

Specification of neuropeptide Y phenotype in visual cortical neurons by leukemia inhibitory factor

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SUMMARY

Building the complex mammalian neocortex requires appropriate numbers of neurochemically specified neurons. It is not clear how the highly diverse cortical interneurons acquire their distinctive phenotypes. The lack of genetic determination implicates environmental factors in this selection and specification process. We analysed, in organotypic visual cortex cultures, the specification of neurons expressing neuropeptide Y (NPY), a potent anticonvulsant. Endogenous brain-derived neurotrophic factor and neurotrophin 4/5 play no role in early NPY phenotype specification. Rather, the decision to express NPY is made during a period of molecular plasticity during which differentiating neurons with the potential to express

NPY compete for the cytokine leukemia inhibitory factor which is produced in the cortex, but is negatively regulated by thalamic afferences. The neurons that fail in this competition are parvalbuminergic basket and chandelier neurons, which express NPY transiently, but will not acquire a permanent NPY expression. They switch into a facultative NPY expression mode, and remain responsive to the neurotrophins which modulate NPY expression later in development.

Key words: Neuropeptide Y, Visual cortex, Leukemia inhibitory factor, Neurotrophins, Rat

INTRODUCTION

The interneurons in the mammalian cortex are highly diverse and may constitute up to 100 distinct cell types characterized by morphology and axon termination, neurochemical and molecular profile, firing properties, and responsiveness to transmitters (DeFelipe, 1993; Parra et al., 1998; Stevens, 1998). To complicate matters, during corticogenesis interneurons not only arise from the telencephalic ventricular zone, but invade the neocortex from the lateral ganglionic eminence, and they display a notoriously nonradial dispersal behavior (Anderson et al., 1997; Tan et al., 1998). No wonder then that the neurochemically defined interneuron types (Demeulemeester et al., 1988, 1989; DeFelipe, 1993; Gonchar and Burkhalter, 1997) are not related by genetic lineage, but apparently become specified locally by environmental factors (Mione et al., 1994; Götz and Bolz, 1994; Götz et al., 1995). Once arrived at their final destination many differentiating interneurons, and also pyramidal neurons, transiently express neuropeptides, calcium-binding proteins or receptors, and subsequently undergo phenotypic shifts which are influenced by afferent innervation, trophic factors and sensory experience (Shaw et al., 1986; Parnavelas and Cavanagh, 1988; Wahle, 1994; Alcantara et al., 1996; Obst and Wahle, 1995, 1997; Obst et al., 1998). These shifts appear essential for the establishment of the adult neurochemical architecture because they shape the laminar- and areal-specific sets of distinct interneuron types likely required for proper function of the mature cortex.

In the adult rat cortex interneurons constitute three families characterized by the expression of parvalbumin, or calretinin, or somatostatin (Gonchar and Burkhalter, 1997). The parvalbuminergic neurons are fast-spiking basket and chandelier cells which do not express neuropeptides or calbindin (Demeulemeester et al., 1989, 1991; Gonchar and Burkhalter, 1997). Calretinin neurons are mostly bipolar cells, while the somatostatin neurons constitute a variety of types which colocalize neuropeptide Y (NPY) and calbindin. In developing rat however, the diversity appeared much higher. Cauli et al. (1997) using single-cell RT-PCR followed by Southern blots reported that in postnatal day-16 to -22 neocortex, many parvalbuminergic neurons coexpressed NPY, somatostatin and calbindin. During this time, parvalbuminergic neurons acquire their fast spiking properties (Massengill et al., 1997) and begin to downregulate their transient calbindin expression (Alcantara et al., 1996). In adult cortical interneurons, calbindin and peptidergic phenotypes (Gonchar and Burkhalter, 1997) are associated with non-fast spiking physiological properties (Kawaguchi and Kubota, 1997). Most likely, the segregation of neurochemical properties observed in the adult has not yet been completed by the age analyzed by Cauli et al. (1997) with a very sensitive technique. With regard to the enormous phenotypic plasticity it must be assumed that sets of neurons of any given phenotype become selected from a larger number of neurons with the genetic potential to express this phenotype. This implies that differentiating neurons must somehow decide which phenotype to adopt.

We studied neurons expressing NPY mRNA in rat visual cortex. In the adult, NPY delineates a subset of non-fast spiking interneurons of small basket and Martinotti cell morphology. Recent studies have emphasized the important cellular and behavioral functions of NPY. It is implicated in feeding behavior, anxiety, the preference for alcohol, and most importantly, NPY is a major endogenous antiepileptic peptide and modulator of excitatory synaptic transmission (Wahlestedt et al., 1993; Erickson et al., 1996a,b; Klapstein and Colmers, 1997; Baraban et al., 1997; Woldbye et al., 1997; Ehlers et al., 1998; Thiele et al., 1998; Tecott and Heberlein, 1998; Vezzani et al., 1999). It is likely then that NPY expression is under tight regulatory control. However, NPY expression is highly dynamic and is easily promoted by neuronal excitation and epileptiform activity (Gall et al., 1990; Carnahan and Nawa, 1995, for review; Nawa et al., 1995; Schwarzer et al., 1996; Marty and Onteniente, 1999). NPY expression is also promoted by brain-derived neurotrophic factor (BDNF), neurotrophin 4/5 (NT-4/5), and the cytokine leukemia inhibitory factor (LIF) and the neurons express tyrosine receptor kinase *trkB* and the LIF receptor β (Carnahan and Nawa, 1995; Marty et al., 1997; Wirth et al., 1998a,b; Marty and Onteniente, 1999; Gorba and Wahle, 1999). In these conditions (high levels of neuronal activity, exposure to exogenous trophic factors) NPY mRNA is upregulated to high levels in many more neurons than normal, including parvalbuminergic cortical interneurons and pyramidal neurons (M. Engelhardt, G. diCristo, N. Berardi, L. Maffei, P. W., unpublished) or hippocampal granule and pyramidal cells (Gall et al., 1990).

