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Differential Effects of Glutamate Agonists and D-Aspartate on Oxytocin Release from Hypothalamus and Posterior Pituitary of Male Rats

Macarena Pampillo,[1] María del Carmen Díaz,[1] Beatriz H. Duvilanski,[1] Valeria Rettori,[2] Adriana Seilicovich,[1] and Mercedes Lasaga[1]

[1]Centro de Investigaciones en Reproducción, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires (1121), Argentina; [2]Centro de Estudios Farmacológicos y Botánicos, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires (1414), Argentina

In order to determine whether ionotropic (iGluRs) and metabotropic (mGluRs) glutamate receptor activation modulates oxytocin release in male rats, we investigated the effect of agonists of both types of glutamate receptors on oxytocin release from hypothalamus and posterior pituitary. Kainate and quisqualate (1 m*M*) increased hypothalamic oxytocin release. Their effects were prevented by selective AMPA/kainate receptor antagonists. NMDA (0.01–1 m*M*) did not modify hypothalamic oxytocin release. Group I mGluR agonists, such as quisqualate and 3-HPG, significantly increased hypothalamic oxytocin release. These effects were blocked by AIDA (a selective antagonist of group I mGluRs). In the posterior pituitary, oxytocin release was not modified by kainate, quisqualate, trans-ACPD (a broad-spectrum mGluR agonist) and L-SOP (a group III mGluR agonist). However, NMDA (0.1 m*M*) significantly decreased oxytocin release from posterior pituitary. D-Aspartate significantly increased oxytocin release from the hypothalamus, while it decreased oxytocin release from posterior pituitary. AP-5 (a specific NMDA receptor antagonist) reduced the D-Aspartate effect in the hypothalamus, but not in the posterior pituitary. Our data indicate that the activation of non-NMDA receptors and group I mGluRs stimulates oxytocin release from hypothalamic nuclei, whereas NMDA inhibits oxytocinergic terminals in the posterior pituitary. D-Aspartate also has a dual effect on oxytocin release: stimulatory at the hypothalamus and inhibitory at the posterior pituitary. These results suggest that excitatory amino acids differentially modulate the secretion of oxytocin at the hypothalamic and posterior pituitary levels.

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Author to whom all correspondence and reprint requests should be addressed: Mercedes Lasaga, Centro de Investigaciones en Reproducción, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Piso 10, Buenos Aires (1121), Argentina, E-mail: mlasaga@fmed.uba.ar

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Introduction

Oxytocin is synthesized in magnocellular neurons of the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) and in accessory magnocellular nuclei of the hypothalamus *(1)*. After synthesis, oxytocin is transported along the axons of these neurons to the neural lobe of the pituitary, where it is released into the bloodstream. Also, magnocellular neurons from the PVN project into the external zone of the median eminence, where oxytocin can be released into portal blood *(2)*. The somatodendritic release of oxytocin is the mechanism by which most of the peptide is released in magnocellular neurons of SON and PVN *(3)*. PVN is also composed of parvocellular neurons, which contribute to the centrally released oxytocin *(4)*.

The effects of oxytocin on male and female sexual behavior, feeding behavior, and grooming, plus its effects on the uterus and the mammary gland, indicate that this neurohormone plays an important role in reproductive functions in both sexes *(5)*. Moreover, oxytocin is involved in social stress-related behaviors *(5)*, the processing of cognitive information *(6)*, and central cardiovascular *(7)* and hydromineral homeostasis regulation *(8)*.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS), where it plays an important role in a number of functions *(9)*. Glutamate acts on two general types of receptors: ion channel-linked ionotropic receptors (iGluRs) and G protein-coupled metabotropic receptors (mGluRs). iGluRs are composed of multiple subunit proteins. Based on their primary sequence and agonist sensitivity, iGluRs are classified into three subtypes: *N*-methyl-D-aspartate (NMDA), kainate, and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) *(10)*. mGluRs are responsible for the modulatory actions of glutamate *(11)*, and eight mGluRs have been identified and

