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REGULATION OF ENDOGENOUS NORADRENALINE RELEASE FROM THE BED NUCLEUS OF STRIA TERMINALIS

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Abstract—The bed nucleus of stria terminalis (BNST) contains the highest concentration of noradrenaline (NA) in the brain. Minislices of the ventral portion of the bed nucleus of stria terminalis (vBNST) were used to study the release of endogenous NA. High K^+ induced a Ca^{2+}-dependent and reserpine-sensitive release of NA. Clonidine (1 μM), an α_2-noradrenergic receptor agonist, significantly decreased K^+-induced release of NA, whereas yohimbine (1 μM), an α_2-noradrenergic antagonist, increased this release. *N*-Methyl-D-aspartate (NMDA), a specific agonist of NMDA-type glutamate receptors, evoked the release of NA from vBNST minislices. In the presence of D-serine (10 μM), an agonist at the glycine site associated with the NMDA receptor, the NMDA effect was significantly higher. Glycine (1 μM) also increased NA release evoked by NMDA. However, glycine exhibited a significant effect by itself, suggesting the existence of strychnine-sensitive glycine receptors in vBNST. Endogenous NA release induced by 40 mM K^+ and NMDA was not additive. Thus, vBNST minislices seem to be a good model to study the release of endogenous NA in the CNS. Such NA release in the vBNST is regulated by α_2-noradrenergic receptors and by glutamate through NMDA receptors.

Key words: BNST; *N*-methyl-D-aspartate receptors; noradrenaline release; presynaptic receptors; glycine

The vBNST† is the brain area with the highest concentration of NA [1, 2]. It has been shown recently that iontophoretic application of NA inhibits the firing rate of BNST neurons and that such an effect is significantly higher in the vBNST [3]. Electrophysiological evidence indicates that inputs from the amygdala and the hypothalamus converge over the same neurons of the vBNST, suggesting an integrative role for this nucleus upon the activity of the limbic system [4]. Reciprocal connections of the BNST with the hypothalamus provide the anatomical basis to propose a role for the BNST in the control that the limbic system exerts upon neurovegetative functions [5]. In addition, several studies have shown that the BNST is related to aggressiveness and maternal behaviors [6, 7]. In spite of this information, very few neurochemical studies have been performed in the BNST. In particular, it would be interesting to learn about transmitter release and its regulation from NA-containing nerve terminals located in the BNST.

Most NA release studies in the CNS have been conducted using isotopes such as [^{3}H]NA or [^{3}H]-tyrosine to label the transmitter release compartment [8, 9]. A few studies, performed mainly in the hippocampus and hypothalamus, have addressed the question of endogenous NA release and its regulation in the brain [10–12]. The vBNST, with its high content of NA, might prove to be a very adequate model to study endogenous NA release in the CNS. Actually, the release of endogenous NA from vBNST has been studied recently by means of fast cyclic voltammetry [13, 14]. The purpose of the present work was to study the release of endogenous NA and its regulation by α_2-noradrenergic and NMDA receptor ligands in the CNS, using superfused minislices from vBNST and HPLC coupled to electrochemical detection to quantify NA. Preliminary accounts of this work have been reported elsewhere.‡§

MATERIALS AND METHODS

Materials. Clonidine hydrochloride, yohimbine, NMDA, glycine and D-serine were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade.

Preparation of vBNST minislices. vBNST mini-

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† Abbreviations: vBNST, ventral region of the bed nucleus of stria terminalis; NA, noradrenaline; and NMDA, *N*-methyl-D-aspartate.

‡ Forray MI, Andrés ME and Gysling K, Liberación de noradrenalina desde minicortes de la estría terminal de la rata. Abstr. XIII, Latinoamerican Congr. Pharmacol. Ther., Montevideo, Uruguay, p. 209, 1990.

§ Gysling K, Forray MI, Andrés ME and Bustos G, Regulation of noradrenaline release by *N*-methyl-D-aspartate receptors in the bed nucleus of the stria terminalis. Abstr. XIV, Annual Meeting Soc. Pharmacol., Valparaiso, Chile, p. 15, 1992.

slices were obtained from 250–300 g male Sprague–Dawley rats, as described previously [15]. Briefly, the animals were decapitated, and the brains were quickly removed and placed in ice-cold saline. The portion of the brain containing the BNST was mounted in a vibratome stage (CAMPDEM INST.) and sectioned in 300 μm coronal slices. The slices having the BNST (from 0.2 to 1.1 mm posterior to the bregma) were placed in a cold glass, and the vBNST was dissected from each coronal slice, with a small blade (1 mm) following the limits indicated by Brownstein and Palkovits [1], the stereotaxic atlas of Paxinos and Watson [16] and as we have carefully described recently [15]. Thus, minislices that follow the boundaries of the vBNST and of 300 μm thickness were obtained.