Together, this suggests the existence of constitutive and facultative NPY expressers. Clearly, the ability of the nervous system to upregulate an anticonvulsant like NPY in a use-dependent way is highly adaptive. Yet, the mechanisms by which developing neurons learn to express NPY in a permanent, constitutive versus a transient, facultative mode are unknown.

MATERIALS AND METHODS

Cultures

Organotypic cortex monocultures and thalamocortical cocultures were prepared from pigmented Long-Evans rats at the day of birth and kept *in vitro* for up to 70 days (DIV; Obst and Wahle, 1995, 1997). Cocultures regenerate the axonal connections within the first 2 weeks *in vitro* (Bolz et al., 1990; Obst and Wahle, 1997). Control cocultures were implanted with lipophilic dyes, and axonal connections in fiber bridges between the explants were seen in all these cocultures. MCM was harvested from 10-30 DIV monocultures every other day. It was either directly applied to cocultures, or preincubated for 1 hour with the anti-LIF antibody before being applied to cocultures. BDNF, NT-4/5, neurotrophin-3, nerve growth factor and LIF (all from Peprotech) were applied at 20 ng/ml medium every third day for the time periods indicated in the legends. The neutralizing anti-LIF antibody (R&D Systems) was applied at 4 μ g/ml medium every second day. Its neutralizing efficiency was proved in a control experiment: we reported (Wirth et al., 1998b) that exogenous LIF stimulates NPY mRNA expression in monocultures grown in the presence of 10 mM $MgSO_4$ to block spontaneous bioelectric activity. When anti-LIF antibody was applied to activity-blocked monocultures supplemented with exogenous LIF, the antibody completely blocked the effect of LIF (2 μ g/ml and 4 μ g/ml anti-LIF antibody were equally effective, the neutralization in the present study was done with a 4 μ g/ml

concentration). *TrkB*-IgGs and *TrkC*-IgGs were kindly provided by Dr Yancopoulos (Regeneron Pharmaceuticals). They were applied at a concentration of 20 μ M, which was shown to effectively neutralize endogenous neurotrophins in tissue cultures (McAllister et al., 1996; Routbort et al., 1997).

Biolistic transfection

Thalamocortical cocultures were transfected at 5 DIV using the Helios Gene Gun (Biorad) and 1.6 μ m gold particles coated with expression plasmid cDNAs encoding either p-EGFP-N1 under cytomegalovirus promoter (Clontech; 1 μ g/mg gold) or p-EGFP-N1 (1 μ g/mg gold) plus pCAGGS-LIF under beta-actin promoter (2 μ g/mg gold; generously provided by Dr Austin Smith, Center of Genome Research, Edinburgh, UK; for vector construction including the LIF vector see Miyazaki et al., 1989; Niwa et al., 1991). Both plasmids were prepared endotoxin-free (Endofree, Qiagen), precipitated onto gold particles according to the manufacturer's recommendation (Biorad), and 0.2 mg gold was loaded per cartridge. The gene gun was mounted into a support, with the distance from muzzle to tissue set at 1 cm, pressure was 160 psi (1.1×10^6 Pa). EGFP expression was checked at 7 DIV, and only well expressing cultures were kept. Expression was observed for about 2 further weeks suggesting that also LIF was expressed through the presumed period of molecular plasticity. At 75 DIV, OTC were checked for visible fiber bridges between the two explants and only cultures passing this examination went into NPY *in situ* hybridization.

In situ hybridization and quantitative analysis

NPY mRNA-expressing cells were labeled by *in situ* hybridization in paraformaldehyde-fixed, freefloating cultures. Digoxigenin-labeled riboprobes complementary to exon 2 and/or the signal peptide of NPY were transcribed (Roche; following the manufacturer's protocol) and used under established conditions and controls (Obst and Wahle, 1997). Both probes yielded identical results. The coculture and the monoculture study were each performed with riboprobes from one transcription to yield probe constancy. For analysis (Obst and Wahle, 1997; Wirth et al., 1998) labeled neurons were plotted (Eutectics Neuron Reconstruction System®, Raleigh, NC, USA), and subsequently total neuron number per culture was determined, after counterstaining with thionin, to calculate the percentage of NPY mRNA-expressing neurons. Non-parametric statistics (Mann-Whitney U-Test) was performed followed by corrections for multiple testing according to Holm (1979).

Fluorescent double-labeling with mouse monoclonal antibodies against parvalbumin (SWANT) followed by biotinylated secondary and Texas Red-conjugated streptavidin was performed as described by Gorba and Wahle (1999) in 40 DIV and 60 DIV cortex monocultures and thalamocortical cocultures. The percentage of cortical parvalbuminergic neurons coexpressing NPY was determined (about 150 neurons were analyzed for every age and experimental condition).

