**Abstract**

D-Aspartate, an abundant D-amino acid enriched in neuroendocrine tissues, can be degraded by D-aspartate oxidase (Ddo). To elucidate the function of D-aspartate, we generated mice with targeted deletion of Ddo (Ddo<sup>−/−</sup>) and observe massive but selective augmentations of D-aspartate in various tissues. The pituitary intermediate lobe, normally devoid of D-aspartate from endogenous Ddo expression, manifests pronounced increases of immunoreactive D-aspartate in Ddo<sup>−/−</sup> mice. Ddo<sup>−/−</sup> mice show markedly diminished synthesis and levels of pituitary proopiomelanocortin/α-MSH, associated with decreased melanocortin-dependent behaviors. Therefore, Ddo is the endogenous enzyme that degrades D-aspartate, and Ddo-deficient organs, low in D-aspartate, may represent areas of high turnover where D-aspartate may be physiologically important.

**Key words:** amino acid; neuroendocrine; knock-out mice; aspartate; turnover; behavior; proopiomelanocortin (POMC)

**Introduction**

D-Enantiomer amino acids, previously thought to occur only in bacteria, have recently been demonstrated in mammals (Dunlop et al., 1986). D-Serine is an apparent glial neurotransmitter/neuromodulator in the brain and is synthesized by serine racemase, a pyridoxal 5′-phosphate-dependent enzyme that racemizes L- to D-serine (Wolosker et al., 1999). In physiological conditions, D-serine is degraded by D-amino acid oxidase (Dao) such that Dao-deficient mice display increased levels of D-serine (Hashimoto et al., 1993).

D-Aspartate is the only other abundant D-amino acid found in mammals, with the highest levels found in the adrenals, brain, pineal, pituitary, retina, and testes (Hashimoto and Oka, 1997). Immunohistochemical studies have identified discrete cellular localizations of D-aspartate including adrenal medulla epinephrine cells, hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, and testicular elongate spermatids (Schell et al., 1997; Sakai et al., 1998). In the pituitary, D-aspartate is concentrated in the posterior lobe (PL) and in scattered cells of the anterior lobe (AL), with negligible levels in the intermediate lobe (IL) (Schell et al., 1997; Lee et al., 1999).

Physiologic activities responsible for metabolizing D-aspartate are not definitively established. In vitro, D-aspartate oxidase (Ddo), a flavin adenine dinucleotide-dependent peroxisomal enzyme, displays selective oxidative activity toward acidic D-amino acids (Simonic et al., 1997). It is expressed at highest levels in the kidney, liver, brain, and pituitary IL (Schell et al., 1997). The absence of potent and selective Ddo inhibitors hinders investigations into the physiologic role of D-aspartate.

In the present study, we describe the generation of mutant mice (Ddo<sup>−/−</sup>) with targeted deletion of Ddo and demonstrate massive, selective augmentations of D-aspartate levels in numerous tissues. In Ddo<sup>−/−</sup> mice, the pituitary IL displays substantial D-aspartate, which is undetectable in wild-type mice. The pituitary IL contains almost exclusively melanotropes, which generate proopiomelanocortin (POMC) as the sole source of pituitary α-melanocyte-stimulating hormone (α-MSH), a member of the melanocortin peptide family (Hadley and Haskell-Luevano, 1999). Melanocortins mediate several behaviors including penile erection (Martin and MacIntyre, 2004), autogrooming (Spruijt et al., 1992), and weight loss (Vergoni et al., 2000). Elevated D-aspartate levels in the Ddo<sup>−/−</sup> pituitary IL lead to diminished POMC/α-MSH as well as melanocortin-dependent behaviors, thereby establishing physiologic functions for D-aspartate.

**Materials and Methods**

Generation of Ddo-deficient mice. A human DDO cDNA (GenBank accession number D89858) was used to identify a bacterial artificial chromosome (BAC) clone that spanned the mouse Ddo gene. The gene-targeting
vector was prepared as follows: the short arm, a 1.9 kb Sph fragment containing ~1.3 kb upstream and ~500 bp downstream from exon 2, was subcloned into EcoRI–BamHI-digested pSLoxPNT. The long arm, a 5 kb NotI–BamHI fragment containing the middle of intron 3 to the 5′ region of intron 4, was subcloned into Smal–BamHI-digested pBluecript II (Stratagene, La Jolla, CA). The latter plasmid was cleaved with EcoRV and NotI, generating a 5 kb insert that was cloned into AscI–NotI-digested pSLoxPNT. The orientation of both arms was verified by DNA sequencing and restriction endonuclease analysis.

