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# An Endogenous Adrenoceptor Ligand Potentiates Excitatory Synaptic Transmission in Cultured Hippocampal Neurons

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**Noradrenergic inputs modulate hippocampal function via distinct receptors. In hippocampal neuronal cultures, mRNA expression of adrenoceptor subtypes is maintained from 1 day *in vitro* (DIV) to 22 DIV. Noradrenaline dose-dependently stimulates phosphoinositide (PI) breakdown in both immature and mature cultures through the activation of  $\alpha 1$  receptors. At 22 DIV, basal PI breakdown depends on excitatory synaptic activity since it is decreased by tetrodotoxin or glutamate receptor antagonists. At 22 DIV, a similar decrease of basal PI breakdown is also observed with  $\alpha 1$ ,  $\alpha 2$  or  $\beta$  adrenoceptor antagonists. These effects are not additive with that produced by tetrodotoxin. Adrenergic antagonists also strongly reduce spontaneous excitatory post-synaptic currents (sEPSC) as evidenced by whole cell recording. Therefore, in hippocampal cultures, excitatory transmission is modulated by a tonic activation of adrenoceptors probably produced by an endogenous ligand. Indeed, (i) the depletion of catecholamine pools by reserpine also decreases both basal PI metabolism and sEPSC; (ii) hippocampal neurons possess both tyrosine hydroxylase (TH) and dopamine- $\beta$ -hydroxylase mRNAs, encoding enzymes required for catecholamine synthesis; and (iii) some hippocampal neurons show TH-immunoreactivity. TH-positive cells are also detected in E18 hippocampal sections. Thus, cultured hippocampal neurons synthesize and release an adrenergic-like ligand, which tonically potentiates excitatory synaptic transmission in mature cultures.**

## Introduction

In adult hippocampal formation, three adrenoceptor subtypes ( $\alpha 1$ ,  $\alpha 2$  and  $\beta$ ) are expressed, with  $\alpha 1D$ ,  $\alpha 2A$ ,  $\alpha 2C$ ,  $\beta 1$  and  $\beta 2$  showing the strongest mRNA expression (Rainbow *et al.*, 1984; McCune *et al.*, 1993; Nicholas *et al.*, 1996). Moreover, the numerous actions of noradrenaline (NA) throughout all hippocampal areas via the activation of these adrenergic receptors have been demonstrated in numerous reports (Vizi and Kiss, 1998). NA profoundly modulates both excitatory and inhibitory synaptic transmission, as demonstrated in slices and in cultures. More precisely,  $\beta$  adrenergic receptor stimulation is always associated with a potentiation of excitatory transmission (Gereau and Conn, 1994; Huang and Kandel, 1996; Katsuki *et al.* 1997; Huang *et al.*, 1998), whereas the stimulation of  $\alpha 2$  adrenoceptors leads to an inhibition of excitatory transmission in cultured hippocampal cells (Boehm, 1999). Alpha-1 adrenergic receptors are associated with both hyperpolarization (Madison and Nicoll, 1986; Pedarzani and Storm, 1996) and depolarization (Bergles *et al.*, 1996) of hippocampal cells. These receptors can be involved either in presynaptic inhibition of glutamate release in area CA3 of the hippocampus (Scanziani *et al.*, 1993) or in excitation of CA1 hippocampal interneurons, leading to an increased inhibitory transmission (Bergles *et al.*, 1996). A similar action of  $\alpha 1$  adrenergic receptors has also been reported in cortical interneurons (Kawaguchi and Shindou, 1998). Conversely, in magnocellular neurons of hypothalamic slices, the activation of  $\alpha 1$  receptors induces an increase of spontaneous excitatory

synaptic transmission *via* the stimulation of glutamate-releasing neurons (Daftary *et al.*, 1998).

Considering the major regulatory effects exerted by adrenoceptor stimulation on both synaptic transmission and synaptic plasticity in mature hippocampus, it is conceivable that adrenoceptor activation could have an influence on hippocampal developmental plasticity. In this respect, adrenergic modulations could be retained during the formation of hippocampal neuronal network *in vitro*. To investigate this hypothesis, we checked the expression of the different adrenergic receptor subtype mRNAs by RT-PCR during *in vitro* development of hippocampal neurons in primary culture. Then we focused on the function of the expressed receptors in the regulation of both phosphoinositide (PI) metabolism and spontaneous excitatory synaptic transmission.

Our data are consistent with the existence in mature hippocampal cultures of a continuous potentiation of excitatory synaptic transmission via the tonic activation of adrenergic receptor subtypes, probably due to the endogenous synthesis and release of an adrenoceptor agonist by some neurons in these cultures. Parts of these data have been previously presented in an abstract form (Aubert *et al.*, 2000).

## Materials and Methods

### Hippocampal Cultures

Hippocampi were dissected from E18 rat fetuses taken from Sprague-Dawley rats ('Centre d'Elevage Dépre', Saint Doulchard, France) and then incubated for 12 min in Versene (Life Technologies, Cergy Pontoise, France). After two washes in phosphate-buffered saline (PBS), cells were mechanically dissociated in culture medium using restricted, fire-polished pipettes. Culture medium contained DMEM/HAM-F12 (Life Technologies) supplemented with glucose (33 mM), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), sodium bicarbonate (13 mM), HEPES (5 mM), insulin (87  $\mu$ M), apo-transferrin (50  $\mu$ g/ml), progesterone (20 nM),  $\beta$ -estradiol (1 pM), 3,5,3'-triiodotyronine (3 nM), putrescine (100  $\mu$ M) and sodium selenite (46 nM). After centrifugation (4 min, 400 g), the pellet of dissociated cells was dispersed in culture medium and viable cells (trypan blue extruding cells) were counted. Then  $7.5 \times 10^5$  cells were then plated in 1.9 cm<sup>2</sup> area culture dishes (Nunc, Roskilde, Denmark) containing 0.5 ml of culture medium. Culture dishes had been previously coated with poly-L-lysine (7.5  $\mu$ g/ml) and then with DMEM/F12 containing 10% foetal calf serum (Life Technologies) before plating cells in culture medium. For electrophysiological experiments,  $2 \times 10^6$  cells were plated on rectangular (10  $\times$  11 mm) Thermanox (Nunc) coverslips in 26  $\times$  33 mm culture dishes containing 2 ml of culture medium. Cells were then maintained until the day of experiment, at 37°C in a 5% CO<sub>2</sub> atmosphere, in the same culture medium, without any changes.

### RT-PCR and Southern Blot

RT-PCR experiments were performed in order to investigate the expression of mRNA encoding the various adrenergic receptor sub-

types and the enzymes involved in catecholamine synthesis, tyrosine hydroxylase (TH) and dopamine- $\beta$ -hydroxylase (DBH).

#### Total RNA Preparation

Total cellular RNAs of hippocampal cell cultures were extracted according to the procedure Salvatori *et al.* (Salvatori *et al.*, 1992) and adapted to 24-well culture plates. For this purpose, each well received 250  $\mu$ l of lysis solution and cell lysate was collected in 500  $\mu$ l fractions; then the protocol was followed as described (Salvatori *et al.*, 1992). RNAs extracted from different tissues were used for some control experiments. Hippocampi of both rat embryos (E18) and 25-day-old rats (P25) and adrenal glands of adult rats were rapidly dissected, frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  until total RNA extraction. Extractions from tissues were performed with Trizol reagent (Life Technologies) according to the protocol provided by the manufacturer.

To completely remove genomic DNA from the various RNA samples, a DNase I treatment step was systematically performed. Two reactions were conducted simultaneously for the reverse transcription (RT) reaction step – one in the presence of RT enzyme and the other in the absence of RT enzyme for negative controls – in order to demonstrate that the PCR products were the results of RNA- but not genomic DNA-amplification.

#### Oligonucleotide Primers

PCR primer sequences for  $\alpha$ 1A,  $\alpha$ 1B,  $\alpha$ 2A,  $\alpha$ 2B and  $\alpha$ 2C mRNA detection were selected according to previously published criteria (Vidovic *et al.*, 1994; Scofield *et al.*, 1995): GTAGCCAAGAGAGAAAGCCG and CAACCCACCACGATGCCAG for  $\alpha$ 1A, GCTCCTTCTACATCCCGCTCG and AGGGGAGCCAACATAAGATGA for  $\alpha$ 1B, GCGCCCCAGAACCTCTCTCTGGTG and GAGTGGCGGGAAAAGGATGACGGC for  $\alpha$ 2A, AAAGCAGCCACTGCAGAGGTCTC and ACTGGCACTCCACATTCTTGCC for  $\alpha$ 2B, CTGGCAGCCGTGGTGGGTTCCTC and GTCGGG-CCGGCGGTAGAAAGAGAC for  $\alpha$ 2C.