PCR

Five monocultured cortices and five cortices from thalamocortical cocultures per age, at the six ages indicated in Fig. 3, were pooled, mRNA was isolated (mRNA Direct kit, Dynal), and single-stranded cDNA was reverse transcribed with AMV reverse transcriptase (20 U/ μ l, Stratagene) at 42°C for 45 minutes. PCR was performed in a total volume of 50 μ l with GoldStar DNA Polymerase (Eurogentec). PCR conditions were kept in the linear range determined for each product (30 cycles for BDNF, 36 cycles for NT-4/5 and LIF). The two sets of cDNA libraries were probed for BDNF (bases 154-393 specific for 3' exon V; Maisonpierre et al., 1991), NT-4/5 (bases 309-605; Berkemeier et al., 1991), and LIF (bases 179-513; Yamamori et al., 1989). Relative band intensities were densitometrically determined with the Eagle Eye System® (Stratagene). Intensities were normalized to glucose-6-phosphate dehydrogenase expression (bases

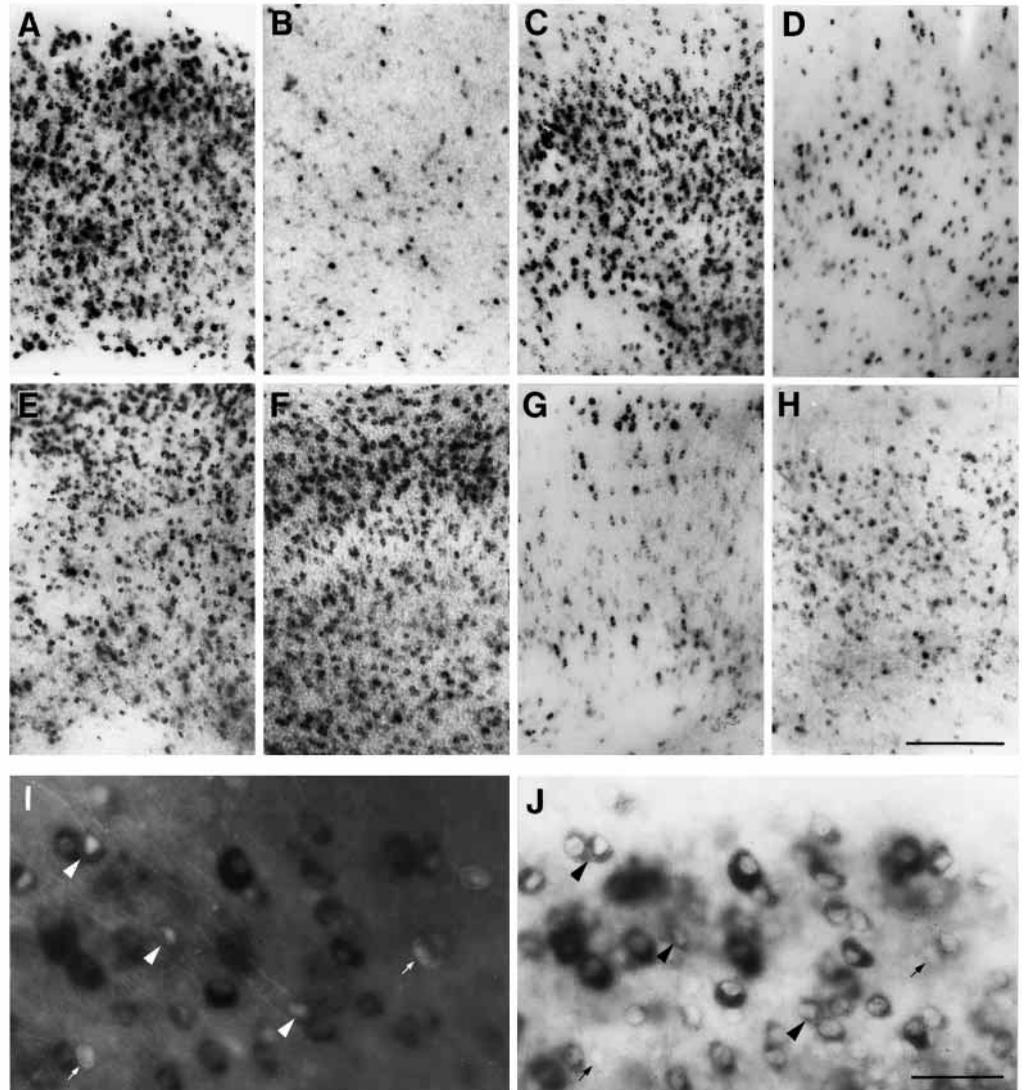


Fig. 1. NPY mRNA expression in representative monocultures (VC) and thalamocortical cocultures (VC-Th). (A) VC at 60 DIV. (B) VC-Th at 70 DIV. (C) VC-Th at 70 DIV, MCM from 3-20 DIV. (D) VC-Th at 70 DIV, MCM from 60-70 DIV. (E) VC-Th at 70 DIV, NT-4/5 from 60-70 DIV. (F) VC-Th at 70 DIV, LIF from 3-20 DIV. (G) VC-Th at 70 DIV, LIF from 60-70 DIV. (H) VC-Th at 70 DIV, MCM plus anti-LIF from 3-20 DIV. The labeling intensity per neuron reflects the amount of mRNA (Emson, 1993). (I,J) High degree of overlap between NPY mRNA and parvalbumin immunofluorescence in 60 DIV monocultures. Scale bar in A-H, 300 μ m; I,J, 50 μ m.

2112-2272; Ho et al., 1988), and were expressed relative to intensities measured at postnatal day 0 (P0), which had been set to 1. Values from at least three PCRs were used to construct the graphs with standard deviation from mean.