Table 1
Effect of Different Concentrations of Kainate and Quisqualate (an AMPA and Group I mGluRs Agonist) on Hypothalamic Oxytocin Release[a]

	0 m*M*	0.01 m*M*	0.1 m*M*	1 m*M*
Kainate	21.00 ± 1.86 (4)	15.95 ± 1.99 (5)	25.12 ± 1.77 (5)	32.86 ± 0.59 (4)**
Quisqualate	14.62 ± 1.21 (6)	14.98 ± 2.52 (4)	15.49 ± 1.42 (4)	23.88 ± 3.32 (5)*

[a]Data are expressed as pg of oxytocin released in 30 min/mg protein. Values represent mean ± SEM. Numbers in brackets represent the number of determinations. Data were evaluated by one-way ANOVA followed by Dunnett´s test, *$p < 0.05$, **$p < 0.01$ vs control (0 m*M*).

classified on the basis of their primary structure and second messenger coupling. Group I receptors (mGlu 1 and 5) stimulate phosphatidylinositol phosphate hydrolysis, whereas group II (mGlu 2 and 3) and group III (mGlu 4,6,7 and 8) receptors inhibit adenylate cyclase activity *(11)*.

Glutamate receptors are localized in key neuroendocrine sites such as the hypothalamus and the pituitary gland. The SON and PVN have a high density of glutamate presynaptic terminals *(12)* that make contact with oxytocinergic magnocellular neurons *(13)*. In these nuclei, glutamate increases peptide release from isolated fragments of magnocellular dendrites *(14)*. Expression of different subunits of kainic and AMPA receptors has been demonstrated in the SON *(15–19)*. Also, the SON and the PVN are located in one of the most densely labeled brain regions for the NMDA receptor subunit NR1 *(20)*. SON magnocellular neurons express group I mGluRs, though at less density than iGluRs *(21,22)*. However, an immunohistochemical study using an anti-mGlu2 receptor antibody has shown that the SON contained densely stained neurons *(23)*. Both NMDA and non-NMDA receptor subtypes were observed in the posterior pituitary *(21)*. Also, the presence of group II metabotropic receptors has been shown in both anterior and posterior lobes of the pituitary gland *(23)*. The expression of ionotropic and metabotropic receptors in the hypothalamic neurohypophysial system suggests a neuroendocrine role of glutamate receptors in the control of posterior pituitary hormone release.

Excitatory amino acids participate in the regulation of the secretion of neurotransmitters such as GABA *(24)* and several neuropeptides, including oxytocin, through different receptor subtypes. However, there is controversy as to which subtype of iGluRs mediates glutamate action on oxytocinergic neurons. Although in vivo studies indicate that oxytocinergic neurons lack functional NMDA receptors *(25)*, in vitro studies suggest that oxytocinergic neurons are controlled by both NMDA and non-NMDA receptors *(26)*. Moreover, non-NMDA receptors have been shown to increase plasma oxytocin concentration in lactating rats *(27)*. The present study was designed to identify the type of glutamate receptor involved in the effect of glutamate on hypothalamic oxytocin release of male rats. Also, we investigated the effect of ionotropic and metabotropic glutamate agonists on oxytocin release from the posterior pituitary.

The presence of D-amino acids has been demonstrated in mammals *(28)*. The localization of D-aspartate (D-Asp) in CNS and endocrine glands suggests that it has a role in neuroendocrine modulation *(29)*. D-Asp has been found in most magnocellular neurons of SON and PVN, and in their nerve terminals at the posterior lobe of the pituitary gland *(29)*. Therefore, we also studied the effect of D-Asp on oxytocin release from hypothalamic explants and the posterior pituitary.