Other PCR primers were chosen from published cDNA sequences (access nos L31771, D00634, X17607, S56481, M10244 and L12407 for  $\alpha$ 1D,  $\beta$ 1,  $\beta$ 2,  $\beta$ 3, TH and DBH, respectively): CGTGTGCTCCTTCTACCT-ACCC and CGACGATGGCCAACGTCTTGGC for  $\alpha$ 1D, TCATCGTGCTG-CTCATCGTAGT and CATGAGGATGGGACGGAAGGA for  $\beta$ 1, TTCTGG-TGCGAGTTCTGGACT and CTGGAAGACCCTGGAATAGAC for  $\beta$ 2, TAG-TTCCCAGCGAGTTTTCATC and GAACTCACTCTGAGCAGAAATCAA for  $\beta$ 3, CACAGCGGAAGAGATTGCTAC and TCCAATGTCTGGGAGAACTG for TH, CTGTCCATCTAGTGATGGGA and TCATACATGATGATGTGGTG for DBH. Potential cross-reactivity between oligonucleotide primers was checked using the software program Amplify (William Engels, University of Wisconsin). The specificity of the individual primer pairs for the respective cDNA target was verified by agarose gel electrophoresis followed in some cases by Southern blot. Oligodeoxynucleotide primers were purchased from Genosys (Cambridge, UK) or from Life Technologies.

#### Reverse Transcription and PCR Amplification

Five microlitres of DNase treated-RNA were reverse transcribed in a 20  $\mu$ l reaction mixture containing 50 mM Tris, 75 mM KCl, 3 mM  $\text{MgCl}_2$ , 10 mM dithiothreitol and 0.5  $\mu$ g oligo(dT)<sub>12-18</sub> (Life Technologies). After incubation at  $95^{\circ}\text{C}$  for 2 min and then  $70^{\circ}\text{C}$  for 5 min, 200 units of superscript II reverse transcriptase (Life Technologies) and 500  $\mu$ M each deoxy-nucleotide (dNTP) (Life Technologies) were added. Incubation was performed for 1 h at  $42^{\circ}\text{C}$  and then for 5 min at  $95^{\circ}\text{C}$ .

Two microlitres of RT products were used for PCR amplifications in a 50  $\mu$ l reaction mixture containing 10 mM TrisHCl pH 9.0, 2 mM  $\text{MgCl}_2$ , 50 mM KCl, 0.1% Triton X100, 200  $\mu$ M each dNTP, 1  $\mu$ M sense and antisense primers and 1 unit Taq DNA polymerase (Promega, Madison, WI), overlaid with mineral oil. The PCR was performed in an Amplifon II Thermal cycler with the following protocol: 3 min at  $95^{\circ}\text{C}$ ; then 35 cycles of 1 min at  $94^{\circ}\text{C}$ , 1 min at annealing temperature ( $58^{\circ}\text{C}$  for DBH;  $60^{\circ}\text{C}$  for  $\alpha$ 1B,  $\alpha$ 1D,  $\alpha$ 2B,  $\beta$ 2 and  $\beta$ 3;  $64^{\circ}\text{C}$  for  $\alpha$ 1A,  $\alpha$ 2A,  $\alpha$ 2C and  $\beta$ 2;  $65^{\circ}\text{C}$  for TH) and 1 min at  $72^{\circ}\text{C}$ , followed by 5 min at  $72^{\circ}\text{C}$ . The PCR products were separated on a 2% agarose gel stained with ethidium bromide.

#### Southern Blot

After electrophoresis, PCR products were transferred onto Nylon membrane (Hybond, Amersham, UK) and then UV cross-linked. Digoxigenin labelling of oligonucleotide probes (ACTGTCCGCCCGTGATTTC and AGTATGTGGTCTCAGTGCTGG for TH and DBH, respectively) was performed using the 'DIG oligonucleotide 3'-end labeling kit' from Roche Diagnostics (Meylan, France), following the protocol of the manufacturer. Hybridization of the membrane was run at  $64^{\circ}\text{C}$ . The detection was performed using an anti-DIG alkaline phosphatase-conjugated antibody and CDP-star as the chemiluminescent substrate (Roche Diagnostics), as described by the manufacturer.

#### Inositol Phosphate (IP) Measurements

IP measurements were performed as previously described (Blanc *et al.*, 1995, 1999). Briefly, PI were labelled for 24 h by incorporating 1.5  $\mu$ Ci/well myo[2- $^3\text{H}$ (N)]inositol (sp. act. 15 Ci/mmol; ICN, Orsay, France). After two washes in Krebs-Ringer buffer (comprising: 125 mM NaCl, 3.5 mM KCl, 25 mM  $\text{NaHCO}_3$ , 1.25 mM  $\text{KH}_2\text{PO}_4$ , 1.5 mM  $\text{CaCl}_2$ , 1.25 mM  $\text{MgSO}_4$ , 10 mM D-glucose and 10 mM HEPES, bubbled with  $\text{O}_2/\text{CO}_2$ ; 95/5) at  $37^{\circ}\text{C}$ , cells were incubated for 15 min with 10 mM LiCl and then for a further 30 min with agonist, or buffer for control. When required, antagonists were added at the same time as LiCl, i.e. 15 min prior to agonist stimulation. IPs were extracted with perchloric acid and separated on Dowex 1X8 formate-form columns (Biorad) as previously described (Blanc *et al.*, 1995, 1999). Inositol monophosphate (IP1) formation was measured.

#### Electrophysiological Measurements

On the day of the experiment, a coverslip with plated cells was transferred to the recording chamber mounted on an inverted microscope (IMT2, Olympus, Japan). Cells were perfused with the extracellular solution containing: 124 mM NaCl, 3 mM KCl, 26 mM  $\text{NaHCO}_3$ , 1.25 mM  $\text{NaH}_2\text{PO}_4$ , 1.5 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgSO}_4$  and 10 mM D-glucose (bubbled with  $\text{O}_2/\text{CO}_2$ ; 95/5), at room temperature. Spontaneous excitatory postsynaptic currents (sEPSC) were measured using whole-cell recording with glass microelectrodes (4–5 M $\Omega$  resistance) filled with a solution containing: 120 mM CsMeSO<sub>3</sub>, 1 mM NaCl, 1 mM  $\text{MgCl}_2$ , 10 mM BAPTA, 5 mM N-(2,6-dimethyl-phenylcarbamoylmethyl)-triethylammonium bromide (QX-314), 5 mM HEPES pH 7.3 and 4 mM Mg-ATP. Neurons were voltage-clamped at  $-60\text{mV}$  and sEPSC recorded in the presence of picrotoxin (50  $\mu$ M) in the perfusate, to eliminate GABA<sub>A</sub>-receptor mediated spontaneous inhibitory currents. In the presence of tetrodotoxin (TTX, 500 nM), miniature EPSCs (mEPSCs) could be recorded. EPSC were filtered at 1 kHz, sampled at 10 kHz with patch-clamp amplifier (Axopatch 200 B, Axon Instruments, USA) and digitized (Digidata 1200 Interface, Axon Instruments, USA). Continuous recording and analysis of both sEPSC and mEPSC were performed with John Dempster's software ('WinCDR'). Drugs were applied for 300 s via the perfusion. In order to analyse sEPSC, a detection threshold was set at 30 pA with a minimal time of 500 ms between detected events. For the calculation of the frequency of bursts, events were analysed for 200 s before and for 200 s during the application of drugs. Miniature EPSC were analysed by setting the threshold detection at 5 pA with a minimal time between events of 100 ms. A paradigm similar to the one set up for the analysis of sEPSC was then used to determine the frequency and the amplitude of mEPSC.

#### TH Immunolabelling

Immunocytochemistry on cultured cells was performed directly on plastic wells. Cells were fixed in 4% paraformaldehyde and then pre-incubated for 2 h at room temperature in PBS containing 0.2% bovine serum albumin (BSA), 20% horse serum and 0.1% Triton X-100. Incubation with monoclonal anti-TH antibody (1/5000 clone TH2 from Sigma, L'Isle d'Abeau Chesnes, France) was performed overnight at  $4^{\circ}\text{C}$  in PBS containing 0.2% BSA and 2% horse serum. After two washes in PBS-BSA 0.2%, cells were incubated in biotinylated secondary anti-mouse antibody (1/100) from Vectastain Elite ABC kit (Vector, AbCys-Valbiotech, Paris, France) and samples were then processed as described by the manufacturer. Horseradish peroxidase activity was revealed using the 'VIP' substrate from Vector. For control experiments, primary antibody

was omitted or replaced by mouse IgG (Sigma) at a concentration similar to anti-TH antibody.