RESULTS

Transient NPY expression and phenotype restriction

Many developing neurons *in vivo* express NPY transiently and stop the expression later in development. This process has been termed phenotype restriction (Obst and Wahle, 1995, 1997). It occurs during the second postnatal month in rat, concurrent with the decline phase of the sensitive period of cortical plasticity (Fagiolini et al., 1994), and shapes the areal- and laminar-specific NPY mRNA expression pattern of the mature visual cortex. Surprisingly, the phenotype restriction fails to occur in visual cortex raised as organotypic monoculture (Figs 1A, 2, bar 1). Apparently, all neurons fated to express NPY become committed to do so under this condition (Fig. 1A). The phenotype restriction is therefore not a cell-autonomous

process, but rather the consequence of a decision which NPY neurons make. The decision likely occurs during an early period of molecular plasticity, and it depends on environmental factors. A major factor is the afferent innervation, since it downregulates NPY mRNA expression. The phenotype restriction occurs only in thalamocortical (Fig. 1B, note also a decline in intensity of labeled neurons; Fig. 2, bar 2) and corticocortical, but not in efferently connected corticotectal cocultures, during the second month *in vitro* (between 30-60 DIV) with a time course precisely as *in vivo* (Obst and Wahle, 1995). Afferents thus influence the neurochemical architecture of their target region.

The phenotype restriction is prevented by diffusible factor(s) present in monoculture-conditioned medium (MCM). MCM was applied to thalamocortical cocultures from 3-20 DIV (which overlaps in time with the *in vitro* regrowth of afferents and neuronal maturation) followed by normal medium until 60 DIV. Despite their temporally limited presence, the MCM factor(s) are able to override the phenotype-restricting capacity of the afferents and to confer a permanent NPY expression (Fig. 1C, note the high levels of expression and the much

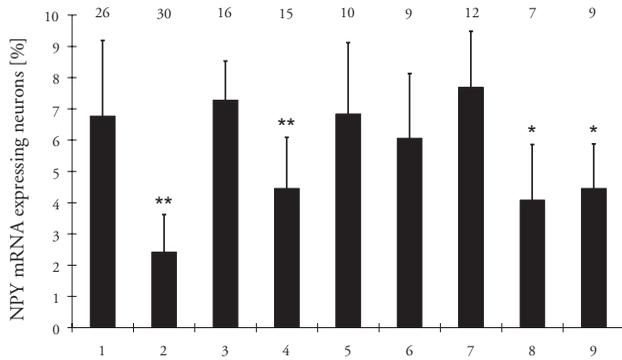


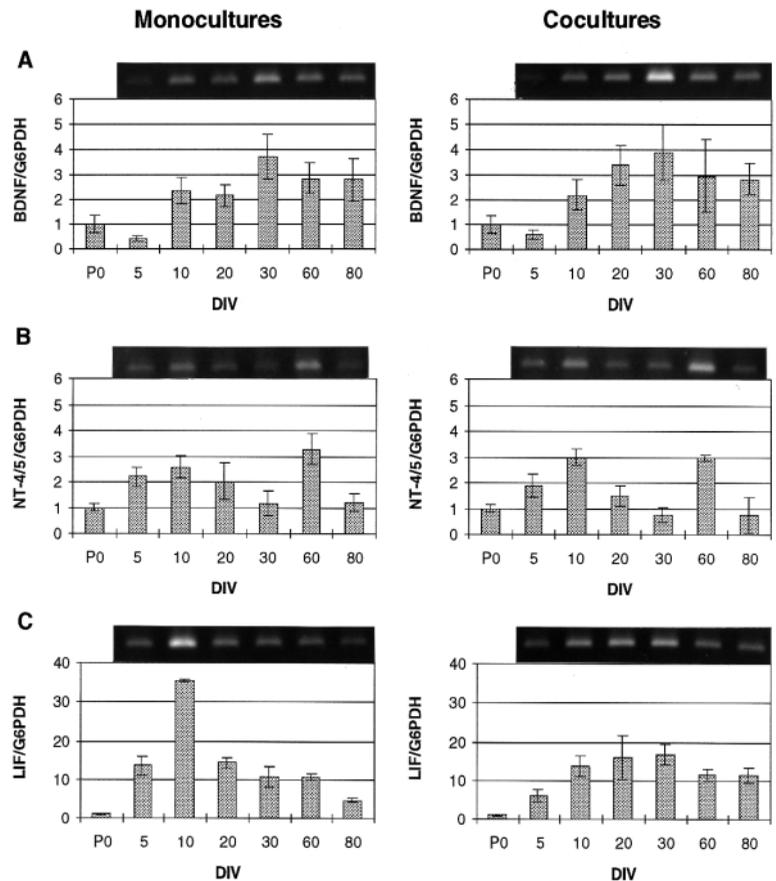
Fig. 2. Percentages of NPY mRNA-expressing neurons in monocultures (VC) and thalamocortical cocultures (VC-Th). Mean values with standard deviation are given, the number of cultures analyzed is given above every bar. Bar 1, VC 60-90 DIV; 2, VC-Th 60-90 DIV; 3, VC-Th 60-70 DIV, MCM from 3-20 DIV; 4, VC-Th 70 DIV, MCM from 60-70 DIV; 5, VC-Th 60-70 DIV, NT-4/5 from 3-20 DIV; 6, VC-Th 70 DIV, NT-4/5 from 60-70 DIV; 7, VC-Th 60 DIV, LIF from 3-20 DIV; 8, VC-Th 70 DIV, LIF from 60-70 DIV; 9, VC-Th 60 DIV, MCM plus anti-LIF from 3-20 DIV. ** $P < 0.01$, * $P < 0.05$, all Mann-Whitney U-tested against monocultures (bar 1) and corrected for multiple testing according to Holm (1979), which is as stringent as the Bonferroni-correction, but minimizes second order errors.

higher density of labeled neurons; Fig. 2, bar 3). In 'old' cocultures, which had already undergone the phenotype restriction, these MCM factor(s) are much less effective (Fig. 1D, note the low density of labeled neurons; Fig. 2, bar 4).

