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Somatostatin-Induced Activation and Upregulation of NMDA Receptor Function: Mediation Through CaMKII, PLC, PKC and Src in Hippocampal Noradrenergic Nerve Endings

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ABBREVIATIONS: BIM-23056, D-Phe-Phe-Tyr-D-TRP-Lys-Val-Phe-D-Nal-NH_2**;** CAKβ/Pyk2, cell-adhesion kinase β/proline-rich tyrosine kinase 2; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; DAG, diacylglycerol; GF109203X, dihydrochloride3-[1-[3-(dimethylamino)propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione; GPCRs, G protein-coupled receptors; H89, N-(2-[p-bromocinnamylamino]ethyl)5-isoquinolinesulfonamide hydrochloride; IP_3, inositoltrisphosphate; KN93, (N-(2-[N-[4-Chlorocinnamyl]-N-methyl-amino-methyl]phenyl)-N-(2-hydroxyethyl)-4-methoxy-benzene-sulfonamide-phosphate salt; L362,855, (c[Aha-Phe-Trp-D-Trp-Lys-Thr-Phe]); Lavendustin A, (5-Amino-[(N-2,5-Dihydroxybenzyl)-N'-2-hydroxybenzyl]salicylic acid; MK801, dizocilpine; NE, norepinephrine; NMDA, N-methyl-D-aspartate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C); PP2, 3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1Hpyrazolo[3,4-d]pyrimidin-4-amine; Src, tyrosine kinase; SRIF, somatostatin; t-TBS, Tris-buffered saline-Tween; U73122, 1-(6-[([17β]-3-methoxyextra-1,3,5[10]-trien-17-yl)amino]hexyl)-1H-pyrrole-2,5-dione

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ABSTRACT

Somatostatin receptors and glutamate NMDA receptors coexist on hippocampal noradrenergic axon terminals. Activation of somatostatin receptors was previously found to positively influence the function of NMDA receptors regulating norepinephrine release. The somatostatin receptors involved were pharmacologically characterized as sst5 type, in experiments in Mg^{2+}-free solutions. Here, we first confirm the pharmacology of these receptors using selective sst5 ligands in Mg^{2+}-containing solutions. Moreover, we show by western blot that the sst5 protein exists on purified hippocampal synaptosomal membranes. We then investigated the pathways connecting the two receptors using as a functional response the release of norepinephrine from rat hippocampal synaptosomes in superfusion. The release of norepinephrine evoked by somatostatin-14 plus NMDA/glycine was partly prevented by the protein kinase C inhibitor GF109203X and by the non-receptor tyrosine kinase (Src) inhibitors PP2 and lavendustin A; it was largely and almost totally abolished by the phospholipase C inhibitor U73122 and by the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN93, respectively; it was unaffected by the protein kinase A inhibitor H89. The norepinephrine release evoked by somatostatin-14/NMDA/glycine was inhibited when anti-phosphotyrosine antibodies had been entrapped into synaptosomes. Entrapping the recombinant activated tyrosine kinase $pp60^{c\text{-}Src}$ strongly potentiated the release of norepinephrine elicited by NMDA/glycine in Mg^{2+}-free medium, but failed to permit NMDA receptor activation in presence of external Mg^{2+} ions. The results suggest the involvement of CaMKII in the sst5 receptor-mediated *activation* of NMDA receptors in presence of Mg^{2+} and of the PLC/PKC/Src pathway in the *upregulation* of the ongoing NMDA receptor activity.

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Glutamate NMDA receptors are ion channel-associated receptors expressed by coassembly of NR1 and NR2(A-D) subunits. These receptors display both pre- and postsynaptic localization and play relevant roles in development, neuroplasticity and excitotoxicity (see, for a review, Engelman and MacDermott, 2004).

Interactions between NMDA receptors and G protein-coupled receptors (GPCRs) coexpressed on membranes have been reported by several laboratories (Lu et al., 1999; Pittaluga et al., 2000; Lan et al., 2001; Heidinger et al., 2002; Kotecha et al., 2003). In particular, activation of GPCRs can affect NMDA receptor function, representing a major mechanism of glutamate transmission modulation. The cross-talks between coexisting GPCRs and NMDA receptors involve intracellular kinase pathways which may differ among the different receptor-receptor interactions.

Depending on the subunits targeted and the kinases involved, phosphorylation can affect either negatively or positively the NMDA receptor function (see Kotecha and MacDonald, 2002). NR1 and NR2 subunits can undergo phosphorylation by several kinases including protein kinases A (PKA; Leonard and Hell, 1997), protein kinases C (PKC; Zheng et al., 1999; Liao et al., 2001), cytosolic tyrosine kinases of the Src family (Yu et al., 1997; Lu et al., 1999; Salter and Kalia, 2004) and Ca^{2+}/calmodulin-dependent kinase II (CaMKII; Leonard et al., 1999; Soderling et al., 2001). While phosphorylation of NR1 subunits generally limits NMDA receptor function (Zukin and Bennett, 1995), phosphorylation of NR2 subunits enhances NMDA receptor-mediated effects (see MacDonald et al., 2001).