Results

Effect of Ionotropic and Metabotropic L-Glutamate Receptor Agonists on Oxytocin Release from Hypothalamic Explants

We first studied the effect of NMDA on oxytocin release from hypothalamic explants that included the SON and PVN in a Mg^{2+}-free medium. NMDA did not significantly affect oxytocin release at any concentration tested (control: 26.70 ± 2.80 pg/mg protein; NMDA 0.01 m*M*: 25.56 ± 5.11 pg/mg protein; NMDA 0.1 m*M*: 21.14 ± 2.46 pg/mg protein; NMDA 1 m*M*: 21.98 ± 1.68 pg/mg protein; $n = 5–10$). Kainate at 1 m*M* significantly increased hypothalamic oxytocin release (Table 1). The stimulatory effect of kainate on oxytocin release was specific, since it was blocked by DNQX (an AMPA/kainate receptor antagonist) (Fig. 1). Also, quisqualate at a dose of 1 m*M* significantly increased oxytocin release from hypothalamic explants (Table 1). The blockade of AMPA receptors by incubation with a relatively specific AMPA receptor antagonist *(30)*, GIKY 52466, significantly reduced the increase of oxytocin release induced by quisqualate (Fig. 2). Because quisqualate is also a potent agonist of group I mGluRs, we investigated the effect of quisqualate on oxytocin release in the presence of AIDA, a specific antagonist of group I mGluRs. This antagonist blocked the stimulatory effect of quisqualate (Fig. 3), suggesting that group I mGluRs are involved in mediating the stimulatory effect of glutamate on hypothalamic oxytocin release. To further investigate this topic, we studied the effect of 3-HPG, a specific group I mGluR agonist, on oxytocin release. 3-HPG significantly increased oxytocin release from hypothalamic explants (control: 31.74 ± 2.91 pg/mg protein, 3-HPG 0.1 m*M*: 50.13 ± 3.24 pg/mg protein, $n = 4–5$,

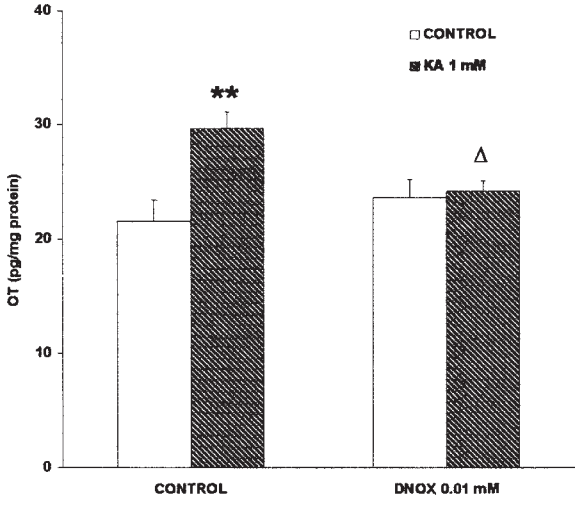

Fig. 1. Effect of kainate (KA) and DNQX (an AMPA/kainate receptor antagonist) on hypothalamic oxytocin release. Values represent mean ± SEM (n = 4–5 per group). Data were evaluated by two-way ANOVA. $^{**}p < 0.01$ vs respective control without kainate; $^{\Delta}p < 0.05$ vs respective control without DNQX.

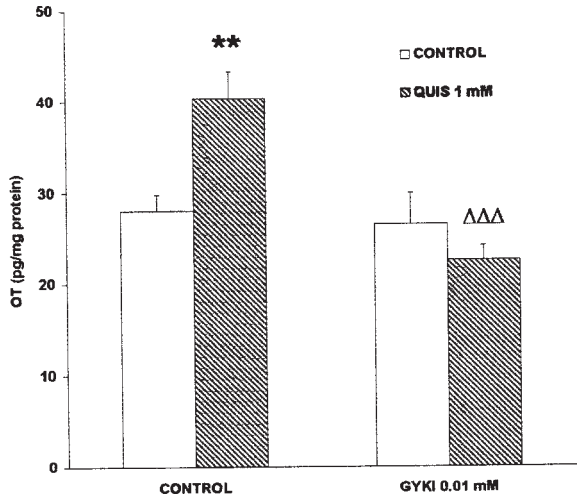

Fig. 2. Effect of quisqualate (QUIS) (an AMPA and group I mGluRs agonist) and GYKI 52466 (an AMPA receptor antagonist) on hypothalamic oxytocin release. Values represent mean ± SEM (n = 6 per group). Data were evaluated by two-way ANOVA. $^{**}p < 0.01$ vs respective control without quisqualate; $^{\Delta\Delta\Delta}p < 0.001$ vs respective control without GYKI 52466.