Superfusion procedure. vBNST minislices from three rats were placed in a superfusion chamber as described by Bustos and Roth [17] and superfused with Krebs-Ringer-phosphate (KRP), saturated with 100% O_2, at a flow rate of 2.0 mL/min. The composition of the KRP solution was (in mM): KCl, 4.85; $CaCl_2$, 0.75; $MgSO_4$, 1.2; NaCl, 128; Na_2HPO_4, 16 (pH 7.4); glucose, 16; and ascorbic acid, 0.5. For high K^+-KRP, equimolar amounts of NaCl were replaced by KCl to maintain an isosmotic condition. After a superfusion period of 60 min, a basal sample of 2 min was collected. Release was evoked by a 2-min exposure to 40 or 55 mM K^+. Superfusates were received in 400 μL of cold 0.2 N perchloric acid. At the end of the experiment, the slices were recovered and sonicated in 0.2 N perchloric acid to determine NA tissue levels.

Quantification of NA. The superfusates were subjected to concentration in alumina. Dihydroxybenzyl amine (DHBA, 0.5 pmol in 20 μL) was added to each sample as the internal standard. Samples were mixed with 15 mg of activated alumina [18] in Tris buffer, pH 8.3 to 8.5, and 1% EDTA. The alumina was rinsed thoroughly with water, which was later aspirated. NA was desorbed with 100 μl of 0.2 N $HClO_4$. Samples were centrifuged, and the supernatants were kept on ice until NA determination by HPLC coupled to electrochemical detection [19]. Twenty microliters of the supernatant was injected by means of a Rheodyne injector valve into an HPLC system with the following configuration: a one piston pump (LDC), a C18 reverse phase column (Lichrospher, 60 RP-select B, Merk) and an electrochemical detector (LC3A, BAS). The mobile phase contained 0.1 M NaH_2PO_4, 1.4 mM sodium 1-octanesulfonate, 0.2 mM EDTA and 1.5% acetonitrile (pH 2.5), and was pumped at a flow rate of 0.8 mL/min. The potential of the amperometric detector was set at 0.8 V. Under these experimental conditions, retention times were 10 min for NA and 16 min for DHBA, which was used to correct for recovery. The routine limit of detection for NA was 20 fmol. Protein in the pellet was determined by the method of Lowry *et al.* [20].

Calculations and statistical analysis. Release of NA was expressed as picomoles of NA released during the stimulation period. When minislices were subjected to two stimulation periods, the results are expressed as the ratio between the second (S2) and the first (S1) stimulation period (S2/S1). A non-parametric Mann-Whitney U test was used for comparison of mean values. For the reserpine study, a two-tailed Student's *t*-test was used.

RESULTS

Potassium-induced release of NA. Superfusion of vBNST slices from three rats for a 2-min period with 40 mM K^+ or 55 mM K^+ evoked the release of 1.44 ± 0.23 (N = 10) and 4.50 ± 0.76 (N = 12) pmol

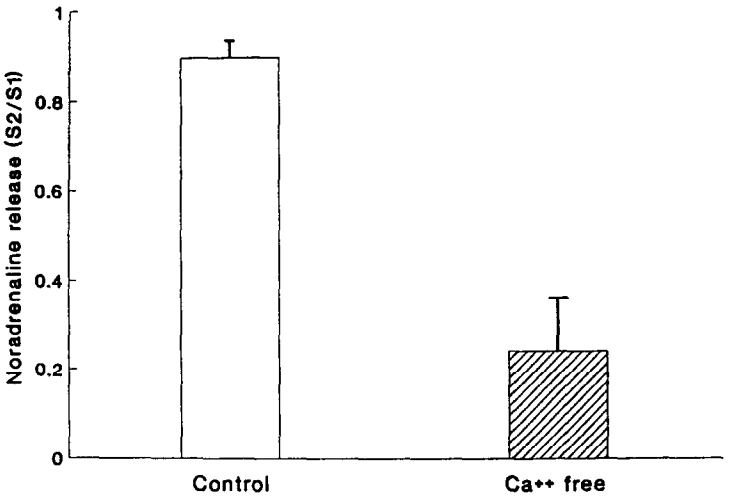

Fig. 1. Release of endogenous NA induced by 55 mM K^+. Minislices from vBNST from three rats were superfused with KRP and subjected to two stimulation periods of 2 min with KRP-K^+ (55 mM) (S1 and S2, respectively). To study the calcium dependency, this ion was omitted from the superfusion medium 20 min before the second stimulation period (S2). The results are expressed as the ratio between S2 and S1 and are the means ± SEM of 12 controls and 3 Ca^{2+}-free independent experiments.