NMDA receptors exist on CNS noradrenergic nerve endings, where they mediate exocytotic release of norepinephrine (NE; Jones et al., 1987; Fink et al., 1992; Raiteri et al., 1992). In rat hippocampus, these receptors, which contain NR2B subunits (Pittaluga et al., 2001), colocalize with somatostatin (SRIF) receptors which are positively coupled to phosphoinositide breakdown and, based on a pharmacological study with selective ligands, belong to the sst5 subtype (Pittaluga et al., 2000). Activation of SRIF receptors fails to affect

NE release, but seems to permit activation and upregulation of NMDA receptors in presence of physiological concentrations of extracellular Mg^{2+} and without depolarization (Pittaluga et al., 2000).

The mechanisms underlying the positive effects of SRIF on NMDA receptor function are not known. We previously suggested the involvement of inositoltrisphosphate (IP_3) and PKC (Pittaluga et al., 2000). The aims of the present work were (i) to investigate the phosphorylative pathway(s) involved in the SRIF-NMDA receptor–receptor interaction using synaptosomes in superfusion, a technique particularly suitable for identifying receptors that coexist on the same nerve terminal and understanding their cross-talks (see Raiteri and Raiteri, 2000); (ii) to distinguish between processes leading to *activation* of release-enhancing NMDA receptors and *upregulation* of receptors already in the open channel state. The results suggest the involvement of CaMKII in the sst5 receptor-mediated activation and of PKC and Src in the sst5 receptor-mediated upregulation of NMDA receptor function.

Materials and Methods

Animals and brain tissue preparation. Adult male rats (Sprague-Dawley, 200-250 g) were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light-dark schedule (light 7 a.m. - 7 p.m.). Food and water were freely available. The experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC).

The animals were killed by decapitation and the hippocampi were rapidly removed at 0-4°C. Crude synaptosomes were prepared according to Raiteri et al. (1992). Briefly, the ventral-medial part of the hippocampus was homogenized in 40 volumes of 0.32 M sucrose, buffered at pH 7.4 with phosphate (final concentration 0.01 M). The homogenate was centrifuged at 1000 x *g* for 5 min, to remove nuclei and cellular debris, and crude synaptosomes were isolated from the supernatant by centrifugation at 12,000 x *g* for 20 min.

In some experiments, the tissue was homogenized in buffered sucrose containing 225 units/ml of Src ($pp60^{c\text{-}Src}$) or 20 µg of antibody anti-phosphotyrosine in order to entrap these agents into subsequently isolated synaptosomes (Raiteri et al., 2000).

Release experiments. The synaptosomal pellets were resuspended in physiological medium having the following composition (mM): NaCl, 125; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1; $NaHCO_3$, 22; glucose, 10 (aeration with 95% O_2 and 5% CO_2); pH 7.2-7.4. Synaptosomes were incubated 15 min at 37°C with [^{3}H]NE (final concentration 30 nM) in presence of 0.1 μM 6-nitroquipazine to avoid false labelling of serotonergic terminals.

Identical portions of the synaptosomal suspension were layered on microporous filters at the bottom of parallel superfusion chambers thermostated at 37°C (Raiteri and Raiteri, 2000). Synaptosomes were superfused at 0.5 ml/min with standard physiological solution aerated with 95% O_2 and 5% CO_2, at 37°C. Synaptosomes were first equilibrated during 36 min of superfusion; subsequently, 9 consecutive 1-min fractions (t = 36 min to t = 45 min) were collected. Synaptosomes were exposed to agonists at the end of the third fraction collected (t = 39 min) till the end of the superfusion, while antagonists were added eight min before agonists. When indicated, the superfusion medium was replaced, at t = 20 min, with a medium from which Mg^{2+} ions were omitted. Fractions collected and superfused synaptosomes were counted for radioactivity.