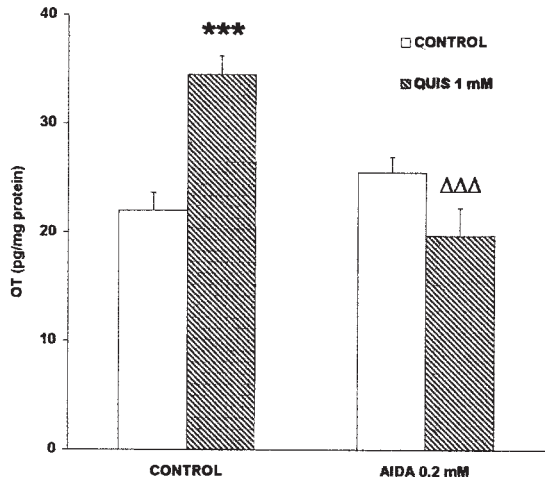

Fig. 3. Effect of quisqualate (QUIS) (an AMPA and group I mGluRs agonist) and AIDA (a group I mGluRs antagonist) on hypothalamic oxytocin release. Values represent mean ± SEM (n = 4–5 per group). Data were evaluated by two-way ANOVA. $^{***}p < 0.001$ vs respective control without quisqualate, $^{\Delta\Delta\Delta}p < 0.001$ vs respective control without AIDA.

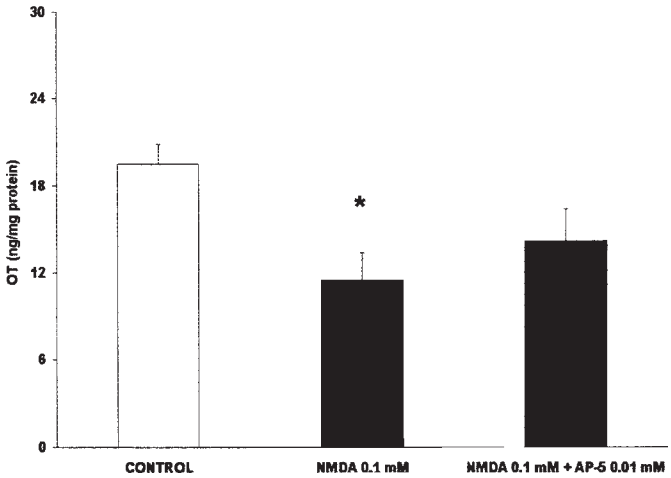

Fig. 4. Effect of NMDA on posterior pituitary oxytocin release. Values represent mean ± SEM (n = 4–6 per group). Data were evaluated by one-way ANOVA, followed by Student–Newman–Keuls multiple comparisons test. $^{*}p < 0.05$ vs control.

$p < 0.01$). 3-HPG did not affect oxytocin release at lower concentrations or in the presence of 0.2 m*M* AIDA (data not shown).

Effect of Ionotropic and Metabotropic L-Glutamate Receptor Agonists on Oxytocin Release from Posterior Pituitary

NMDA (0.1 m*M*) significantly decreased oxytocin release from the posterior pituitary in a Mg^{2+}-free medium. This inhibitory effect was reduced in the presence of AP-5, a NMDA receptor antagonist (Fig. 4). Kainate, quisqualate, trans-ACPD (a group I and II mGluR agonist), and L-SOP (a group III mGluR agonist), at the concentrations tested, (0.1–1 m*M*) had no effect on oxytocin release from posterior pituitaries (Table 2).