Parvalbuminergic neurons frequently coexpress NPY in monocultures, but rarely in cocultures

The high number of constitutive NPY expressers in monocultures suggests that additional subsets of interneurons display NPY mRNA. Quantitative evaluation of NPY in situ hybridization followed by parvalbumin immunofluorescence reveals that in 60 DIV monocultures about 40% of the parvalbuminergic neurons coexpressed NPY mRNA (Fig. 1I,J). In contrast, in 40-60 DIV thalamocortical cocultures displaying 3-4% NPY neurons we observed that about 10% of the parvalbuminergic neurons colocalized NPY, while in cocultures older than 60 DIV, which usually display about 2-3% NPY neurons, the parvalbumin-NPY coexpression was no longer observed. The degree of coexpression is thus inversely related to the effectiveness of the phenotype restriction. This suggests that differentiating parvalbuminergic basket and chandelier cells initially express NPY, but then undergo the phenotype restriction and stop the NPY expression.

Fig. 3. Developmental expression of (A) BDNF, (B) NT-4/5 and (C) LIF in a set of cDNA libraries prepared from monocultured cortex (left) and a set of libraries prepared from the cortex of thalamocortical cocultures (right). Note that LIF was highly expressed in 5 DIV and 10 DIV monocultures, but not in cocultures. Postnatal day 0 (P0) is the day of explantation. Expression at P0 was set to 1.



Thalamic innervation regulates LIF expression

Thus, the phenotype restriction is an essential process in the neurochemical maturation of visual cortex, and it occurs in vivo and in afferently innervated cultures, but not in monocultures. We therefore set out to identify the factor(s) which in monocultures prevented the correct phenotype decision in interneurons. The neurons express *trkB* and LIF receptor β . BDNF, NT-4/5 and LIF are potent regulators of the NPY phenotype. We therefore analyzed whether the expression of these trophic factors is influenced by afferences. We have screened by semiquantitative PCR, sets of cDNA libraries prepared from the cortical explants in monoculture and in thalamocortical coculture. BDNF expression initially declines in vitro due to the explantation but then increases in both mono- and cocultures during the second week to reach a plateau thereafter (Fig. 3A). The BDNF increase depends on spontaneous activity which develops in mono- and cocultures during the second and third week (Gorba et al., 1999). NT-4/5 expression is not depressed by explantation; it increases in both mono- and cocultures until 10 DIV, then decreases to a lower level, and displays again a transient peak at 60 DIV (Fig. 3B). The mRNA expression of the neurotrophins was apparently not influenced by the presence of thalamic afferences. In contrast, a clear difference has been observed for the LIF mRNA. It is highly expressed at 5 DIV and 10 DIV in monocultures. In contrast, LIF mRNA is low in 5 DIV and 10 DIV cocultures (Fig. 3C). During this time period, the afferent connections in cocultures become reestablished (Bolz et al., 1990). This suggested that during an early postnatal period the LIF

mRNA expression and thus the amount of LIF peptide is negatively regulated by afferences.

Exogenous LIF prevents the phenotype restriction in cocultures

Next, thalamocortical cocultures are supplemented with trophic factors (separately) for the period of molecular plasticity from 3-20 DIV followed by normal medium until 60 DIV. Despite their temporally limited presence, NT-4/5 (Fig. 2, bar 5) and LIF (Fig. 1F, note high density and high levels of expression; Fig. 2, bar 7) are both able to override the phenotype-restricting capacity of the afferents. Both factors prevent the phenotype restriction and confer a permanent NPY mRNA expression. In contrast, BDNF (Obst and Wahle, 1997), neurotrophin-3, and nerve growth factor (not shown) are not effective in preventing the phenotype restriction.

To test LIF further, we have transfected the cortex in thalamocortical cocultures at 5 DIV with an expression plasmid for LIF under control of a constitutive promoter. Green fluorescent protein (EGFP) served as reporter and as control plasmid. After 75 DIV the EGFP transfected control cocultures ($n=5$) display $2\% \pm 0.6\%$ NPY mRNA-expressing neurons in the cortex, which is typical for thalamocortical cocultures of that age. In contrast, the LIF/EGFP transfectants ($n=8$) display $5\% \pm 1.2\%$ NPY mRNA expressing neurons. The transient endogenous overexpression of LIF although maximally in 300 cells per culture has resulted in a partial, but significant rescue of the NPY mRNA expression (Mann-Whitney U-Test: $P=0.006$) in the presence of thalamic afferents.

In 60 DIV thalamocortical cocultures which had already undergone the phenotype restriction, exogenous NT-4/5 reinforces NPY expression (Fig. 1E; Fig. 2, bar 6) as does exogenous BDNF (Obst and Wahle, 1997). This rules out cell death of putative NPY neurons. Rather, the NPY expression has stopped in the course of the phenotype restriction especially in the parvalbuminergic cells. These neurons have switched to a facultative expression mode, and upregulate NPY only in response to exogenous neurotrophins. The endogenous TrkB ligands are apparently no longer able to promote or sustain the NPY expression. In contrast, exogenous LIF is much less effective in 60 DIV cocultures (Fig. 1G; Fig. 2, bar 8), and thus displays a profile very similar to the MCM. This suggests a role for LIF in early specification of the NPY phenotype, especially the decision to constitutively express NPY.