Western blot of sst5 protein. To obtain a cellular preparation particularly enriched in isolated nerve endings, synaptosomes were prepared by homogenizing hippocampi in 40 volumes of 0.32M sucrose buffered at pH 7.4 with Tris (final concentration 0.01M) and then purified by Percoll gradient. Briefly, the homogenate was centrifuged at 1000 x *g* for 5 min, to remove nuclei and debris, and synaptosomal fraction was purified by Percoll-sucrose density (2-20%; vol/vol) gradient centrifugation for 5 min at about 33500 x *g*. The 10-20% Percoll interface was removed, washed to eliminate Percoll and synaptosomes were isolated from the supernatant by centrifugation at 12,000 x *g* for 20 min. Synaptosomes were then lysed in 1 ml of ice-cold water and pellets isolated by centrifugation at 7000 x *g*. The pellets were solubilized in a lysis buffer containing 20 mM HEPES (pH 7.4), 5 mM EDTA, 3 mM EGTA, 150 mM NaCl, and 4 mg/ml dodecyl-B-d-maltoside for 1h at 4°C and then ultracentrifuged at 100,000 x *g* for 1 h at 4 °C. The supernatant containing solubilized synaptic membrane proteins was subjected to standardized colorimetric analysis to evaluate the protein content. 200 μg of membrane proteins were denatured and fractionated under reducing conditions on 10% SDS-PAGE, then transferred electrophoretically to Hybond C-Extra nitrocellulose membranes (Amersham Pharmacia Biotech, Oakville, Canada). After transfer, nonspecific binding sites were blocked by Tris-buffered saline-Tween (t-TBS: 0.02 M Tris, 0.137 M NaCl, 0.1% Tween-20) containing 5% non-fat dried milk. After three washes with t-TBS, membranes were incubated for 16 h at 4° C with a 1:150 dilution of goat anti-rat SST5r polyclonal antibody (Santa Cruz Biotechnology, Inc.) in t-TBS containing 3% BSA. Membranes were washed three times with t-

TBS, then incubated for 2 h at 22 ^{o} C with a 1:5000 dilution of the antigoat horseradish peroxidase-linked IgG. After three washes with t-TBS, immunoreactive bands were detected by the chemiluminescence detection system ECL Western blot analysis system (Amersham Pharmacia Biotech, Little Chalfont, UK). The immunoreactive bands were visualized by autoradiography after 0.5 min exposure to Hyperfilm ^{TM} MP (Amersham Pharmacia).

Calculations and statistics. The radioactivity released into each superfusate sample was expressed as a percentage of the total synaptosomal tritium content at the start of the fraction collected (fractional efflux). When the time-course of the effect is reported, drug effects are expressed as percent of effect and evaluated as the ratio between the percentage of tritium released into each fraction after the first and that in the first fraction collected. This ratio was compared with the corresponding ratio obtained under control conditions (no drug added).

Analysis of variance was performed by ANOVA followed by Dunnett's test or Newman Keuls multiple-comparisons test or Student's *t*-test as appropriate. Data was considered significant for $p < 0.05$ at least. Appropriate controls with antagonists and inhibitors were always run in parallel.

Chemicals. 1-[7,8-^{3}H]norepinephrine (specific activity 39 Ci/mmol) RPN-800 pre-stained molecular mass marker, chemiluminescence detection system ECL Western blot analysis system, Hyperfilm ^{TM} MP were from Amersham Radiochemical Center (Buckinghamshire, UK). Hybond C-Extra nitrocellulose membranes (Amersham Pharmacia Biotech, Oakville, Canada). N-methyl-D-aspartate (NMDA), 3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1Hpyrazolo[3,4-d]pyrimidin-4-amine (PP2) from Tocris-Cookson (Bristol, UK). SRIF-14 from Peninsula Lab. Inc. (Merseyside, UK). Glycine, dihydrochloride3-[1-[3-(dimethylamino)propyl]-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione (GF109203X), 1-(6-[([17β]-3-methoxyextra-1,3,5[10]-trien-17-yl)amino]hexyl)-1H-pyrrole-2,5-dione (U73122), (5-Amino-[(N-2,5-Dihydroxybenzyl)-N'-2-hydroxybenzyl]salicylic acid

(Lavendustin A), N-(2-[p-bromocinnamylamino]ethyl)5-isoquinolinesulfonamide hydrochloride (H89), (N-(2-[N-[4-Chlorocinnamyl]-N-methyl-amino-methyl]phenyl)-N-(2-hydroxyethyl)-4-methoxy-benzene-sulfonamide-phosphate salt (KN93) were purchased from Sigma-RBI (Milan, Italy). Src ($pp60^{c\text{-}Src}$) and mouse anti-phosphotyrosine (clone 4G10), were from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase conjugated anti goat secondary antibody was purchased from Chemicon (Temecula, CA). Goat anti-rat sst5R polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). MK801 was from Merck-Sharp and Dohme (Harlow, Essex, UK) while 6-nitroquipazine maleate was from Duphar, Amsterdam, The Netherlands. BIM-23056 and L362,855 were gifts from Dr. P.P.A. Humphrey (Cambridge, UK) and Dr D. Hoyer (Basel, Switzerland).

