

# SHORT COMMUNICATION

## Neurons in the Rat Occipital Cortex Co-expressing the Substance P-Receptor and GABA: a Comparison Between *In Vivo* and Organotypic Cultures

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### Abstract

The morphology and the distribution of neurons expressing the NK1-receptor (NK1R) and the co-expression of  $\gamma$ -aminobutyric acid (GABA) in these neurons were studied in the rat occipital cortex and in organotypic cultures (OTCs) derived from this structure. By employing immunohistochemistry, we demonstrate that the NK1R-expressing neurons are non-pyramidal neurons and co-express GABA. Some differences were noted between *in vivo* and OTCs. NK1R-expressing neurons in OTCs had larger somata and longer dendrites and the proportion stained with an anti-GABA-antibody (~50%) was smaller than *in vivo* (90%). The preferential location of NK1R-expressing neurons in layers II/III and VI, seen *in vivo* is not present in OTCs where these neurons distribute rather homogeneously through layers II–VI. Our findings imply that in contrast to the cat and monkey, in the rat occipital cortex the effects of substance P are almost exclusively mediated via inhibitory interneurons.

### Introduction

The tachykinin peptide family whose members are known to be present in the neocortex comprise substance P (SP), neurokinin A (NKA), neuropeptide K (NPK), neuropeptide  $\gamma$  and neurokinin B (NKB) (Arai and Emson, 1986; Takeda *et al.*, 1990). The tachykinin peptides interact differentially with three tachykinin receptors: the NK1-receptor (NK1R) prefers interaction with SP, the NK2-receptor with NKA, NPK and neuropeptide  $\gamma$  and the NK3-receptor with NKB (Helke *et al.*, 1990). Radioimmunoassay and binding studies have demonstrated the presence of these receptors in the cortex (Dam *et al.*, 1988). The presence of mRNA coding for functional NK1R in the visual cortex has been demonstrated by means of the oocyte expression system (Matute *et al.*, 1993a). By means of microiontophoretic techniques it has been shown that in the cortex exogenous tachykinins depolarize, i.e. excite, neurons (for review see Albus *et al.*, 1992). Tachykinin-induced suppressions, or hyperpolarizations, were attributed to an indirect action of the tachykinins mediated via inhibitory interneurons (Olpe *et al.* 1987; Albus *et al.*, 1992).

Recent investigations employing *in situ* hybridization histochemistry (Maeno *et al.*, 1993; Matute *et al.*, 1993b) or immunohistochemistry (Liu *et al.*, 1994; Nakaya *et al.*, 1994) have revealed species differences in the morphology and laminar distribution of neurons expressing the NK1R. In the cat visual cortex most of these neurons are pyramidal cells in layers II, III and V; in the rat neocortex they are non-pyramidal neurons which distribute through all layers. In

order to substantiate and extend the latter findings we have studied the morphology, transmitter identity and intracortical location of neurons expressing the NK1R in the rat occipital cortex. In addition, as a first step to analyse NK1R-receptor functions at a cellular level, we wondered whether the characteristic properties of NK1R-expressing neurons are maintained in organotypic cultures (OTCs) of the rat occipital cortex. Due to a high degree of accessibility these long term culture systems have been claimed to be particularly well suited to study basic cellular mechanisms of neuronal functions (Gähwiler, 1981).

### Materials and methods

Sprague–Dawley rats and Wistar rats (240–260 g) were deeply anaesthetized with sodium pentobarbitone (60 mg/kg) and perfused through the ascending aorta with PBS (145 mM NaCl, 10 mM sodium phosphate, pH 7.4), followed by a solution of 4% paraformaldehyde and 0.1% glutaraldehyde in PBS. After perfusion, the brain was removed and postfixed in 4% paraformaldehyde in PBS for a period of 2–6 h. Vibratome sections of neocortex were cut at 45–50  $\mu$ m thick.