The sequence-replacement gene-targeting vector was linearized with NotI and electroporated into mouse embryonic stem (ES) cells (strain 129/SvJae). Mouse ES cells were cultured on mitomycin C-treated STO feeder cells in medium containing G418 (250 μg/ml) and 1-2′(2′-fluoro-B-β-arabinofuranosyl)-5-iodomuracil (0.2 μM). Drug-resistant ES cell colonies were picked 10 d after electroporation. Targeted colonies (Ddo1/−) were identified by Southern blot analysis of BamHI-digested genomic DNA with a 5′-flanking probe amplified from BAC DNA with primers 5′-AGG GTG AAA AGC TAT CTG TGG GTA A-3′ and 5′-GCA GAG AGG CAG TTC CAC AGA GCT T-3′. Four targeted clones, each with a single integration event, were injected into C57Bl/6 blastocysts to produce chimeric mice, which were bred to establish lines of Ddo knockout mice.

**Northern blot and in situ hybridization.** [α-32P]dCTP (-All-in-One random prime labeling mix; Sigma, St. Louis, MO) and/or digoxigenin-labeled (Dig-labeling kit; F. Hoffmann-La Roche, Basel, Switzerland) mouse full-length POMC or Ddo (spanning exons 2–4) probes were synthesized. Mouse Ddo cDNA template was amplified by reverse transcriptase-PCR from mouse liver RNA with primers 5′-CTG TGG AGC TGG CGT GAT AGG-3′ and 5′-TCC AGC CAC GGG AGG TAG GC-3′. Total RNA was isolated from mouse tissue (Tri Reagent; Sigma), and 25 μg was separated by electrophoresis on a 1% agarose/formaldehyde gel and transferred onto Nytran SuPerCharge membrane (Schleicher & Schuell, Dassel, Germany). An 18S cDNA probe was used as controls for RNA integrity and loading.

Fresh-frozen mouse pituitary sections (20 μm) were fixed in 4% paraformaldehyde and acetylated in 0.1 M triethanolamine, pH 8, with 0.25% acetic anhydride. Probe binding was determined by alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1:5000; F. Hoffmann-La Roche). Standard prehybridization, hybridization, washing, and visualization procedures were used.

**Immunoblotting.** Kidney/ liver and pituitary were respectively homogenized in (1) 5X vol of 50 mM Tris, pH 8.5, and 250 mM sucrose or (2) 100 μl of 50 mM Tris, pH 7.5, 2 mM EDTA, 100 μM PMSF, 100 mM NaCl, 1 mM DTT, and 1 protease inhibitor cocktail tablet (E. Hoffmann-La Roche) with EcoRV. Kidney/ liver cDNA template was amplified by reverse transcriptase-PCR from mouse liver RNA with primers 5′-CTG TGG AGC TGG CGT GAT AGG-3′ and 5′-TCC AGC CAC GGG AGG TAG GC-3′. Total RNA was isolated from mouse tissue (Tri Reagent; Sigma), and 25 μg was separated by electrophoresis on a 1% agarose/formaldehyde gel and transferred onto Nytran SuPerCharge membrane (Schleicher & Schuell, Dassel, Germany). An 18S cDNA probe was used as controls for RNA integrity and loading.

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**Immunohistochemistry.** Kidney/liver and pituitary were respectively homogenized in (1) 5X vol of 50 mM Tris, pH 8.5, and 250 mM sucrose or (2) 100 μl of 50 mM Tris, pH 7.5, 2 mM EDTA, 100 μM PMSF, 100 mM NaCl, 1 mM DTT, and 1 protease inhibitor cocktail tablet (E. Hoffmann-La Roche) with EcoRV. Kidney/liver cDNA template was amplified by reverse transcriptase-PCR from mouse liver RNA with primers 5′-CTG TGG AGC TGG CGT GAT AGG-3′ and 5′-TCC AGC CAC GGG AGG TAG GC-3′. Total RNA was isolated from mouse tissue (Tri Reagent; Sigma), and 25 μg was separated by electrophoresis on a 1% agarose/formaldehyde gel and transferred onto Nytran SuPerCharge membrane (Schleicher & Schuell, Dassel, Germany). An 18S cDNA probe was used as controls for RNA integrity and loading.