TH immunolabelling was also performed on brain sections of E18 rat embryos. Brains were dissected, fixed in 4% paraformaldehyde, incubated in 20% sucrose and then frozen. Cryostat frontal sections (16  $\mu$ m thick) were treated for 30 min with 0.3% H<sub>2</sub>O<sub>2</sub> in PBS and preincubated for 2 h at room temperature in PBS containing 0.2% BSA, 20% horse serum and 0.3% Triton X-100. Sections were then processed as described for cultures, except that secondary antibody concentration was reduced to 1/500. Some parts of these sections were photographed, and the cellular localization of the staining was then checked by nuclear counterstain with Harris's hematoxylin dye.

#### Drugs

D-AP5 [D(-)-2-amino-5-phosphopentanoic acid], CGP55845 [(2S)-3-[[[(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl] (phenylmethyl)phosphinic acid], DNQX (6,7-dinitroquinoxaline-2,3-dione), HEAT [BE 2254 or 2-[[[ $\beta$ -(4-hydroxyphenyl)ethyl]aminomethyl]-1-tetralone], (S)-MCPG [(S)- $\alpha$ -methyl-4-carboxyphenylglycine], NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*l*]quinoxaline-7-sulphonamide), rauwolfscine (17 $\alpha$ -hydroxy-20 $\alpha$ -yohimban-16 $\beta$ -carboxylic acid, methyl ester) and SCH 23390 [R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine] were obtained from Tocris Cookson (Bristol, UK). Noradrenaline [( $-$ )-arterenol: 3,4-dihydroxyphenylethanolamine], picrotoxin, prazosin (1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl)piperazine), D,L-propranolol [1-(isopropylamino)-3-(1-naphthoxy)-2-propanol], QX314 [*N*-(2,6-dimethyl-phenyl)carbamoylmethyl]triethylammonium bromide] and ( $-$ )-sulpiride [( $-$ )-*N*-1-(ethylpyrrolidin-2-yl-methyl)-2-methoxy-5-sulfamoylbenzamide] were purchased from Sigma-Aldrich (France). Reserpine (methyl reserpate 3,4,5-trimethoxy-benzoic acid ester) was obtained from RBI (Natick, USA) and tetrodotoxin from Latoxan (Valence, France).

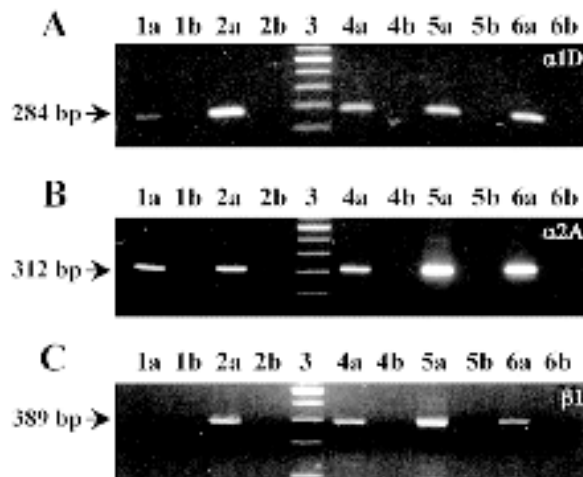
## Results

### Adrenergic Receptors mRNA Expression during *In Vitro* Development

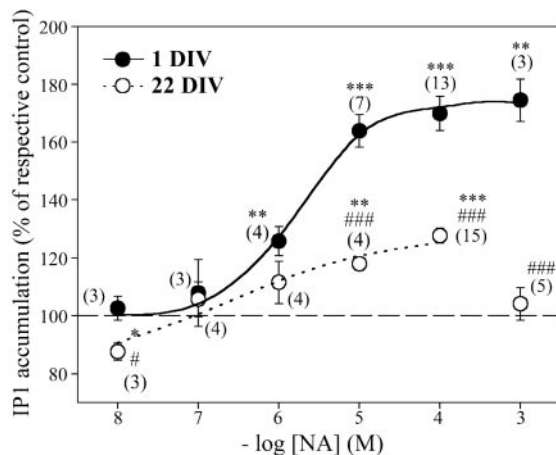
RT-PCR studies demonstrate that mRNAs encoding the different adrenergic receptor subtypes, i.e.  $\alpha$ 1A,  $\alpha$ 1B,  $\alpha$ 1D,  $\alpha$ 2A,  $\alpha$ 2B,  $\alpha$ 2C,  $\beta$ 1,  $\beta$ 2 and  $\beta$ 3, are expressed in cultured hippocampal cells.  $\alpha$ 1D,  $\alpha$ 2A and  $\beta$ 1 subtypes mRNAs are strongly expressed (Fig. 1), as well as  $\alpha$ 1A and  $\alpha$ 2C mRNAs, while  $\alpha$ 1B,  $\alpha$ 2B,  $\beta$ 2 and  $\beta$ 3 mRNAs are weakly detected (data not shown). All these adrenergic receptor mRNAs are also found in both E18 and P25 rat hippocampi, except for  $\beta$ 1 receptor mRNA which is not detectable in E18 hippocampi. In hippocampal cultures, the expression of these mRNAs is maintained throughout *in vitro* development (at 1, 7 and 22 DIV).

### Effect of NA on PI Breakdown in Hippocampal Neurons at 1 and 22 DIV

NA dose-dependently elicits IP1 accumulation in 1 and 22 DIV hippocampal neurons (Fig. 2). At 1 DIV, the maximal accumulation of 170  $\pm$  6% ( $n$  = 13) expressed as a percentage of basal accumulation is reached for a NA concentration of 100  $\mu$ M. A significantly lower maximal IP1 stimulation (128  $\pm$  3%,  $n$  = 15) is obtained by the same concentration of NA in 22 DIV old neurons.  $EC_{50}$  values for NA-induced IP1 accumulation at 1 and 22 DIV are 1.6 and 0.2  $\mu$ M, respectively. While a typical dose-response curve is obtained at 1 DIV (Hill coefficient close to 1), the slope of this curve is lower at 22 DIV (Hill coefficient = 0.42), suggesting a more intricate effect of NA at this stage. Moreover, at 22 DIV, the dose-response curve is bell-shaped and 1 mM NA does not induce any significant accumulation of IP1, which is consistent with a previous report (Blanc *et al.*, 1995).



**Figure 1.** Expression profile of mRNAs encoding  $\alpha$ 1D (A),  $\alpha$ 2A (B),  $\beta$ 1 (C) adrenergic receptors in both cultured hippocampal cells from 1 to 22 DIV and hippocampus at stages E18 and P25 by RT-PCR. Agarose gel electrophoresis of 25  $\mu$ l of PCR products amplified from 1/10<sup>6</sup> cDNA that were reverse-transcribed from 5  $\mu$ l of DNase I treated-RNA. DNase I (Life Technologies) treatment was performed following the supplier's protocol adapted for 4  $\mu$ g of RNA in 10  $\mu$ l with 2 units of enzyme. RT-PCR reactions were performed with (a) or without (b) reverse transcriptase (as a negative control) for the following RNA samples: E18 rat embryo hippocampi RNA (lane 1), RNA from P25 rat hippocampi (lane 2), RNA from hippocampal cultures at 1, 7 and 22 DIV (lanes 4, 5 and 6, respectively). A 100 bp DNA ladder (Life Technologies, brightest band at 600 bp) was loaded in lane 3. Bands of 284 bp, 312 bp and 389 bp in length have the expected sizes of the cDNA fragments defined respectively by the  $\alpha$ 1D (A),  $\alpha$ 2A (B) and  $\beta$ 1 (C) specific oligonucleotide primers.