To test this, we have preabsorbed the MCM with neutralizing antibodies against LIF before applying it to thalamocortical cocultures for 3-20 DIV followed by normal medium until 60 DIV. Now the MCM is no longer effective in preventing the phenotype restriction (Fig. 1H, note that density and labelling intensities clearly differ compared to e.g. Fig. 1C; Fig. 2, bar 9).

Modulating endogenous LIF, but not TrkB receptor ligands, elicits a phenotype restriction in the absence of afferences

The above findings led us to conclude that limited amounts of LIF during the period of molecular plasticity forced potential NPY neurons to make a phenotype decision. A crucial test for this assumption is the following. Could we induce a phenotype restriction in monocultures by reducing the amount of endogenous LIF during the first weeks in vitro? Further, is there

an early role for the endogenous neurotrophins, which are generally believed to regulate neuropeptide expression? Monocultures (Fig. 5, bar 1) were treated with neutralizing anti-LIF antibody for 3-30 DIV, and at 30 DIV they displayed about 3.5% NPY neurons (Fig. 4A; Fig. 5, bar 2). In contrast, monocultures treated with anti-LIF antibody for 3-20 DIV followed by normal medium until 30 DIV displayed 7% NPY neurons (Fig. 4B, note the higher density of cells and increased labeling intensity; Fig. 5, bar 3) indicating the ability of many neurons to transiently upregulate NPY expression after an early LIF deprivation. However, without sufficient LIF signalling during the early period of molecular plasticity these neurons fail to acquire a permanent, constitutive NPY expression: monocultures treated with anti-LIF antibody from 3-20 DIV followed by normal medium until 60 DIV display only 4% NPY neurons (Fig. 4C, note the faint expression in most neurons; Fig. 5, bar 4) and therefore had undergone a phenotype restriction. Thus, reducing or preventing LIF signalling in monocultures during the period of molecular plasticity elicits a phenotype restriction which would normally occur only in an afferently innervated cortex. In contrast, a late postnatal withdrawal of LIF from 30-40 DIV does no longer alter the phenotype (Fig. 4D; Fig. 5, bar 5), indicating that the NPY expression by this age is no longer dependent on endogenous LIF.

An early withdrawal of endogenous BDNF and NT-4/5 with TrkB-IgGs from 2-20 DIV followed by normal medium until 50 DIV results in about 7% NPY neurons, and in considerable contrast to the early LIF withdrawal does not elicit a phenotype restriction (Fig. 4E, note the high density of labeled cells, many of which however expressed NPY mRNA at a slightly lower level compared for instance to figure 1A; Fig. 5, bar 6). However, treating 30 DIV monocultures (Fig. 5, bar 1) for a further 10 days with TrkB-IgGs reduces NPY neurons to about 4% (Fig. 4F; Fig. 5, bar 7). As expected, TrkC-IgGs are ineffective (Fig. 4G, Fig. 5, bar 8), because NPY neurons do not express trkC receptors. Together this indicates that BDNF and/or NT-4/5 are required for the transient upregulation of NPY to peak levels at around the end of the first month in vitro. A treatment of monocultures from 3-30 DIV with anti-LIF antibody and TrkB-IgGs also results in 4% NPY neurons (Fig. 4H, note the low density, and that a majority of the neurons now expressed very low levels of NPY mRNA; Fig. 5, bar 9). Although all neutralization experiments yield significant reductions in NPY cell numbers, they appear less effective than the direct action of afferents, which could be due to an incomplete penetration of the reagents into the explants. Alternatively, the results suggest the presence of a core set of NPY neurons which expresses NPY independent of LIF and neurotrophins. Indeed, a LIF null mutant displays NPY neurons in the cortex (Bugga et al., 1998). Yet, high NPY expression levels and especially the expression in the parvalbuminergic neurons are gained only when these neurons are primed by LIF signalling during differentiation, and switched to trkB ligands later in development.

DISCUSSION

Competition for LIF specifies NPY neurons

A high percentage of cortical interneurons has the genetic potential to express the neuropeptide NPY, and does so during development, but the majority of those cells do not express