A Northern blot of Ddo was performed with a probe generated against the gene using cDNA template from mouse liver RNA with primers 5′-CTG TGG AGC TGG CGT GAT AGG-3′ and 5′-TCC AGC CAC GGG AGG TAG GC-3′. Total RNA was isolated from mouse tissue (Tri Reagent; Sigma), and 25 μg was separated by electrophoresis on a 1% agarose/formaldehyde gel and transferred onto Nytran SuPerCharge membrane (Schleicher & Schuell, Dassel, Germany). An 18S cDNA probe was used as controls for RNA integrity and loading.

Amino acid analysis demonstrates striking increases in Ddo levels in kidney, brain, adrenals, and ovaries (Figs. 2A–E, S1, available at www.jneurosci.org as supplemental material). In contrast, there is only a twofold increase in Ddo levels (with a parallel decline in D-aspate levels) in the testes, a tissue...
in which wild-type mice normally display considerable D-aspartate levels (Fig. 2F). In Ddo−/− mice, kidney D-aspartate levels elevate threefold, in contrast to a 150-fold increase in Ddo−/− mice (Fig. 2A). Although rat adrenals possess D-aspartate (Hashimoto and Oka, 1997), no D-aspartate is detectable in wild-type mouse adrenals (Fig. 2D). HPLC analysis reveals no changes in the levels of any other D- or L-amino acid in the above measured tissues (data not shown).

Given the high levels of wild-type testicular D-aspartate, we wondered whether the testes might serve as the sole source of mouse D-aspartate. However, castration of 4-month-old Ddo−/− mice does not diminish Ddo−/− kidney D-aspartate levels [castrated (n = 6), 598 ± 129 nmoles/g wet weight; sham-castrated (n = 3), 581 ± 69 nmoles/g wet weight; p = 0.93]. Additionally, although some studies have suggested a role for testicular D-aspartate in regulating testosterone synthesis (Nagata et al., 1999), we fail to detect altered serum testosterone concentrations in Ddo−/− mice (Ddo+/+, 1.30 ± 0.24 ng/ml; Ddo−/−, 1.01 ± 0.14 ng/ml; p = 0.23; n = 10).

Immunohistochemical localizations of D-aspartate in wild-type mice resemble results described previously in rats (Schell et al., 1997; Sakai et al., 1998). D-Aspartate in wild-type mice localizes to cerebral cortical neurons, hippocampal CA3/CA2 neurons, neurons in the hilum of the dentate gyrus, hypothalamic PVN and SON neurons, pinealocytes, and elongate spermatids (Figs. S2, S3, available at www.jneurosci.org as supplemental material). Unlike the juvenile rat (Schell et al., 1997), D-aspartate is not immunohistochemically detected in the 4-week-old wild-type mouse adrenals (Fig. S3, available at www.jneurosci.org as supplemental material). In Ddo−/− mice, stronger D-aspartate labeling is found in cerebral cortical neurons, hippocampal neurons, pinealocytes, and elongate spermatids (Figs. S2, S3, available at www.jneurosci.org as supplemental material). New D-aspartate labeling in the Ddo−/− mice, undetectable in wild-type mice, is observed in adrenal medulla epinephrine cells, renal Bowman’s capsule, renal thin limbs of the loops of Henle, and testicular Leydig cells (Figs. S2, S3, available at www.jneurosci.org as supplemental material). At our level of microscopic resolution, D-aspartate in wild-type and Ddo−/− brain and peripheral tissues appears diffusely throughout cells and neuronal processes.

In the pituitary IL, we observe a striking phenotype in the Ddo−/− mice. In wild-type mice, Ddo is normally expressed in the IL (Fig. 3A), explaining the negligible levels of D-aspartate in the wild-type IL (Fig. 3B). Wild-type D-aspartate staining is instead dense in fibers of the pituitary PL that arise from the D-aspartate-enriched hypothalamic SON and PVN cell bodies and in isolated cells of the AL (Fig. 3B). In Ddo−/− mice, which lack pituitary IL Ddo (Fig. 3A), D-aspartate increases dramatically in the IL but only modestly in the AL and PL (Fig. 3B). Because pituitary IL melanotropes generate POMC as the source of pituitary α-MSH, we wondered whether the augmented IL D-aspartate influences POMC/α-MSH. In Ddo−/− pituitary IL, POMC expression is substantially reduced as judged by in situ hybridization (Fig. 3C). This reduction is specific, because we observe no changes in either POMC expression or the number of POMC-positive neurons in Ddo−/− pituitary AL (data not shown) or arcuate nucleus of the hypothalamus (Fig. 3D). Moreover, pituitary POMC protein (Fig. 3E) is markedly diminished by Western blot, and α-MSH levels are decreased as judged by both dot blot assays and immunofluorescence (Fig. 3F, G).

