

Regulation of rat magnocellular neurosecretory system by D-aspartate: evidence for biological role(s) of a naturally occurring free D-amino acid in mammals

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Abstract

Little evidence is available for the physiological function of D-amino acids in species other than bacteria. Here we demonstrate that naturally occurring free D-aspartate (D-Asp) is present in all magnocellular neurons of rat hypothalamus. The levels of this naturally occurring D-amino acid were elevated during lactation and returned to normal thereafter in the magnocellular neurosecretory system, which produces oxytocin, a hormone responsible for milk ejection during lactation. Intraperitoneal injections

of D-Asp reproducibly increased oxytocin gene expression and decreased the concentration of circulating oxytocin *in vivo*. Similar changes were observed in the vasopressin system. These results provide evidence for the role(s) of naturally occurring free D-Asp in mammalian physiology. The findings argue against the conventional concept that only L-stereoisomers of amino acids are functional in higher species.

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Introduction

It is widely accepted that D-amino acids do not have physiological function in species other than bacteria. Recently, the existence of some D-amino acids in some organs of higher animals has been demonstrated by several groups using HPLC techniques (D'Aniello & Giuditta 1977, Dunlop *et al.* 1986, Neidle & Dunlop 1990, Hashimoto *et al.* 1992, 1993, Kera *et al.* 1995, D'Aniello *et al.* 1995, Imai *et al.* 1995, Hamase *et al.* 1997, Sakai *et al.* 1997). Although extensive in number, the above studies have not provided an answer to the question as to whether these D-amino acids are biologically functional. On the basis of this assumption, many D-isomers have been used to investigate various physiological and pathological processes *in vivo*. For example, D-aspartate (D-Asp) is widely used as a putatively metabolically inert analogue of L-glutamate (L-Glu) to study glutamate transporter systems. Only recently, with the advance of immunohistochemistry for detecting single free amino acids, has the cellular localization of D-Asp and D-serine pinpointed the possible sites of action for these D-isomers (Schell *et al.* 1995, D'Aniello *et al.* 1996, Di Fiore *et al.* 1998, Sakai *et al.* 1998). Therefore, evidence for the function of naturally occurring free D-amino acids in higher animals is eagerly awaited.

By using an antiserum highly specific to free D-Asp, this D-isomer was localized to various regions of the brain and numerous endocrine glands in the rat in our previous study (Schell *et al.* 1997). To elucidate further the physiological functions of naturally occurring D-Asp, we focused on the hypothalamic magnocellular system, which has been well studied as a model for neuropeptide secretion. Mammalian magnocellular neurosecretory neurons are primarily clustered in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus. They principally secrete either oxytocin or vasopressin from their nerve terminals in the posterior pituitary into the systemic circulation in response to a variety of different physiological stimuli. Oxytocin release is mainly caused by orgasm, parturition and suckling, whereas vasopressin release occurs in response to stimuli that change or threaten the water status of the body (Gainer & Wray 1994). Therefore, two experimental conditions to stimulate synthesis and release of the respective peptides have been widely used: lactation for oxytocin and salt loading for vasopressin, although in either case changes in the other neuropeptide are not absent entirely (Windhager 1992).

By experimentally manipulating this model system in the present study, we provide several lines of evidence

indicating that naturally occurring D-Asp is biologically functional in the regulation of mammalian neurosecretion.

Materials and Methods

Animals

Female Long-Evans rats (2–10 months old) were purchased from Charles River (Wilmington, MA, USA), housed under conditions of 14 h light:10 h darkness, administered food and water *ad libitum*, and mated and bred normally. Experimental groups of littermates were established; different experimental groups were from different litters. Tissues from each experimental group of animals were processed together throughout the entire experimental manipulation. Different groups were processed separately. For all experiments using injections, vaginal smears were performed daily from 8 days before the treatment until the animals were killed following the experimental treatments. Only rats with two regular oestrous cycles were used. The animals within each group were also matched by the stages of their oestrous cycles on the day starting the injections.

In all the immunohistochemistry and *in situ* hybridization experiments, the cutting angle for the brains was adjusted so that sections would correspond to those in the stereotactic atlas of Paxinos and Watson (1982). All sections from around interaural levels 9.00 to 6.20 mm (containing virtually all the SON and PVN) were collected. Adjacent slices were processed for experiments and for technical controls without the primary detection agents (antibody against D-Asp or oligo probes for oxytocin and vasopressin) respectively.