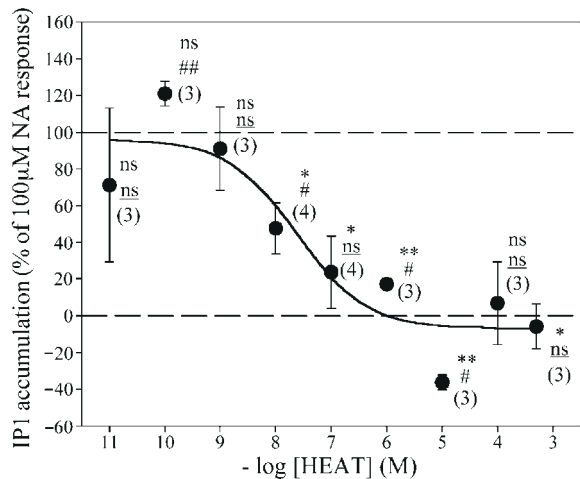


**Figure 2.** Dose-response curves for NA stimulation of [<sup>3</sup>H]inositol monophosphate (IP1) accumulation in hippocampal cultures at 1 DIV (closed circle) and 22 DIV (open circle). Cells were incubated for 30 min with increasing concentrations of NA, as described in Materials and Methods. The data are expressed as percentages of respective basal IP1 accumulation obtained in control cells. These basal IP1 accumulations are 515  $\pm$  17 and 11 936  $\pm$  556 d.p.m./well at 1 and 22 DIV, respectively. The results are means  $\pm$  SEM of 3–15 separate experiments conducted in triplicate (numbers in parentheses refer to the number of experiments). Where absent, the SEM is smaller than the symbol size. Fitting curves were obtained using SigmaPlot software (Jandel Scientific) using the equation:

$$y = y_{\min} + (y_{\max} - y_{\min}) / ([NA]^n / [NA]^n + EC_{50})$$

where  $n$  is equivalent to the Hill coefficient and  $y_{\min}$  and  $y_{\max}$  are the minimal and the maximal responses, respectively. Fitting at 22 DIV was performed omitting the last dot (1 mM NA).  $y_{\min}$  was fixed at 100 and 80% for 1 and 22 DIV, respectively. Values obtained are:  $EC_{50}$  = 1.6  $\mu$ M,  $n$  = 0.92 at 1 DIV and  $EC_{50}$  = 0.2  $\mu$ M,  $n$  = 0.42 at 22 DIV. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 versus respective basal IP1. # $P$  < 0.05, ### $P$  < 0.001 for 22 versus 1 DIV values with Student's *t*-test.





**Figure 3.** Inhibition of the 100  $\mu\text{M}$  NA-stimulated IP1 accumulation in 1 DIV hippocampal neurons by increasing concentrations of HEAT. HEAT was added 15 min prior to NA stimulation which lasted 30 min. Data are expressed as percentages of the 100  $\mu\text{M}$  NA-response and are means  $\pm$  SEM of values from at least three experiments conducted in triplicate (numbers in parentheses refer to the number of experiments). The 100% line corresponds to the NA-stimulated IP1 accumulation. The IP1 response to HEAT alone at the various concentrations tested was taken at 0%. Whatever the concentration used, HEAT *per se* has no significant effect on basal IP1 accumulation in 1 DIV cell cultures. The curve is fitted according to the equation:

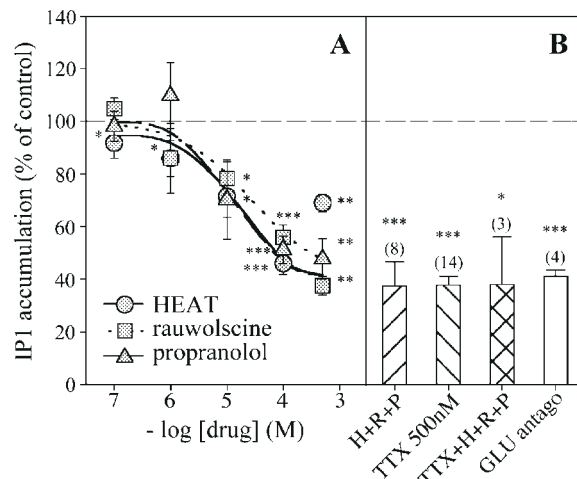
$$y = y_{\min} + (y_{\max} - y_{\min}) / (1 + (IC_{50} / [HEAT])^n)$$

where  $IC_{50} = 24$  nM and the Hill coefficient is  $n = 0.7$ . ns, not significant,  $*P < 0.05$ ,  $**P < 0.01$  versus 100  $\mu\text{M}$  NA-stimulated IP1 accumulation; ns, not significant,  $\#P < 0.05$ ,  $\#\#P < 0.01$  versus corresponding concentration of HEAT using Student's *t*-test.

#### Effects of Adrenergic Receptors Antagonists on Basal and NA-induced PI Breakdown at 1 and 22 DIV

In order to verify that NA effects on PI hydrolysis are mediated by  $\alpha 1$  receptor activation, we used HEAT, a specific  $\alpha 1$  receptor antagonist. At 1 DIV, the 100  $\mu\text{M}$  NA-stimulation is dose-dependently inhibited by HEAT, with an  $IC_{50}$  value of 24 nM. Full inhibition is obtained with 10  $\mu\text{M}$  HEAT (Fig. 3). HEAT *per se* has no significant effect on basal IP1 accumulation at any concentration tested in hippocampal neurons at 1 DIV (data not shown). Antagonists of other adrenergic receptor subtypes (rauwolscine and propranolol,  $\alpha 2$  and  $\beta$  antagonists, respectively) at concentrations up to 10  $\mu\text{M}$  affect neither basal- nor NA-induced IP1 accumulation at 1 DIV (data not shown). This strongly suggests that NA-stimulated PI hydrolysis mainly involves  $\alpha 1$  receptor activation.

A similar inhibition by HEAT of NA-induced IP formation is also observed on neurons at 22 DIV (data not shown). However, at this stage, a dose-dependent decrease of basal IP1 accumulation is observed in the presence of HEAT alone (Fig. 4A). This inhibition is already significant with 100 nM of HEAT and reaches a  $54 \pm 4\%$  inhibition at 100  $\mu\text{M}$  (Fig. 4A). In addition, both  $\alpha 2$  and  $\beta$  antagonists (rauwolscine and propranolol, respectively) also induce a strong decrease in basal IP1 accumulation on their own (maximal effects:  $62 \pm 4$  and  $52 \pm 8\%$  inhibition, respectively). The combination of these three antagonists (100  $\mu\text{M}$  each) does not further decrease the basal IP1 accumulation (Fig. 4B). Therefore, in 22 DIV cells, basal PI hydrolysis partly stems from a tonic stimulation of  $\alpha 1$ ,  $\alpha 2$  and  $\beta$  adrenergic receptor subtypes.



**Figure 4.** Effects of adrenergic receptor and synaptic transmission blockades on basal IP1 accumulation in hippocampal cells at 22 DIV. (A) Dose-response curves of adrenergic receptor antagonists: HEAT ( $\alpha 1$  antagonist, circle), rauwolscine ( $\alpha 2$  antagonist, square) and propranolol ( $\beta$  antagonist, triangle). (B) Effect of adrenoceptor antagonist combination (H + R + P: HEAT, rauwolscine and propranolol, 100  $\mu\text{M}$  each), tetrodotoxin (TTX, 500 nM) and glutamate receptors antagonist cocktail (GLU antago: 500  $\mu\text{M}$  D-AP5, 100  $\mu\text{M}$  DNQX and 1 mM MCPG). Cells were incubated for 45 min with 10 mM LiCl, either in the absence (control) or in the presence of blockers. Data are expressed as percentages of the basal IP1 accumulation obtained in control (dotted line,  $11936 \pm 556$  d.p.m./well). Each point is the mean  $\pm$  SEM of values from 3–14 separate experiments, each performed in triplicate. The dose-response curves are the results of fitting according to the equation:

$$y = y_{\min} + (y_{\max} - y_{\min}) / (1 + (IC_{50} / [DRUG])^n)$$

where  $IC_{50} = 16$ , 28 and 13  $\mu\text{M}$  for HEAT, rauwolscine and propranolol, respectively.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  versus basal IP1 accumulation with Student's *t*-test.

#### Effect of Tetrodotoxin (TTX) and Glutamate Antagonists on Basal IP1 Accumulation

Antagonists of the three subtypes of adrenergic receptors lead to a similar inhibition of basal PI metabolism at 22 DIV, whereas only  $\alpha 1$  adrenergic receptors can directly activate phospholipase C (PLC) and induce inositol triphosphate formation. During *in vitro* development, neurites growth occurs and neurons contact each other, as described previously (Vicario-Abejón *et al.*, 1998), with the potential appearance of synaptic transmission. In this case, endogenously released substances, such as glutamate, could stimulate PI breakdown and thus could participate in the maintenance of basal IP1 accumulation level. In fact, a strong increase in basal IP1 accumulation is observed between 1 DIV ( $515 \pm 17$  d.p.m./well) and 22 DIV ( $11936 \pm 556$  d.p.m./well), as previously observed (Blanc *et al.*, 1999). Moreover, TTX (500 nM), though having no significant effect at 1 DIV on basal IP1 accumulation (data not shown), strongly reduces it at 22 DIV (Fig. 4B). This suggests that synaptic activity contributes to basal PI breakdown.

