Amino Acids (2010) 38:229–235
DOI 10.1007/s00726-008-0231-6

ORIGINAL ARTICLE

D-Aspartate binding sites in rat Harderian gland

Marcello Di Giovanni · Enza Topo · Alessandra Santillo · Antimo D'Aniello · Gabriella Chieffi Baccari

Received: 10 November 2008 / Accepted: 29 December 2008 / Published online: 20 January 2009

Abstract Radioligand binding of D-[^{3}H]aspartic and L-[^{3}H]glutamic acids to plasma membranes from rat Harderian gland was evaluated. Binding was optimal under physiological conditions of pH and temperature, and equilibrium was reached within 50 min. Specific binding for D-Asp and L-Glu was saturable, and Eadie–Hofstee analysis revealed interaction with a single population of binding sites (for D-Asp $K_d = 860 \pm 28$ nM, $B_{max} = 27.2 \pm 0.5$ pmol/mg protein; for L-Glu, $K_d = 580 \pm 15$ nM and $B_{max} = 51.3 \pm 0.8$ pmol/mg protein). L-[^{3}H]glutamate had higher affinity and a greater percentage of specific binding than did D-[^{3}H]aspartate. The pharmacological binding specificity of L-[^{3}H]glutamate indicated an interaction with NMDA-type receptors. Specifically, the order of potency of the displacing compound tested was L-Glu > D-Asp > NMDA > MK801 > D-AP5 > glycine. For D-[^{3}H]aspartate, the data revealed an interaction of D-Asp with either NMDA-type receptors or putative specific binding sites.

Keywords Harderian gland · D-Aspartate · Binding sites · Rat

M. Di Giovanni (✉) · A. Santillo · G. Chieffi Baccari (✉)
Dipartimento Scienze della Vita,
Seconda Università degli Studi di Napoli,
Via Vivaldi 43, Caserta, Italy
e-mail: marcello.digiovanni@unina2.it

G. Chieffi Baccari
e-mail: gabriella.chieffi@unina2.it

M. Di Giovanni · E. Topo · A. D'Aniello
Laboratory Animal Physiology and Evolution,
Stazione Zoologica Anton Dohrn, Villa Comunale, Naples, Italy

Introduction

The anatomical site of the orbital Harderian gland (HG) originally suggested that its main function was to lubricate the eye. However, numerous studies in either mammals or low vertebrates have indicated that it may be involved in several different functions (for reviews see Payne 1994; Chieffi et al. 1996), including roles in thermoregulation (Thiessen and Kittrell 1980; Thiessen 1988) or photoprotection, as part of the retinal-pineal axis (Hoffman et al. 1989), and as a source of either pheromones (Payne 1979; Thiessen and Harriman 1986) or growth factors (for review, see Chieffi et al. 1996). The rat HG synthesizes lipids, which pour into the conjunctival sac (Seyama et al. 1992), as well as porphyrins, which accumulate as solid luminal accretions (Spike et al. 1990). In a recent study, we demonstrated that substantial amounts of D-aspartate (D-Asp) are contained endogenously within the HG, along with aspartate racemases that generate D-Asp from L-Asp and vice versa (Raucci et al. 2005; Santillo et al. 2006; Monteforte et al. 2008). Furthermore, this gland is capable of uptaking and of accumulating exogenously administered D-Asp (Raucci et al. 2005; Santillo et al. 2006; Monteforte et al. 2008). Although the excitatory amino acid D-aspartate plays crucial roles in many functions of the central nervous system (see for review, D'Aniello 2007), its presence in the peripheral tissues is still unclear. Moreover, our recent studies have indicated that D-Asp plays a prominent role in eliciting exocrine secretion, and thus it could represent a novel secretagogue molecule for exocrine tissues (Raucci et al. 2005; Santillo et al. 2006; Monteforte et al. 2008). Particularly, we proved that D-Asp acute treatment markedly increases secretory activity in the HG through extracellular signal-regulated protein kinase (ERK) pathway (Raucci et al. 2005; Monteforte et al. 2008), a finding

that led us to hypothesize the presence of receptor-binding sites for D-Asp in the rat HG.

Therefore, in the present study we investigated the specificity, kinetics, and stoichiometry of D-Asp binding sites in plasma membranes from rat HG. In addition, since it is known that D-Asp in the rat nervous system is recognized by L-glutamate receptors (Foster and Fagg 1987), we also examined the presence of receptor-binding sites for L-Glu.