All quantitative analyses were performed blind. A paired *t*-test was used to evaluate the significance of differences, because all the comparisons in the study were between two paired groups and all the data could be considered (directly or after arcsin transformation) to be normally distributed.

Immunohistochemistry

Of the three animals in each group, one remained virgin ('virginal' group) throughout; the other two were mated, underwent pregnancy, and delivered on the same day. Four days after the delivery, the litter was removed from one lactating rat ('lactating-stop' group), whereas the other was kept lactating with her pups ('lactating' group); litters were never removed until the continuously lactating rats became unconscious after anaesthetization. Fourteen days after the litters had been born, all three animals were anaesthetized by overdose of pentobarbital and perfused intracardially with 5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) between 1000 and 1100 h. In such manipulations, the circulating levels (mean \pm S.E.M.;

pg/ml) of oxytocin ($n=5$) were: virginal, 410 ± 72 ; lactating, 811 ± 104 ; and lactating-stop, 397 ± 65 . The oxytocin mRNA levels (mean \pm S.E.M.; $n=3$; relative to virginal taken to be 100%) in SON and PVN respectively, were: lactating $346\% \pm 41\%$ and $278\% \pm 36\%$; and lactating-stop, $114 \pm 8\%$ and $98\% \pm 6\%$. Additional rats were used for the double immunolabelling for D-Asp and oxytocin/vasopressin. Brains were removed, immersed in the same fixative overnight at 4 °C, and cut into 70 μ m-thick coronal sections on a vibratome. All pituitaries of these rats were also removed, cryoprotected in 30% sucrose in 0.1 M phosphate buffer overnight and then frozen at -70 °C. Coronal sections (30 μ m) were cut using a cryostat and thaw-mounted onto polylysine-coated glass slides. All sections of the pituitary were collected.

The rabbit anti-D-Asp antibody was produced by immunizing the animals using D-aspartate coupled to bovine serum albumin with glutaraldehyde and the colloidal gold technique, and then affinity purified. Its high specificity for the single D-isomer (not L-Asp, its analogues, or a variety of aspartate-containing peptides) has been established, and pre-absorption with free-D-Asp completely abolishes the immunoreactivity (Schell *et al.* 1997). The antibody was pre-incubated with 0.5 mM L-Asp liquid-phase glutaraldehyde conjugate overnight at 4 °C before the brain and pituitary sections were processed for immunohistochemistry as previously described (Schell *et al.* 1997). A pre-absorption control was included in all experiments using the antibody as a technical control in the present study. After immunocytochemistry, all sections were mounted onto gelatin-coated slides, dried at room temperature overnight, and then coverslipped with DePeX. Double-labelling was carried out as previously described (Eichmüller *et al.* 1996) with the D-Asp antibody and one for oxytocin or vasopressin (Peninsula Lab, Belmont, CA, USA). The antibody for oxytocin does not cross-react with vasopressin and *vice versa*. Pre-absorption with these peptides abolished immunoreactivity. Quantitative comparisons of D-Asp immunoreactivity were performed by measuring the optical density (OD) of the immunohistochemical staining using an image analysis system (MCID, Imaging Research, CA, USA), as described by Wang & Morris (1999).

Injection experiments and *in situ* hybridization

The matched rats in each group were injected i.p. with 0.5 M sodium D-Asp solution, pH 7.4 (1.0 mmol/kg body weight), 0.5 M sodium L-Asp (1.0 mmol/kg body weight), or an equal volume of 0.9% NaCl at 10 00 h each day for 7 days. Blood concentrations of both D- and L-Asp rose significantly ($P<0.02$) above basal levels 5 h after a single such injection and returned to basal levels within 24 h (before the following injection), confirming reasonable bioavailability of each isomer in our experiments. On the eighth day, all animals were killed between 1000 and

1100 h by stunning and decapitation. Each brain was removed, rapidly frozen on dry ice and stored at -70°C . Coronal sections ($15\ \mu\text{m}$) were cut at -20°C using a cryostat, thaw-mounted onto polylysine-coated glass slides, maintained at -70°C while awaiting further processing and then used for *in situ* hybridization.