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Results

The function of NMDA receptors, in presence of Mg^{2+} ions, is facilitated by the action of somatostatin sst5 receptors present in the hippocampus

As previously shown (Pittaluga et al., 2000) exposure of hippocampal synaptosomes to SRIF-14 plus NMDA/glycine evokes release of preloaded [^{3}H]NE, in presence of a physiological concentration of Mg^{2+} ions (1.2 mM). When added alone, neither SRIF-14 (1 nM) nor NMDA(100 μM)/glycine (1 μM) can affect the release of [^{3}H]NE. The release induced by SRIF-14 plus NMDA/glycine depends on NMDA receptor activation since MK801, a selective NMDA channel blocker inactive on its own, almost totally prevented the releasing effect (1 μM MK-801 = 2.65 ± 3.01%; 1 nM SRIF-14 + 100 μM NMDA + 1 μM glycine = 73.08 ± 6.56%; + 1 μM MK-801 = 15.60 ± 7.89%, $p < 0.05$ at least; results expressed as percentage of increase over basal release).

In the work by Pittaluga et al. (2000), the positive effect of SRIF-14 on the NMDA-evoked release of NE was related to activation of SRIF receptors exhibiting sst5 pharmacology. Since that pharmacological characterization was carried out in Mg^{2+}-free solutions, it was important to ascertain if sst5 receptor ligands behaved similarly in solutions containing a physiological concentration of Mg^{2+} ions. As shown in Fig 1, BIM-23056, a selective sst5 receptor antagonist, totally prevented the release of [^{3}H]NE induced by SRIF-14/NMDA/glycine, suggesting that only sst5 receptors participate to the somatostatin-NMDA receptor-receptor interaction. The figure also shows that the sst5 partial agonist L362,855 mimicked in part SRIF-14.

The pharmacological characterization of the receptors involved as sst5 type may appear surprising .Based on morphological results, the presence of receptors of the sst5 type in some CNS regions is in fact controversial (Fehlmann et al., 2000; Rocheville et al., 2000a;

Schulz et al., 2000; Kang et al., 2003). We therefore analyzed proteins from purified synaptosomal membranes for the presence of the sst5 receptor protein. As shown in Fig. 1, western blot analysis with anti-sst5 antibodies recognized a component with an apparent mass of 60-70 kDa, suggesting that sst5 protein is expressed in nerve ending membranes. The prominent band with the apparent mass of 60-70 kDa should correspond to the monomeric form of the sst5 receptor (Rocheville et al., 2000b).

Somatostatin-NMDA receptor-receptor interaction: involvement of PLC and PKC, but not PKA

Table 1 shows that U73122 (0.1 μM), a selective inhibitor of PLC function, largely prevented the release evoked by SRIF-14/NMDA/glycine indicating the involvement of PLC present in noradrenergic nerve terminals. It is known that activated PLC promotes phosphoinositide breakdown followed by IP_3 and diacylglycerol (DAG) production and possible consequent activation of intraterminal PKCs. Accordingly, GF109203X, a selective PKC blocker, inhibited in part the release of [^{3}H]NE provoked by SRIF-14/NMDA/glycine in Mg^{2+}-containing medium (Table 1).

Little is known about the relations between PKA activity and NMDA receptor function. PKA-targeted sites were proposed to reside within the NR1 subunit, although PKA was shown to influence also the functional activity of NMDA receptors by modifying NR2 subunits (Leonard and Hell, 1997). These events, however, have been mainly related to inhibitory effects on NMDA receptor function. Under our experimental conditions, the PKA selective inhibitor H89, added at 0.5 μM, failed to affect the release of [^{3}H]NE induced by SRIF-14/NMDA/glycine (Table 1), suggesting that PKA-mediated phosphorylation of the NMDA receptor is unlikely to modulate NE release. Under very similar experimental conditions, H89 had been found to inhibit the upregulation of NMDA receptors provoked by

nicotine receptor activation in noradrenergic axon terminals of the hippocampus (Risso et al., 2004). At the concentrations applied, the enzyme blockers used did not modify, on their own, the spontaneous release of tritium (see legend to table 1).

Involvement of Src

It is known that PKC can phosphorylate NMDA NR2 subunits directly, on serine and threonine, as well as indirectly, on tyrosine, by activating cytosolic tyrosine kinases of the Src family (Yu et al., 1997; Lu et al., 1999; MacDonald et al., 2001; Salter and Kalia, 2004). The possibility that the SRIF-NMDA receptor-receptor interaction involves the Src signalling was evaluated by studying the effects of the selective Src inhibitors PP2 and lavendustin A. As shown in Fig. 2, addition to the superfusion medium of PP2 (1 μM) or lavendustin A (5 μM) inhibited in part the [^{3}H]NE release induced by SRIF-14/NMDA/glycine from hippocampal synaptosomes. The spontaneous release of tritium was not affected by the kinase inhibitors used (see legend to Fig. 2, panel A).