Because α-MSH influences autogrooming (Spruijt et al., 2002), penile function (Martin and MacIntyre, 2004), and weight homeostasis (Vergoni et al., 2000), we monitored these parameters in Ddo−/− mice (Fig. 4A–D). At 2, 7, and 12 months of age, Ddo−/− mice are significantly heavier than wild-type litter-mate controls (Fig. 4A). Ddo−/− males also display diminished sexual behavior, as evidenced by increased latencies by males to mount and intromit females (Fig. 4B) and by severe failure of ejaculation (Ddo−/−, 75 ± 16.4%; Ddo−/−, 12.5 ± 12.5% of mice that successfully ejaculate during first hour of testing; p = 0.009; n = 8). During the resident–intruder aggression test (Fig. 4C) and elevated plus maze test (Fig. 4D), Ddo−/− mice spend nearly fourfold less time autogrooming than wild-type controls. This effect is selective as judged by the absence of changes in α-MSH-independent autogrooming (Fig. 4D).

Discussion

Through genetic inactivation of Ddo in mouse, we observe massive and selective elevation of D-aspartate across the body. Ddo enzymatic activity was first identified before D-enantiomer amino acids were believed to be endogenously synthesized in
Elevated D-aspartate in mammals (Still et al., 1949). Therefore, the D-amino acid oxidizing property of Ddo was considered nonphysiologic, and unsuccessful efforts were made to find possible endogenous functions (Burns et al., 1984). With orders of magnitude D-aspartate elevation in Ddo<sup>−/−</sup> mice, this study clearly demonstrates that Ddo is the endogenous enzyme responsible for metabolizing D-aspartate.

Reciprocally high and low tissue concentrations occur for Ddo and D-aspartate, resembling Dao and D-serine (Snyder and Kim, 2000). Negligible levels of endogenous D-aspartate in Ddo-enriched organs had implied that D-aspartate lacks important physiologic functions in these areas (Schell et al., 1997). The pronounced increase in Ddo<sup>−/−</sup> mice of D-aspartate in Ddo-enriched organs suggests that D-aspartate is physiologically relevant and turns over rapidly because of high Ddo activity. Conversely, the source of D-aspartate may come from endogenous synthesis by an unidentified aspartate racemase, because Ddo<sup>−/−</sup> testes show increased D-aspartate concomitant to decreased l-aspartate. Conversion of l-aspartate to D-aspartate has been reported in rat cerebral cortical cultures (Wolosker et al., 2000) and invertebrates (D’Aniello et al., 2003).

One potential function for D-aspartate is observed in the pituitary IL. The Ddo<sup>−/−</sup> IL displays elevated D-aspartate levels with diminished POMC expression and α-MSH formation. Introcerebroventricular injection of α-MSH increases autogrooming (Spruijt et al., 1992), enhances penile erection (Martin and MacIntyre, 2004), and elicits anorexia/weight loss (Vergoni et al., 2000). Genetic inactivation of a principal brain α-MSH receptor, melanocortin receptor 4, leads to adult-onset hyperphagia-induced obesity (Huszár et al., 1997) and sexual deficits resembling Ddo<sup>−/−</sup> mice (Van der Ploeg et al., 2002). Genetic inactivation of the other major brain α-MSH receptor, melanocortin receptor 3, leads to increased fat mass and decreased lean mass (Butler et al., 2000; Chen et al., 2000). The elevated body mass, sexual deficits, and decreased autogrooming in Ddo<sup>−/−</sup> mice are consistent with their diminished α-MSH levels.

How might D-aspartate influence POMC expression and other targets? Nagata and coworkers (1999) showed that D-aspartate stimulation of testosterone formation in Leydig cell cultures is abolished by inhibition of glutamate transport, implying that D-aspartate is transported into target cells rather than acting at surface receptors. Conceivably, a similar mechanism mediates D-aspartate actions on melanotropes.

