH) modulates nitric oxide signalling by catalysing the hydrolysis of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of all three isoforms of nitric oxide synthase (Leiper, 2005). It is, however, becoming apparent that DDAH may also have roles independent of nitric oxide signalling, perhaps through association NF1 (Tokuo *et al*., 2001) or Sp1 (Hasegawa *et al*., 2006).

In mammals, there are two isoforms of DDAH, expressed in most human and murine fetal tissues (Tran *et al*., 2000; Tran *et al*., 2003). The role of DDAH in development is largely unknown, but rat DDAH1 RNA has been shown to be developmentally regulated (Mishima *et al*., 2004). Nitric oxide is generated at high levels during implantation of the blastula (Saxena *et al*., 2000). Angiogenesis and vasodilatation in the developing placenta are also postulated to be nitric oxide dependent (Lyall, 2003). Embryonic implantation and development of the placental vasculature are disrupted by experimental manipulation of nitric oxide levels (Lyall, 2003). Despite these observations, deletion of all three nitric oxide synthase isoforms does not appear to have a major impact on fetal development (Morishita *et al*., 2005).

The aim of the present study was to determine whether DDAH is important in normal fetal development. We have generated null alleles for mouse DDAH1 and 2. Here we report that heterozygote DDAH1$^{wt/del}$ embryos and pups are macroscopically indistinguishable from wild-type embryos while adult mice exhibit endothelial dysfunction and pulmonary hypertension (Leiper *et al*., 2007). Early homozygous DDAH1$^{del/del}$ embryos are generated at very low frequencies, and fail to progress through early embryonic development. DDAH1 protein is expressed in the embryonic trophoblast, but not in the early embryo. In contrast, DDAH2 null

Abbreviations used in this paper: ADMA, asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase; NO, nitric oxide.

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mice exhibit are viable and fertile apparently with no abnormal phenotype. DDAH1 and DDAH2 RNA are expressed in dynamic, overlapping but distinct patterns throughout embryonic development, partially overlapping with NF1 RNA expression. We hypothesise that DDAH plays a role in embryonic development, which may in part be nitric oxide dependent.

Results

Heterozygote DDAH1$^{(wt/del)}$ embryos are morphologically indistinguishable from wild-type embryos

Heterozygous DDAH1$^{(wt/del)}$ embryos express DDAH1 RNA and protein at approximately 50% of wild-type levels, while circulating plasma ADMA levels of heterozygotes are ~20% higher than those of wild-type mice. Heterozygotes are morphologically indistinguishable from wild-type littermates. This has been reported in detail elsewhere (Leiper *et al*., 2007).

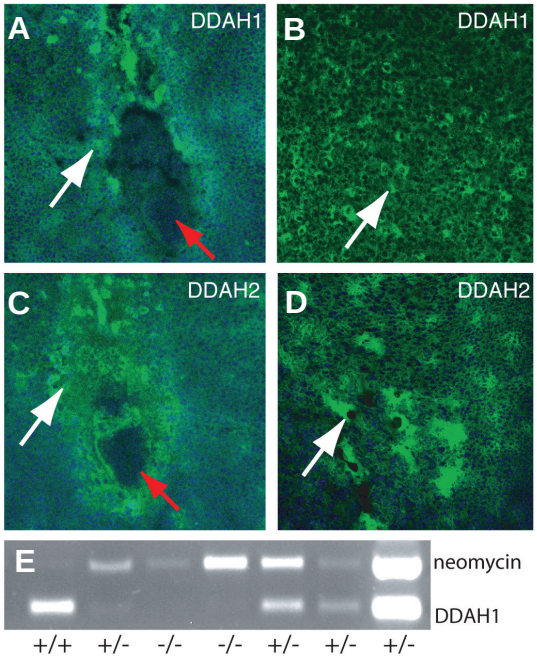
Homozygote DDAH2 $^{(del/del)}$ mice exhibit normal development and survival