Since the effects of PKC and Src inhibitors (used at the maximally effective concentration; not shown) were only partial, we tested a combination of the two inhibitors. As reported in Table 2, addition of GF109203X together with PP2 did not produce additive inhibitory effect on the SRIF-14/NMDA/glycine-evoked release of [^{3}H]NE.

Involvement of CaMKII

NMDA receptor subunits have been identified as CaMKII anchoring proteins in postsynaptic densities. Upon kinase autophosphorylation, CaMKII can bind the cytosolic C-terminal region of NR2B subunits and affect the function of the NMDA-associated ionic channel (Leonard et al., 1999; Soderling et al., 2001). We therefore investigated the possibility that the SRIF receptor-NMDA receptor interaction that occurs presynaptically

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involves CaMKII-mediated phosphorylative processes by analyzing the effect of the selective CaMKII inhibitor KN93 on the release of [^{3}H]NE induced by SRIF-14/NMDA/glycine. As shown in Fig. 2, panel B, KN93, added at 1 μM, almost totally inhibited the evoked [^{3}H]NE release. The spontaneous release of tritium was not affected by 1 μM KN93 (see legend to Fig. 2).

Synaptosomal entrapping of antibodies anti-phosphotyrosine prevents the SRIF-14/NMDA/glycine-evoked release of [^{3}H]NE

The finding that kinases of the Src family participate in the interaction between SRIF and NMDA receptors implies that the cross-talk between the two receptors includes phosphorylation of tyrosine residues. To evaluate the involvement of a tyrosine phosphorylative pathway, antibodies raised against phosphotyrosines were entrapped into synaptosomes. We had previously shown that synaptosomes isolated after homogenization of brain tissue in the presence of anti-syntaxin or anti-SNAP25 antibodies exhibited decreased transmitter release when exposed to depolarizing stimuli, indicating that the above antibodies had been entrapped into nerve endings (Raiteri et al., 2000). As shown in Fig. 3, entrapping antibodies anti-phosphotyrosine into hippocampal synaptosomes decreased by about 50% the release of [^{3}H]NE elicited by SRIF-14/NMDA/glycine. Entrapping anti-phosphotyrosine antibodies did not modify the spontaneous release of [^{3}H]NE (control synaptosomes = 0.71 ± 0.02 % ; entrapped synaptosomes = 0.66 ± 0.05%; results expressed as % of tritium released into the first fraction collected).

Src mediates upregulation, but not activation, of NMDA receptors

The finding that Src inhibitors prevented only in part (~ 50%) the NE release elicited by SRIF-14/NMDA/glycine (Fig. 2, panel A) may suggest that Src does not mediate the

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permissive role played by SRIF on the activation of NMDA receptors in presence of Mg^{2+}ions. To shed light on this aspect, we entrapped into synaptosomes the recombinant tyrosine kinase $pp60^{c\text{-}Src}$, an activated form of Src (Yu et al., 1997; Lu et al., 1999), and studied the effects of NMDA/glycine on the release of [^{3}H]NE in presence or absence of external Mg^{2+} ions.

Table 3 shows that entrapped $pp60^{c\text{-}Src}$ failed to permit NMDA receptor activation, since no releasing effect could be observed when synaptosomes staffed with $pp60^{c\text{-}Src}$ were exposed to NMDA/glycine in presence of a physiological concentration (1.2 mM) of Mg^{2+} ions. On the contrary, $pp60^{c\text{-}Src}$ strongly potentiated the ongoing NMDA-induced release, as shown by the results obtained when $pp60^{c\text{-}Src}$-staffed synaptosomes were exposed to NMDA/glycine in absence of external Mg^{2+} ions. Entrapping $pp60^{c\text{-}Src}$ did not modify the spontaneous release of [^{3}H]NE (control synaptosomes = 0.56 ± 0.05 %; entrapped synaptosomes = 0.64 ± 0.07%; results expressed as % of tritium released into the first fraction collected).

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Discussion

The main findings of the present study are that (1) activation of sst5 receptors present on hippocampal noradrenergic terminals positively affects the function of coexisting NMDA receptors through pathways involving CaMKII, PLC, PKC and Src; (2) somatostatin seems to trigger NMDA receptor activation through CaMKII; (3) the PLC/PKC/Src pathway upregulates NMDA receptor function once the receptor is gated to an open state.

