

A peptide nucleic acid (PNA) is more rapidly internalized in cultured neurons when coupled to a *retro-inverso* delivery peptide. The antisense activity depresses the target mRNA and protein in magnocellular oxytocin neurons

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ABSTRACT

A peptide nucleic acid (PNA) antisense for the AUG translation initiation region of prepro-oxytocin mRNA was synthesized and coupled to a *retro-inverso* peptide that is rapidly taken up by cells. This bioconjugate was internalized by cultured cerebral cortex neurons within minutes, according to the specific property of the vector peptide. The PNA alone also entered the cells, but more slowly. Cell viability was unaffected when the PNA concentrations were lower than 10 μ M and incubation times less than for 24 h. Magnocellular neurons from the hypothalamic supraoptic nucleus, which produce oxytocin and vasopressin, were cultured in chemically defined medium. Both PNA and vector peptide–PNA depressed the amounts of the mRNA coding for prepro-oxytocin in these neurons. A scrambled PNA had no effect and the very cognate prepro-vasopressin mRNA was not affected. The antisense PNA also depressed the immunocytochemical signal for prepro-oxytocin in this culture in a dose- and time-dependent manner. These results show that PNAs driven by the *retro-inverso* vector peptide are powerful antisense reagents for use on cells in culture.

INTRODUCTION

The antisense oligodeoxynucleotides are promising tools for manipulating gene function and may provide new ways of treating hereditary, viral and cancer diseases. They inhibit gene expression by binding to a complementary mRNA sequence, so preventing the translation of this RNA into a protein (1–3). However, their efficacy is limited by their poor uptake by cells and their rapid degradation by nucleases (4,5). Chemical modifications that increase their lipophilicity, such as conjugation to cholesterol and lipids, have led to increased intracellular

concentrations and, in some cases, increased activity (6,7). Chemical modifications of the oligodeoxynucleotide backbone also increase the resistance to nucleases while preserving the biological activity (4).

A new type of DNA analogue, a peptide nucleic acid (PNA), was designed recently by Nielsen *et al.* (8). In PNA, the phosphodiester backbone of DNA or RNA is replaced by a homomorphous backbone consisting of (*N*-2-aminoethyl)glycine units bearing the nucleobases attached through methylenecarbonyl linkers. PNAs are resistant to proteases and nucleases and thus much more stable in cells than is DNA and RNA (9). PNAs also bind DNA and RNA more tightly than the natural nucleic acids bind to each other, while retaining and even improving the sequence specificity (10). PNAs are very potent molecules *in vitro* (11–14) but were believed to be poorly transported across cell membranes (12,15) until a recent paper demonstrated their internalization (16). To date, only PNAs microinjected into living cells have had any biological effect (11,17), except for their *in vivo* effect on the brain after intracerebral administration (18).

The present report describes the uptake and action of an antisense PNA coupled to a *retro-inverso* vector peptide that is very rapidly taken up by cells (19). The target of this PNA is prepro-oxytocin mRNA. The neuropeptide oxytocin is synthesized in the brain essentially by the magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei. There are two types of magnocellular neurons in the hypothalamus, present in roughly equal proportions; one produces oxytocin and the other vasopressin. These neuropeptides are released into the bloodstream from the neurohypophysial terminals of the magnocellular neurons to exert multiple peripheral hormonal actions. Both peptides also act as neurotransmitters or neuromodulators in the central nervous system. In the brain, oxytocin is particularly active in controlling maternal and sexual behaviour, but most of its central actions remain unclear, particularly in males (20–22).