During *in vitro* maturation, endogenously released glutamate might increase basal PI hydrolysis by acting on both ionotropic and PLC-linked metabotropic receptors (Blanc *et al.*, 1995, 1999). To test the contribution of endogenous glutamate to basal PI metabolism, we used a cocktail of glutamate receptor antagonists, i.e. AP5 (500  $\mu\text{M}$ ), DNQX (100  $\mu\text{M}$ ) and MCPG (1 mM), antagonists of NMDA, AMPA/kainate and PLC-linked metabotropic receptors, respectively. The addition of this antagonist cocktail induces a strong reduction of basal IP1 accumu-

lation ( $41 \pm 2\%$ ,  $n = 4$ ) at 22 DIV (Fig. 4B), while it does not significantly affect it at 1 DIV (data not shown). Therefore, basal IP1 accumulation in 22 DIV cultures is strongly dependent on endogenously released glutamate and thus, at least partly reflects spontaneous excitatory synaptic transmission.

Furthermore, it should be emphasized that the level of inhibition of basal PI breakdown induced either by TTX or by the glutamate receptor antagonist cocktail is close to that obtained with the combination of adrenergic antagonists (Fig. 4B). Moreover, the combination of TTX and adrenergic antagonist cocktail does not elicit a further decrease in basal IP1 accumulation (Fig. 4B), indicating that these effects are not additive and probably depend on each other. This leads us to hypothesize that adrenergic antagonists decrease basal PI hydrolysis by reducing, directly or indirectly, the amount of endogenously released glutamate. To check this, we measured sEPSC, which reflect glutamate-mediated excitatory spontaneous synaptic transmission.

#### **Action of Adrenergic Antagonists on Spontaneous Excitatory Synaptic Transmission**

sEPSC were recorded in mature cells (from 15 up to 40 DIV). As indicated in Materials and Methods, sEPSC were isolated in the presence of the GABA<sub>A</sub> antagonist picrotoxin (50  $\mu$ M). Under these conditions, sEPSC occur as large bursts (Li *et al.*, 1998), with a frequency ranging from 0.05 to 0.3 Hz. These bursts are fully blocked by AMPA receptor antagonist NBQX (10  $\mu$ M) and display a linear voltage-dependency with a reversal potential of  $0.7 \pm 4.3$  mV ( $n = 3$ ) (not shown). Perfusion of adrenergic antagonists, i.e. HEAT, propranolol and rauwolscine, at concentrations of 1 or 10  $\mu$ M, induces a remarkable reduction of the burst frequency (Fig. 5). A similar effect is obtained with another  $\alpha$ 1 adrenergic receptor antagonist, prazosin (10  $\mu$ M; not shown). At 1  $\mu$ M, HEAT, rauwolscine and propranolol elicit inhibitions of control frequency of  $31 \pm 10\%$  ( $n = 6$ ),  $28 \pm 10\%$  ( $n = 4$ ) and  $33 \pm 8\%$  ( $n = 4$ ), respectively (Fig. 5C). At 10  $\mu$ M, HEAT reduces control frequency by  $70 \pm 8\%$  ( $n = 11$ ), propranolol by  $72 \pm 7\%$  ( $n = 10$ ) and rauwolscine by  $82 \pm 10\%$  ( $n = 6$ ) (Fig. 5C). It must be noticed that in some cells, these antagonists at a concentration of 10  $\mu$ M elicit a complete blockade of sEPSC (3/11 cells for HEAT, 2/10 for propranolol and 3/6 for rauwolscine) (Fig. 5B). However, not all the cells are sensitive to these antagonists. Indeed, when tested at 1 or 10  $\mu$ M, HEAT has an effect on 67% of the cells (20/30, including experiments performed at 3  $\mu$ M), propranolol on 78% (14/18) and rauwolscine on 77% (10/13). Similar inhibitory effects of adrenoceptor antagonists on sEPSC were recorded in the absence of picrotoxin (not shown). The inhibition of sEPSC by rauwolscine is partly reduced by CGP55845, a selective GABA<sub>B</sub> antagonist ( $59 \pm 11\%$  inhibition induced by rauwolscine in the presence of CGP55845,  $n = 4$ ; Fig. 5D). None of these adrenergic antagonists alters passive properties of the membrane of the recorded neurons, since no change in input resistance ( $R_{in}$ ) is observed in the presence of these antagonists (data not shown). Therefore, it appears that, on their own, adrenergic antagonists alter spontaneous excitatory synaptic transmission dose dependently in cultured hippocampal neurons.

In order to examine the dependence on action potentials of the effects of adrenergic antagonists on spontaneous glutamatergic synaptic transmission, mEPSC were recorded in the presence of TTX (500 nM) and picrotoxin (50  $\mu$ M). The three adrenergic antagonists tested, at a concentration of 10  $\mu$ M,

modify neither the amplitude nor the frequency of miniature events (data not shown).

Altogether, these results suggest that during *in vitro* development, adrenergic receptors become tonically activated and potentiate excitatory synaptic transmission. Tonic activation of adrenergic receptors could stem from either a constitutive receptor activity or receptor stimulation by an endogenous catecholaminergic ligand synthesized and released in culture.

#### **Effect of Reserpine on both Basal IP1 Accumulation and sEPSC**

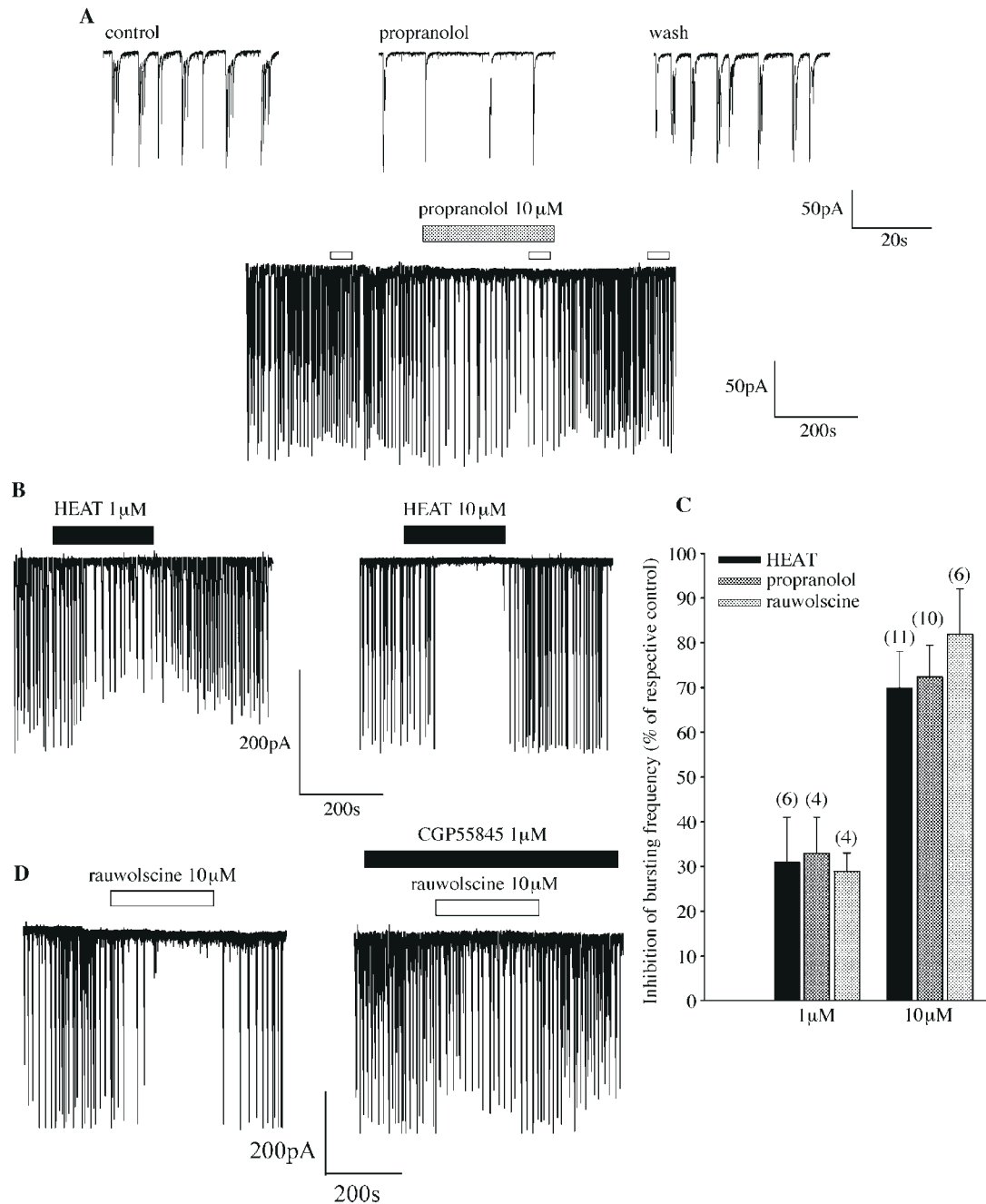
To investigate the latter hypothesis, the effect of reserpine, which depletes catecholamine stores, was tested on both basal PI hydrolysis and sEPSC. PI breakdown was measured at 1 and 22 DIV. Reserpine (10  $\mu$ M) has no effect on IP1 accumulation in 1-DIV-old culture, whereas at 22 DIV it induces a strong decrease in basal PI hydrolysis (Fig. 6A). Dimethylsulphoxide (DMSO; 0.1%), the solvent used to dissolve reserpine, had no effect at either 1 or 22 DIV (Fig. 6A). It should be noted that, at 22 DIV, the reserpine-induced reduction of basal IP1 accumulation is close to the decrease obtained with the cocktail of adrenergic receptor antagonists (Fig. 6A). Moreover, the combination of both reserpine and adrenergic antagonist cocktail does not further reduce basal IP accumulation (Fig. 6A), suggesting that the effects of reserpine and adrenergic receptor antagonists are interdependent.