Noradrenergic axon terminals in the hippocampus possess NMDA receptors mediating exocytotic-like NE release (Jones et al., 1987; Fink et al., 1992; Raiteri et al., 1992). In absence of depolarizing stimuli, activation of these receptors by NMDA/glycine only occurs when external medium is Mg^{2+}-free. However, if SRIF-14, inactive on its own on NE release, is added together with NMDA/glycine, release of NE occurs also in medium containing physiological concentrations of Mg^{2+}. Due to the characteristics of the technique used to monitor release (a monolayer of synaptosomes up-down superfused in conditions minimizing indirect effects; see Raiteri and Raiteri, 2000), sst5 receptors and NMDA receptors are likely to coexist and interact in noradrenergic axon terminals (Pittaluga et al., 2000).

The release of NE elicited by SRIF-14/NMDA/glycine, in presence of external Mg^{2+}, was completely prevented by the sst5 receptor antagonist BIM-23056 and by the NMDA receptor antagonist MK-801. Furthermore, it was largely reduced when PLC was inhibited by U73122. It was reported that SRIF receptors of the sst5 type can couple to PLC and to enhancement of phosphoinositide metabolism (Wilkinson et al., 1997), with consequent production of IP_3 and DAG. Thus it seems that PLC activation by SRIF acting at sst5 receptors plays an important role in the function of NMDA receptors, in presence of extraterminal Mg^{2+}. The mechanism is probably indirect and mediated by downstream signalling molecules of PLC, in particular PKC. Accordingly, the release of NE provoked by

SRIF/NMDA/glycine was in part reduced by selective PKC inhibitors (Table 1; see also Pittaluga et al., 2000). PKC can phosphorylate NR1 or NR2 subunits leading, respectively, to inhibition or potentiation of NMDA receptor responses (MacDonald et al., 2001; Kotecha and MacDonald, 2002). The finding that PKC blockade inhibited the evoked NE release is compatible with the view that PKC participates in the phosphorylation of NR2 subunits of the NMDA receptor. A number of PKA phosphorylation sites exist on NMDA receptors (Leonard and Hell, 1997). We here found that inhibition of PKA by H89 had no effect on the release of NE evoked by SRIF-14/NMDA/glycine, excluding the involvement of the enzyme.

Our results suggest that Src-mediated tyrosine phosphorylation plays a role. This is supported by the inhibition of the evoked NE release observed in presence of two Src inhibitors as well as by the finding that anti-phosphotyrosine antibodies prevented the effect of SRIF-14/NMDA/glycine.

The release-enhancing NMDA receptor present on hippocampal noradrenergic terminals is an NR2B-containing receptor (Pittaluga et al., 2001). NMDA receptor subunit NR2B is the major tyrosine phosphorylated protein in the postsynaptic density (Moon et al., 1994). Src is associated with NMDA receptors and phosphorylation by Src upregulates NMDA receptor currents (Yu et al., 1997; Lu et al., 1999). Several studies have addressed the signalling mechanisms controlling Src-family kinase activation during glutamatergic transmission. One likely signal is the cell-adhesion kinase β/proline-rich tyrosine kinase 2 (CAKβ/Pyk2; Huang et al., 2001) which can be stimulated by PKC; in turn CAKβ binds and activates Src-family kinases (Dikic et al., 1996). Thus, the PLC/PKC/CAKβ/Src pathway may well couple sst5 receptor activation and NMDA receptor function in hippocampal noradrenergic neurons. This cascade has been proposed to mediate upregulation of NMDA currents by various GPCRs (Lu et al., 1999; Heidinger et al., 2002; Kotecha et al., 2003; Salter and Kalia, 2004).

The sst5-NMDA receptor-receptor interaction seems to strictly depend on CaMKII activity, since KN93 completely abolished the evoked NE release. Increased cytosolic Ca^{2+} availability from various sources, including stimulation of IP_3 receptors consequent to PLC activation, could trigger rapid CaMKII autophosphorylation and translocation to NR2B subunits of the NMDA receptor (Leonard et al., 1999; see Soderling et al., 2001). The interaction between NR2B and CaMKII was reported to lock the enzyme in an active conformation (Bayer et al., 2001); according to the authors, binding of CaMKII to NR2B and enzyme autophosphorylation can function synergistically, thus constituting a feed-forward pathway able to positively affect NMDA-mediated transmission.

Antagonists at sst5 and NMDA receptors completely abolished the SRIF-14/NMDA/glycine-evoked NE release. Inhibition of CaMKII also totally blocked the evoked release of the catecholamine. In contrast, PKC and Src inhibitors prevented only in part the SRIF-14/NMDA/glycine effect. The finding that PKC and Src inhibitors, when combined, did not elicit additive effect is consistent with PKC and Src working in series.

At this point, it seems important to recall that NMDA and glycine, added in presence of Mg^{2+} ions and without depolarization, are unable to elicit NE release, while release occurs when SRIF-14 is added with the NMDA receptor coagonists. One could therefore distinguish two aspects of the SRIF action: a permissive role on the *activation* of the NMDA receptors, followed by *upregulation* of the receptor ongoing activity.