Materials and methods

Chemicals

D-[2,3-^{3}H]aspartic acid (cod. TRK606, 10–30 Ci/mmol; 1 mCi/ml) and L-[3,4-^{3}H]glutamic acid (cod. TRK445, 55.0 Ci/mmol; 1 mCi/ml) were obtained from Amersham Biosciences UK. All common analytic reagents and drugs (unless otherwise indicated) were from Sigma, UK Ltd.

Animals and preparation of membranes

Adult Wistar male rats, *Rattus norvegicus albinus* (2 months old, 300–350 g) were purchased from Charles River laboratory. Animals were kept under regulated conditions of temperature (28°C), exposed to an automatically regulated light–dark (LD) cycle of 12:12, and received laboratory food pellets and water ad libitum. The experimental protocol, as well as the housing conditions, were in accordance with the Italian guidelines (D.Lvo116/92) and authorized by the local Animal Care Committee (Servizio veterinario ASL 44, Prot. Vet. 22/95). The animals were killed by decapitation, and the Harderian glands were rapidly dissected. For plasma membrane preparations, glands were homogenized according to the method described by Guerrero et al. (1996), with some modifications. In brief, glands were first homogenized using Ultra-Turrax T25 homogenizer with Krebs-Ringer solution (NaCl 110 mM; KCl 4.6 mM; $CaCl_2$ 10 mM, $MgCl_2$ 6.6 mM; $NaHCO_3$ 25 mM; NaH_2PO_4 14 mM; Glucose 15 mM, HEPES 10 mM, pH 7.4, and a cocktail of protease inhibitors from Sigma Chemical Company, code P 8340) containing 0.25 M sucrose, 0.5 mM EDTA, pH 7.5. They were then centrifuged at 1,500 g for 15 min at 2–4°C. Next, the supernatant was centrifuged at 30,000 g for 30 min and washed twice with Krebs-Ringer solution. Last, the final pellet was resuspended in Krebs-Ringer to obtain a membrane protein concentration of 2 mg/ml that was used for the binding assay. Protein concentration was determined by Bradford method (Bradford 1976) using bovine serum albumin as standard.

For binding studies in nuclei, we used a protocol described by previous authors (Coto-Montes et al. 2003). In brief, glandular tissues (1.2–1.5 g) were homogenized in 3 ml of Tris-HCl 10 mM, pH 7.5 containing $CaCl_2$ 3 mM, $MgCl_2$ 2 mM, dithiothreitol 0.5 mM, sucrose 0.3 M, 0.15% Triton X–100 in a glass/Teflon homogenizer and layered over 3 ml of the same buffer containing 0.4 M sucrose. The samples were then centrifuged at 2,500 g for 10 min. The resulting nuclear pellet was resuspended without vortexing in 1 ml of the same buffer and centrifuged once again. All the procedures described above were performed at 4°C. Finally, the nuclei were resuspended in 1 ml of Tris-HCl 50 mM, pH 7.5, disrupted using a Polytron homogenizer, and frozen to −80°C until the binding assay was performed (usually within 48 hr).

Binding assay

The binding assay was carried out by using D-[2,3-^{3}H]aspartic acid or L-[3,4-^{3}H]glutamic acid as radioligand (Foster et al. 1981). In this procedure, membrane-bound radioligands are separated from free ligands by centrifugation. Optimal binding parameters were performed with 150 nmoles of D-[^{3}H]aspartate incubated with 200 μg of membrane protein for 1 h at 4°C in Krebs solution (pH 7.4). Experiments were performed three times; each data point was the mean of triplicate determinations. Binding data at equilibrium were obtained by two different processes: (1) saturation experiments, in which radioactive ligands concentrations varied and (2) competition experiments, in which concentrations of unlabeled ligands varied.