OTCs of the occipital cortex of rats (Wistar) were prepared and maintained *in vitro* by employing the protocol described by Gähwiler (1981). Culture tubes were supplied with 0.75 ml semi-artificial medium (50% Eagle's basal medium, 25% Hank's balanced salt

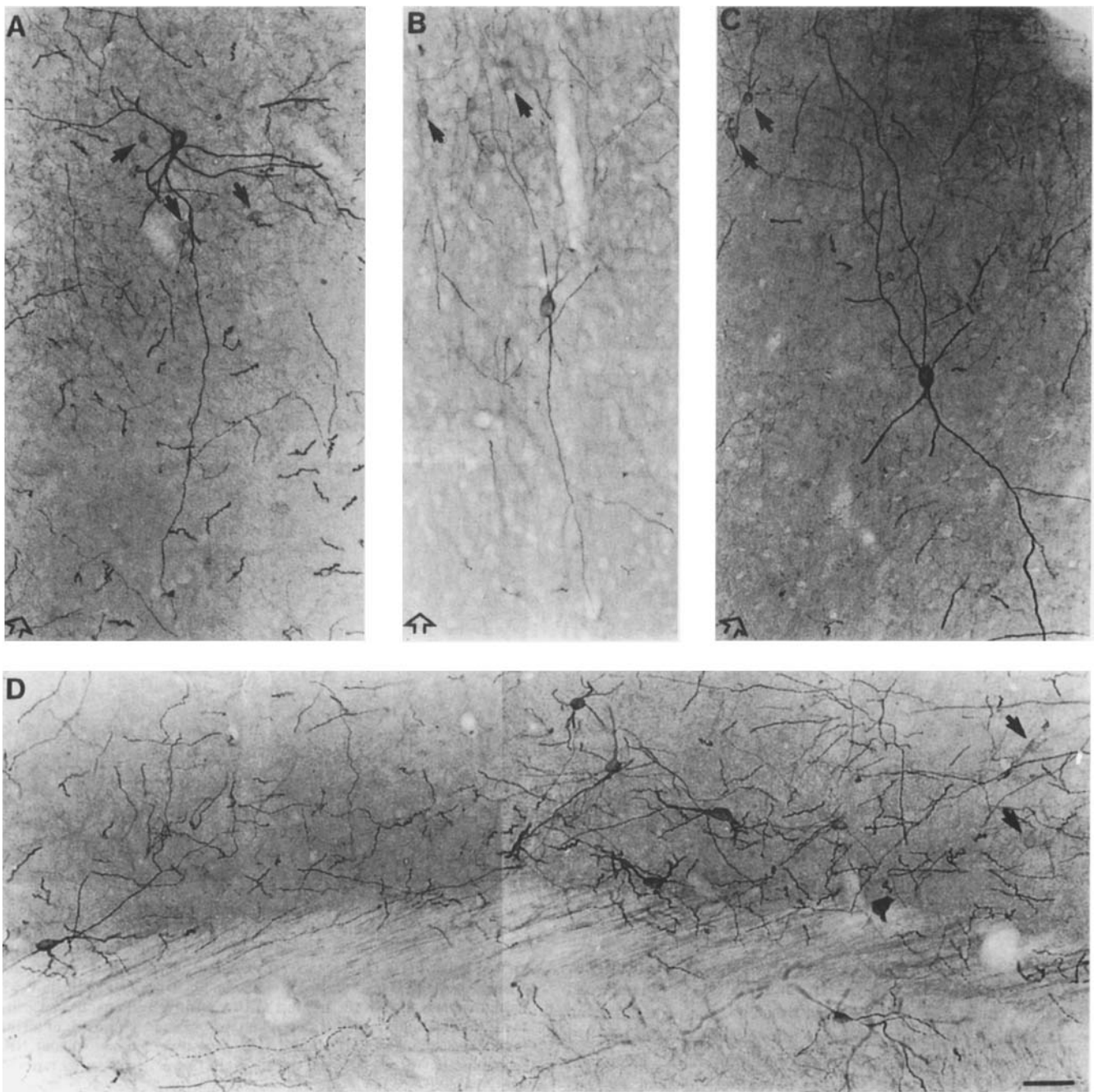


FIG. 1. Morphology of non-pyramidal neurons in the rat occipital cortex expressing the NK1R. Intensely stained neurons and weakly stained neurons (arrowheads) in layers III (A), V (B), II (C) and VI and adjacent white matter (D). Open arrows point toward cortical surface. Scale bar, 50  $\mu$ m.

solution, 25% inactivated horse serum, 1 mM L-glutamine, 0.65% D-glucose and 1.26 mM  $\text{Ca}^{2+}$ ). All procedures were carried out in sterile conditions. The medium was changed every 4–5 days. For immunohistochemical experiments OTCs about 3 weeks *in vitro* were immersed in 4% paraformaldehyde and 0.1% glutaraldehyde for 1 day.