The oxytocin probe was a 39-mer complementary to bases 246–284 that overlap the start of exon A of the rat oxytocin sequence (Ivell & Richter 1984). The vasopressin probe was a 27-mer directed against amino acids 110–118 of rat pre-propressophysin (Ivell & Richter 1984). Both probes have been previously characterized, and their specificity has been reported (Albeck *et al.* 1997, Luckman *et al.* 1997). *In situ* hybridization using ^{35}S -labelled oligonucleotides was performed as previously described (Kadowaki *et al.* 1994). All slides from each group of rats were exposed to the same Hyperfilm- β max autoradiography film (Amersham, Arlington Heights, IL, USA). Quantification of the changes in the relative amounts of oxytocin/vasopressin mRNA in the SON and PVN was performed by measuring the optical density of autoradiographic films, as described by Kadowaki *et al.* (1994).

Radioimmunoassay of oxytocin

Groups of littermates were used and different groups were from different litters. The animals in each group were injected i.p. with D-Asp, L-Asp, or 0.9% NaCl at 10 00 h. Alternatively, L-Glu or 0.9% NaCl was injected into other animals. Five hours after the injection, the animals were anaesthetized. Blood was taken from the left ventricle and then processed for the oxytocin assay using a kit (Peninsula Lab, Belmont, CA, USA) and following the manufacturer's protocol, including extraction of the peptide from plasma. Different groups of animals were processed independently using different batches of the injection solutions and radioimmunoassay materials. The intra- and interassay variations were 4.0% and 9.1% respectively.

Results

Presence of naturally occurring D-Asp in all the neurons of the magnocellular neurosecretory system

In order to simplify the search for a possible function for D-Asp, we first determined precisely which cells contained D-Asp in the hypothalamus by double immunolabelling for either oxytocin or vasopressin plus free D-Asp. D-Asp-positive neurons in the SON and PVN were either oxytocin- or vasopressin-genic (oxytocin, $43\% \pm 3\%$ in SON, $51\% \pm 7\%$ in PVN; vasopressin, $55\% \pm 3\%$ in SON, $48 \pm 1\%$ in PVN; $n=4$). In addition to the areas previously reported (Schell *et al.* 1997), the accessory nuclei of the magnocellular neurosecretory system were found to contain D-Asp within either oxytocin or vasopressin neurons. D-Asp was also colocalized with

vasopressin neurons in the dorsomedial part of the supra-chiasmatic nucleus (data not shown), which, in part, led our primarily manipulating oxytocin system in the following experiments (see below). All oxytocin-/vasopressin-positive neurons were also D-Asp-positive.

Physiological association of naturally occurring D-Asp with lactation

We then focused primarily on the oxytocin system because, unlike that of vasopressin, it exists predominantly within the magnocellular system, and because lactation is a purely physiological event occurring during normal life.

To observe any physiological association of naturally occurring D-Asp in the rat magnocellular system, five groups of three littermates each were examined. In each group, the three rats were manipulated to either: (a) retain virginal status throughout; (b) undergo pregnancy and lactate for 14 days; or (c), undergo pregnancy, lactate for 4 days and then cease lactating by removal of their pups (suckling stimulus). Quantitative image analysis showed significantly ($P<0.05$) elevated D-Asp immunoreactivity (optical density, mean \pm s.e.m.) in the SON (0.62 ± 0.02) and PVN (0.60 ± 0.02) of lactating rats, as compared with that in the same nuclei of virginal rats (SON, 0.57 ± 0.03 ; PVN, 0.54 ± 0.03) (Fig. 1). Rats that underwent pregnancy and lactation but stopped lactating 10 days before being killed did not show any significant differences in D-Asp levels in the two magnocellular nuclei (SON, 0.57 ± 0.02 ; PVN, 0.53 ± 0.02) from their virginal counterparts; as with the virgins, they exhibited a lower level ($P<0.05$) of D-Asp immunostaining than that of continuously lactating rats.

Similarly, in the posterior pituitary, lactating rats exhibited significantly stronger immunoreactivity ($P<0.05$, $n=5$) of the D-amino acid than did virgin rats (OD, 0.202 ± 0.004 vs 0.166 ± 0.003 ; Fig. 1G and H). Again, the level of D-Asp (OD, 0.177 ± 0.008) in transiently lactating rats (stopped after 4 days lactation) did not differ significantly from the virgins.

