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Acetylcholine synthesis and neuron differentiation

STEFANO BIAGIONI, ADA MARIA TATA, ANTONELLA DE JACO and GABRIELLA AUGUSTI-TOCCO*

Dipartimento di Biologia Cellulare e dello Sviluppo. Università "La Sapienza", Roma, Italia.

ABSTRACT Development of the nervous system is dependent on the co-operation between cell determination events and the action of epigenetic factors; in addition to well known factors, e.g. growth factors, neurotransmitters have been assigned a role as "morphogens" and modulators of neuronal differentiation in an early developmental phase. The possible role of acetylcholine as a modulator of neuronal differentiation has been considered in two experimental systems. A neuroblastoma cell line, which does not synthesise any neurotransmitter, has been transfected with a choline acetyltransferase construct; activation of acetylcholine synthesis, thus achieved, is followed by a higher expression of neuronal specific traits. The presence in these cells of muscarinic receptors is consistent with the existence of an autocrine loop, which may be responsible for the more advanced differentiation state observed in the transfected cells. Expression of cholinergic markers appears as a common feature of DRG sensory neurons, independently of the neurotransmitter used. Choline acetyltransferase can be detected in DRG at early developmental stages. The distribution of muscarinic receptors in DRG has suggested that activation of acetylcholine synthesis may be related in an early developmental phase to the interaction between neurons and non-neuronal cells and to modulation of cell differentiation. Both systems suggest that acetylcholine may have a role as a modulator of neuronal differentiation.

KEY WORDS: *Neuronal differentiation, neurite outgrowth, neurotransmitters, acetylcholine.*

Introduction

The developmental program of the nervous system begins with the induction of the neural plate. Following this initial event, cooperation between a cell determination process and epigenetic factors occurs. As for the first, after the discovery of proneural genes in *Drosophila*, their homologues in Vertebrates, such as Mash-1 and neurogenin, have been identified; they are expressed in early proliferating neural cells and appear responsible for neural commitment. At later stages "downstream genes", as NeuroD, can be considered as responsible for the activation of a differentiation program, while homeotic genes are responsible for the definition of neural phenotypes at the regional and cellular levels (Edelman and Jones, 1998).

As far as the epigenetic factors, the role of growth factors (Calof, 1995), adhesion molecules (Cunningham, 1995) and neurotransmitters (Levitt *et al.*, 1997; Bignami *et al.*, 1997) is well known; they act on committed cells, modulating cell survival and proliferation, fibre elongation and the expression of neurospecific genes. It is interesting to mention that homeotic genes may play a role also on committed cells. In fact, it has been reported that the Antennapedia homeodomain peptide is capable of translocating across the plasma membrane, via a non-receptor mediated process (Derossi *et al.*, 1996) and promotes the expression of neurospecific genes, as neurofilament and choline acetyltransferase (Biagioni *et al.*, 2000); it thus appears to act as an inducer of neuronal cell differentiation and a paracrine mechanism has been proposed for its action (Joliot *et al.*, 1997).

A major question is whether and how these multiple external signals, so far identified, cooperate throughout development to build the nervous system in the diversity of its structures.

Neurotransmitters as "morphogens"

A large body of evidence, emerging from diverse experimental systems and approaches, indicates that neurotransmitter molecules are present in a wide variety of animal species throughout development, thus giving support to their role as signal molecules controlling various basic cellular processes. In this view, as development proceeds, neurotransmitters take up new functions, ending up in the nervous system as mediators of synaptic communication (Buznikov *et al.*, 1996).

Abbreviations used in this paper: mAChR, muscarinic acetylcholine receptors; bFGF, basic Fibroblast Growth Factor; ChAT, Choline Acetyltransferase; DRG, Dorsal Root Ganglia; GABA, γ-Aminobutyric Acid; GAD, Glutamic Acid Dehydrogenase; HACU, High Affinity Choline Uptake; IGF, Insulin Growth Factor; NMDA, N-methyl-D-aspartate.