Effect of D-Aspartate on Oxytocin Release from Hypothalamic Explants and Posterior Pituitary

D-Aspartate significantly increased oxytocin release from hypothalamic explants only at 1 m*M* (control: 21.12 ± 0.87 pg/mg protein; D-Asp 0.1 m*M*: 20.25 ± 3.53 pg/mg protein, D-Asp 1 m*M*: 36.05 ± 2.86 pg/mg protein, n = 5–8,

Table 2
Effect of Kainate, Quisqualate (an AMPA and Group I mGluR Agonist), Trans-ACPD (a Group I and II mGluR Agonist), and L-SOP (a Group III mGluR Agonist), on Oxytocin Release from Posterior Pituitary[a]

	0 m*M*	0.1 m*M*	1 m*M*
Kainate	18.72 ± 1.80 (7)	31.16 ± 4.91 (5)	26.47 ± 5.11 (6)
Quisqualate	25.08 ± 3.11 (7)	26.84 ± 4.86 (7)	25.81 ± 5.16 (7)
trans-ACPD	11.25 ± 5.64 (4)	17.97 ± 0.52 (5)	10.41 ± 2.58 (5)
L-SOP	16.96 ± 3.00 (5)	17.56 ± 2.18 (5)	11.30 ± 0.73 (5)

[a]Data are expressed as ng of oxytocin released in 30 min/mg protein. Values represent mean ± SEM. Numbers in brackets represent the number of determinations. Data were evaluated by one-way ANOVA.

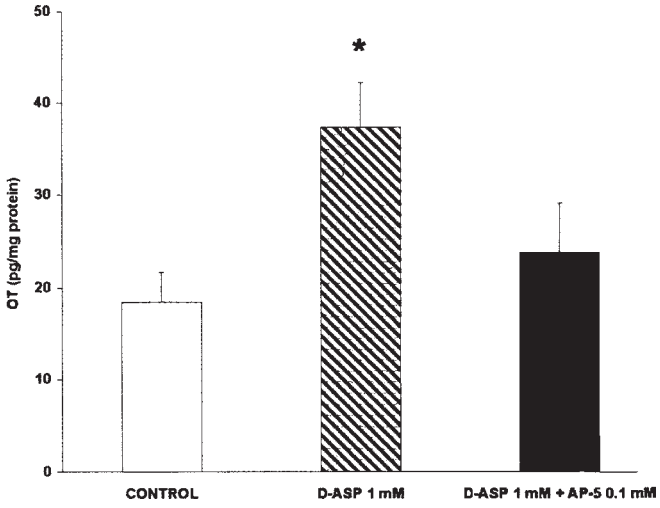

Fig. 5. Effect of D-aspartate (D-Asp) on hypothalamic oxytocin release. Values represent mean ± SEM (n = 5–6 per group). Data were evaluated by one-way ANOVA, followed by Student–Newman–Keuls multiple comparisons test. *$p < 0.05$ vs control.

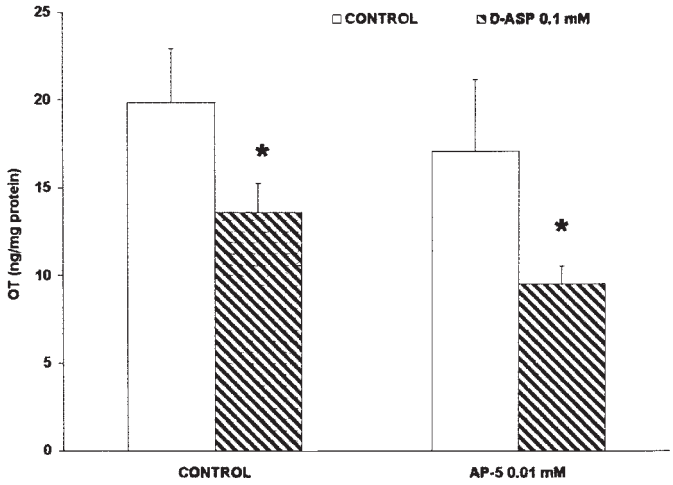

Fig. 6. Effect of D-aspartate (D-Asp) and AP-5 (a NMDA receptor antagonist) on posterior pituitary oxytocin release. Values represent mean ± SEM (n = 5–6 per group). Data were evaluated by two-way ANOVA. *$p < 0.05$ vs respective control without D-aspartate.