Saturation analyses

For the saturation binding assay, 200 μg of membranes was incubated with concentrations of labeled D-Asp (or L-Glu) ranging between 0 and 2,000 nM. The tubes were incubated under shaking at 18°C for 60 min. Then after centrifugation for 10 min at 13,000 g at 2–6°C, the supernatant was aspirated. Next, the tubes were washed twice with 2 ml of ice-cold Krebs-Ringer solution without disturbing the pellets. The pellets containing bound radioligands were first dissolved in 0.2 ml 1 M NaOH and then mixed with 3 ml liquid scintillation fluid. Finally, the radioactivity was determined. To investigate the effect of Na^+, K^+ and Mg^{2+} on specific D-[^{3}H]aspartate and L-[^{3}H]glutamate bindings, 200 μg of HG membranes were incubated in Tris-HCl buffer (0.05 M, pH 7.4) with increasing concentrations of tested cations. Final concentrations of labeled D-Asp or L-Glu were 150 nM. To calculate the specific bindings of D-[^{3}H]aspartic acid

(or L-[^{3}H]glutamic acid), the non-specific value consistently present in 1 mM unlabeled ligands was subtracted from the amount of the total binding (occurring in the absence of non-radioactive ligands).

Competition experiments

For the competitive binding assays, 100 µl of the membrane suspension was placed in a 2 ml Eppendorf (polypropylene) microcentrifuge tube along with 50 µl of D-[^{3}H]aspartic acid (10 pmol) and 50 µl of one of the following unlabeled acids: D-Asp or L-Glu, NMDA, Gly, MK-801, D-AP5 and DNQX at final concentrations ranging between 0 and 2,500 µM. Assay conditions were the same as those used in the saturation assays. Total binding of D-[^{3}H]aspartic acid was determined using 50 µl of Krebs-Ringer instead of cold D-Asp. To determine the binding affinity for receptors to L-glutamate, L-[^{3}H]glutamic acid was used instead of D-[^{3}H]aspartic acid. Finally, total binding of labeled L-Glu was assayed by using 50 µl of Krebs-Ringer instead of cold L-Glu.

Statistical analysis

Data from binding experiments were analyzed using GraphPad Prism, (GraphPad software, San Diego, CA, USA), which yields the equilibrium binding constants (i.e., B_{max}, maximal number of binding sites; K_D, dissociation constant, and IC_{50}, median inhibition concentration). Statistical evaluation of data was performed with this software program using Student paired *t*-tests. Values of $P < 0.05$ and $P < 0.01$ were considered statistically significant.

Results

D-aspartate and L-glutamate bindings to HG membranes

Effects of temperature, pH and time course of equilibrium binding

Specific D-[^{3}H]aspartate and L-[^{3}H]glutamate bindings to HG membranes were affected by temperature and pH changes. As shown in Fig. 1a, temperatures higher than 30°C inhibited both D-[^{3}H]aspartate and L-[^{3}H]glutamate from binding to HG membranes. As regards pH changes, higher affinity was detected at pH 7.5 (Fig. 1b). When the time course was examined, we found that equilibrium was attained after approx. 60 min (Fig. 1c). The specific binding, which was measured at concentration of 150 nM D-[^{3}H]aspartate and 150 nM L-[^{3}H]glutamate, represented about 40 and 50% of total binding, respectively.