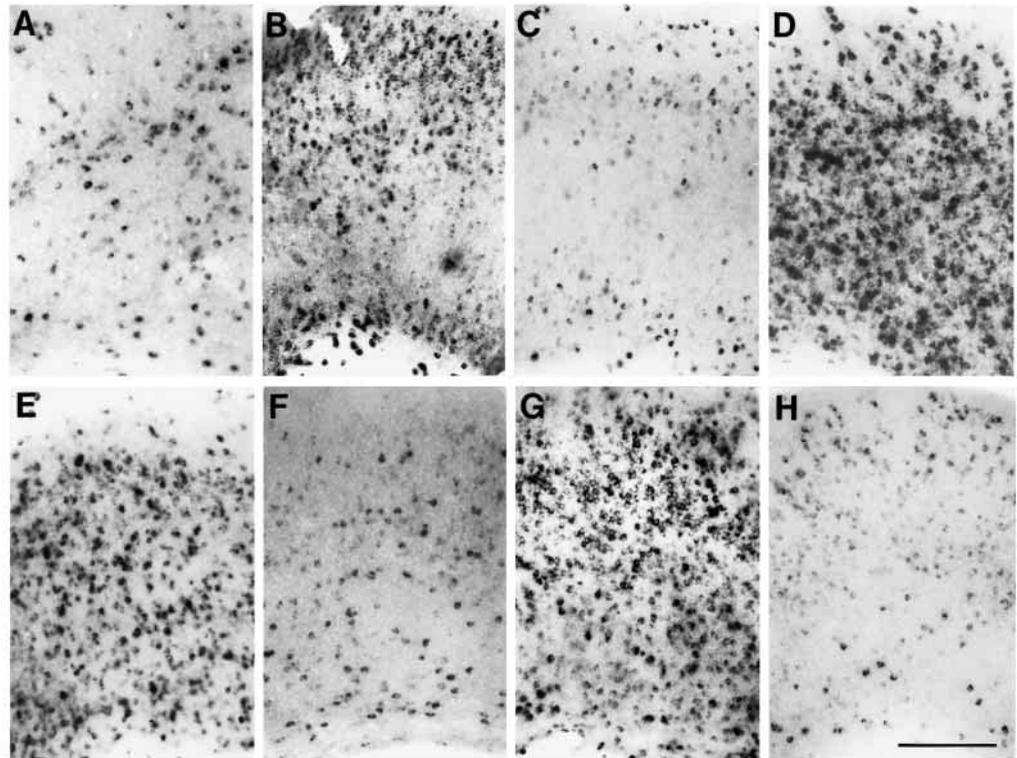


Fig. 4. NPY mRNA expression in representative monocultures (VC) supplemented with neutralizing agents. (A) VC 30 DIV, anti-Lif from 3-30 DIV. (B) VC 30 DIV, anti-LIF from 3-20 DIV. (C) VC 60 DIV, anti-LIF from 3-20 DIV. (D) VC 40 DIV, anti-LIF from 30-40 DIV. (E) VC 50 DIV, TrkB-IgG from 3-20 DIV. (F) VC 40 DIV, TrkB-IgG from 30-40 DIV. (G) VC 40 DIV, TrkC-IgG from 30-40 DIV. (H) VC 40 DIV, TrkB-IgG plus anti-LIF from 30-40 DIV. Scale bar: 300 μ m.

NPY in the normal adult cortex. They can be committed to express NPY permanently when exposed to sufficient LIF signalling during differentiation. Yet, LIF is low in concentration during the period of molecular plasticity and this forces the parvalbuminergic interneurons into the phenotype restriction.

LIF, ciliary neurotrophic factor and other ligands signalling through gp130/LIF β -receptor complex are known to alter phenotypes of CNS neurons. For instance, in embryonic raphe precursor cells and a raphe-derived cell line, both factors (exogenous application) reduce the number of serotonin-expressing neurons by switching them to a cholinergic phenotype, while BDNF counteracts and partially rescues the expression of serotonergic traits (Rudge et al., 1996).

Our study now shows that LIF influences the phenotype choice of neocortical neurons. The amount of endogenous LIF produced in thalamocortical cocultures appears to stabilize the NPY expression in 2-3% of the neurons, which is close to, but slightly higher than in vivo percentages (Obst and Wahle, 1995). Yet, LIF mRNA levels in cocultures were higher than in vivo (although still much lower than e.g. BDNF mRNA levels; not shown). The higher LIF expression in vitro might be due to the lack of most cortical afferences because in vivo several afferent systems may act in synergy to downregulate LIF. Indeed, lowest percentages of NPY neurons have been obtained in thalamus-cortex-cortex cultures which include two afferent systems (Obst and Wahle, 1997). This suggests that during the period of molecular plasticity neurons with the genetic potential to express NPY are competing for LIF. Application of exogenous (pure factor) or endogenous LIF (the MCM) or a temporally limited overexpression makes larger amounts available, eliminates competition, and confers a high level of expression to all putative NPY neurons. The factor is

effective only when applied during the period of interneuronal maturation (Meyer and Ferres-Torres, 1984; Miller, 1986). In contrast, a reduction of LIF level either by the action of thalamic afferents or by neutralizing antibodies in the MCM experiments with cocultures as well as in monocultures aggravates the competition. Many neurons especially the parvalbuminergic cells will transiently express NPY in response to endogenous BDNF or NT-4/5 signalling until the end of the first postnatal month, but will stop the expression

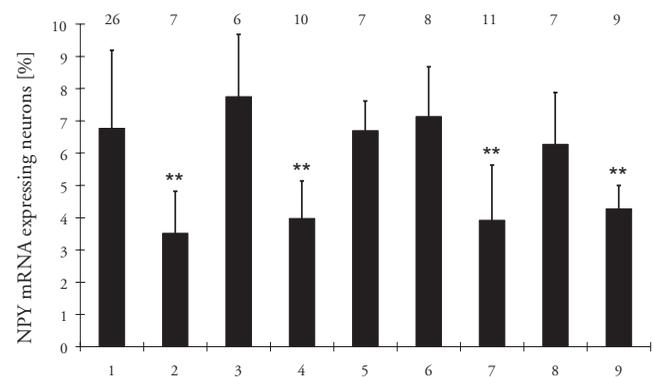


Fig. 5. Effects of neutralizing agents on NPY mRNA expression in monocultures. Bar 1, VC 60-90 DIV (same as in Fig. 2). 2, VC 30 DIV, anti-LIF from 3-30 DIV. 3, VC 30 DIV, anti-LIF from 3-20 DIV. 4, VC 60 DIV, anti-LIF from 3-20 DIV. 5, VC 40 DIV, anti-LIF from 30-40 DIV. 6, VC 50 DIV, TrkB-IgG from 3-20 DIV. 7, VC 40 DIV, TrkB-IgG from 30-40 DIV. 8, VC 40, TrkC-IgG from 30-40 DIV. 9, VC 30 DIV, TrkB-IgG plus anti-LIF from 3-30 DIV. ** $P < 0.01$, * $P < 0.05$, all Mann-Whitney U-tested against monocultures (bar 1) and corrected for multiple testing according to Holm (1979).