For immunolocalization of the NK1R, a rabbit polyclonal NK1R antibody (Vigna *et al.*, 1994) was used. Sections (free floating) of occipital cortex and OTCs (on coverslips) were pre-incubated with  $\text{H}_2\text{O}_2$  in PBS for a period of 20 min and then blocked over 30–45 min in 2% normal goat serum with 0.5% Triton X-100 (or in the case of OTCs 1% Triton X-100) in PBS. Next, they were incubated overnight at 4°C with the NK1R primary antibody diluted 1:500.

After washing thoroughly with PBS, a biotinylated goat anti-rabbit (dilution 1:200) secondary antibody was added for 60 min and later incubated for 60 min in avidin–biotin–peroxidase complex (Vectastain, Burlingame, CA). The immunoreaction product was visualized with diaminobenzidine and  $\text{H}_2\text{O}_2$ . Sections and cultures were then washed in PBS, mounted onto gelatinized slides, dehydrated and coverslipped.

For the colocalization experiments, a double immunofluorescence staining method was performed. Following the preincubation and blocking procedure (see above), sections and OTCs were incubated overnight with the NK1R primary antibody at 1:500 dilution. Labelling was achieved by using a fluorescein isothiocyanate-labelled goat anti-

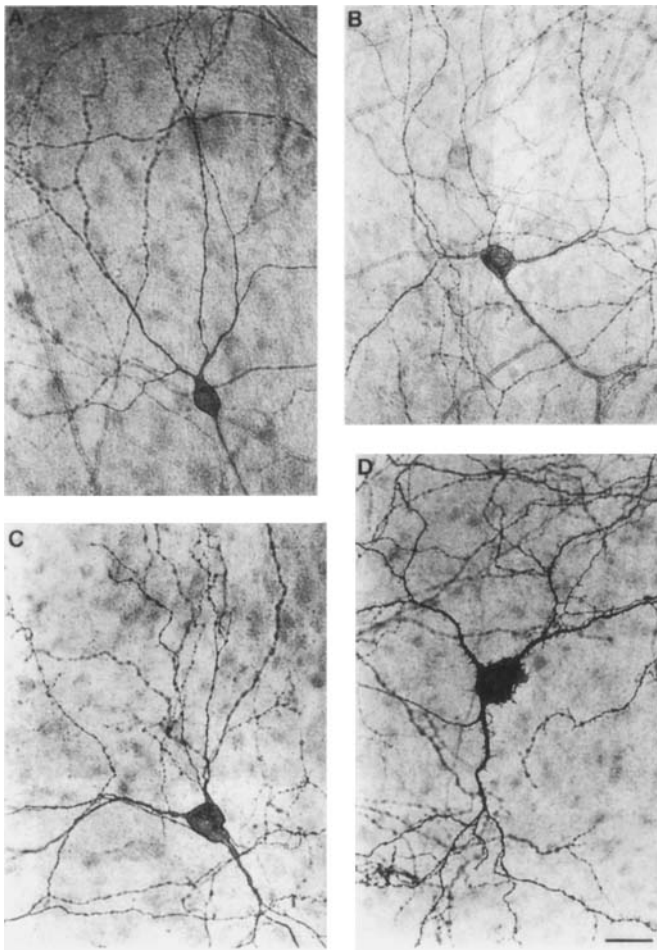


FIG. 2. Morphology of non-pyramidal neurons in OTCs from the rat occipital cortex expressing the NK1R. Neurons may display beaded dendrites (A–D) and hairlike processes (D). Position in layers: II (A,C), VI (B), V (D). Scale bar, 30  $\mu$ m.

rabbit antibody (dilution 1:200; Sigma). Subsequently, after several washes in PBS the same sections were incubated overnight at 4°C with a mouse monoclonal anti-GABA antibody (3A12; Matute and Streit, 1986) diluted 1:20 000 followed by incubation of biotinylated horse anti-mouse secondary antibody diluted 1:100 and labelled with Texas Red conjugated streptavidin (dilution 1:100; Chemicon International Inc., Temecula, CA). As a control, in some sections GABA was visualized with fluorescein isothiocyanate and the NK1R primary antibody with Texas Red. The results of both staining procedures were the same. As a further control, primary antibodies were omitted from these immunohistological procedures, and no staining was observed. Labelled cells were viewed under the corresponding UV excitation frequency and photographed.