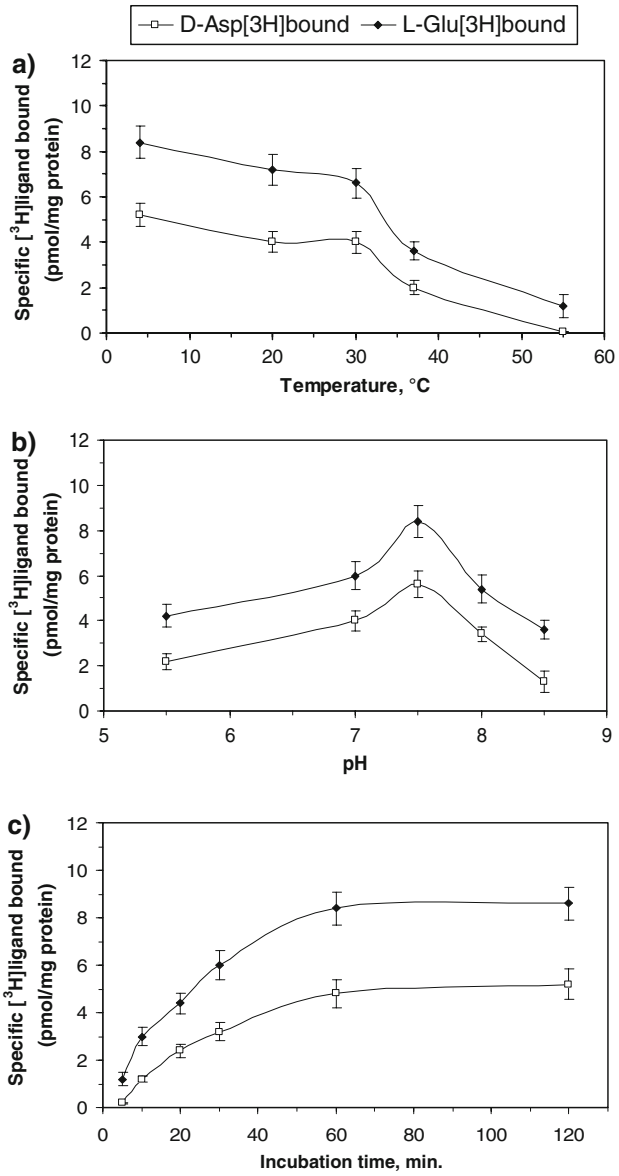

Fig. 1 Effect of temperature (**a**), pH (**b**) and incubation time (**c**) on D-[^{3}H]aspartate and L-[^{3}H]glutamate bindings to Harderian gland membranes. Labeled ligand concentration was 150 nM for both amino acids. The results are means ± SEM of triplicate determinations for each point

Saturability of specific D-[^{3}H]aspartate and L-[^{3}H]glutamate bindings

HG membranes were incubated with increasing concentrations of labeled D-Asp or L-Glu (from 0 to 2,000 nM). The specific bindings were saturable: for D-Asp we found $K_d = 860 \pm 28$ nM and $B_{max} = 27.2 \pm 0.5$ pmol/mg protein (Fig. 2a); for L-Glu, we found $K_d = 580 \pm 15$ nM and $B_{max} = 51.3 \pm 0.8$ pmol/mg protein (Fig. 2b). As demonstrated by the straight line on the graphical representation of the data (Eadie–Hofstee) (Fig. 3), only a homogeneous population of binding sites was involved.

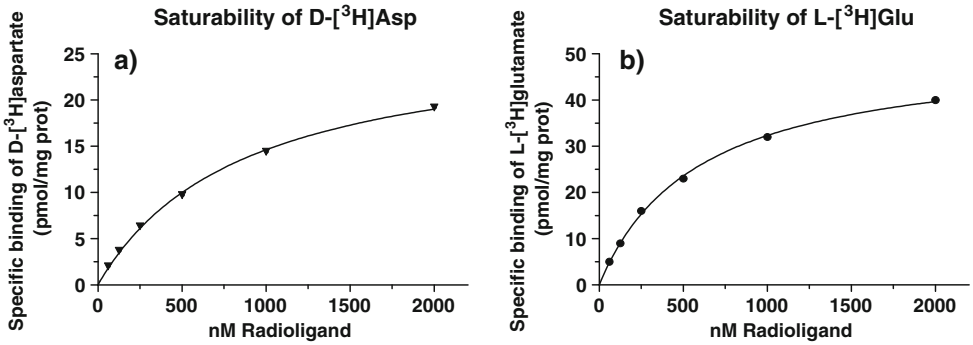

Fig. 2 Saturability of specific D-[^{3}H]aspartate (**a**) and L-[^{3}H]glutamate (**b**) bindings to rat HG membranes. Only specific binding is shown. The results are mean values obtained from triplicate determinations for each point. SEM values are too small to be shown

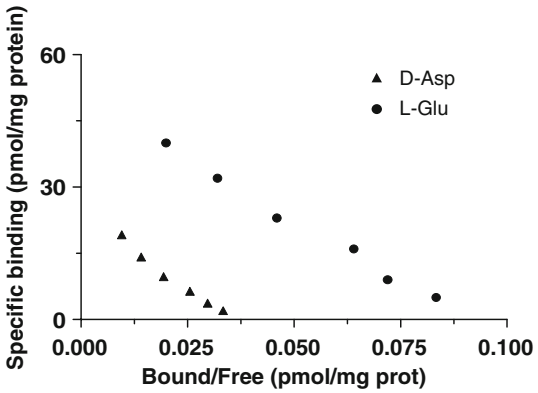

Fig. 3 Eadie–Hofstee plot of D-[^{3}H]aspartate and L-[^{3}H]glutamate bindings. Each linear plot indicates the homogeneity of binding sites. Each point is the mean of triplicate experiments. SEM values are too small to be shown