during the second month. Parvalbuminergic neurons thus are the 'losers' of the competition, because they acquire a permanent NPY expression only with sufficient LIF signalling. Once the decision to downregulate NPY has been executed, during the second month, neither MCM nor exogenous LIF were able to relicit the NPY expression in parvalbuminergic neurons. In the adult cortex the NPY phenotype is present in morphologically fairly distinct subset of interneurons (Martinotti cells, small basket neurons) which could be regarded as being the most effective competitors for LIF. In addition this subset contains neurons which appear independent of LIF signalling, because they expressed NPY constitutively even in the LIF/neurotrophin-deprived cultures. The latter assumption supports findings in the LIF knockout mouse: in the adult, the null mutant neocortex displays a set of NPY-immunoreactive neurons which were reported to be not significantly different from those of wild-type animals (Bugba et al., 1998). It would be interesting to see whether the absence of LIF has altered the phenotypic potential of the facultative expressers.

Functional implications

Interestingly, the parvalbuminergic interneurons switch to a facultative expression mode. The amount of endogenously produced neurotrophins is obviously not sufficient to maintain a permanent NPY expression in this set of interneurons. Further, the concentration of secreted endogenous neurotrophins in the MCM apparently was still below threshold. In contrast, exogenous BDNF or NT-4/5 (20 ng/ml medium) are able to promote a high NPY reexpression. In other words, only much higher than normal steady state amounts of BDNF or NT-4/5 can promote NPY reexpression in the parvalbuminergic basket and chandelier cells. The developmental competition for LIF thus has rendered about half of all putative NPY interneurons, especially the parvalbuminergic neuron types, unable to respond to endogenous steady state neurotrophin levels in the adult cortex.

Exogenous NT-4/5 has also prevented the afferent-induced NPY phenotype restriction. In contrast to earlier reports (Carnahan and Nawa, 1995), BDNF failed probably because the neuronal activity required as cofactor for a BDNF effect is very low in young cultures (McAllister et al., 1996; Wirth et al., 1998a,b; Klostermann and Wahle, 1999). However, an early temporally limited withdrawal of endogenous neurotrophins does not elicit a phenotype restriction indicating that NPY expression and phenotype specification initially is *trkB*-ligand independent. Further, the time course of BDNF and NT-4/5 mRNA expression is similar in monocultures and thalamocortical cocultures. Together this suggests that neurotrophins are involved in a use- and activity-dependent modulation of neuropeptide expression during later postnatal and adult life (Carnahan and Nawa, 1995; Marty et al., 1997; Marty and Onteniente, 1999). Visual stimulation for instance upregulates BDNF expression (Thoenen, 1995) and directly or via BDNF stabilizes the NPY phenotype selectively in neurons in supragranular layers of area 17 (Obst et al., 1998) which also retain a high level of coexpression of *trkB* receptors (Gorba and Wahle, 1999). Overexcitation increases neurotrophin expression, and directly or in synergy with neurotrophins stimulates NPY expression: epileptiform activity elicits NPY

expression in a much higher than normal proportion of cortical and hippocampal neurons, including excitatory cells (Gall et al., 1990; Nawa et al., 1995; Schwarzer et al., 1996). BDNF infusion *in vivo* during the critical period elicits NPY mRNA in pyramidal cells and in up to 40% of the parvalbuminergic neurons (M. Engelhardt, G. diCristo, N. Berardi, L. Maffei, P. W., unpublished data). NPY in turn acts as a potent anticonvulsant and inhibits glutamatergic synaptic transmission by reducing presynaptic calcium influx and thus transmitter release (Klapstein and Colmers, 1997; Vezzani et al., 1999). In the suprachiasmatic nucleus, NPY mediates long-term depression in electrical activity and in calcium levels (Van den Pol et al., 1996). In contrast, the absence of NPY is proconvulsant: knockout animals develop epileptiform activity (Erickson et al., 1996b), and the animals are unable to terminate kainic acid-induced seizures and die (Baraban et al., 1997).

The ontogenetic period where many cortical neurons are transiently expressing NPY overlaps with the period where the maturation of inhibition lags behind the maturation of excitation. Thus, a higher than normal NPY expression at this developmental stage might be neuroprotective. Switching many of the putative NPY neurons after a transient period of expression into a facultative expression mode may then serve two aims. First, it reduces the NPY peptide level concurrent with the decline phase of the sensitive period of cortical development, and this might gradually allow more efficient glutamatergic and possibly GABAergic transmission. Second, the specification by competition and transient expression might have imprinted a presumably neuroprotective mechanism: the parvalbuminergic neurons (which have transiently expressed NPY) will later in postnatal life 'remember' their ability to express NPY. As we showed by the late postnatal application of neurotrophins to 60 DIV cocultures they are capable of reexpressing this endogenous anticonvulsant when exposed to overexcitation and higher than normal steady state amounts of neurotrophins.

Conclusions

Genetic mechanisms do not appear to deliver the complete set of adult cortical interneuron types, but rather a few basic models of GABAergic neurons with an enormous potential to differentiate into various interneuron types. A postmigratory specification by competitive local mechanisms could guarantee qualitatively and quantitatively an appropriate laminar-specific composition of NPY neurons (the constitutive expressers) without eliminating molecular plasticity (use-dependent NPY expression in the facultative expressers). It remains to be shown whether this concept holds for other neurochemically, morphologically, and functionally differentiated interneuron types. Temporal or spatial variations in factor production, or release under the control of afferent fiber systems, or the availability of cooperating factors during differentiation could then account for the area-, and even species-specific neurochemical diversity, and plasticity, of cortical interneuron types.

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