To shed light on the question, we entrapped into synaptosomes $pp60^{c\text{-}Src}$, a recombinant Src in the activated form (Yu et al., 1997; Lu et al., 1999), having assumed that Src comes last in the PLC/PKC/CAKβ/Src sequence. Should Src permit NMDA receptor activation, NE release would be observed when $pp60^{c\text{-}Src}$-staffed synaptosomes are exposed to NMDA/glycine in presence of Mg^{2+}. However, if Src only potentiates the ongoing NMDA activity, NMDA/glycine would release NE from $pp60^{c\text{-}Src}$-staffed synaptosomes only in Mg^{2+}-

free medium. Entrapped $pp60^{c\text{-}Src}$ failed to permit NMDA receptor activation in Mg^{2+}-containing medium, but potentiated the ongoing activity of NMDA suggesting that the PKC/CAKβ/Src cascade mediates upregulation of the function of NMDA receptors already activated. This idea does not exclude the participation of other agents, considering the impressive number of proteins constituting the NMDA receptor complex (Husi et al., 2000).

How sst5 receptors mediate *activation* of NMDA receptors on noradrenergic neurons remains to be established. Multiple mechanisms can be envisaged. Based on our results, a likely possibility is that phosphorylation of NR2B subunits by CaMKII leads to removal of Mg^{2+} from the NMDA receptor channel and to activation of the receptor in absence of depolarization. Another possibility stems from the increasing evidence that GPCRs can interact with NMDA receptors by physically associating with their subunits (Liu et al., 2000; Fiorentini et al., 2003; Salter, 2003). SRIF receptors of the sst2 type were found to physically associate with a scaffolding protein which can also bind to NR subunits (Peineau et al., 2003). One may speculate that also sst5 can perform a similar interaction, resulting in NMDA receptor activation.

Kotecha et al. (2003) have found that, in cultured hippocampal neurons, co-activation of mGluR5 and NMDA receptors upregulate NMDA receptor currents through the PKC/CAKβ/Src cascade. Interestingly, NMDA channels must be gated to an open state during stimulation of mGluR5 for the upregulation to occur; in fact, no potentiation could be observed in presence of Mg^{2+}. Differently, co-application of SRIF-14 and NMDA/glycine elicited NE release in presence of external Mg^{2+}, compatible with the idea that somatostatin initially permits NMDA receptor activation in presence of physiological concentrations of Mg^{2+} and without depolarization and subsequently mediates upregulation of receptor function through the PKC/CAKβ/Src pathway. Together with previous reports (see Kotecha and MacDonald, 2002 and references therein), the present results confirm the multiplicity of the

pathways that can be implicated in GPCR-NMDA receptor interactions, indicating the importance of their careful characterization.

To our knowledge, a clear distinction between activation and upregulation of NMDA receptor function by GPCRs has not been previously considered. The reason may be that, in the experimental systems generally used, one can measure a 'basal channel activity' which may be decreased or, more frequently, augmented by GPCR activation. In our system, the endogenous agonists released are immediately removed by the medium up-down superfusing the synaptosomal thin layer, so that NMDA and SRIF receptors remain virtually ligand-free and functionally silent. Addition of NMDA/glycine can not elicit any NE release if Mg^{2+} is present, but release occurs if SRIF and NMDA/glycine are added concomitantly. Thus our technique permits to identify conditions leading to NMDA receptor activation or to potentiation of the receptors already activated in absence of Mg^{2+}.

Considering the involvement of NMDA receptors, somatostatin and norepinephrine in memory and learning (Olias et al., 2004 and references therein), the effects of SRIF on the NMDA-evoked release of NE in the hippocampus deserves further investigation, also in view of the development of selective somatostatin receptor agonists (see, for instance, Rohrer et al., 1998) to be employed in conditions of cognitive impairments.

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Footnote

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Legends to the Figures

Figure 1. Upper panel: The effect of SRIF-14 on the NMDA/glycine-evoked release of [^{3}H]NE, in presence of 1.2 mM Mg^{2+}, is mimicked by the partial sst5 agonist L-362,855 and prevented by the selective sst5 antagonist BIM-23056. Results are expressed as percentages of increase over basal release. Data are means ± S.E.M. of three experiments run in triplicate. * $p < 0.05$ vs. control. Lower panel: Representative western blot of sst5 receptor proteins located in purified rat hippocampal synaptosomal membranes. Each lane corresponds to a synaptosomal membrane suspension prepared from a different animal. The presence of sst5 protein was detected by western blot technique using selective antibodies raised against sst5 receptor protein.