In order to determine the proportion of GABA-positive neurons expressing the NK1R, four sections were analysed in detail. Labelled neurons were counted in square areas with 330  $\mu$ m sidelength centred on upper, middle or lower layers of occipital cortex. A total of 40 square areas was analysed, of which 16 comprised layers I–IV, 12 layer V and 12 layer VI including the adjacent white matter.

## Results

NK1R-specific immunoreactivity was observed exclusively in non-pyramidal neurons and was associated with the soma and the complete

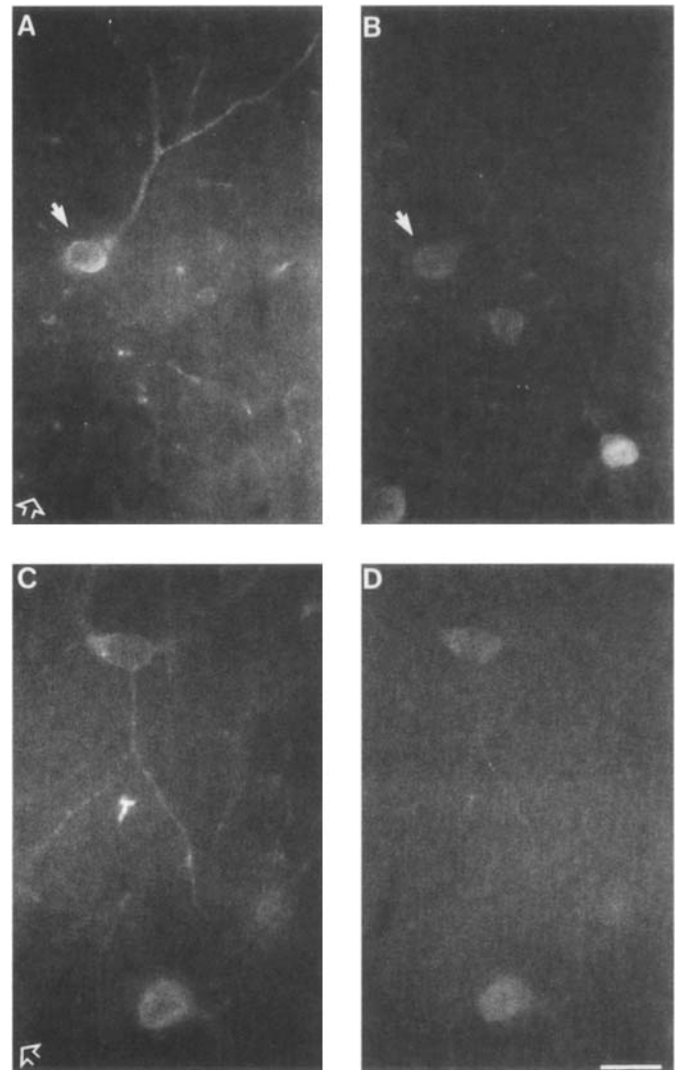


FIG. 3. Double immunofluorescence labelling of coronal sections of rat occipital cortex with antibodies against the NK1R (A,C) and GABA (B, D). Each row shows the same field in layers II/III (top) and layer V (bottom) viewed under FITC fluorescence (left column) or Texas Red (right column). Arrows in A and B mark the same cell; note GABA-positive cells in B which do not express the NK1R. In most GABA-positive neurons, the intensity of the GABA stainings does not depend on the presence or absence of a NK1R-immunoreaction. Intensely stained GABA-positive neurons without NK1R-immunoreactivity as the one shown in B (lower right) were rare. Scale bar, 20  $\mu$ m.

dendritic tree (see also Liu *et al.*, 1994). With regard to the staining intensity, two populations of neurons were found: intensely stained neurons with large somata and weakly stained neurons which usually had smaller somata and which were more numerous in upper than in lower layers (Fig. 1). Most neurons had multipolar dendritic patterns, the dendrites being spine-free or sparsely spinous. A few of the other types of non-pyramidal neurons present in the visual cortex of the rat (Feldman and Peters, 1978), namely bitufted and bipolar neurons (Fig. 1B, C), were also found to express the NK1R; these neurons presented spine-free or very sparsely spinous dendrites as well. In layer VI and in the adjacent white matter as well as in layer II dendrites of some neurons were oriented parallel to the cortical surface (Fig. 1C, D).