$p < 0.01$). This stimulatory effect was reduced by the presence of AP-5 (0.1 m*M*) (Fig. 5). On the other hand, D-Asp (0.1 m*M*) had an inhibitory effect on oxytocin release from the posterior pituitary (Fig. 6); this effect was not modified by addition of AP-5 to the incubation medium (Fig. 6).

Discussion

The present results indicate that in male rats the activation of kainate and AMPA iGluRs and group I mGluRs stimulates hypothalamic oxytocin release, whereas only NMDA inhibits oxytocin release from the axon terminals in the posterior pituitary. We observed no effect of NMDA on oxytocin release from hypothalamic nuclei. The involvement of NMDA receptors in the control of oxytocinergic neurons remains a matter of debate. Although oxytocinergic neurons in the SON express functional NMDA receptors *(19,21, 27,31,32)*, the in vivo local application of NMDA agonists in the SON did not activate these neurons *(33,34)*. Also, it has been suggested that NMDA receptors are not involved in the generation of the bursting activity of oxytocinergic neurons induced by suckling *(26)*. It has also been suggested recently that NMDA receptors colocalize with AMPA receptors in the SON, and that this colocalization could result in a negative modulation of NMDA receptors during glutamate receptor activation *(35)*. Thus, NMDA may act only on the fine tuning of bursting behavior and have no effect on basal activity of oxytocinergic neurons. Very recently, Morsette et al. *(36)* showed that NMDA elicited a late increase, after approximately 90 min, in oxytocin release from rat hypothalamo-neurohypophysial explants containing only the SON. These authors suggested an indirect effect of NMDA on oxytocin release from the explants.

We found that both kainate and quisqualate (an AMPA agonist) stimulate hypothalamic oxytocin release. These results are consistent with previous reports indicating that the major excitatory input to oxytocinergic neurons takes place essentially via non-NMDA receptors *(35,37,38)*. Pre-

vious studies showed a significant increase in oxytocin RNA in the PVN following kainic acid–induced seizures *(39)*. It has also been reported that kainate depolarizes oxytocinergic neurons in culture, and that injection of AMPA into the SON increases plasma oxytocin levels *(27)*. However, Morsette et al. *(36)* reported that oxytocin release from hypothalamo-neurohypophysial explants is mediated via AMPA receptors, rather than kainate receptors.

Our data also indicate that group I mGluRs participate in the stimulatory effect of glutamate on hypothalamic oxytocin release, since the stimulatory effects of 3-HPG (a selective group I mGluR agonist) and quisqualate, which also acts via group I mGluRs, were blocked by a specific group I mGluR antagonist (AIDA). Jourdain et al. *(40)* found that trans-ACPD (a group I and II agonist) did not activate oxytocinergic neurons in culture, and therefore ruled out the presence of metabotropic glutamate receptors in these neurons. However, Schrader and Tasker *(41)* found that the activation of group I metabotropic receptor subtype reduced K^+ currents in SON magnocellular neurons, whereas the activation of group II and III receptors had no direct effect on them, suggesting the presence of group I mGluRs in this hypothalamic area. Our results indicate that group I mGluRs may have a physiological role in the control of hypothalamic oxytocin release.

Glutamate immunoreactivity was observed in both pituicytes and neurosecretory endings of the neural lobe of the pituitary *(21)*. Significant binding of glutamate to NMDA and non-NMDA receptor subtypes was observed in the posterior pituitary, whereas binding of glutamate to the metabotropic receptor subtypes was lower than to the ionotropic subtypes *(21)*. Here, we report that only NMDA decreased oxytocin release from the posterior pituitary of male rats. The activation of other iGluRs and mGluRs did not affect oxytocin release from the posterior pituitary.