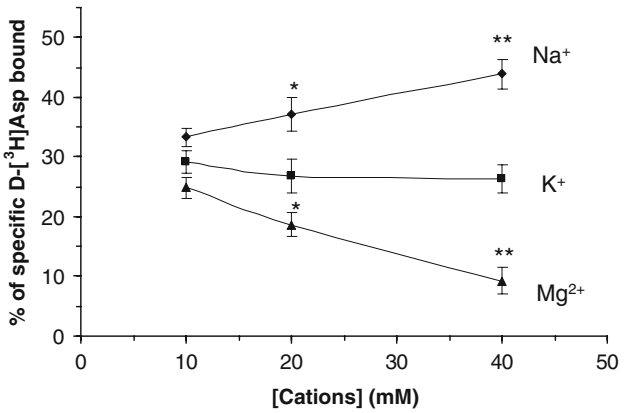

Fig. 4 Effect of cations on specific D-[^{3}H]aspartate binding. Final concentration of D-[^{3}H]aspartate was 150 nM. The results are means ± SEM obtained from triplicate determinations for each point. * $P < 0.05$ versus 10 mM, ** $P < 0.01$ versus 10 mM

Although the dissociation constants (K_ds) of D-Asp and L-Glu were similar, the density of binding sites for L-glutamate was approximately two times higher than that for D-aspartate.

Effect of cations on D-[^{3}H]aspartate and L-[^{3}H]glutamate specific bindings

Na^+, K^+ and Mg^{2+} were tested to verify their ability to modify specific D-aspartate and L-glutammate bindings to Harderian gland membranes. When D-[^{3}H]aspartate was used, Mg^{2+} produced a dose-dependent inhibition of aspartate binding sites, whereas Na^+, at concentration up to 20 mM, produced an enhancement of the same binding sites (Fig. 4). The presence of Na^+, K^+ and Mg^{2+} did not modify the percentage of L-glutamate binding on HG membranes (about 50%).

Displacement of D-[^{3}H]aspartate and L-[^{3}H]glutamate specific bindings

To obtain information about specificity of glutamate and aspartate binding sites, the ability of a number of compounds to displace radioligand bindings was tested. These parameters were expressed as IC_{50} values, i.e., Log dose/percentage inhibition plots for active compound at 150 nM labeled amino acid concentrations. Figure 5 shows the percentage of inhibition of amino acids (L-Glu, D-Asp, NMDA and Gly) and of both NMDAR (MK801, D-AP5) and non-NMDAR (DNQX) antagonists at D-[^{3}H]aspartate and L-[^{3}H]glutamate binding sites. When D-[^{3}H]aspartic acid was used as the radioligand, both D-Asp and L-Glu showed a displacing activity toward the D-[^{3}H]aspartic acid bind (Fig. 5a). IC_{50} was 28 μM for D-Asp and 32 μM for L-Glu, whereas NMDA and glycine showed low affinity in binding inhibition (IC_{50} 150, 200 μM, respectively) (Fig. 5a). When MK801 (potent selective and non-competitive NMDA receptor antagonist), D-AP5 (NMDA receptor antagonist) and DNQX (selective non-NMDA receptor antagonist) were used as antagonists, they did not have any significant displacement effect ($IC_{50} > 1$ mM) (not shown). On the contrary, when L-[^{3}H]glutamate acid was used as the radioligand, both L-Glu and D-Asp showed significant binding displacement (Fig. 5b), with L-Glu showing higher binding affinity than D-Asp (IC_{50} was 10.7 μM for L-Glu and 23.6 μM for D-Asp). NMDA also showed significant displacing activity toward L-[^{3}H]glutamate (IC_{50} 50 μM)