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The supraoptic nuclei of 30–50 2-day-old rats were aseptically microdissected out and incubated for 20 min at room temperature in dissection medium (4 ml HBSS, 1 mM sodium pyruvate, 10 mM HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin) plus DNase I (0.5 mg/ml, 1100 U/ml), protease X (1 mg/ml, 40 U/ml) and protease XIV (1 mg/ml, 5.6 U/ml). The nuclei were then rinsed four times with dissection medium, left for 15 min in the last wash and transferred to 6 ml of culture medium for gentle mechanical dissociation by a few passages through a pipette. The cells were centrifuged for 8 min at 60 g, gently resuspended in culture medium, layered in 4-well plates onto a 1-week-old culture of cortical neurons and cultured for 3–28 days. The culture medium was changed by half volumes twice a week. For each culture experiment, the number of oxytocin-positive cells was counted on a control coverslip 4 h after seeding and used to normalize the semi-quantitative immunocytochemical study performed 5 days later.

Incubation of cells with PNA or vector peptide–PNA

The uptakes of PNA, scrambled PNA and vector peptide–PNA (compounds P2, sP2 and P3, respectively) by cortical neurons cultured in either 4- or 96-well plates were measured. The biotinylated compounds were dissolved in culture medium and incubated with the cultured neurons for 3 min to 2 days (all ending at 5 days in culture) at 0.1–30 µM. The incubation medium was then removed, the cells washed with culture medium and fixed with ethanol/glacial acetic acid (95:5 v/v) for 10 min at –20°C.

For light microscopy, the coverslips were washed three times with PBS, flooded for 10 min with PBS containing 5% bovine serum albumin (PBS/BSA) and incubated at 4°C for 16 h with streptavidin–peroxidase complex (diluted 1:200 in PBS/BSA). They were then washed three times in PBS and incubated for 15 min with 0.05% 3,3'-diaminobenzidine (DAB) containing 0.01% H₂O₂ in PBS. Finally, the coverslips were washed thoroughly with PBS, dehydrated and mounted in Entellan (Merck, Darmstadt, Germany).

Semi-quantitative assays of uptake were done on 96-well plates treated as above with streptavidin–peroxidase complex and washed three times with PBS. The plates were then read at 405 nm in a Dynatech MR5000 plate reader, incubated with DAB for 15 min, washed three times with PBS and read again. The corrections for the absorbance of cells, non-specific adsorption of the compounds and non-specific staining were made by comparing empty wells and wells containing cells with or without incubation with the biotinylated compounds, before and after incubation with DAB. Each experimental value is the mean of triplicate determinations. The final data are the means of three experimental values obtained with three cell cultures performed on different days.

The effect of PNA, scrambled PNA and vector peptide–PNA (compounds P2, sP2 and P3, respectively) on the prepro-oxytocin mRNA in supraoptic magnocellular neurons cultured over a layer of cortical neurons in 4-well plates without glass coverslips was studied. The compounds were incubated with the cells in culture medium for a 30 min to 24 h period, ending on 5 days of culture. The incubation medium was then removed, the cells were washed with culture medium and the total RNA immediately extracted using an RNeasy™ kit (Qiagen, Courtaboeuf, France).

The effect of PNA on the immunocytochemical signal for prepro-oxytocin on cultured supraoptic magnocellular neurons was studied using compound P1 (Fig. 1) because it is not

biotinylated and thus does not interfere with immunocytochemistry. The compound was incubated with the cells in culture medium for 4–24 h ending on 5 days of culture. The incubation medium was then removed, the cells washed with culture medium and the coverslips processed for immunocytochemistry and image analysis. The viability of the cells after incubation with PNA was assessed using fluorescein diacetate and propidium iodide (28).

Reverse transcription and PCR

The reverse transcription was performed on total RNA (digested with DNase I; Life Technologies) using Superscript™ II reverse transcriptase (Life Technologies) according to the manufacturer's instructions, with RNasin™ (Promega, Charbonnières, France) as RNase inhibitor. The reverse primer used for reverse transcription was specific for both oxytocin and vasopressin, i.e. covering a region of the prepro-oxytocin mRNA and prepro-vasopressin mRNA sequences that are identical (sequence 5'-CCT CCG CTT CCG CAA GGC TTC TGG C-3', GenBank/EMBL accession no. X59496, nt 3013–3037 for oxytocin and 13760–13784 for vasopressin).