On mature cells, sEPSC are strongly blocked by reserpine ( $70 \pm 5\%$  inhibition;  $n = 5$ ) (Fig. 6B). The inhibition of sEPSC by reserpine appears to be slower than that induced by adrenergic receptor antagonists (a 10 min treatment is prerequisite). Moreover, reserpine action is long-lasting, even after washing. These data indicate that the release of catecholamines is an important step in the regulation of both basal PI metabolism and sEPSC.

#### **Expression of TH and DBH in Hippocampal Cell Cultures and in Hippocampal Formation of E18 Rat Embryos**

Synthesis of NA and adrenaline require enzymatic machinery of catecholamine biosynthesis pathways, including TH and DBH, which catalyse the synthesis of 3,4-hydroxyphenylalanine from tyrosine and NA from dopamine, respectively. Thus, the ability of hippocampal neurons in culture to synthesize an adrenergic ligand was tested by measuring the expression of mRNAs encoding TH and DBH. RT-PCR experiments, followed by Southern blots, were performed on RNA extracted from both adrenal tissue (positive control) and developing hippocampal neuronal cultures at 1, 7, 14 and 22 DIV. At each culture stage tested, mRNAs encoding TH and DBH are detected (Fig. 7). Specificity of amplified fragments is confirmed by the labelling of an unique band on Southern blot using a DIG-labelled internal oligonucleotide probe (Fig. 7B,D). During *in vitro* development, TH and DBH mRNA expressions apparently increase. At 1 DIV, TH and DBH expressions are faint, whereas at 22 DIV, TH mRNA expression is close to that obtained in adrenal tissue. For the measurement of TH mRNA expression, we also used a second set of oligonucleotides and obtained the same results (data not shown).

In order to verify the expression of TH protein in 22 DIV cultures, we performed immunocytochemical labelling of this protein using a monoclonal anti-TH antibody (Fig. 8A). Few neurons were strongly labelled, but those that were had very large widespread and branched processes (Fig. 8A). On neuronal



**Figure 5.** Effects of adrenergic receptor antagonists on excitatory spontaneous synaptic activity. (A, lower) Single example illustrating the inhibitory action of propranolol (10  $\mu$ M) on sEPSC. (A, upper) Sample traces have been extracted to show synaptic activity on a smaller time scale before (control), during (propranolol) and after (wash) the application of propranolol at the times indicated by small open squares. (B) Records of synaptic activity illustrating the effects of HEAT at 1 and 10  $\mu$ M. (C) Pooled data of the inhibitory effect of HEAT, propranolol and rauwolscine at 1 and 10  $\mu$ M on burst frequency. In order to quantify the inhibitory effect of adrenergic antagonists, burst frequency during the application of the drugs was normalized to control frequency. Data are expressed as mean  $\pm$  SEM (numbers in parentheses represent the number of cells recorded). (D) Effect of CGP55845 (GABA<sub>B</sub> antagonist) on inhibition of sEPSC by rauwolscine. Traces illustrate the effect of rauwolscine alone (10  $\mu$ M, left panel) and rauwolscine (10  $\mu$ M) in the presence of CGP55845 (1  $\mu$ M, right panel) on a single cell.

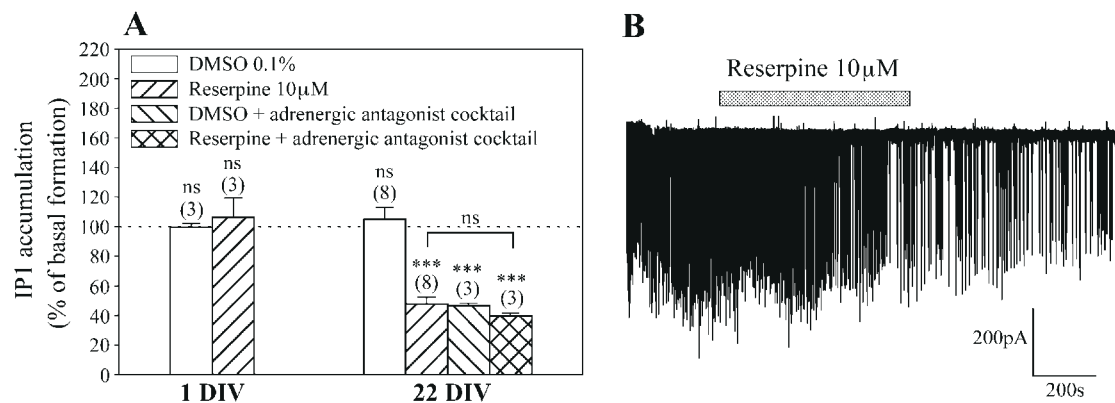
processes, labelling appears as punctate varicosities which sometimes are very close to cellular body of other neurons.

Therefore, *in vitro*, some hippocampal neurons express TH and DBH enzymes, and thus can synthesize an adrenergic ligand, NA or adrenaline.

Finally, we tested whether TH and DBH expressions observed in hippocampal cultures could also be found *in situ* in E18 rat hippocampi, and thus would not stem from an artifact of the

culture procedure. The expression of mRNAs encoding both TH and DBH in E18 rat hippocampi was confirmed by RT-PCR and Southern blot experiments (Fig. 7). Moreover, in frontal sections of E18 rat brain, some cells were positively labelled with an anti-TH antibody (Fig. 8B). It should be noted that labelled cells are mostly localized at the limit between fimbria area and Ammon's horn.

Therefore, in E18 rat hippocampal formation, some cells



**Figure 6.** Effects of reserpine (10  $\mu$ M) on basal IP1 accumulation and sEPSC in hippocampal cell cultures. (A) Effects of reserpine and DMSO (0.1%, solvent used to dissolve reserpine) at 1 and 22 DIV. In addition, adrenoceptor antagonist cocktail including HEAT, rauwolscine and propranolol (100  $\mu$ M each), and a combination of reserpine and the adrenoceptor antagonist cocktail were tested in cultures at 22 DIV. Experiments were performed as described in Materials and Methods. Reserpine or antagonists were added at the same time as LiCl. The data are expressed as percentages of respective basal IP1 accumulation (dotted line). The results are means  $\pm$  SEM of 3–8 separate experiments conducted in triplicate (numbers in parentheses refer to the number of experiments). ns, not significant; \*\*\* $P$  < 0.001 versus basal IP accumulation using Student's  $t$ -test. (B) Single representative recording illustrating the effect of reserpine (10  $\mu$ M) on sEPSC measured in mature cells. Reserpine is applied for 10 min. A long lasting decrease in the occurrence of sEPSC is observed. On average, the basal frequency of sEPSC is inhibited by  $70 \pm 5\%$  ( $n = 5$ ) after treatment with reserpine. *Per se*, DMSO (0.1%) has no effect on sEPSC.