Table 1. Effect of reserpine administration on tissue content and K^+-induced NA release from vBNST

	NA content (nmol/mg protein)	NA release (pmol)
Control	0.43 ± 0.04	3.46 ± 0.53
Reserpine	0.03 ± 0.01*	1.12 ± 0.06*

Rats were injected intravenously with 1 mg/kg of reserpine 90 min before killing them. Minislices of vBNST dissected from control (saline injected) and treated rats were superfused and subjected to a 2-min stimulation period with KRP-high K^+. NA tissue content was measured in homogenates of minislices from control and treated rats. Results are the means ± SEM of 5 independent experiments.

* $P < 0.005$, compared with control.

of NA, respectively. In the present experimental conditions, basal release of NA was below the detection limit of the method used to measure NA. Figure 1 shows the Ca^{2+} dependence of the K^+-evoked release of NA. In these last experiments, superfused slices were subjected to two stimulation periods with 55 mM K^+. The ratio between the second and first K^+-stimulation period (S2/S1) was equal to 0.85 ± 0.05 (N = 11). The omission of Ca^{2+} during the second stimulation period resulted in a marked decrease of the S2/S1 ratio (Fig. 1).

Effect of prior reserpine administration on tissue levels and NA release. The intravenous administration of 1 mg/kg of reserpine 90 min prior to killing the animals resulted in a 93% depletion of the NA tissue content in the vBNST (Table 1). In this condition, the 55 mM K^+-induced NA release from vBNST slices was reduced significantly (Table 1).

Effect of α_2-noradrenergic receptor ligands on potassium-induced NA release. Further experiments were conducted to test whether stimulated NA release from vBNST slices could be modulated by α_2-noradrenergic receptors. As shown in Fig. 2, the presence of the α_2-agonist clonidine (1 μM) significantly inhibited the K^+-evoked release of NA from vBNST slices. Under similar experimental conditions, it was also observed that the addition of the α_2-antagonist yohimbine (1 μM) provoked a significant increase in the potassium-induced release of NA (Fig. 2).

Effect of NMDA receptor ligands on NA release. Figure 3 shows that NMDA (250 μM), in the absence of Mg^{2+}, induced the release of NA from superfused vBNST slices. As it has been shown that the NMDA receptor has an allosteric site for glycine (Gly), we added exogenous Gly to make sure that this site was fully occupied. The addition of 1 μM Gly produced a marked release of NA (Fig. 3). NMDA in the presence of 1 μM Gly induced a significant release of NA. Due to the fact that Gly *per se* presented an effect, we decided to use instead D-serine (D-Ser), a selective exogenous agonist at the strychnine-insensitive Gly site associated with the NMDA receptor complex. D-Ser (1 or 10 μM) produced no detectable effect in NA release from vBNST slices. However, NMDA (250 μM) in the presence of 10 μM D-Ser induced a significantly higher release of NA as compared with NMDA alone (Fig. 3).

Effect of NMDA on potassium-induced NA release. Experiments were performed to study, under depolarizing conditions, the effects of NMDA receptor ligands on NA release. As shown in Fig. 4, the release of NA from vBNST slices induced by NMDA plus D-Ser in the presence of high K^+ (40 mM) was not additive to the endogenous NA release induced by 40 mM K^+ alone.