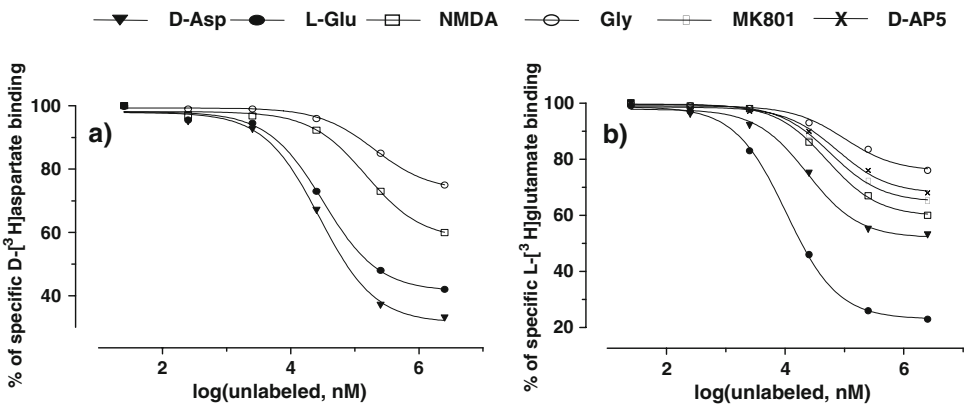

Fig. 5 Competitive inhibition of D-[^{3}H]aspartate (**a**) and L-[^{3}H]glutamate (**b**) bindings by amino acids (L-Glu, D-Asp, NMDA and Gly) and NMDAR (MK801, D-AP5) and non-NMDAR (DNQX) antagonists. Each point is the mean of triplicate experiments. SEM values are too small to be shown

(Fig. 5b). Glycine had little effect in displacing L-[^{3}H]glutamate (IC_{50} 100 μM) (Fig. 5b). In addition, NMDA receptor antagonists (MK801, D-AP5) displayed a consistent displacer activity (IC_{50} 60, 75 μM, respectively) for L-[^{3}H]glutamate acid (Fig. 5b) whereas DNQX did not show any binding affinity (not shown).

Discussion

We here report for the first time evidence that D-aspartate and L-glutamate-binding proteins are present in the rat Harderian gland. These findings should not be surprising if we consider that high endogenous concentrations of D-Asp are present in rat HG and that this amino acid accumulates in the gland when it is administered in vivo (Monteforte et al. 2008). Specific binding sites of both D-Asp and L-Glu were detected in HG plasma membranes but not in nuclear membranes.

We found that D-[^{3}H]aspartic acid and L-[^{3}H]glutamate acid bind saturably to an apparently homogeneous population of sites on HG membranes. The binding of both D-Asp and L-Glu occurred optimally under physiological conditions of pH and temperature. Our results indicate that the K_d for D-Asp binding sites is higher than that for L-Glu binding sites, and, in addition, D-Asp shows fewer binding sites with respect to L-Glu in rat HG. What makes these findings particularly intriguing is the fact that although D-Asp is largely distributed in the tissues of several species of vertebrates and invertebrates (for review, see D'Aniello 2007), binding sites for this amino acid have been described only in the rat's nervous system (Foster and Fagg 1987; Takamoto et al. 2002). Although it is not clear how endogenous D-Asp is transported, stored and released in vivo, it has been demonstrated that D-Asp can be taken up into cells through high affinity L-glutamate and L-aspartate transporters (Davies and Johnston 1975; Albus and Habermann 1983; Palmer and Reiter 1994) thus accumulating in astrocytes and neurons, including synaptosomes (Gundersen et al. 1995; Fleck et al. 2001; Waagepetersen et al. 2001). In addition, Anderson and Vickroy (1990) demonstrated a selective interaction of D-[^{3}H]aspartic acid with excitatory amino acid transporters, but not with GluRs, in rat forebrain. High D-Asp levels have been described during embryonic development, but as yet the functional significance of this is unclear. Notably, since D-Asp is an agonist for L-Glu receptor, it may be involved in the development and neurogenesis of the brain. Furthermore, since the brain is a steroidogenic tissue like the testis and adrenal gland, D-Asp may modulate the production of neurosteroids in the brain, similar to that in latter tissues (Furuchi and Homma 2005).

Our studies of the pharmacological characteristics of D-Asp binding sites revealed that the binding of D-[^{3}H]aspartic acid is Na^+-dependent. Particularly, we found that the addition of Na^+ in a binding assay substantially enhanced binding activity for D-Asp, whereas the addition of Mg^{2+} inhibited it. Moreover, the addition of Na^+ or Mg^{2+} in the binding assay did not modify L-Glu binding.

Intriguingly, when L-[^{3}H]glutamate was used for the binding assay, we observed that all compounds, viz, the amino acids L-Glu, D-Asp, NMDA, glycine, and the putative antagonists (MK801 and D-AP5) displaced L-[^{3}H]glutamate with different activity (L-Glu > D-Asp > NMDA > MK801 > D-AP5 > glycine). Finally, when DNQX was used as a displacer, it did not display any binding affinity. Thus, these data indicate that the HG membranes of rats possess putative glutamate binding sites of NMDA-type to which D-Asp binds, though to a lesser extent than L-Glu. Accordingly, Foster and Fagg (1987) described in rat brain specific binding sites for L-Glu receptors which also bind efficiently D-Asp. The hypothesis that rat HG membranes possess NMDA receptors is supported by our recent study in which we demonstrated expression of NMDAR subunits (NR1-NR2A-NR2B-NR2D) in this gland (Monteforte et al. 2008). According to studies carried out in rat brain (Watkins and Evans 1981; Mayer and Westbrook 1985; Kiskin et al. 1990), our

previous study suggested that D-Asp is able to activate NMDARs in rat HG (Monteforte et al. 2008).