We have also generated mice carrying an inactivated DDAH2 locus. These mice have reduced DDAH2 expression at both the RNA and protein levels (not shown) in all tissues studied. Breeding of these mice indicates that both heterozygous and homozygous inactivation of the DDAH2 locus does not impact on embryonic survival, with wild type, heterozygous and null mice produced in Mendelian ratios. 21 litters were collected from heterozygote DDAH2$^{(wt/del)}$ parents, 176 mice in all. 41 (23.3%) were found to be DDAH2$^{(wt/wt)}$, 95 (54.0%) DDAH2$^{(del/wt)}$and 40 (22.7%) DDAH2$^{(del/del)}$. A detailed characterisation of these mice will be presented elsewhere.

Reduction of DDAH1 expression levels affects early embryonic development at several points

Thirty-four DDAH1$^{(wt/del)}$/ DDAH1$^{(wt/del)}$ crosses were carried out. We observed that approximately 33% of the resulting pups were wild-type DDAH1$^{(wt/wt)}$ and 66% heterozygote DDAH1$^{(wt/del)}$ (72 vs. 166, 238 pups scored). Genotyping of embryonic day 2 (e2) blastocysts obtained from DDAH1$^{(wt/del)}$/ DDAH1$^{(wt/del)}$ crosses indicated that homozygous embryos were present, albeit at low frequency ~5% (4 out of 79 scored). See Fig. 1 for representative genotyping results.

We observed significant numbers of resorbing embryos at e15 in DDAH1$^{(wt/del)}$ female/ DDAH1$^{(wt/wt)}$ male crosses (21 resorbing



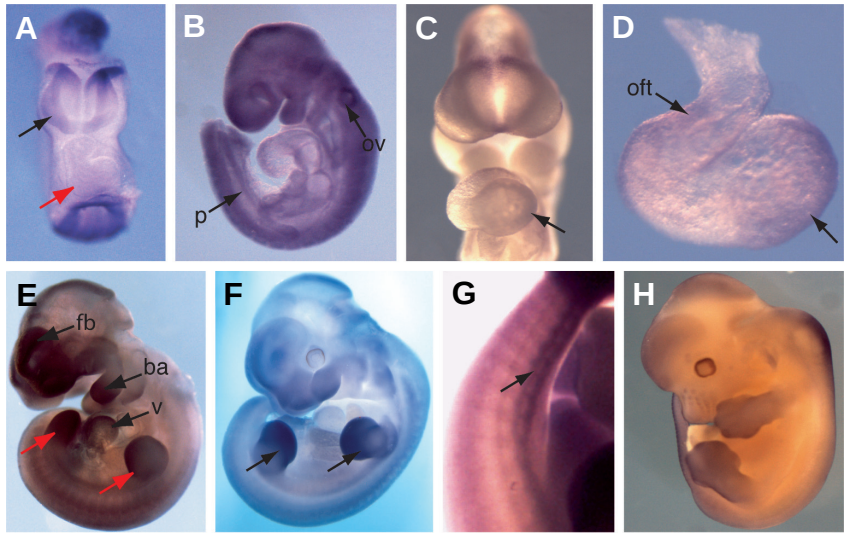


Fig. 1 (Left). Immunohistochemistry of 6-day pregnant mouse cryostat sectioned uterus with polyclonal anti-DDAH serum. (A) *Immunofluorescence of cryosectioned e6.5 embryo and placenta for DDAH1 (green), showing anti-DDAH1 activity in the trophoblast (white arrow), with absent activity in the implanted embryo (red arrow) (blue stain is nuclear DAPI).* **(C)** *Anti-DDAH2 immunofluorescence in e6.5 embryo and uterus (white arrow) showing higher levels of anti DDAH2 activity surrounding the embryo (embryo marked by red arrow). Note higher levels of embryonic DDAH2 than DDAH1 in (A).* **(B)** *Anti-DDAH1 immunofluorescence throughout the 6.5-day pregnant mouse placenta (arrowed), in contrast to anti-DDAH2 immunofluorescence activity, which is associated with developing blood vessels (D, arrowed).* **(E)** *Representative agarose gel of blastocyst genotyping by PCR of Neomycin and DDAH-1 amplicons. Blastocycts collected at day 2-post fertilization from a heterozygous DDAH-1 null female crossed to a heterozygous null DDAH-1 male. Genotypes indicated are "+/-"= DDAH-1 heterozygous null, "+/+"= wild-type, "-/-"= DDAH-1 homozygous null.*