Although L-amino acids predominate in living organisms, substantial levels of D-Asp occur in mammals *(42)*. D-Asp is endogenously present as a natural molecule in rat CNS and endocrine glands such as adrenals, testes, pineal, and pituitary, and in almost all peripheral tissues of rats *(28,29)*. D-Asp immunoreactivity was observed in the anterior pituitary gland and in axon terminals of the posterior pituitary *(43)*. It has been suggested that D-Asp is produced in certain body tissues and transferred to other tissues via the vascular system *(44)*. D-Asp has been shown to stimulate GnRH release and modulate LH and GH secretion *(45)*. D-Asp functions as a neurotransmitter activating NMDA and, probably, other types of receptors *(29)*.

The current study provides evidence that D-Asp stimulates hypothalamic oxytocin release. It has recently been demonstrated that D-Asp administration increases oxytocin gene expression in the SON and PVN, and decreases the concentration of circulating oxytocin *(46)*. We found a differential effect of D-Asp depending on the tissue: D-Asp increases hypothalamic oxytocin release and decreases oxytocin release from the posterior pituitary.

D-Asp seems to exert its stimulatory effect on hypothalamic oxytocin release through NMDA receptors, since its effect was reduced in the presence of AP-5 (a NMDA receptor antagonist). The expression of functionally distinctive NMDA receptor subtypes has been reported in oxytocinergic cells *(47)*, which may be formed from various NR1-NR2 combinations, depending on subunit availability, with functional differences depending on the ease with which the channel can be opened *(48)*. Since we found no effect of NMDA on hypothalamic oxytocin release, it is possible to speculate that D-Asp could activate a functional variation of the NMDA receptor at the hypothalamic level. However, at the posterior pituitary, the inhibitory effect of D-Asp on oxytocin release was not reduced by AP-5, suggesting that the effect of D-Asp on the posterior pituitary is not mediated through NMDA receptors. D-Asp could act in the posterior pituitary independently of glutamate receptor activation, as has been shown in other tissues such as testes *(49)* and ovary *(50)*. Regardless of the receptor type through which D-Asp exerts its effects on oxytocinergic neurons, the modulation of D-Asp on oxytocin release may have an impact on the multiple functions controlled by oxytocin, such as reproductive functions, maternal, sexual, and social bonding behaviors *(51,52)*.

It has been established that oxytocinergic neurons release the peptide not only from their axon terminals, but also from somatodendritic membranes by exocytosis within the SON and PVN nuclei *(3,53,54)*. Tallying with this, exocytotic profiles were reported in the somatodendritic membrane of magnocellular neurons *(54)*. The release of oxytocin from the axon terminals occurs in response to electrical activity, which is regulated, in turn, by the locally released oxytocin. On the contrary, the dendritic release of oxytocin may be uncoupled from electrical activity *(53)*. Moreover, endogenous oxytocin, released from magnocellular dendrites, acts as a retrograde transmitter to affect afferent excitation *(55)*. Our results suggest that glutamate, acting through both ionotropic and metabotropic receptors and D-aspartate, could be controlling local oxytocin release, and, in this way, would be modulating the activity of oxytocinergic neurons. Therefore, region-specific effects of glutamate agonists and D-Asp on oxytocin release may reflect differential actions on the pattern of secretion from dendrites and axon terminals. The frequency of firing probably depends on a balance between excitatory and inhibitory inputs at both levels.

In summary, our findings suggest that glutamate has a stimulatory influence on the local somatodendritic release of oxytocin via kainate and AMPA receptors and group I mGluRs, whereas secretory activity is decreased in axon terminals in the posterior pituitary via NMDA receptors. D-Aspartate also has a dual effect on oxytocin release: stimulatory

at the hypothalamic level, and inhibitory at the posterior pituitary level. This differential effect may have some physiological relevance, since both amino acids could affect several physiological functions by modulating oxytocin release.