The prepro-oxytocin and prepro-vasopressin mRNAs were amplified using MJ Research PTC-100/60 or PTC-150/16 thermal controllers (Watertown, MA) and Taq DNA polymerase (Life Technologies) according to the manufacturers' instructions. The settings were 3–10 µl reverse transcription product, 25–35 cycles, 60 s denaturation at 94°C, 30 s annealing at 62°C and 90 s extension at 72°C. The forward primer was 5'-CTG CCC CAG TCT CGC TTG-3' for oxytocin (GenBank/EMBL accession no. X59496, nt 2536–2553) and 5'-TCG CCA TGA TGC TCA ACA CTA C-3' for vasopressin (GenBank/EMBL accession no. X59496, nucleotides 15203–15224). The reverse primer was the same for both amplifications (5'-GCG AGG GCA GGT AGT TCT CC-3', GenBank/EMBL accession no. X59496, nt 2981–3000 for oxytocin and 13797–13816 for vasopressin). The intron between the forward and reverse primers ensures rejection of any genomic DNA which could have resisted the DNA digestion preceding reverse transcription. The PCR products were electrophoresed on a 2% agarose gel in TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8) containing 0.5 µg/ml ethidium bromide and the bands digitized under UV light. The lengths of the PCR products were as expected (245 nt for oxytocin, 258 for vasopressin). The specificity of the DNA bands obtained by PCR was also verified by digesting the PCR products with *Hha*I restriction enzyme (Life Technologies): the lengths of the main fragments were as expected (91 nt for oxytocin, 157 for vasopressin).

The sequence recognized by the antisense PNA was chosen in a 5' position with respect to all the primers used in reverse transcription and PCR to avoid any interference with the RT–PCR by PNA co-purified with the RNA. We also checked that micromolar concentrations of PNA had no effect on the RT and PCR reactions.

Immunocytochemistry and image analysis

The cultured supraoptic neurons were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, 15% picric acid for 20 min at room temperature. The coverslips were then washed three times with PBS, flooded for 10 min with PBS/BSA and incubated for 16 h at 4°C with anti-oxytocin/neurophysin PS38 monoclonal antibody (29) diluted 1:50 in PBS/BSA. They were

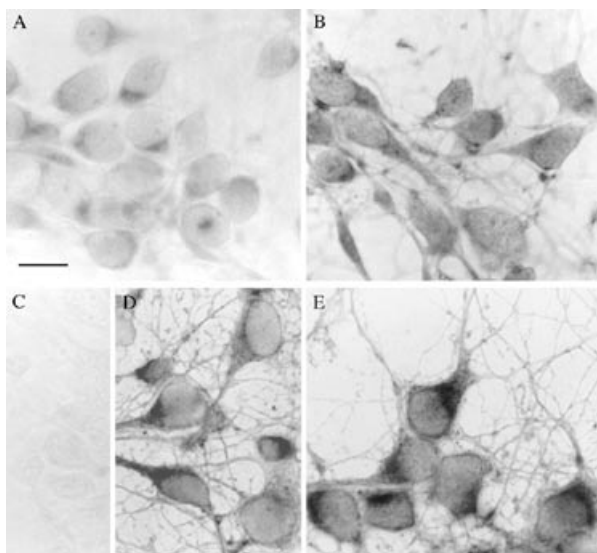


Figure 2. Uptake of PNAs by cerebral cortical neurons cultured for 5 days in a chemically defined medium. (A) 10 min and (B) 2 h incubation with 3 μ M nude PNA (compound P2) showed that PNA can cross the cell membrane. (D) 10 min and (E) 1 h incubation with 3 μ M vector peptide-PNA (compound P3) showed that the vectorized form of the PNA is much more rapidly internalized. (C) Control labelling (incubation without PNA). Bar in (A) 10 μ m.

washed three times in PBS, once in PBS/BSA, flooded with the biotinylated sheep anti-mouse antibody (diluted 1:100 in PBS/BSA) for 1 h, washed three times in PBS, once in PBS/BSA, incubated for 1 h with streptavidin-peroxidase complex (diluted 1:200 in PBS/BSA) and processed as described above.