Fig. 2 (Right). *In situ* hybridisation to DDAH1 antisense RNA, revealing expression pattern of DDAH1 RNA during embryonic development. (A) *e8.5 embryo, showing strong DDAH1 RNA expression in the neural folds (black arrow) but not the heart (red arrow).* **(B)** *Widespread DDAH1 RNA expression in e9.5 embryo (ov-otic vesicle, p-pronephros)* **(C)** *Anterior view of e9.5 embryo, showing DDAH1* in situ *hybridisation staining in the ventricle (arrowed).* **(D)** *Dissected e9.5 heart, showing DDAH1 RNA expression in the ventricle (arrowed) and outflow tract (oft).* **(E)** *e10.5 embryo, showing DDAH1 RNA expression in the fore-brain (fb), cardiac ventricle (v), branchial arches (ba) and limb bud (red arrows).* **(F)** *Strong expression of DDAH1 RNA is seen in the limb of e12.5 embryo (arrowed).* **(G)** *High-power view of tail vasculature (arrowed) of e12.5 embryo.* **(H)** *e14.5 embryo, showing DDAH1 RNA staining in the vibrissae, brain and limbs.*

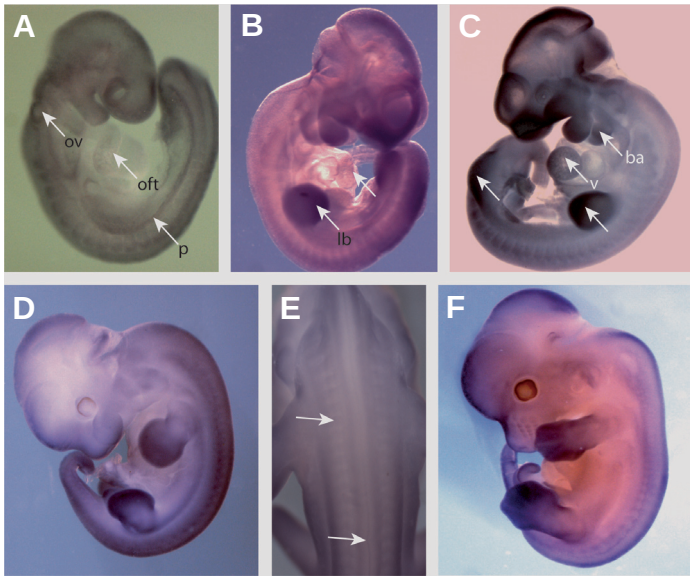


Fig. 3. *In situ* hybridisation to DDAH2 antisense RNA, revealing expression pattern of DDAH2 RNA during embryonic development. **(A)** *Expression of DDAH2 in e9.5 embryo, visible in the otic vesicle (ov), cardiac outflow tract (oft) and pronephros (p).* **(B,C)** *e10.5 embryo revealing expression in the limb buds (arrowed), branchial arches (ba), cardiac ventricle (v).* **(D,E)** *expression in e12.5 embryo, revealing strong limb bud expression, and peripheral nervous system staining (arrowed in the dorsal view of the embryo in E). In e14.5 embryo* **(F)** *expression is seen in the limbs, and peripheral nervous system.*