These results demonstrate that the increased amount of D-Asp in the magnocellular system is associated with lactation, i.e. elevated oxytocin synthesis and release, and that this change of the naturally occurring D-amino acid levels occurs under entirely physiological circumstances *in vivo*. The increased immunoreactivity of D-Asp appeared to occur in all parts of the SON, i.e. also in the vasopressin neurons (Fig. 1). This is not unexpected because the levels of vasopressin are also increased during pregnancy and lactation, although such changes are believed to be a secondary response to the higher settings of water balance during the period (Gainer & Wray 1994). It was interesting to note that in the magnocellular neurons, the light microscopic immunostaining for D-Asp appeared to occur in the nuclei as well as in the cytoplasm and the nuclei showed strong labelling (Fig. 1J and K).

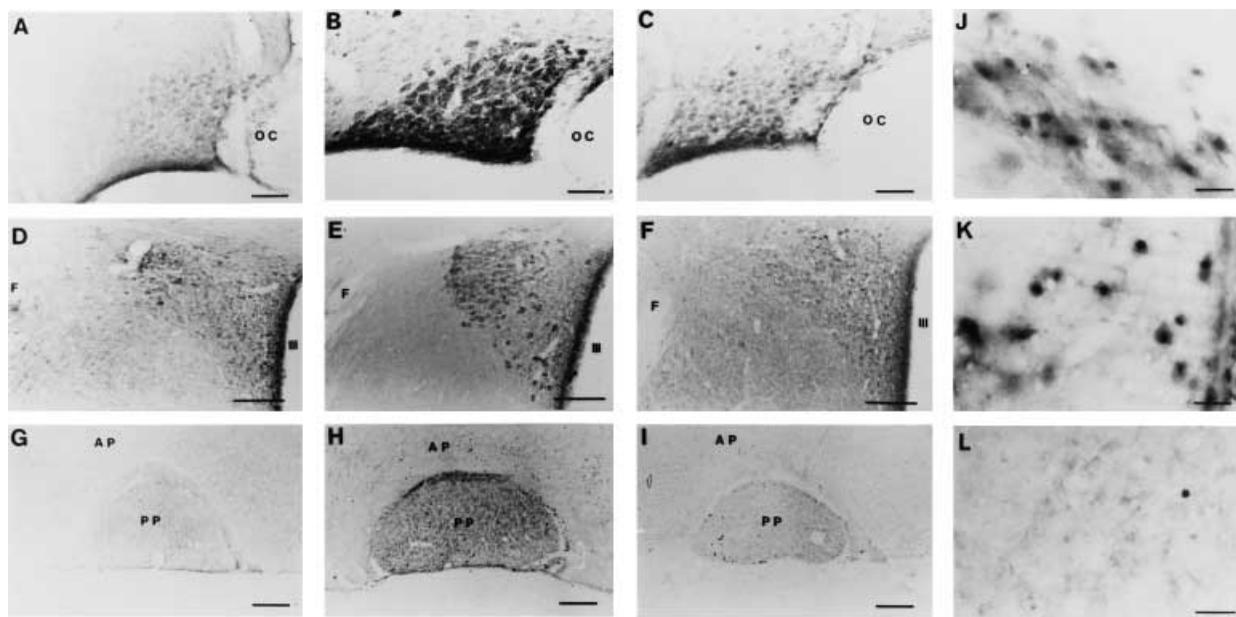


Figure 1 Photomicrographs of D-aspartate immunostaining in the rat SON (A–C) and PVN (D–F) of the hypothalamus, and in the posterior pituitary (G–I) of a group of three female littermates manipulated to either remain virginal throughout (A, D and G), undergo pregnancy and lactate for 14 days (B, E and H), or undergo pregnancy, lactate for 4 days and then stop lactating (C, F and I). The tissue from each group of three rats was processed together, including for image analysis. Higher magnification revealed apparent staining in the nuclei as well as cytoplasm of magnocellular neuron in both SON (J) and PVN (K) of a virginal rat. The immunoreactive structures in the posterior pituitary were not clearly defined at the light microscopic level (L). III, third ventricle; AP, anterior pituitary; F, fornix; OC, optic chiasm; PP, posterior pituitary. Scale bars A–C, 0.2 mm; D–F, 0.5 mm; G–I, 1 mm; J–L, 0.05 mm.

However, in the posterior pituitary, the nature of the immunoreactive structures was difficult to determine at this level of resolution (Fig. 1L).