The immunocytochemical signal obtained with the PS38 antibody in magnocellular neurons incubated with PNA was analysed under a Reichert Polyvar light microscope equipped with a Sony video camera connected to a Macintosh 8100 computer running the public domain NIH Image program (developed at the US National Institutes of Health). Cell images were captured on the blue channel of the digitizer, the most sensitive for detecting the DAB brown staining, under standardized conditions of light intensity. Cell labelling was measured by delimiting the labelled cells and averaging the pixel intensities; the very low mean background staining was measured on 10 unstained cells of the same coverslip, averaged and subtracted from the crude values to give the individual mean cell labelling values. The results were expressed as the distribution of these values, after normalization using the number of oxytocin-positive magnocellular neurons seeded in the 14 mm wells at each culture. The final results are the means of three different cultures.

RESULTS

Vector peptide-PNA but also nude PNA are internalized by cultured neurons

The experiment was designed to introduce an antisense PNA into cultured cells by coupling it to a vector peptide which crosses the cell membrane. Surprisingly, we found that the PNA alone (compound P2) was also internalized. The first images showed positive staining

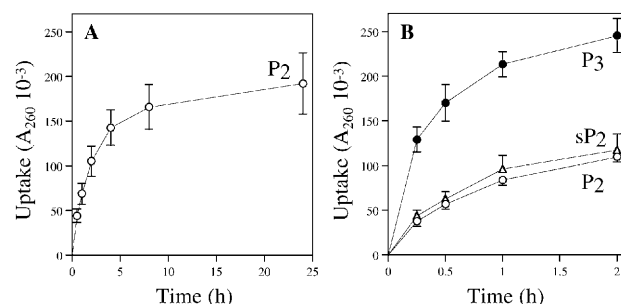


Figure 3. Semi-quantitative analysis of PNA uptake by cerebral cortical neurons cultured for 5 days in a chemically defined medium. (A) 24 h uptake of 3 μ M nude PNA (compound P2); (B) short-term comparative study of the uptake of 3 μ M nude PNA (compound P2), scrambled PNA (compound sP2) and vector peptide-PNA (compound P3). Means \pm SEM. Two-way analysis of variance (product versus time) in (B), P3 curve significantly different ($P < 0.01$) from both P2 and sP2 curves.

of the apical part of the neuronal cytoplasm after only 10 min incubation with 3 μ M P2 (Fig. 2A). The labelling increased markedly thereafter and was punctate at high magnification (which is compatible with penetration by endocytosis), and spread over the entire cell, including the nucleus and neurites (Fig. 2B, 2 h incubation). The vector peptide-PNA (compound P3) was taken up much more rapidly by the cortical neurons, labelling both the cell body and the cell processes within minutes. The signal was already stronger after 10 min incubation with vector peptide-PNA (Fig. 2D) than the signal obtained after incubation with the PNA alone for 2 h (Fig. 2B) and it further increased thereafter (Fig. 2E, 1 h incubation). PNA molecules were not taken up only by neuronal cells, but also by the few remaining astrocytes in the culture (not shown).

Maintaining neuronal cultures at 4°C for 5 min and then incubating them at the same temperature for 10 min with the vector peptide-PNA (compound P3) did not prevent internalization (not shown), which is consistent with the hypothesis of direct penetration across the cell membrane, a tentative hallmark of the vector peptide (36), rather than via endocytosis. The PNA alone was internalized too slowly for it to be studied by this method without causing cell damage.