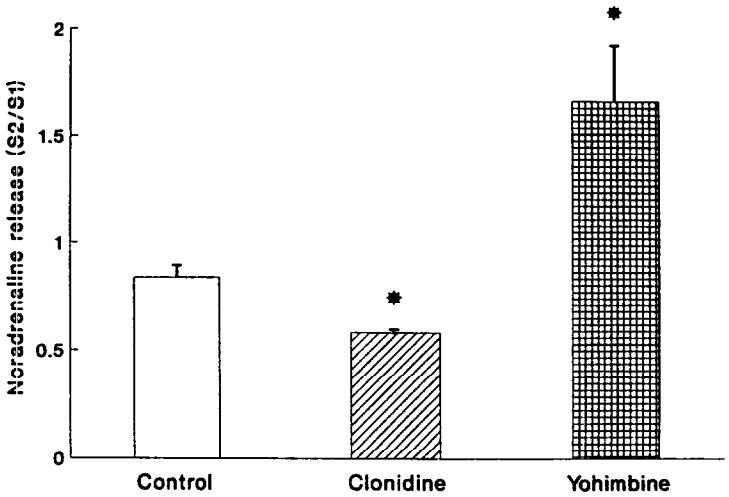

Fig. 2. Effects of clonidine and yohimbine on the K^+-induced release of NA. Minislices from vBNST were superfused with KRP and subjected to two stimulation periods of 2 min with 40 mM K^+. Clonidine or yohimbine (1 μM) was added 5 min previous to the second stimulation. Results are expressed as the ratio of S2/S1 and are the means ± SEM of 4–10 independent experiments. Key: (*) $P < 0.02$, compared with control.

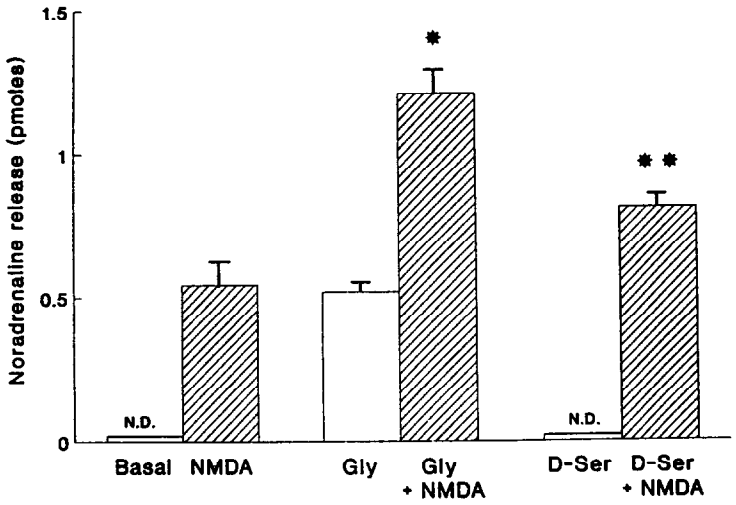

Fig. 3. Effect of NMDA on NA release. Minislices from vBNST were superfused with KRP without Mg^{2+}. Slices were exposed to 250 μM NMDA for a period of 2 min. To test the effect of 1 μM Gly or 10 μM D-Ser, these drugs were added during the 2-min basal period and together with NMDA. Results are expressed as picomoles released during the 2-min period and represent the means ± SEM of 3–10 independent experiments. Key: (*) $P < 0.001$, compared with Gly alone; and (**) $P < 0.02$, compared with NMDA without D-Ser.

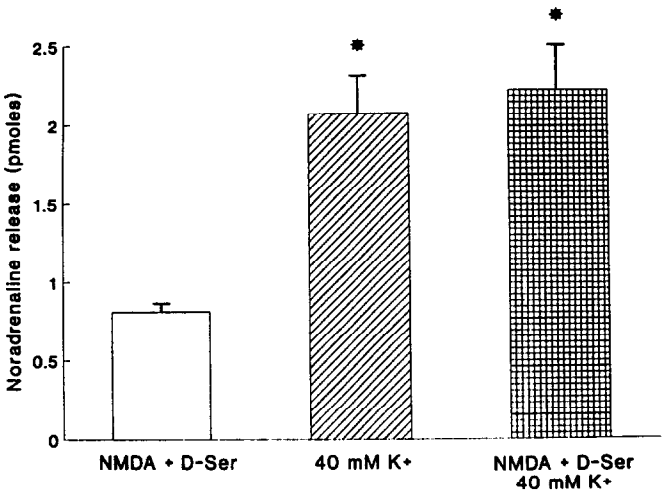

Fig. 4. Effect of NMDA on K^+-induced NA release. Minislices from vBNST were superfused with KRP without Mg^{2+}. Slices were exposed for 2 min to 250 μM NMDA + 10 μM D-Ser, 40 mM K^+ or both. Results are expressed as picomoles of NA released during the stimulation period and represent the means ± SEM of 5–10 independent experiments. Key: (*) $P < 0.001$, compared with NMDA + D-Ser.

DISCUSSION

The results described in this paper show that minislices of vBNST are a good model to study the release of endogenous NA in the CNS. The very high concentration of NA, equivalent to striatal DA levels, in the vBNST [1] and the fact that there are no noradrenergic cell bodies in this region make it very suitable to conduct endogenous NA release studies. Potassium-induced release of NA from superfused vBNST minislices was calcium dependent and was reduced drastically by the previous administration of reserpine to rats. Thus, under the experimental conditions of this study, the high K^+-induced release of NA in the vBNST originated from a calcium-dependent and reserpine-sensitive intracellular NA pool.