embryos out of a total of 73 embryos, 28.8%). In contrast, resorbing embryos were rare in DDAH1$^{(wt/wt)}$ female/ DDAH1$^{(wt/del)}$ male crosses (2 out of 42, 4.8%). The ratio of wild-type to heterozygote DDAH1$^{(wt/del)}$ pups was approximately 1:1 in both of these crosses however litters from DDAH1$^{(wt/del)}$ female/ DDAH1$^{(wt/wt)}$ male crosses were significantly smaller than from DDAH1$^{(wt/wt)}$ female/ DDAH1$^{(wt/del)}$ male crosses (6.53±0.6 vs. 10.42±0.7, p≤0.001 n=19). This implies a role for DDAH1 in utero-placental function. No overall subfertility was observed in either male or female DDAH1$^{(wt/del)}$ heterozygotes.

Immunohistochemistry of cryostat sectioned uteri from wild-type 6.5-day pregnant female (CBA/Bl10) mice using rabbit anti-mouse DDAH1 polyclonal serum revealed that DDAH1 is present in the trophoblast, but not the embryo itself (Fig. 1A). Expression of DDAH1 was evident throughout the uterus (Fig. 1B). Immunohistochemistry with anti-DDAH2 polyclonal serum revealed that DDAH2 protein is also present in the trophoblast (Fig. 1C), and is additionally associated with the developing placental vasculature (Fig. 1D).

DDAH isoforms are expressed in a dynamic, overlapping pattern during embryonic development

RNA *in situ* analysis of wild-type embryos with digoxigenin-labelled RNA antisense probes for DDAH1 and DDAH2 was carried out. DDAH1 RNA expression was observed in the developing forebrain of the e8.5 embryo, with weak expression visible in the heart (Fig. 2A). Cardiac expression of DDAH1 RNA was seen in the outer curvature of the cardiac ventricle and cardiac outflow tract at e9.5 (Fig. 2 C,D). Expression is visible in the forebrain, branchial arches and cardiac ventricle at e10.5 (Fig. 1E). At e12.5, the dorsal aortas and tail blood vessels were stained for DDAH1 RNA (Fig. 2 F,G). Strikingly, DDAH1 RNA expression was seen in the developing limb buds from e10.5 onwards (Fig. 2 E,F,H), concentrated in the distal regions of the limb at e12.5 (Fig. 2F), less pronounced by e14.5 (Fig. 2H).

In situ hybridisation of wild-type mouse embryos to digoxigenin-labelled DDAH2 antisense RNA probes revealed that DDAH2 RNA expression was present in the cardiac outflow tract, otic vesicle and pronephros at e9.5 (Fig. 3A). Expression in the left ventricle and cardiac outflow tract, branchial arches and limb buds was observed at e10.5 (Fig. 3 B,C). At e12.5, DDAH2 RNA expression was detected in the limb buds and peripheral nervous system (Fig. 3 D,E). This pattern was also visible at e14.5, albeit weakly (Fig. 3F).

Expression of NF1, a RASGTPase activating factor shown to coimmunoprecipitate with rat DDAH1 *in vitro* (Tokuo *et al.*, 2001), was also investigated. *In situ* hybridisation to a digoxigenin-labelled antisense NF1 RNA probe revealed overlapping expression with the two DDAH isoforms. At e9.5, NF1 was expressed in the branchial arches, early limb bud and otic placode (Fig. 4A). At e12.5, NF1 RNA was detectable in the limb bud and developing brain (Fig. 4 B,C), overlapping with DDAH1 and 2 RNA expression (Figs. 2 and 3).