Interestingly, when the binding of D-[^{3}H]aspartic acid was tested in the presence of L-Glu and D-Asp, these amino acids showed high displacing activity, with D-Asp showing a lower IC_{50} than did L-Glu. On the contrary, glycine and NMDA showed low displacing activity, whereas NMDAR antagonists (MK801 and D-AP5) and non-NMDAR antagonist (DNQX) did not show any displacer activity.

In conclusion, on the basis of the present findings, we cannot rule out the hypothesis that the rat HG, besides possessing L-glutamate receptors to which D-Asp binds, also possesses specific high-affinity binding sites for D-Asp. Undoubtedly, further molecular research is warranted to provide a more thorough characterization of these sites.

Acknowledgment We thank Dr. Paola Merolla for English editorial assistance.

References

Albus U, Habermann E (1983) Tetanus toxin inhibits the evoked outflow of an inhibitory (GABA) and an excitatory (D-aspartate) amino acid from particulate brain cortex. Toxicon 21:97–110. doi:10.1016/0041-0101(83)90053-3

Anderson KJ, Vickroy TW (1990) Anatomical and pharmacological heterogeneity of D-[^{3}H]aspartate binding sites. Eur J Pharmacol 185:119–121. doi:10.1016/0014-2999(90)90220-Z

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Chieffi G, Chieffi Baccari G, Di Matteo L, d'Istria M, Minucci S, Varriale B (1996) Cell biology of harderian gland. Int Rev Cytol 168:1–80. doi:10.1016/S0074-7696(08)60882-7

Coto-Montes A, Tomás-Zapico C, Escames G, León J, Tolivia D, JosephaRodríguez-Colunga M, Acuña-Castroviejo D (2003) Characterization of melatonin high-affinity binding sites in purified cell nuclei of the hamster (*Mesocricetus auratus*) harderian gland. J Pineal Res 34(3):202–207. doi:10.1034/j.1600-079X.2003.00029.x

D'Aniello A (2007) D-Aspartic acid: an endogenous amino acid with an important neuroendocrine role. Brain Res Brain Res Rev 53:215–234. doi:10.1016/j.brainresrev.2006.08.005

Davies LP, Johnston GA (1975) D-aspartate oxidase activity in extracts of mammalian central nervous tissue. J Neurochem 25:299–304. doi:10.1111/j.1471-4159.1975.tb06970.x

Fleck MW, Barrionuevo G, Palmer AM (2001) Synaptosomal and vesicular accumulation of L-glutamate, L-aspartate and D-aspartate. Neurochem Int 39:217–225. doi:10.1016/S0197-0186(01)00018-3

Foster AC, Fagg GE (1987) Comparison of L-[^{3}H]glutamate, D-[^{3}H]aspartate, DL-[^{3}H]AP5 and [^{3}H]NMDA as ligands for NMDA receptors in crude postsynaptic densities from rat brain. Eur J Pharmacol 133:291–300. doi:10.1016/0014-2999(87)90025-2

Foster AC, Fagg GE, Mena EE, Cotman CW (1981) L-glutamate and L-aspartate bind to separate sites in rat brain synaptic membranes. Brain Res 229:246–250. doi:10.1016/0006-8993(81)90765-4

Furuchi T, Homma H (2005) Free D-aspartate in mammals. Biol Farm Bull 28:1566–1570. doi:10.1248/bpb.28.1566

Guerrero JM, Osuna C, Molinero P, Caraballo MI, Harmouch A, Pozo D, Rafii-el-Idrissi M, Garcia-Macias JF, Calvo JR (1996) Characterization of binding sites for beta-adrenergic agonists and vasoactive intestinal peptide in the rat harderian gland. Microsc Res Tech 34:139–143. doi:10.1002/(SICI)1097-0029(19960601)34:2<139::AID-JEMT7>3.0.CO;2-N