In OTCs from the rat occipital cortex, almost all neurons with

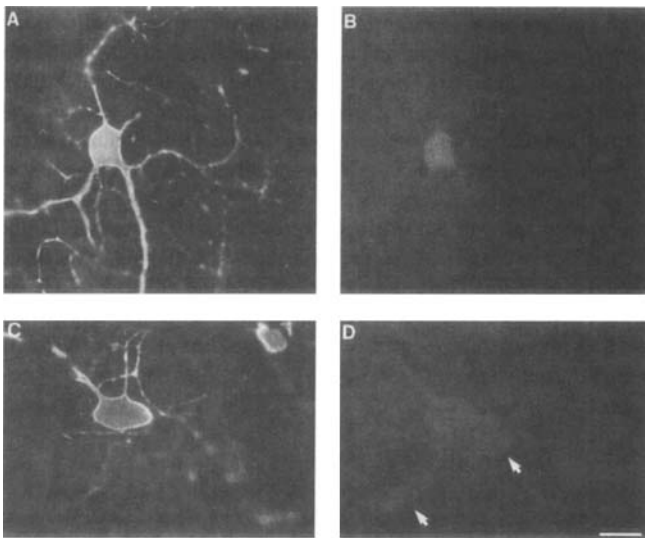


FIG. 4. Double immunofluorescence labelling of OTCs from the rat occipital cortex with antibodies against GABA (A,C) and the NK1R (B,D). Each row shows the same field in layer III (top) and layer VI (bottom) viewed under Texas Red (left column) or FITC fluorescence (right row). Arrows in D point to NK1R-positive somata which do not contain GABA. Note the small GABA-positive neuron in C which does not express the NK1R. Scale bar, 20  $\mu\text{m}$ .

NK1R-specific immunoreactivity were intensely stained and had a non-pyramidal morphology either with multipolar or bitufted or a bipolar dendritic pattern (Fig. 2). Dendrites were either spine-free or carried fine hair-like appendages, which were seen occasionally also on the soma (Fig. 2D). Quite a number of dendrites were beaded (Figs. 2A, C, D). Hairlike processes on dendrites and soma and beaded dendrites were not observed *in vivo*. The most striking difference in the morphology of NK1R-positive neurons *in vivo* and in OTCs, however, was soma size which was smaller *in vivo* than in OTCs (*in vivo*: mean  $\pm$  SD = 13.5  $\pm$  4  $\mu\text{m}$ ;  $n$  = 225; upper and lower 95% confidence interval 15.3  $\mu\text{m}$ , and 11.7  $\mu\text{m}$  respectively; *in vitro*: mean  $\pm$  SD = 20  $\pm$  4.3  $\mu\text{m}$ ;  $n$  = 168; upper and lower 95% confidence interval 20.7  $\mu\text{m}$ , and 19.4  $\mu\text{m}$  respectively). The difference was statistically significant (Mann–Whitney test, two-tailed  $P$  value < 0.0001). In addition, in OTCs dendrites were longer (at  $\sim$ 16%) than those observed *in vivo* (*in vitro*: mean  $\pm$  SD = 358.4  $\pm$  118.4  $\mu\text{m}$ ;  $n$  = 21; upper and lower 95% confidence interval: 412.3 and 304.5; *in vivo*: mean  $\pm$  SD = 309  $\pm$  89.4  $\mu\text{m}$ ;  $n$  = 21 upper and lower 95% confidence interval 349.9 and 268.2). The difference was not significant, however (Mann–Whitney test, two-tailed  $P$  value > 0.05).