NADPH diaphorase staining overlaps DDAH *RNA expression patterns*

NADPH-diaphorase staining relies on the fact that nitric oxide synthase isoforms are NADPH-diaphorases, and will thus hydrolyse nitro-blue-di-tetrazolium in the presence of NADPH, giving a chromogenic reaction (Weinberg *et al.*, 1996). NADPH-diaphorase staining of e9.5 embryos revealed nitric oxide synthase activity throughout the heart, and in the limb-bud, pronephros, branchial arches, otic placode and forebrain (Figs. 5 A,D). At e10.5 diaphorase staining was observed in the cardiac ventricle and outflow tract, limb bud, somites, branchial arches and brain (Fig. 5 B,E). By e11.5, strong staining was

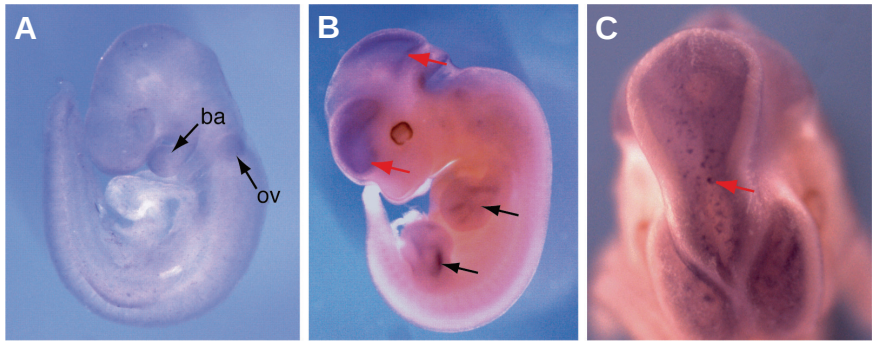


Fig. 4. Developmental expression profile of the RASGTPase *NF1* overlaps with *DDAH1* and *2*. *At e9.5* **(A)**, *expression of* NF1 *RNA is evident in the otic vesicle (ov) and branchial arches (ba).* **(B,C)** *At e12.5, expression is seen in the limb buds (black arrows in B) and brain (red arrows in B, C).*

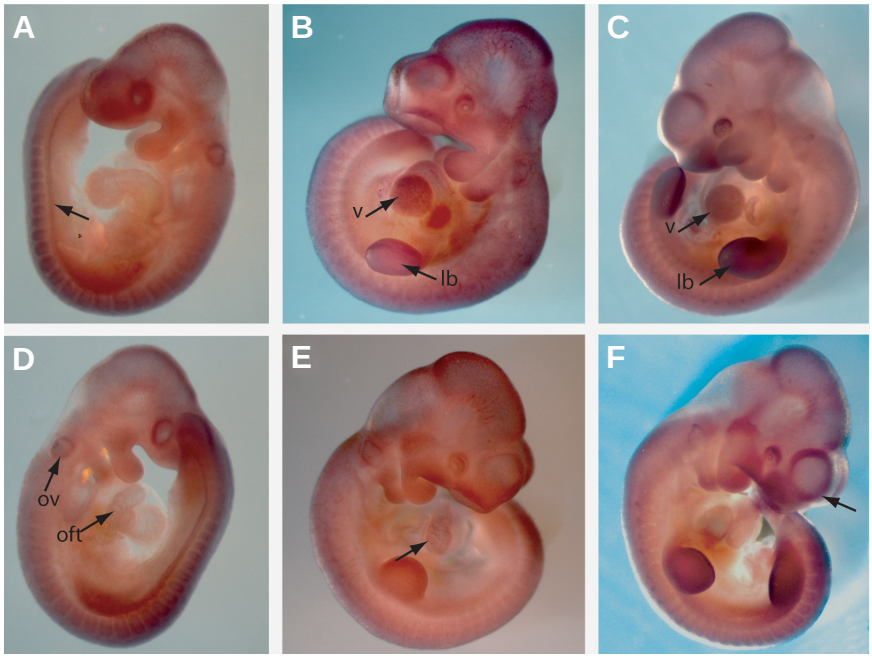


Fig. 5. NADPH diaphorase staining pattern of embryos, revealing nitric oxide synthase activity at e9.5 (A,D), e10.5 (B,E) and e12.5 (C,F). *Strong diaphorase activity is seen at e9.5 in the pronephros (A, arrowed), cardiac outflow tract (D, oft) and otic vesicle (D, ov). (B) Limb diaphorase activity is seen at e10.5 (lb), and staining is evident in the cardiac ventricle (v) and cardiac outflow tract (E, arrowed). At e12.5, strong activity is evident in the limb buds (C, lb), as well as cardiac ventricle (C, v) and forebrain (E, arrowed).*

observed in the developing limbs, left ventricle and forebrain (Fig. 5 C,F).