Materials and Methods

Male Wistar rats weighing 200–250 g were used. The animals were fed lab chow and water ad libitium and kept in controlled conditions of light (12 h light/dark) and temperature (20–25ºC). The animals were treated according to the NIH Guide for the Care and Use of Laboratory Animals. After decapitation and removal of the posterior pituitary and the brain, a hypothalamic explant including SON and PVN was dissected by making a transversal cut just forward of the optic chiasm, extending dorsally 2.5 mm. A horizontal cut extended from this point caudally to behind the pituitary stalk, where another frontal cut was made. Bilateral longitudinal cuts were made at the hypothalamic sulci.

In order to investigate oxytocin release, two hypothalamic explants or one posterior pituitary were preincubated for 15 min in a Dubnoff shaker (60 cycles per min) at 37ºC, in an atmosphere of 95% O_2–5% CO_2, in 0.5 mL of Krebs-Ringer bicarbonate buffer (KRB) (118.46 m*M* NaCl, 5 m*M* KCl, 2.5 m*M* $CaCl_2$, 1.18 m*M* NaH_2PO_4, 1.18 m*M* $MgSO_4$, 24.88 m*M* $NaHCO_3$, pH 7.4), containing 10 m*M* glucose, 10 m*M* Hepes, 1 m*M* ascorbic acid, 0.1 m*M* bacitracin, and 0.1% bovine serum albumin. Then, the medium was replaced with fresh KRB containing the substances to be tested, and the tissues were incubated for 30 min. At the end of the incubation period, the media were removed, and after heating for 10 min at 100ºC, were centrifuged at 10000*g* for 10 min. The supernatants were stored at −70ºC until determination of oxytocin concentration. The tissues were quickly frozen on dry ice and homogenized in distilled water. Protein concentration in tissue homogenates was determined by the method of Lowry et al., using bovine serum albumin as standard. The incubations performed in the presence of NMDA were done in a Mg^{2+}-free medium.

Drugs and Solutions

L-Quisqualic acid (quisqualate), *O*-phospho-L serine (L-SOP), (±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid (trans-ACPD), and (*RS*)-1-aminoindan-1,5-dicarboxy-lic acid (AIDA) were purchased from Tocris Cookson Ltd., Bristol, UK. *N*-Methyl-D-Aspartic acid (NMDA), kainic acid (kainate), D-aspartic acid (D-aspartate), DL-2-amino-5-phosphonovaleric acid (AP-5), and 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) from Sigma-Aldrich Co. (+)-α-amino-3-hydroxy-benzeneacetic acid (3-HPG) and 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride (GYKI 52466) were purchased from RBI. Oxytocin was purchased from Peninsula Laboratories Inc., Belmont, CA, and ^{125}I radionucleide from New England Nuclear, Boston, MA.

Oxytocin Determination

Oxytocin concentration in the incubation medium was measured on duplicate samples by radioimmunoassay, using ^{125}I-oxytocin as tracer and anti-oxytocin antiserum (Sigma, final dilution 1:36), as previously described *(56)*. Oxytocin was used as standard preparation, as well as for iodination with ^{125}I. The reaction was stopped with cold 2% albumin in phosphate-buffered saline and 96% ethanol. The intraassay coefficient of variation was lower than 9%, and assay sensitivity was 7 pg/tube. All samples from animals tested within a specific experimental paradigm were measured in the same RIA, to avoid interassay variability.

Data Analysis and Statistics

Results were expressed as mean ± SEM. The significance of the differences between means was determined by Student's *t* test, one-way analysis of variance (ANOVA), followed by Dunnett´s test for comparisons against the control group, or Student–Newman–Keuls test for multiple comparisons, or two-way ANOVA with interaction terms. Differences were considered significant when $p < 0.05$. All experiments were performed at least twice. Figures represent results of individual experiments.

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