The semi-quantitative study of cortical neurons cultured in 96-well plates confirmed that PNA uptake by cultured neurons was relatively rapid and was maximal in \sim 8 h (Fig. 3A). The vector peptide-PNA (compound P3) penetrated more rapidly than PNA alone (compounds P2 and sP2) at the same molar concentration (Fig. 3B). Fitting the time curves to the equation uptake = $A \times t / (B + t)$ (t = time) (30) allowed comparison of the initial speed of the uptake (equal to A/B) and showed that the vector peptide increased the speed of PNA internalization 5.6-fold.

These results were obtained for a wide range of PNA concentrations (0.1–30 μ M) and incubation times (10 min–48 h). Cell viability was monitored in parallel by the green staining of living cells following fluorescein diacetate uptake and red staining of the nuclei of damaged cells by propidium iodide (28). The viability of cells incubated with 3 μ M PNA or vector peptide-PNA for up to 1 day was unchanged. Cells incubated

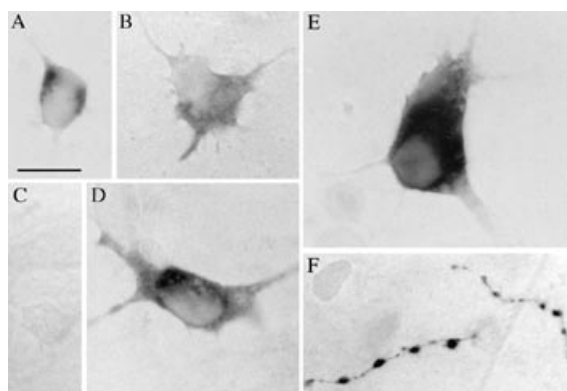


Figure 4. Development of magnocellular oxytocin neurons cultured in a chemically defined medium. The immunoreactive cells at the onset of culture (A) rapidly developed neurites (B) (3 days in culture) and increased in size and labelling intensity (D) (5 days in culture) for up to 21 days in culture (E). From day 5, stained axons bearing regularly arranged varicosities ran throughout the culture well (F) (21 days in culture); they are characteristic of mature magnocellular neurons. (C) Control labelling (PS38 antibody omitted). Bar in (A) 10 μ m.

with 10 μ M PNA or vector peptide–PNA for 24–48 h showed some damage, which became greater at higher concentrations.

Culture of magnocellular oxytocin neurons

Studies on the biological activity of the antisense molecules on the target mRNA and protein required a cell culture procedure providing sufficient well-developed magnocellular oxytocin neurons. After initial attempts based on published methods (31–33), successful cultures were obtained by layering enzymatically and mechanically dissociated neurons from supraoptic nuclei of 2-day-old pups over a layer of cerebral cortex neurons plated 1 week earlier. These conditions gave a 20% immunoreactive neuron survival at 5 days (~40 neurons/well) (Fig. 4A). These 20 μ m neurons were intensely immunoreactive to the monoclonal anti-oxytocin/neurophysin PS38 antibody characterized by Ben-Barak *et al.* (29) and were thus clearly identifiable at this stage as magnocellular oxytocin neurons (Fig. 4D). Nearly 50% of these neurons (20 neurons/well) survived thereafter and became mature magnocellular neurons (Fig. 4E), displaying a strong immunocytochemical signal all over the cell, from the cell body, the thick, short dendritic processes, to the characteristic thin axon bearing regularly distributed varicosities which ran for a few millimetres throughout the culture well (Fig. 4F). The effects of the antisense molecules were studied on 5-day-old cultures.

Antisense molecules affect specifically their target mRNA and protein

Because of the very small number of oxytocin magnocellular neurons in the 14 mm culture wells, we used reverse transcription (RT) and PCR to detect the target mRNA in the total RNA extracted from the cultures. All the RNA extracts of each experiment underwent RT–PCR simultaneously with identical parameters (amount of RT product used, number of cycles). These parameters were modified to give several degrees of amplification, ranging from the first appearance to saturation of the signals. The first lanes to display detectable bands were always those of the control wells, incubated with culture medium.