Discussion

In this study we report that DDAH1 null mouse embryos are generated at low efficiency, and fail to complete embryonic development. Embryonic DDAH1 and DDAH2 RNA expression patterns are dynamic and overlapping but distinct, with DDAH1 RNA expression detectable in the developing vasculature, and DDAH2 RNA in the embryonic peripheral nervous system. Nitric oxide synthase activity overlaps DDAH1 and 2 RNA expression. Interestingly, expression of NF-1 RNA, which encodes a protein reported to coimmunoprecipitate with DDAH1 in rat, overlaps in the developing limb and brain, consistent with a potential interaction of NF1 and DDAH in development.

Circumstantial evidence supports a role for nitric oxide in blastocyst implantation, in that nitric oxide synthase isoforms are expressed at high levels in the trophoblast (Gouge *et al*., 1998; Purcell *et al*., 1999; Gagioti *et al*., 2000; Saxena *et al*., 2000). Nitric oxide has been postulated to have several roles in embryonic implantation and placentation, for example as a phagocytic toxin in trophoblast giant cells (Gagioti *et al*., 2000) and an angiogenic signal in the developing placenta (Cooke and Losordo, 2002; Lyall, 2003). Administration of inhibitors of nitric oxide synthesis to *in vitro* assays of implantation and trophoblast outgrowth suggest that nitric oxide synthesis is tightly regulated during implantation (Sengoku *et al*., 2001). eNOS/iNOS double knockout mice produce fewer homozygous double null mice than expected, consistent with an implantation defect (Tranguch and Huet-Hudson, 2003). The triple eNOS/iNOS/nNOS null mouse has reduced fertility, but interestingly some triple null embryos are viable (Morishita *et al*., 2005). Thus the fertilisation and implantation defect we observe in DDAH1null blastocysts could be nitric oxide dependent, with a greater magnitude than predicted but the survival of the triple NOS knockout mouse seems to argue against this.

We found that DDAH1-null pre-implantation embryos are produced at very low frequency (5%) indicating that DDAH protein and/or activity is involved in fertilisation or the earliest stages of embryogenesis. The ratio of heterozygote to wild-type DDAH1 pre-implantation embryos was similar to that of viable pups, suggesting that heterozygous deletion of DDAH in the embryo does not have any effect on embryonic viability. However, we did observe significant embryonic resorption in DDAH1 $^{(wt/del)}$ females that was not seen in wild-type females. This observation suggests that elevated maternal ADMA (raised in DDAH1$^{(wt/del)}$ heterozygotes (Leiper *et al*., 2007)) may negatively impact embryonic viability post-implantation. Consistent with this suggestion, elevated plasma ADMA levels have been reported in human pre-eclampsia (Savvidou *et al*., 2003) and intrauterine growth retardation (Rutherford *et al*., 1995).

Even though DDAH1 and DDAH2 protein expression patterns in the placenta overlap (Fig. 1), we observed no implantation of DDAH1$^{(del/del)}$ blastocysts and no detectable phenotype in DDAH2$^{(del/del)}$ embryos and mice, implying no functional redundancy in the trophoblast between DDAH1 and DDAH2. However, the normal development of DDAH2$^{(del/del)}$ mice suggests that DDAH1 can compensate of the loss of DDAH2.