Figure 2. Effects of kinase inhibitors on the released [^{3}H]NE induced by SRIF-14 + NMDA + glycine from rat hippocampal synaptosomes. **Panel A:** Effects of Src inhibitors. Open square: control (no drug added); grey square: SRIF-14 (1 nM) + NMDA (100 μM) + glycine (1 μM); black diamond: + PP2 (1 μM); grey diamond: + lavendustin A (5 μM). PP2 and lavendustin A failed on their own to affect the basal release of [^{3}H]NE (1 μM PP2 = 6.34 ± 5.87; 5 μM lavendustin A = -2.51 ± 3.67). **Panel B:** Effect of the CaMKII inhibitor KN93. Open square: control (no drug added); grey square: SRIF-14 (1 nM) + NMDA (100 μM) + glycine (1 μM); black square: + KN93 (1 μM). KN93 failed on its own to affect the basal release of [^{3}H]NE (1 μM KN93 = 4.87 ± 5.32). Results are expressed as percentages of increase over basal release. Data are means ± S.E.M. of at least four experiments run in triplicate. * $p < 0.05$ vs. control; # $p < 0.05$ vs. SRIF-14 + NMDA + glycine.

Figure 3. Effect of entrapped anti-phosphotyrosine antibodies on the [^{3}H]NE release induced by SRIF-14 + NMDA + glycine from rat hippocampal synaptosomes. Synaptosomes with or without entrapped antiphosphotyrosine antibodies were exposed to SRIF-14 (1 nM) + NMDA (100 μM) + glycine (1 μM). Empty bar: control synaptosomes; black bar: antibody-staffed synaptosomes. Results are expressed as percentage of increase over basal release. Data are means ± S.E.M. of three experiments run in triplicate. * $p < 0.05$ vs. control.

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Table 1. PLC and PKC, but not PKA, mediate the [^{3}H]NE release induced by SRIF-14 + NMDA + glycine from rat hippocampal synaptosomes

	[^{3}H]NE release
SRIF-14 + NMDA + glycine	71.16 ± 8.95
SRIF-14 + NMDA + glycine + U73122 (0.1 μM)	18.77 ± 7.33[a]
SRIF-14 + NMDA + glycine + GF109203X (0.1 μM)	24.36 ± 3.09[a]
SRIF-14 + NMDA + glycine + H89 (0.5 μM)	66.01 ± 15.34

SRIF-14 (1 nM), NMDA (100 μM) and glycine (1 μM) were added 8 min after the enzyme inhibitors. Enzyme inhibitors failed, on their own, to affect the basal release of [^{3}H]NE (0.1 μM U73122 = 4.72 ± 3.56; 0.1 μM GF109203X = -3.89 ± 2.34; 0.5 μM H89 = 6.78 ± 5.43). Results are expressed as percentage of increase over basal release. Data are means ± S.E.M. from three to eight experiments run in triplicate (three superfusion chambers for each experimental condition). [a] $p < 0.05$ vs. control.

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Table 2. Effects of PKC and Src inhibitors, alone or in combination, on the [^{3}H]NE release induced by SRIF-14 + NMDA + glycine from rat hippocampal synaptosomes

	[^{3}H]NE release
SRIF-14 + NMDA + glycine	72.97 ± 13.36
SRIF-14 + NMDA + glycine + GF109203X (0.1 μM)	29.84 ± 6.51[a]
SRIF-14 + NMDA + glycine + PP2 (1 μM)	27.47 ± 6.73[a]
SRIF-14 + NMDA + glycine + GF109203X (0.1 μM) + PP2 (1 μM)	28.36 ± 8.94[a]

SRIF-14 (1 nM), NMDA (100 μM) and glycine (1 μM) were added 8 min after the kinase antagonists. Results are expressed as percentage of increase over basal release. Data are means ± S.E.M. from five experiments run in triplicate. [a] $p < 0.05$ vs. control.

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Table 3. Effects of entrapped pp60$^{c\text{-}Src}$ on the NMDA-evoked [^{3}H]NE release in presence or absence of extraterminal Mg^{2+} ions

	[^{3}H]NE release	
	Control	pp60$^{c\text{-}Src}$
NMDA(100 μM) + glycine(1 μM)		
[Mg^{2+}(1.2 mM)]	6.34 ± 3.25	0.57 ± 4.86
NMDA(100 μM) + glycine(1 μM)		
[Mg^{2+}(0 mM)]	64.32 ± 5.81	142.24 ± 8.65^a

Synaptosomes with or without entrapped pp60$^{c\text{-}Src}$ were prepared as described in Materials and Methods and superfused with physiological medium or Mg^{2+}-free medium. Results are expressed as percentage of increase over basal release. Data represent the media ± S.E.M. of three experiments run in triplicate. ^{a} $p < 0.05$ at least vs. respective control.