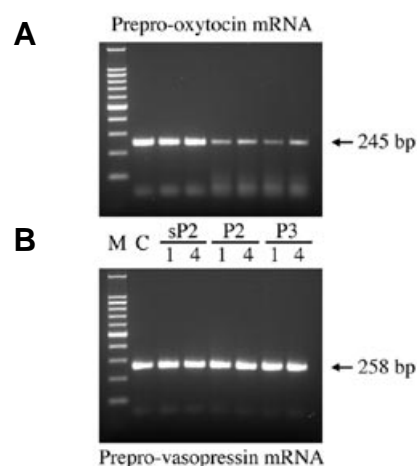


Figure 5. The effect of PNAs on the prepro-oxytocin (A) and prepro-vasopressin (B) mRNA contents of cultured magnocellular neurons. mRNA was detected by RT–PCR with settings giving a signal roughly representative of the mRNA content. (A) Control culture (C) showed that 3 μ M scrambled PNA (compound sP2) incubated for 1 or 4 h had no effect on the prepro-oxytocin mRNA content, which, on the contrary, is depressed by 3 μ M nude PNA (compound P2) and by vector peptide–PNA (compound P3) applied for 1 or 4 h. (B) In the same cultures, the prepro-vasopressin mRNA contents did not react to the PNAs. M, molecular weight scale.

Stepwise adjustment thus gave gel images in which the PCR signals roughly reflect the initial prepro-oxytocin mRNA content of the culture wells. Incubation for 1 or 4 h with 3 μ M antisense PNA (compound P2) or vector peptide–PNA (compound P3) reduced the signal to below control values (Fig. 5A). In contrast, incubation with the scrambled PNA (compound sP2) gave band intensities similar to those of the control wells, showing that this compound had no effect and consequently that the effect of the P2 and P3 PNAs was sequence specific (Fig. 5A).

This was also checked by exploiting the fact that the supraoptic nucleus has nearly equal numbers of oxytocin and vasopressin magnocellular neurons. Amplification of prepro-vasopressin mRNA in the RNA extracts previously studied for prepro-oxytocin mRNA showed no change in the amount of prepro-vasopressin mRNA after incubation with PNA (P2), scrambled PNA (sP2) or vector peptide–PNA (P3), confirming that the antisense effects on the prepro-oxytocin mRNA are specific for the target mRNA (Fig. 5B).

The effect of PNA (compound P1) on the immunocytochemical signal in cultures with the PS38 antibody, i.e. on prepro-oxytocin, was also studied. Incubation for 24 h with increasing concentrations of antisense PNA reduced the number of cells scored as immunoreactive in each labelling class (Fig. 6A). The effect was visible at 1 μ M and more marked at 3 μ M. It was discernible after only 4 h incubation at 3 μ M and reached a maximum after ~8 h incubation (Fig. 6B).

DISCUSSION

PNAs were initially considered to be very potent molecules *in vitro* but almost unable to cross the cell membrane (12,15). The present report clearly shows that nude PNAs are taken up by cultured neurons, as described recently for cultured human myoblasts (16) and rat neurons *in vivo* (18). However, conjugation