Expression patterns of DDAH1 and 2 RNA suggest roles in several developing organ systems. It has previously been shown at that *all-trans* retinoic acid, which has multiple roles in cardiac and neural development induces DDAH2 *in vitro* (Achan *et al*., 2002). We observe DDAH2 RNA expression in the developing heart and neural systems (Fig. 3). A role for nitric oxide signalling in neurogenesis has been suggested in *Xenopus laevis, drosophila* and chick (Peunova and Enikolopov, 1995; Kuzin *et al*., 1996; Peunova *et al*., 2001; Plachta *et al*., 2003). We observe expression of DDAH1 and 2 in the developing brains of mouse embryos in patterns overlapping NF1 RNA expression. Interestingly, these molecules continue to be expressed in overlapping patterns in the adult mouse brain (www.brainatlas.org/ala/). Use of inducible, organ specific Cre lines in conjunction with conditional alleles of DDAH isoforms and NF1, and generation of double conditional DDAH1 and 2 null mutants will allow investigation of potential nitric oxide independent functions of DDAH isoforms in brain development and function.

Unexpectedly, expression of both DDAH isoforms was observed in the developing limb (Figs. 2, 3). Furthermore, we observed localisation of nitric oxide synthase RNA and nitric oxide synthase activity in the limb buds at several developmental stages (Figs. 5, 6). Administration of the nitric oxide synthesis inhibitor *l-*

NAME to pregnant rats has been reported to cause a haemorrhagic fetal limb phenotype (Fantel *et al*., 1999), and sporadic limb loss in eNOS null mice has been reported (Gregg *et al*., 1998), suggesting that nitric oxide signalling functions in limb development. We observed an overlapping distribution of expression of both DDAH isoforms in the limb with RNA encoding the RASGTPAse activating factor NF1, shown to coimmunoprecipitate with DDAH1 in the rat (Tokuo *et al*., 2001). At the moment, the functional significance *in vivo* of the association between NF1 and DDAH1 is unknown, as is whether this is related functionally to nitric oxide or RAS signalling. In the context of the developing limb and brain in our study, there is evidence of nitric oxide synthase activity from NADPH-diaphorase staining overlapping DDAH1/2 and nitric oxide synthase isoform RNA expression domains.

The co-localisation we observed of nitric oxide synthase and DDAH isoform RNA with nitric oxide synthase activity is circumstantial evidence for a nitric oxide synthase-dependent role of DDAH1 and 2 during development. However, the (albeit poor) survival and seemingly normal gross phenotype of the triple eNOS/iNOS/nNOS null transgenic mouse challenges the hypothesis that nitric oxide signalling is an essential developmental regulator in the mouse. The triple nitric oxide synthase mouse reportedly exhibits low levels of fertility but grossly normal morphology, albeit with the development of diabetes insipidus, and poor survival (Morishita *et al*., 2005). It is not known whether nitric oxide itself is absent in these embryos and mice. The low level of generation and subsequent failure to develop of DDAH1 null embryos in our study therefore suggests a nitric-oxide independent role for DDAH in fertilisation and early embryonic development. The previous reports of association of DDAH isoforms with NF1 and Sp1 suggests mechanisms of nitric oxide-independent activity for the DDAH isoforms, regulating these respective signalling pathways. Obviously there may be more dimerisation partners for DDAH, and a potential role for DDAH proteins as transcriptional modifiers is currently under investigation in our laboratory. Generation of conditional double null DDAH1 and 2 mice will address the issue of the importance of these nitric oxide independent actions in normal development, and redundancy of DDAH isoforms suggested by the survival of our DDAH2 null mice. The survival of the triple eNOS/iNOS/nNOS null mouse obviously raises fundamental questions about the role of nitric oxide in mammalian development. Further reports of the phenotype of the triple eNOS/iNOS/nNOS null are awaited.

Our results demonstrate a role for DDAH1 in early mouse development and suggest involvement of DDAH1 and 2 in development of several organ systems. The reported normal development of the triple eNOS/iNOS/nNOS null mouse suggests that any role of DDAH in development may be nitric oxide independent. We are currently generating mice bearing conditional null alleles for both DDAH isoforms. In conjunction with tissue-specific *Cre* lines, this will allow detailed investigation of the roles of DDAH isoforms in organ development.