Double immunofluorescence labelling revealed that NK1R-positive neurons express the inhibitory transmitter GABA: *in vivo* >90% of these neurons were stained with an antibody against GABA (Fig. 3), whereas in OTCs this proportion was less,  $\sim$ 50% (Fig. 4). In order to determine the proportions of GABA-positive neurons containing the NK1R *in vivo*, a total of 426 GABA-positive cells was analysed (see above); 95 of these (22.3%) were NK1R-positive. The proportions of double labelled neurons were higher in lower parts (mean  $\pm$  SD: 38.7  $\pm$  24.1) as compared to upper parts (mean  $\pm$  SD: 19.3  $\pm$  10.3) of occipital cortex (Mann–Whitney test, two-tailed  $P$  value < 0.05). Differences in proportions between lower and middle, and middle and upper parts of cortex were statistically not significant. Since in the rat visual cortex about  $\sim$ 16% of the neurons express glutamic acid decarboxylase, the synthetic enzyme for GABA (Penny *et al.*,

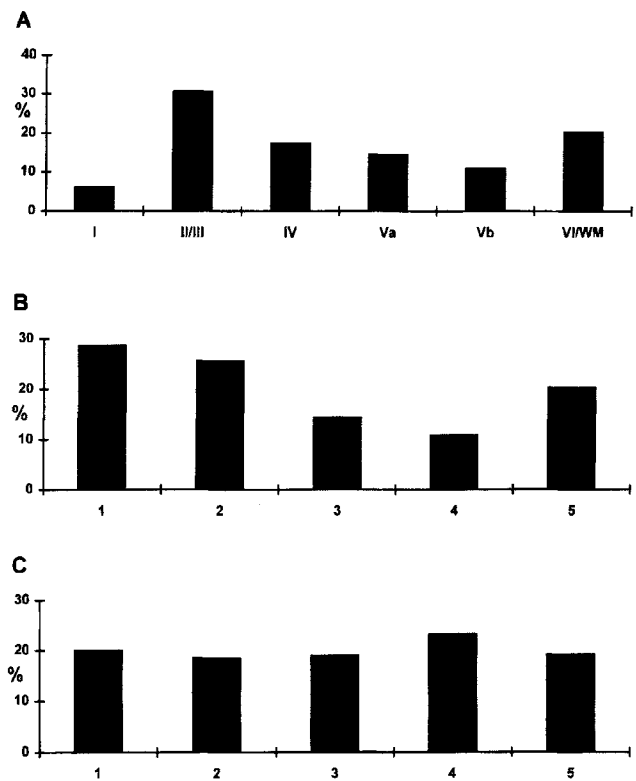


FIG. 5. Representation of the laminar distribution of NK1R-expressing neurons *in vivo* and in OTC preparations of occipital cortex. (A) Laminar distribution *in vivo*. Layers II and III are presented as one lamina. Layer V was divided into an upper (Va) and lower (Vb) part. VI/WM: Layer VI including adjacent white matter. B, C: In order to facilitate comparison, the grey matter (from the pial surface to the layer VI/white matter border) *in vivo* (B) and OTCs (C) was divided into five compartments of equal width and length; the number of neurons in each compartment was then calculated as a proportion (%) of all neurons counted. Cortical layers (roman numerals) relate to compartments (co) as follows: co 1 = I+II; co 2 = III + IV; co 3 = upper V; co 4 = lower V; co 5 = VI. The proportions in compartment 1 refer to neurons in layer II; in layer I only very few neurons were stained. Fourteen *in vivo* sections were analysed and 2196 NK1R-expressing neurons counted; in OTCs the respective numbers were 3 and 520.

1986), one can estimate the population of neurons expressing both the NK1R and GABA to make up  $\sim$ 3% of all neurons.

Pyramidal neurons containing NK1R specific immunoreactivity were detected neither *in vivo* nor *in vitro*. However, these negative findings do not definitely prove that the NK1R is absent in pyramidal neurons of the rat's occipital cortex. The level of NK1R could be much lower in pyramidal neurons than in non-pyramidal neurons and thus escape detection by immunohistochemistry.

The laminar distributions were similar in Wistar and Sprague–Dawley rats, and between the primary visual area and surrounding visual areas (Swanson, 1992) and data therefore were pooled. As can be seen (Fig. 5), *in vivo* proportions of NK1R-expressing neurons in layers II/III and VI, including adjacent white matter, are two to three times higher than in layer I, IV or V. In OTCs the NK1R-expressing neurons distribute throughout all layers in a rather homogenous manner.

## Discussion

Our results confirm earlier findings that neurons expressing the NK1R distribute through all layers of the rat neocortex (Maeno *et al.*, 1993;

Liu *et al.*, 1994; Nakaya *et al.*, 1994). We demonstrate that these neurons reside preferentially in layers II/III, and in layer VI including adjacent parts of the white matter, and that most of them are inhibitory interneurons co-expressing GABA. The latter result confirms suggestions on an inhibitory functions of NK1R expressing neurons which were made on the basis of morphological criteria (Liu *et al.*, 1994; Nakaya *et al.*, 1994). According to our findings, a few NK1R expressing neurons do not express GABA.