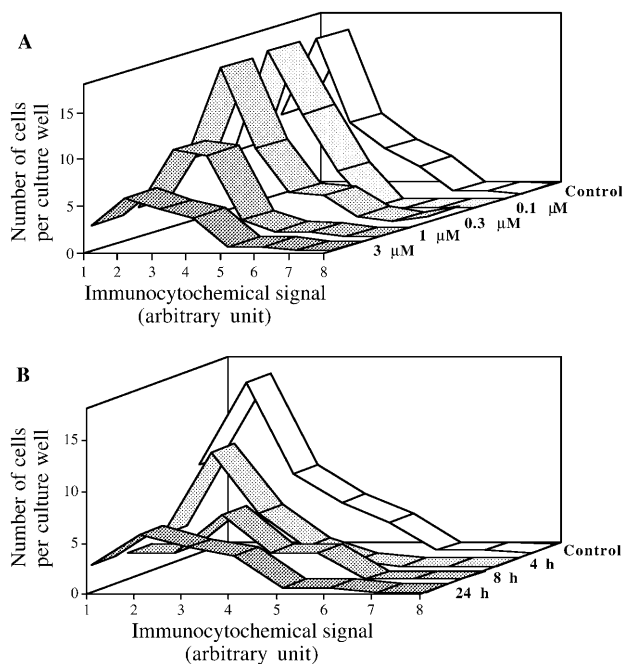


Figure 6. A semi-quantitative study of the prepro-oxytocin content of cultured magnocellular neurons treated with PNA (compound P1). (A) Dose–response curve of the effect; (B) time course of the effect. Two-way analysis of variance (dose or time versus labelling distribution): dose effect in (A), $P < 0.05$; time effect in (B), $P < 0.05$.

with a vector peptide, in the present case a *retro-inverso* peptide that is rapidly internalized by cells (19), increases the speed of PNA uptake by the cultured cells by a factor of 5.6. A very similar experiment recently described the penetration of a PNA conjugated to peptides derived from the third helix of the homeodomain of Antennapedia into human prostate tumour-derived cells (34). A D-peptide analogue of insulin-like growth factor 1 has also been used to target a PNA to cells bearing the corresponding membrane receptor (35). The use of a vector peptide made of D-amino acids, and therefore resistant to proteases, as is the PNA moiety, is a logical improvement of the PNA molecule. The marked enhancement of the internalization rate by the *retro-inverso* peptide used in the present study appears interesting in terms of usefulness for *in vivo* studies, since lower concentrations can be used. Another interesting feature of the conjugation with the *retro-inverso* peptide arises from the current hypothesis on its internalization mechanism: this peptide is believed to cross the cell membrane directly, by an energy-independent mechanism, in which the positive charges regularly distributed along the peptide are thought to cause a local destabilization of the membrane molecular structure by acting on the negatively charged phospholipids (36). In agreement with this, the *retro-inverso* peptide alone (19) and the vector peptide–PNA (compound P3) crossed the neuronal membrane as rapidly at 4 as at 37°C. In contrast, the nude PNA is probably internalized by endocytosis, as are the other antisense molecules. Therefore, if the hypothesis on the internalization mechanism is true, the vector peptide–PNA could be instantaneously available everywhere in the cell instead of being trapped in the endosomal compartment and slowly released into the cytoplasm. This is particularly important for neurons, where some mRNAs are precisely targeted to distal compartments

of the cell, such as the dendrites (37), axons (38) or nerve terminals (39). There, in response to local events, these mRNAs can become involved in the rapid synthesis of proteins which adjust the local cell function to the incoming signalling events. The almost immediate availability of a PNA at these sites when it is driven by the vector peptide should be a good way to act on these proteins.

The second aim of the present paper was to show a biological effect of a PNA in living cells without recourse to cell microinjection (as used previously; 11,17), until the effect recently described *in vivo* was observed, where injection of an antisense PNA into the periaqueductal grey region of the brain resulted in the specific loss of behavioural responses to neurotensin and morphine (18). Our target was the synthesis of oxytocin, a neuropeptide mainly involved in milk ejection and uterine contraction, but also in anterior pituitary, testis and ovary function, as well as in the control of maternal and sexual behaviour (22). The antisense PNA selected was complementary to the AUG translation initiation region of the mRNA, so as to increase the probability that the PNA would interfere with translation of the mRNA (40,41). A procedure was established for maintaining and developing in culture the rare oxytocin neurons easily accessible to dissection, i.e. the magnocellular neurons of the hypothalamic supraoptic nucleus. The procedure ultimately adopted used freshly dissociated neurons from the supraoptic nuclei of 2-day-old rats grown on a layer of cortical neurons cultured 1 week earlier. The chemically defined medium used was based on the Neurobasal™ medium designed for culturing neurons at low cell density (42). This method allowed the survival of up to 20% of the dissociated magnocellular oxytocin neurons after 5 days of culture. The development of magnocellular oxytocin neurons can be followed, from the small cells prepared from 2-day-old rats up to the large mature neurons. Although it can still be improved, the culture procedure provides a sufficient number of cells to illustrate the effect of antisense PNAs on the oxytocin mRNA and peptide.