Influence of oxytocin gene expression and its peripheral release by D-Asp

To ascertain whether there is a causal relation between lactation and the associated increase of D-Asp, we determined whether D-Asp was able to influence the synthesis and secretion of oxytocin. Because it is not possible to demonstrate such a relationship under absolutely endogenous conditions on the basis of available information about D-amino acids in high animals, administration of exogenous D-aspartate needs to be used. Intraperitoneal injection was chosen because it has been suggested that naturally occurring D-aspartate originates from the diet (Imai *et al.* 1997, Fukushima *et al.* 1998), enters the circulation and is retrogradely transported into the brain via the posterior pituitary (Schell *et al.* 1997). Therefore, i.p. administration is more likely to have physiological relevance for the purpose of this study than the usual intracerebroventricular route; the latter could also definitely lead to confusing results when D-Asp is directly applied to the brain because it can interact with most types of L-glutamate receptors and transporters (MacDonald *et al.* 1998). The kinetics and clearance of such peripheral

administered D- and L-Asp have been reported (Stegink 1976, D'Aniello *et al.* 1993).

Groups of three littermates at the same stages of their oestrous cycle were given single i.p. injections of either 0.9% NaCl, L- or D-Asp. Alternatively, two additional groups were injected with either L-Glu or 0.9% NaCl. Circulating oxytocin concentrations were measured after 5 h by radioimmunoassay. Compared with those of the controls treated with 0.9% NaCl or L-Asp, large and significant decreases of oxytocin were detected in the peripheral circulation of the rats treated with D-Asp. No difference in oxytocin levels was observed between animals injected with L-Asp and NaCl (Table 1). The effective dose was 1 mmol/kg body weight. Injection of L-Glu at this dose via the same route did not produce any changes in oxytocin concentration in the circulation (Table 1). These results demonstrate that the effects of D-Asp are not attributable to its similarity to L-glutamate, a well-known neurotransmitter.

We then quantitated the effects of repetitive D-Asp administration on oxytocin gene expression in the magnocellular nuclei. Littermates at the same stages of their oestrous cycle were administered either 0.9% NaCl, L- or D-Asp (1 mmol/kg) every day for 7 days. All the rats exhibited unaltered oestrous cycles, as they had before injection. However, a significantly higher level of oxytocin mRNA in the SON and PVN was detected selectively in

Table 1 Plasma concentrations (mean \pm S.E.M.; pg/ml) of circulating oxytocin in rats 5 hours after i.p. injection of various doses of L-Asp, L-Asp, or L-glutamate vs 0.9% NaCl. Paired *t*-test was employed for the statistical analysis

	NaCl	L-Asp	D-Asp	L-glutamate
0.1 mmol/kg body weight	480 \pm 78 (8)	378 \pm 50 (8)	320 \pm 47 (8)	—
0.5 mmol/kg body weight	391 \pm 68 (8)	420 \pm 144 (8)	475 \pm 64 (8)	—
1 mmol/kg body weight	520 \pm 112 (6)	462 \pm 74 (6)	158 \pm 25 (6)***	—
	438 \pm 69 (8)	—	—	447 \pm 96 (8)
5 mmol/kg body weight	409 \pm 78 (8)	354 \pm 63 (8)	424 \pm 81 (8)	—

* $P < 0.05$, vs NaCl; ** $P < 0.05$, vs L-Asp. All other differences were not significant. Number of animals used are in parentheses.

animals treated with D-Asp; injection of L-Asp showed no such effect (Fig. 2). Repeated (daily \times 7) injections of L-Glu at the same dose caused no change of oxytocin mRNA levels in the two magnocellular nuclei, as compared with saline-injected negative controls (SON, 289 \pm 71 vs 246 \pm 61 fg/mg; PVN, 91 \pm 38 vs 126 \pm 47 fg/mg (mean \pm S.E.M.; $n = 7$)). These data indicate that D-Asp regulates the synthesis and secretion of oxytocin *in vivo*, and these effects are not due to its analogy to L-Glu and a consequent action on the glutamatergic pathway.

We also found that injecting D-Asp at 1 mmol/kg for 7 days induced elevated levels of vasopressin mRNA (mean \pm S.E.M., $n = 7$) in both SON (NaCl : L-Asp : D-Asp,

236 \pm 60 : 243 \pm 69 : 415 \pm 96 fg/mg; $P < 0.05$) and PVN (NaCl : L-Asp : D-Asp, 137 \pm 48 : 98 \pm 27 : 406 \pm 64 fg/mg; $P < 0.01$). Although vasopressin and oxytocin are closely related, these data could also imply that D-Asp might have a general role in hormone production, especially when considering the fact that D-Asp is present in almost all other endocrine glands (Hashimoto *et al.* 1993, Schell *et al.* 1997).