In this study, clonidine significantly inhibited the

release of NA induced by K^+-depolarization from vBNST minislices. It was also observed that the presence of yohimbine, a specific α_2-receptor antagonist, in the superfusion medium significantly increased the induced release of NA. These findings support the conclusion that under our experimental conditions the released NA may be acting upon α_2-noradrenergic receptors. These observations are in agreement with a recent study using fast cyclic voltammetry [14] and which provided pharmacological evidence suggesting that α_2-receptors of the α_{2A} or α_{2D} subtype may be involved in the regulation of NA release. Although the voltammetry methodology has the advantage of a good temporal resolution, other electrochemically active substances may interfere with the electrical signal generated by NA [21]. In the periphery, it is well substantiated that NA release is regulated by presynaptic α_2-noradrenergic receptors. This also may be the case in the CNS; however, most of the studies in the CNS have been performed with radiolabeled noradrenaline [22]. The use of the vBNST as a model has permitted us to provide evidence regarding the release of endogenous NA in the CNS and its regulation by α_2-adrenergic receptors.

There are several reports that NMDA receptor activation induces the release of [^{3}H]NA previously taken up by slices or synaptosomes from cortex and hippocampus [23–27]. We decided to study whether NMDA could induce the release of endogenous NA from vBNST. A significant release of endogenous NA was observed when vBNST minislices were superfused in the presence of NMDA. D-Serine, a well known agonist of the glycine site in the NMDA receptor, did not have any effect by itself but significantly increased the release of NA induced by NMDA. Interestingly, glycine, the endogenous agonist for the allosteric site of the NMDA receptor, produced a significant stimulatory effect on NA release *per se*, probably due to an effect on strychnine-sensitive glycine receptors. Radioautographic evidence indicates a significant presence of NMDA receptor binding in the BNST [28, 29]. Moreover, there is anatomical evidence indicating the existence of glutamatergic projections from the subiculum and hippocampus to the BNST [30]. These last observations together with our present results support a possible functional role for NMDA receptors in the regulation of NA release in the BNST nucleus.

The stimulatory effect of NMDA plus D-Ser on NA release was not additive with the release of NA induced by potassium alone. Thus, under depolarizing conditions, NMDA is not able to evoke NA release in the vBNST, similarly to what has been observed in the CA1–CA3 area of the hippocampus [27]. At the present time, there is no evidence available to explain these observations. However, there are some recent reports in the literature that give some directions to explore further. The first and probably the simplest explanation is that glutamate or other endogenous substances could be acting on NMDA receptors. It is possible that glutamate is released when vBNST minislices are subjected to a high K^+ superfusion medium. Second, it has been shown that most of the Ca^{2+} current flowing into granular cerebellar neurons exposed to K^+-depolarization is due to the entrance of Ca^{2+} through the NMDA receptor [31]. This might also be the case for vBNST neurons enriched with NMDA receptors. This last speculation awaits experimental support.

More complex interactions at the presynaptic level have also been proposed and may account for the present findings of NMDA under depolarizing conditions. Chernevskaya and co-workers [32] have provided electrophysiological evidence that the activation of NMDA receptors in the CA1 region of the hippocampus inhibits Ca^{2+} currents mediated by N-type calcium channels. The calcium channels of the N type are the most commonly related to Ca^{2+} inflow triggered by depolarization into nerve terminals [33, 34]. Thus, a possible blockade of N-type calcium channel by NMDA could also explain the fact that this glutamatergic agonist could not increase NA release further when vBNST slices were subjected to depolarizing conditions.

It has been reported very recently that the microinjection in oocytes of syntaxin cRNA led to the expression of a functional channel protein with an electrophysiological and pharmacological profile similar to that of the "classical" NMDA receptor obtained by the expression of the NMDA receptor R1 subunit [35]. Syntaxin is a protein located in presynaptic neuronal elements tightly related with N-type Ca^{2+} channels [36]. These observations support the possibility that eventual presynaptic NMDA receptors could be regulating the influx of Ca^{2+} through N-type Ca^{2+} channels as suggested previously [32].

In conclusion, the findings reported here indicate that the vBNST is a good model to study endogenous NA release in the CNS. The results show that NA release in the BNST is induced upon depolarization in a Ca^{2+}-dependent manner and that this release may be regulated by α_2-noradrenergic and NMDA receptors.

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