The failure of double staining with GABA in quite a number of NK1R neurons in OTCs could indicate higher proportions of NK1R neurons without GABA in OTCs than *in vivo*. Downregulation of the GABAergic phenotype in a particular class of neurons has been reported to occur in OTCs of the retina (Rowerendleman *et al.*, 1996). On the other hand, Mihály *et al.* (1991) have suggested that low proportions of neuronal somata with GABA-like immunoreactivity present in acute slices of hippocampus and also in some OTCs of neocortex (Caesar *et al.*, 1989) could be attributed to an efflux of GABA from the soma into the dendrites and axon, during the slicing procedure or later, during incubation. The latter suggestion is supported by our observation that in OTCs, dendrites and axons of neurons displaying GABA-like immunoreactivity have occasionally no label in their soma. The proportion of NK1R-expressing neurons colocalizing with GABA in OTCs is therefore possibly underestimated using the present methodology.

The finding that most NK1R-expressing neurons in the rat neocortex are inhibitory interneurons implies differences in the cellular networks and in the functions mediated by substance P between rat and other species like cat and monkey. NK1R-expressing cells in the cat are preferentially pyramidal neurons (Matute *et al.*, 1993b). In the monkey an expression of the NK1R in pyramidal neurons is suggested by the observation that SP-containing axon terminals make synaptic contacts preferentially with spines and dendritic shafts of pyramidal cells (Jones *et al.*, 1988). Thus, in cat and monkey neocortex substance P seems to act preferentially on excitatory neurons projecting to other cortical and subcortical areas, whereas in the rat neocortex it exerts its functions almost exclusively via intrinsically projecting inhibitory neurons. Interestingly, species differences also exist with respect to the morphology of substance P expressing neurons. In the cat somatosensory cortex, ~10% of these neurons have been claimed to be pyramidal neurons (Conti *et al.*, 1992). In the rat somatosensory cortex all neurons expressing SP are non-pyramidal neurons which co-express GABA (Penny *et al.*, 1986).

In a number of studies it has been shown that many of the unique properties of neocortex are maintained in organotypic cultures of this tissue. Pyramidal and non-pyramidal neurons from OTCs of rat neocortex and hippocampus display differentiation of morphology and transmitter phenotypes as well as connectivity and physiological properties comparable to the *in vivo* situation (Caesar *et al.*, 1989; Caesar and Schüz, 1992; Finsen *et al.*, 1992; Götz and Bolz, 1994). Here we add the finding that basic properties of NK1R expressing neurons, namely the non-pyramidal shape and the co-expression of GABA, are maintained in OTCs. Some differences were noted, however, between *in vivo* and OTCs. *In vitro* the somata of NK1R-expressing neurons are significantly larger and their dendrites tended to be longer than *in vivo*. An increase in dendritic length in OTCs from neocortex has also been reported by Caesar and Schüz (1992). The laminar distribution of NK1R-expressing neurons in OTCs is rather homogeneous and does not show the preferential location for upper and deep layers as seen *in vivo*. The homogeneous laminar distribution of NK1R-expressing neurons in our monocultures could be due to the lack of interaction of cortex with thalamic or cortical tissue. This suggestion is based on the observation that the layer

specific phenotype restriction of NPYmRNA expressing cells occurring postnatally in the rat visual cortex occurs in co-cultures of cortex with cortex or thalamus but not in monocultures (Obst and Wahle, 1996). Others have reported changes in distribution and size of synapses in OTCs of rat neocortex; for example, in pyramidal neurons the proportion of axospinous over axodendritic synapses was higher *in vivo* than in OTCs (Caesar and Schüz, 1992). Another interesting finding was that of considerable sprouting of cut axons in the OTCs which would increase the probability of making contacts to neighbouring cells. Therefore, in order to decide whether OTCs are a useful model for studying NK1R functions, the axonal connections of NK1R expressing neurons should be studied in more detail in these culture system.

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## Abbreviations

GABA	$\gamma$ -aminobutyric acid
NK1R	NK1 receptor
NK	neurokinin
NP	neuropeptide
OTC	organotypic culture
SP	substance P

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