Incubating these neurons with 3 μM PNA for 1–4 h depressed their prepro-oxytocin mRNA content. The effect was obtained with the nude PNA (compound P2) and the vector peptide–PNA (compound P3). It is sequence specific, since the scrambled PNA (compound sP2) was without effect, and also gene specific, since the similar prepro-vasopressin mRNA, transcribed on a gene located nearby (only 6 kb away) on the same chromosome in the second type of magnocellular neurons of the supraoptic nucleus, was not affected. Preliminary observations suggest that the effect is less marked with longer incubation times (8 or 24 h), as if the cells were able to partially escape from the antisense molecules by increasing production of the corresponding mRNA. However, this requires further study in other experimental models, using many more cells than the mean 40 magnocellular neurons available in our model and more accurate quantitative methods than the RT–PCR used here because of its necessary sensitivity. Similarly, we have not shown that conjugation to the vector peptide improves the biological activity of the resulting PNA compound, in spite of its increased speed of internalization. Therefore, we can only be sure that the antisense molecules tested (compounds P2 and P3) decrease the prepro-oxytocin mRNA content of magnocellular neurons and the mechanism(s) by which this reduction occurs is still not clear. Since degradation by RNase H is not induced by PNA binding to the mRNA (11), it can be supposed that the mRNA physically blocked by the antisense

molecules in the cell may cause it to enter a metabolic pathway and be degraded.

Immunocytochemistry using the PS38 monoclonal antibody, specific for the prepro-oxytocin, was used to determine whether the effect of the PNA on the mRNA was reflected in the oxytocin protein. The number of labelled cells decreased markedly after incubation for 24 h with at least 1 μ M PNA (compound P1), showing that the effect of the antisense molecule on the mRNA is great enough to alter the cell content of the corresponding protein. The effect is visible after only 4 h incubation with 3 μ M PNA. Preliminary observations indicate that the vector peptide-PNA (compound P3) also reduces the number of immunoreactive cells in cultures (not shown). Again, as for the effect on the mRNA, our experimental model did not allow the demonstration of improved PNA activity due to the delivery peptide conjugation. Nevertheless, our results show that an antisense PNA, with or without a vector peptide, disturbs the synthesis of prepro-oxytocin by magnocellular neurons in a sequence-specific manner. However, incubation with PNA at the concentrations and incubation times used depresses the cell immunocytochemical labelling, but by no means produces a real knock-out of the oxytocin gene, since all types of magnocellular cells, from the heavily marked to the faintly labelled ones, were still observable in the culture. This is perhaps because these secretory neurons produce and retain a large amount of neuropeptide in culture, as they do *in vivo* where they store a large number of oxytocin-containing synaptic vesicles in the neurohypophysial terminals, released at very different rates according to various physiological demands. Nevertheless, our data show, for the first time to our knowledge, that a PNA can depress the synthesis of its target protein, even in such an unfavourable condition, and is thus an efficient and useful antisense reagent for use on cultured cells when added to the culture medium. *In vivo* studies will show whether and how the electrical activity of the magnocellular oxytocin neurons *in situ* responds to intracerebral administration of very low doses of the vector peptide-PNA.

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