Discussion

In our present study, we provide physiological evidence that naturally occurring free D-Asp is able to regulate the rat magnocellular neurosecretory system. Our findings enhance the argument against the conventional concept that only L-stereoisomers of amino acids are functional in higher species.

Because injection of D-Asp in our study caused decrease in circulating oxytocin, D-Asp could exert an inhibitory effect on the levels of peripheral oxytocin, either directly or indirectly. Repeated injections of D-Asp could induce sustained low levels of oxytocin in the periphery, which could then trigger autocrine/paracrine feedback to the magnocellular neurons in the brain and upregulate oxytocin gene expression in the hypothalamus, as observed. It might also be possible that administration of D-Asp at systems level could induce increased release (and therefore gene expression) of the hormone but, at the same time, could also enhance the use of oxytocin in peripheral endocrine organs (D-Asp is present in almost all the endocrine glands). Thus, as an overall result, a decrease in circulating oxytocin might be observed.

Independent experiments reproducibly showed that the dose-response relationship in our injection study was peculiar. This implies that the mechanism of the D-Asp effect is unlikely to be a ligand-receptor-mediated action, which should exhibit an 'S' shaped dose-response curve. In addition, D-Asp oxidase exists widely throughout the body (D'Aniello *et al.* 1993) and its localizations are reciprocal to those of naturally occurring D-Asp (Schell *et al.* 1997). Thus, it has been proposed that too much D-Asp could be toxic to the body and that D-Asp oxidase might act as the 'housekeeper' to maintain D-Asp at the

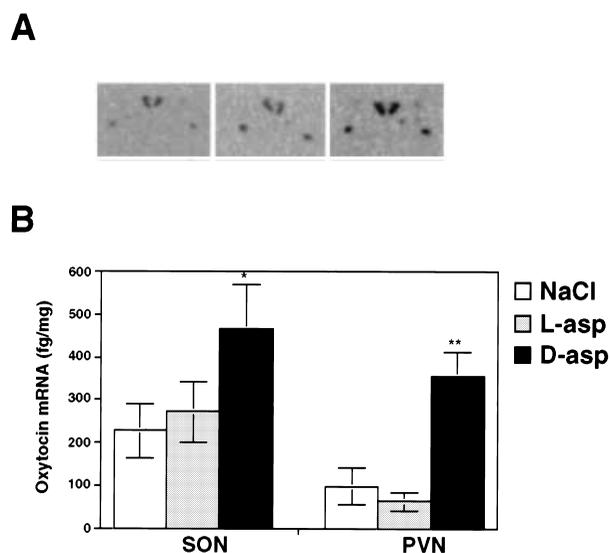


Figure 2 Autoradiographic optical density (mean \pm S.E.M.) of oxytocin mRNA in the SON and PVN of rats treated with physiological saline ($n = 7$), L-Asp ($n = 7$) or D-Asp ($n = 7$). (A) Example of original autoradiography of three female littermates injected (1 mmol/kg) with NaCl (left), L-Asp (middle) and D-Asp (right). The optical density of oxytocin mRNA was selectively increased in D-Asp-treated animals in both the SON (lower dots) and the PVN (upper wedges). (B) Quantitative analysis demonstrates the significant differences in oxytocin mRNA levels resulting from the D-Asp treatment. * $P < 0.05$ vs NaCl and $P < 0.05$ vs L-Asp; ** $P < 0.01$ vs NaCl and $P < 0.01$ vs L-Asp.

appropriate levels and at the proper locations (Schell *et al.* 1997). Larger-than-acceptable doses of D-Asp peripheral injection might therefore trigger the oxidation clean-up mechanism before it could initiate any effects. Thus, D-Asp oxidase could also contribute to the unusual dose-response curve reported in our study. A similar bell-shaped dose-response relationship of D-asp effects has been shown in an *in vitro* system for testosterone release (D'Aniello *et al.* 1996).

In summary, we have provided functional evidence for the biological roles of D-Asp in the rat hypothalamic magnocellular neurosecretory system. Future investigation should be directed towards the precise mechanism of the actions of the D-amino acid. On the basis of the wide presence of the D-Asp and its unusual dose-response reported independently in the regulation of different hormones (the present study and D'Aniello *et al.* 1996), we propose that D-Asp might have a more general role in the modulation of gene expression and/or protein production.

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