Figure 2. Ddo<sup>−/−</sup> mice display elevated α-aspartate levels. A, HPLC amino acid analysis of 4-week-old kidney α-aspartate (Ddo<sup>+/+</sup> vs Ddo<sup>−/−</sup>; p = 0.021; Ddo<sup>+/+</sup> vs Ddo<sup>+/−</sup>; p = 0.008). B, Four-month-old kidney α-aspartate (Ddo<sup>+/+</sup> vs Ddo<sup>−/−</sup>; p = 0.004). C, Four-month-old brain α-aspartate (Ddo<sup>+/+</sup> vs Ddo<sup>−/−</sup>; p = 0.0009). D, Four-month-old adrenal α-aspartate (Ddo<sup>+/+</sup> vs Ddo<sup>−/−</sup>; p = 0.00001). E, Four-month-old ovarian α-aspartate (Ddo<sup>+/+</sup> vs Ddo<sup>−/−</sup>; p = 0.000004). F, Four-month-old testicular α-aspartate (Ddo<sup>+/+</sup> vs Ddo<sup>−/−</sup>; p = 0.0001) and l-aspartate (Ddo<sup>+/+</sup> vs Ddo<sup>−/−</sup>; p = 0.013). *p < 0.05; **p < 0.01; ***p < 0.001; comparison with two-tailed Student’s t test; data are expressed as mean ± SEM.

Figure 3. Elevated α-aspartate in Ddo<sup>−/−</sup> mice pituitary intermediate lobe is associated with diminished melanocortin levels. A, Ddo in situ hybridization (n = 2). B, α-Aspartate immunohistochemistry (n = 4). C, D, POMC in situ hybridization in the pituitary (C; n = 3) and arcuate nucleus (D; n = 2). E, POMC Western blot of crude pituitary lysate (n = 2). F, G, Crude pituitary lysate dot blot (F; n = 4) and immunofluorescence (G; n = 2) of α-MSH. Scale bars, 100 μm. a, Anterior lobe; i, intermediate lobe; p, posterior lobe.
Many studies of melanocortin-dependent behaviors focus on hypothalamic arcuate-synthesized α-MSH acting on central melanocortin receptors (Cone et al., 2001). Ddo−/− mice do not display altered POMC expression in the arcuate nucleus, suggesting that decreased melanocortin-dependent behaviors in Ddo−/− mice do not reflect centrally derived melanocortins but may involve pituitary α-MSH influencing central α-MSH tone.

Pituitary signaling back to the brain and hypothalamus, a controversial concept, may involve either direct retrograde blood flow from the pituitary back to the hypothalamus or filtration/secretion of peptides into the CSF via subarachnoid or choroid plexus routes (Assies et al., 1978; Page, 1982). Injection of radiolabeled α-MSH4–9, the bioactive portion of the 12 amino acid peptide, into the pituitary leads to its accumulation across the brain with highest levels in hypothalamic areas such as the arcuate nucleus, SON, and median eminence (Mezey et al., 1978). After pituitary stalk sectioning, accumulation of radiolabeled peptide diminishes selectively in the hypothalamus but then returns after vascular regeneration (Mezey et al., 1978). Lesion studies imply that high levels of α-MSH in portal plasma arise from the pituitary (Oliver et al., 1977; Paradisi et al., 1993). Various conditions that elevate α-MSH in serum also augment α-MSH in the brain (De Rotte et al., 1980).

Lesion studies also establish a role for the pituitary in melanocortin-dependent behaviors. Hypophysectomized rats display reduced melanocortin-regulated erections and autogrooming (Dunn et al., 1979; Argiolas et al., 1987). Exitoxic ablation of the arcuate nucleus has no effect (Dunn et al., 1985; Argiolas et al., 1987).

Although pituitary α-MSH may influence behavior, arcuate-derived α-MSH is better established as a dominant mediator of melanocortin-dependent behaviors, which is evident in our study. Body weight doubles in POMC-deficient mice reflecting obesity in the melanocortin-3 receptor-deficient mouse. Endocrinology 141:3518–3521.


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Figure 4. Ddo−/− mice display diminished melanocortin-dependent behaviors. A. Body mass measured at indicated ages (2 months, p = 0.03; 7 months, p = 0.007; 12 months, p = 0.004). B. Latency of male mouse to mount (p = 0.01) or intromit (p = 0.025) estrous CD-1 female. C. D. Time spent autogrooming during resident–intruder aggression test (C: n = 8 pairs; p = 0.04) and elevated plus maze test (D: p = 0.02). *p < 0.05; **p < 0.01; comparison by two-tailed t-test; data are expressed as mean ± SEM. Sig., Significance.

References


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