Gundersen V, Shupliakov O, Brodin L, Ottersen OP, Storm-Mathisen J (1995) Quantification of excitatory amino acid uptake at intact glutamatergic synapses by immunocytochemistry of exogenous D-aspartate. J Neurosci 15:4417–4428

Hoffman RA, Johnson LB, Reiter RJ (1989) Regulation of melatonin in the harderian glands of golden hamsters. J Pineal Res 6:63–71. doi:10.1111/j.1600-079X.1989.tb00403.x

Kiskin NI, Krishtal OA, Tsyndrenko AY (1990) Cross-desensitization reveals pharmacological specificity of excitatory amino acid receptors in isolated hippocampal neurons. Eur J NeuroSci 2:461–470. doi:10.1111/j.1460-9568.1990.tb00437.x

Mayer ML, Westbrook GL (1985) The action of N-methyl-D-aspartic acid on mouse spinal neurones in culture. J Physiol 361:65–90

Monteforte R, Santillo A, Di Giovanni M, D'Aniello A, Di Maro A, Chieffi Baccari G (2008) D-aspartate affects secretory activity in rat Harderian gland: molecular mechanism and functional significance. Amino Acids (epub ahead of print, 27 Sept)

Palmer AM, Reiter CT (1994) Comparison of the superfused efflux of preaccumulated D-[^{3}H]aspartate and endogenous L-aspartate and L-glutamate from rat cerebrocortical minislices. Neurochem Int 25:441–450. doi:10.1016/0197-0186(94)90020-5

Payne AP (1979) The attractiveness of harderian gland smears to sexually naive and experienced male golden hamsters. Anim Behav 27:897–904. doi:10.1016/0003-3472(79)90027-7

Payne AP (1994) The harderian gland: a tercentennial review. J Anat 185:1–49

Raucci F, Santillo A, D'Aniello A, Chieffi P, Chieffi Baccari G (2005) D-Aspartate modulates transcriptional activity in Harderian gland of frog, *Rana esculenta*: morphological and molecular evidence. J Cell Physiol 204:445–454. doi:10.1002/jcp.20316

Santillo A, Monteforte R, Raucci F, D'Aniello A, Chieffi Baccari G (2006) Occurrence of D-aspartate in the Harderian gland of *Podarcis s. sicula* and its effect on gland secretion. J Exp Zool 305:610–619. doi:10.1002/jez.a.301

Seyama Y, Kasama T, Yasugi E, Park SH, Kano K (1992) Lipids in Harderian glands and their significance. In: Webb SM, Hoffman RA, Puig-Domingo ML, Reiter RJ (eds) Harderian glands: porphyrin metabolism, behavior and endocrine effects. Springer, Berlin

Spike RC, Payne AP, Thompson GG, Moore MR (1990) Porphyrin profiles in hamster harderian glands. Biochem Soc Trans 18:630–631

Takamoto A, Quiggin LB, Lieb I, Shave E, Balcar VJ, Yoneda Y (2002) Differences between D- and L-aspartate binding to the Na^+-dependent binding sites on glutamate transporters in frozen sections of rat brain. Life Sci 70:991–1001. doi:10.1016/S0024-3205(01)01478-3

Thiessen DD (1988) Body temperature and grooming in the Mongolian gerbil. Ann N Y Acad Sci 525:27–39. doi:10.1111/j.1749-6632.1988.tb38593.x

Thiessen DD, Kittrell EM (1980) The Harderian gland and thermoregulation in the gerbil (*Meriones unguiculatus*). Physiol Behav 24:417–424. doi:10.1016/0031-9384(80)90229-2

Thiessen DD, Harriman AE (1986) Harderian gland exudates in the male *Meriones unguiculatus* regulate female proceptive behavior, aggression, and investigation. J Comp Psychol 100:85–87. doi:10.1037/0735-7036.100.1.85

Waagepetersen HS, Shimamoto K, Schousboe A (2001) Comparison of effects of DL-threo-beta-benzyloxyaspartate (DL-TBOA) and L-trans-pyrrolidine-2, 4-dicarboxylate (t-2, 4-PDC) on uptake and release of [3 h]D-aspartate in astrocytes and glutamatergic neurons. Neurochem Res 26:661–666. doi:10.1023/A:1010939304104

Watkins JC, Evans RH (1981) Excitatory amino acid transmitters. Annu Rev Pharmacol Toxicol 21:165–204. doi:10.1146/annurev.pa.21.040181.001121