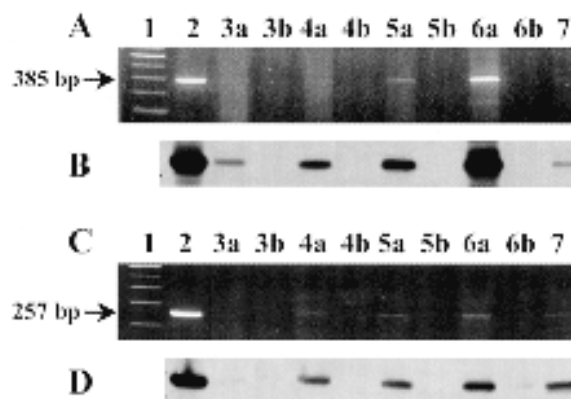
potentially synthesize an adrenergic ligand. This property appears to be maintained in hippocampal neuronal culture.

### Discussion

The major finding of this study is that a tonic activity of adrenoceptors potentiates glutamate-mediated excitatory synaptic transmission in mature cultured hippocampal neurons. The occurrence of this tonic activity is related to the ability of these cells to synthesize and to release an endogenous adrenergic receptor ligand.

Various subtypes of adrenoceptors are expressed in hippocampal cultures throughout their *in vitro* development, as demonstrated by RT-PCR experiments (Fig. 1). Furthermore, pharmacological evidence supports that these adrenoceptors are tonically active and potentiate excitatory synaptic transmission in mature hippocampal cultures: on their own, antagonists of the different adrenoceptor subtypes decrease both basal PI breakdown and sEPSC. It must be emphasized that these two events (basal PLC activity and spontaneous excitatory synaptic transmission) are intermingled in this preparation. Indeed, (i) during *in vitro* development, basal PI metabolism increases, as synaptic transmission takes place; (ii) basal IP formation is decreased by TTX; (iii) spontaneous excitatory synaptic transmission is supported by the release of glutamate and the subsequent activation of post-synaptic glutamate receptors, since it is blocked by NBQX (data not shown); and (iv) a similar decrease of basal PI metabolism is induced by either TTX or glutamate receptor blockade (Fig. 4B). Thus, in mature hippocampal neurons, part of the basal PLC activity stems from the activation of post-synaptic glutamate receptors, via spontaneously released glutamate.

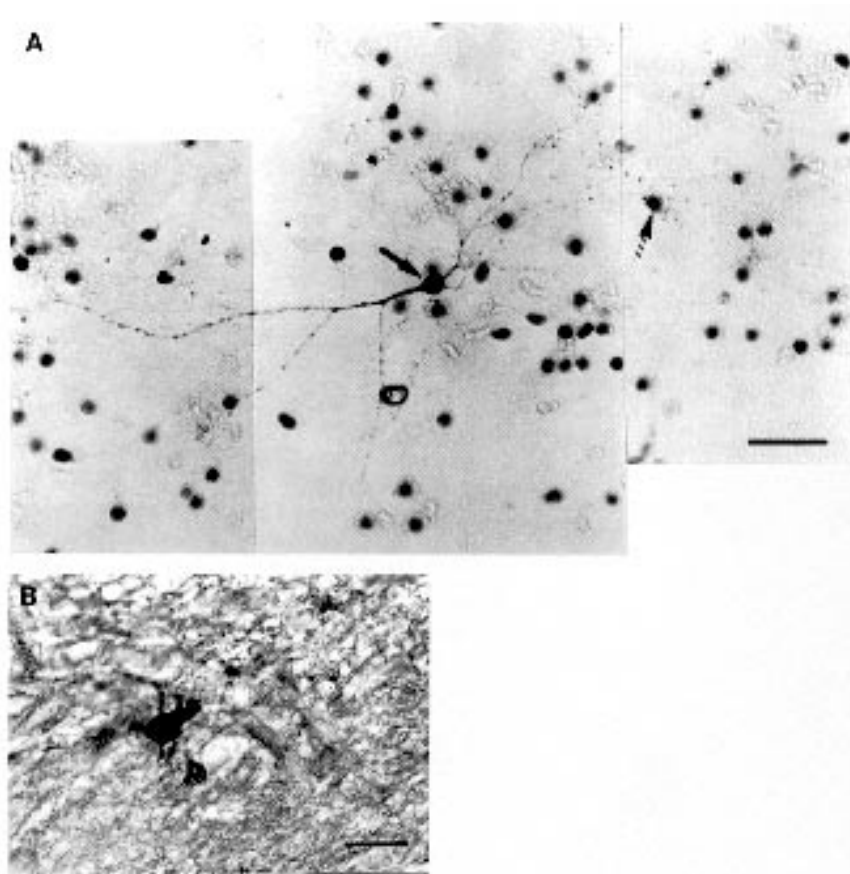
A decrease in basal IP accumulation, similar to that induced by TTX or glutamate receptor antagonists, is also observed with adrenergic antagonists (Fig. 4). Moreover, these two inhibitory effects are not additive. This suggests that inhibition of basal IP accumulation induced by adrenoceptor blockade mainly reflects the action of these antagonists on excitatory synaptic transmission, directly evidenced by measuring sEPSC. Indeed, all these antagonists, HEAT, rauwolscine and propranolol, depress sEPSC (Fig. 5). These effects cannot be attributed to non-specific actions of these antagonists, since: (i) the inhibitory effects of



**Figure 7.** RT-PCR (A,C) and Southern blot (B,D) analysis of mRNAs encoding TH (A,B) and DBH (C,D) during *in vitro* development of hippocampal cultures and in E18 rat hippocampi. RT-PCR analysis were performed as described in Materials and Methods with (a) or without (b) reverse transcriptase (negative control) on 2  $\mu$ g of DNase-treated RNA extracted from adrenal of adult rat (positive control, lane 2), from 1, 7, 14 and 22 DIV hippocampal cultures (lanes 3, 4, 5 and 6, respectively) and from E18 rat hippocampi (lane 7). Lane 1: 100 bp DNA ladder, the brightest band is at 600 bp. Southern blots were performed using specific DIG-labelled oligonucleotide probes as described in Materials and Methods. For DNase treatment, total RNAs were incubated for 4 h at 25°C with RNase free-DNase (Eurogentec, Belgium).

adrenoceptor antagonists are not observed in all the cells recorded, which eliminates the possibility of a non-specific action located, for example, at the level of the membrane; (ii) these antagonists do not induce any changes in the membrane input resistance (data not shown); (iii) the inhibitory effect of these antagonists at 10  $\mu$ M is reversed by the application of adrenoceptor agonists (100  $\mu$ M of isoproterenol, 100  $\mu$ M of clonidine and 1  $\mu$ M of NA for propranolol, rauwolscine and HEAT, respectively); (iv) at the concentrations used, adrenoceptor antagonists do not affect voltage-dependent ion channels, i.e. Na<sup>+</sup>, Ca<sup>2+</sup> and K<sup>+</sup> channels (data not shown); (v) the inhibition of EPSC by adrenoceptor antagonists is fully prevented by TTX (data not shown), indicating that these substances do not block AMPA-mediated spontaneous excitatory transmission simply by acting like glutamate receptor antagonists; (vi) measurements of sEPSC have been performed in the presence of picrotoxin,





**Figure 8.** TH-immunolabelling of hippocampal culture at 22 DIV (A) and of frontal sections of E18 rat brain (B). Experiments were performed as described in Materials and Methods, using a monoclonal anti-TH antibody (1/5000). (A) Scale bar represents 50  $\mu\text{m}$ . Full arrow shows a TH-positive neuron, with a strong labelling all along neurites and in cellular body. Open arrow shows putative release site. (B) Scale bar represents 10  $\mu\text{m}$ .

which excludes the involvement of a non-specific effect of adrenoceptor antagonists on GABA<sub>A</sub> receptors; (vii) these antagonists are not chemically related to each other; and (viii) D1- and D2-like dopamine receptor antagonists (SCH23390 and sulpiride, respectively) have no effect either on basal IP1 accumulation or on sEPSC (data not shown).