Materials and Methods

Generation of mouse lines: generation of DDAH1 null mouse is described in Leiper *et al.,* Nature Medicine 2007. We have disrupted exon 3 of the DDAH2 gene. This exon contains the initiating ATG and encodes the N-terminal 100 amino acids of the protein. Disruption of exon 3 generates a null allele that is unable to express functional protein (as confirmed by western blotting). A full description of this transgenic line will be given elsewhere (manuscript in preparation).

DNA extraction and genotyping of DDAH-1 genetically modified animals

At time of weaning, tail sections were taken from animals for genotyping. Genomic DNA was prepared using DNAeasy Kit (Qiagen) following the manufacturer's instructions. Primers used were
DDAH-1 exon forward (5'- CGAGCCACCGACTCAAAC -3'),
DDAH-1 exon 1 reverse (5'-GAGCGAAATCCACCTCCTC-3'), and
Neomycin reverse (5'-GCTTGGCTGGACGTAAACTC-3')
(all from Sigma-Genosys). DDAH-1 forward and DDAH-1 reverse amplified a 175 base pair fragment indicating the wild-type DDAH-1 gene while DDAH-1 forward and Neomycin reverse amplified a fragment of 290 base pairs indicating the presence of the DDAH-1 knockout. For PCR amplification, 26 μL of 1.1x Reddy-Mix (VWR) was combined with 200 μmol of each of the three primers and 2 μg of genomic DNA. Thermal cycling conditions were 95C for 3 minutes, followed by 40 cycles of 95ºC for 20 seconds, 60ºC for 40 seconds, and 72ºC for 1 minute, and finally 72ºC for 5 minutes. PCR products were run on a 2% agarose gel, stained with ethidium bromide, and visualized using a UV illuminator and GeneSnap software (Syngene). Genotypes were scored by presence or absence of bands for DDAH-1 or Neomycin.

Isolation of blastocysts, and scoring of resorbing embryos

Placing two female mice into the cage of a male performed trio mating. Timed mating was performed by checking for the presence of a vaginal plug in the morning shortly after the end of the dark phase of the animal facilities light cycle. For blastocyst isolation, on the third day following vaginal plug presence, females were sacrificed by cervical dislocation. The oviducts and uterus were removed and gently rinsed with sterile PBS to flush out pre-implanted blastocysts. Isolated blastocysts were rinsed in sterile PBS to remove maternal contamination. To isolate genomic DNA, blastocysts was placed in lysis buffer (50 mM Tris-HCl pH 8-8.5, 1mM EDTA, and 0.5% Tween-20) and 2 μL of 2 mg/ml Proteinase K (Sigma-Aldrich) for 5 hours at 55ºC followed by 95ºC for 5 minutes and then stored at -20ºC.

For resorption studies, following the identification of the vaginal plug, pregnant females were phenotypically screened for abdominal distension at 12-14 days of gestation to confirm pregnancy. At day e15, pregnant females were sacrificed using cervical dislocation and embryos were dissected to score phenotypes. Embryos were sacrificed by approved procedures. Following sacrifice, tail sections of all animals used for blastocyst and resorption studies were taken and post mortem genotyping was performed to confirm genotype.

Immunohistochemistry on cryostat sections

This was carried out as previously described (Leu *et al*., 2001).

In situ *hybridisation*

This was carried out essentially as previously described (Riddle *et al*., 1993). All cDNAs were cloned unidirectionally into the vector pSport6 from the relevant IMAGE clone; DDAH1 (accession number BQ963666), DDAH2 (BQ899468), eNOS(CB181220), nNOS(CF744713), iNOS (BG974882). Plasmids were linearised, and antisense RNA labelled with digoxigenin-UTP (Boehringer Ingelheim) was prepared.

Diaphorase staining was carried out as previously described (Weinberg *et al*., 1996).

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