Inhibitory effects of adrenoceptor antagonists on EPSC are dependent on action potentials since they are fully prevented by TTX (data not shown). This suggests that adrenergic receptor actions on excitatory transmission require synaptic relays which can be excitatory and/or inhibitory. This may explain why the blockade of all adrenoceptor subtypes leads to the same inhibitory effect, while these receptors are known to be linked to different transduction pathways. For instance, the unexpected excitatory effect of  $\alpha_2$  receptors could stem from the inhibition of inhibitory cells. In fact, even if EPSC are measured in the presence of picrotoxin, inhibitory relays may involve, for instance, gamma-aminobutyric acid B (GABA<sub>B</sub>) receptor activation (Scanziani, 2000). A specific GABA<sub>B</sub> antagonist (CGP55845) partly reduced the inhibitory effect of rauwolscine (Fig. 5D), indicating that at least a GABA<sub>B</sub> inhibitory relay is involved in the  $\alpha_2$  modulation of sEPSC. Other inhibitory mediators, such as serotonin, adenosine or somatostatin, which have been described in hippocampal cultures, could also be involved.

The fact that adrenoceptor antagonists have effects on their own suggests that adrenergic receptors are tonically active in our experimental model. This tonic activity may be due either to a constitutive activity of the receptors themselves or to the

activation by an endogenous ligand. The discrimination between these two hypotheses generally remains a matter of debate (Baxter and Tilford, 1995).

Constitutive activities of some receptors, including adrenoceptors, have been mainly reported in systems expressing either a high level of receptors or mutant receptors, and particularly in transfected cells (Lefkowitz *et al.*, 1993; Kenakin, 1995). In these systems, the constitutive activation could be explained by the presence of pre-coupled receptors to G-proteins, even in the absence of ligand (Lefkowitz *et al.*, 1993). In this case, some antagonists may have an inverse agonism activity by uncoupling the receptor from the G-protein (Milligan and Bond, 1997; Jansson *et al.*, 1998; García-Sáinz and Torres-Padilla, 1999). However, inverse agonist efficacy is strongly dependent on the experimental model used. Indeed, propranolol was reported as an inverse agonist in human  $\beta_2$  receptor-transfected Sf9 cells (Chidiac *et al.*, 1993), but it behaved as a neutral antagonist in neuroblastoma-glioma hybrid cells overexpressing a constitutively active  $\beta_2$  receptor (Milligan and Bond, 1997). While we cannot completely rule out that some modifications of receptor expression targeting or coupling, due to our experimental conditions, might be responsible for the appearance of constitutive activity in our model, it seems to be unlikely that the three adrenoceptor subtypes become constitutively active. In addition, the level of adrenoceptor mRNA expression in hippocampal cultures does not appear to be higher than in adult hippocampus.

In fact, several data strongly suggest that in our experimental model, adrenergic receptors are tonically activated by an en-

ogenous ligand. Indeed, like adrenergic antagonists, reserpine depresses sEPSC (Fig. 6B). This effect is delayed as compared with that of adrenergic antagonists, and almost irreversible by washout, but can be reversed by the application of 1  $\mu$ M NA (data not shown). This is consistent with the hypothesis that reserpine acts through catecholamine store depletion, the effects of which are expected to be delayed and long-lasting. In addition, reserpine inhibits basal PI metabolism in a manner similar to that elicited by adrenoceptor antagonists (Fig. 6A). These effects are not additive, suggesting that these compounds share a common pathway to decrease basal PI breakdown. Since the inhibitory effect of reserpine is equal to that of adrenoceptor antagonist cocktail, it should be assumed that the action of an endogenous catecholamine ligand fully accounts for the tonic activity of these receptors.

Moreover, we show here that hippocampal neurons in culture express mRNAs encoding TH and DBH, the enzymes responsible for the synthesis of adrenergic ligand (Fig. 7). Furthermore, in mature hippocampal cultures, few neurons show strong TH-immunoreactivity in the cell body as well as along the length of very extended neurites, particularly in numerous restricted areas, which may be presynaptic terminals (Fig. 8A). This suggests that, even if adrenergic ligand were released by only a few neurons, the latter may contact a lot of other neurons. Thus, the adrenergic ligand released by one TH-positive neuron can act on a huge number of cells. This action could then be further amplified due to network neuronal activity. Hence, the extracellular agonist concentration is probably highly diluted, whereas the concentration of ligand reached in the synapse might be very high, due to the small volume of the synaptic cleft. This could explain why: (i) so far, we have not been able to quantitate directly extracellular NA or adrenaline by HPLC; and (ii) the concentrations of antagonists required to reverse synaptically released ligand effects (potentiation of sEPSC and the consequent increase of basal PI metabolism) are rather high. Indeed, the comparison of dose-response curves (Figs 3 and 4A) indicates that the concentration of HEAT required to reduce basal IP accumulation at 22 DIV is ~1000-fold higher than that required to inhibit 100  $\mu$ M NA-stimulated IP formation at 1 DIV. As indicated above, this could be related to a very high concentration of endogenous ligand in the synaptic cleft, but might also stem from an increase of receptor efficiency during *in vitro* development. This increase might result from the putative clustering of adrenergic receptors in the synapse or from a modification of receptor coupling efficiency during development. It should be noted that the  $EC_{50}$  of NA is ~10-fold lower at 22 DIV than at 1 DIV (Fig. 2).

It must be emphasized that in mature hippocampal cultures basal IP formation reflects electrical activity, while exogenously added NA-evoked IP formation stems from the direct activation of extra-synaptic PLC-linked adrenoceptors, i.e.  $\alpha$ 1, localized either on neurons or on glial cells. At the synapse, adrenergic receptors are probably saturated by endogenous NA and cannot be further activated by the addition of exogenous agonists. Accordingly, among adrenoceptor agonists, only the  $\alpha$ 1 agonist cirazoline is able to partly mimic the effect of NA on IP synthesis, while clonidine and isoproterenol,  $\alpha$ 2 and  $\beta$  agonists respectively, are ineffective (data not shown). Conversely, antagonists of the three subtypes are effective blockers of basal IP accumulation since they prevent the synaptic activation of PLC.

The release of an adrenergic ligand in hippocampal cultures suggests that hippocampal cells *in vitro* are expressing a particular phenotype since in adult hippocampus, NA is mainly

released by fibres originating from the locus coeruleus (Loy *et al.*, 1980) that begin to innervate the hippocampal formation at stage E18 (Loy and Moore, 1979). However, some authors have reported the presence of TH-immunoreactivity in hippocampal cells under specific conditions, e.g. in colchicine-treated rats, during development (Siddiqui *et al.*, 1995) and after complex partial seizure in human (Zhu *et al.*, 1990). In addition, a novel expression of TH was triggered by the synergistic effect of growth factors and protein kinase C activator in cultured rat striatum neurons (Du and Iacovitti, 1997) or after nigrostriatal dopaminergic deafferentation of primate striatum (Betarbet *et al.*, 1997). Therefore, TH expression in developing cultured hippocampal cells could reflect the onset of mechanisms taking place during development or on specific conditions. In fact, mRNAs encoding TH and DBH are expressed in E18 rat hippocampus (Fig. 7). Furthermore, TH-immunoreactive cells are evidenced in brain sections of E18 rat hippocampus (Fig. 8B). It could be hypothesized that these adrenergic ligand synthesizing cells, both *in vivo* or *in vitro*, compensate for the absence of adrenergic afferences by intrinsically supplying an adrenergic ligand.

This ligand could help neurons to express adrenergic receptors (Haddock and Malbon, 1993), as reported for  $\alpha$ 2A (Sakaue and Hoffman, 1991) and  $\beta$ 2 receptors (Collins *et al.*, 1988). It could also regulate neuronal survival or development. In fact, preliminary data indicate that the blockade of  $\alpha$ 1 adrenergic receptors induces neuronal death in hippocampal culture. Trophic roles of  $\alpha$ 1 (Zhang *et al.*, 1996; Pabbathi *et al.*, 1997) or  $\beta$  adrenoceptors (Kwon *et al.*, 1996; Canova *et al.*, 1997) have also been reported in other experimental models. Adrenoceptors are expressed throughout *in vitro* hippocampal neuron development. Therefore, trophic functions of adrenergic receptors could take place in immature cells before the appearance of synaptic activity. Later, the modulation of synaptic activity by adrenergic ligand may also participate in the regulation of neuronal development (Mattson and Kater, 1989).

As a conclusion, our data indicate that some hippocampal cells in culture can express an adrenergic phenotype to maintain a tonic control of excitatory synaptic transmission. This neuronal plasticity allows cultured cells to mimic the *in vivo* noradrenergic control of excitatory transmission in the hippocampus, which could be important for neuronal network function.

## Notes

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