***Address correspondence to:** Gabriella Augusti-Tocco. Dipartimento di Biologia Cellulare e dello Sviluppo. Università "La Sapienza" Roma, P.le Aldo Moro, 5, 00185 Roma, Italy. FAX: 39 06 49912351. e-mail: tocco@axcasp.caspur.it

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Monoamines (as serotonin and norepinephrine) are released by yolk granules at fertilisation in sea urchin and chick and are still present at blastula (Emanuelsson *et al.*, 1988; Buznikov, 1991); experiments using agonists and antagonists for monoamines and acetylcholine have suggested a role for neurotransmitter molecules in the regulation of cell division during cleavage, although the mechanism of their action is still unclear (Lauder, 1993). A quite different approach has provided further evidence of a possible role of muscarinic receptors in the control of cell proliferation. 3T3 cells have been transfected with individual subtypes of muscarinic receptor construct; m1, m3 and m5 transfected cells produced foci of transformation when exposed to carbachol, suggesting that, in a specific cellular context, these receptors can act as "conditional oncogenes". Transformation was not observed in m2 and m4 transfected cells, indicating that the action of acetylcholine on cell proliferation is mediated through the phosphatidylinositol cascade, which eventually leads to fluctuations in cellular Ca^{2+} level (Gutkind *et al.*, 1991). Evidence for coupling of both serotoninergic and cholinergic receptors to this cascade has been reported also in sea urchin embryos (Buznikov *et al.*, 1996).

Administration of antagonist molecules to embryos has also led to propose a role for neurotransmitters in the control of cell motility. Both monoamines and acetylcholine stimulate ciliary activity in sea urchin before hatching. As gastrulation starts serotonin may control migration of primary mesenchyme cells and later acetylcholine appears to control archenteron invagination. Monoamines are involved also in the regulation of morphogenetic movements also in vertebrate embryos, as suggested by malformations (e.g. neural tube defects in chick and craniofacial defects in rodents) caused by inhibitors of monoamine uptake or by receptor antagonists (Lauder, 1993).

As far as nervous system development, it is well known that neurotransmitter synthesis as well as neurotransmitter receptor expression are activated in an early phase of neurogenesis, before the formation of synaptic contacts, in several regions of the nervous system. Immunoreactivity for glutamate and GABA has been found in the developing cortical marginal zone (Del Rio *et al.*, 1992) and subplate cells (Chun and Shatz, 1989) respectively, both examples of transient populations in nervous system development. GABA immunoreactivity is also found in hypothalamic neurons at the beginning of hypothalamus neurogenesis (Van den Pol, 1997). As far as the cholinergic system, ChAT immunoreactivity has been demonstrated in dividing cells of mouse ventricular germinal zones (Schambra *et al.*, 1989) and in pre- or early migratory neurons of rat spinal cord (Phelps *et al.*, 1990). Muscarinic receptors have been revealed in rat central nervous system by autoradiography, using ^{3}H-methyl scopolamine as ligand, as early as day 14 of embryonic life (Schlumpf *et al.*,1991). These observations, together with data arising from *in vivo* and *in vitro* experiments, have led to propose that neurotransmitter molecules may play alternative roles in the development of nervous system as regulators of neuron differentiation, e.g. by acting on fibre outgrowth. Examples of diverse effects of neurotransmitters also in neurogenesis are known, e.g. on proliferation of neural precursor, fibre elongation and cytoarchitecture of specific structures (Lipton and Kater, 1989; Lauder, 1993).

Neurotransmitter effects on neural precursor proliferation have been extensively reviewed by Cameron *et al.* (1998) and their interaction with growth factors has been considered. In rat embryo explants cortical progenitors show a reversible decrease of proliferation rate in the presence of glutamate, mediated by non-NMDA receptors (Lo Turco *et al.*, 1995). A similar response is displayed also by O-2A cells in culture (Gallo *et al.*, 1996). NMDA receptors mediate the decrease of proliferation in dentate gyrus granule cell precursors in rat post-natal development (Gould *et al.*, 1994). Although these data have been obtained in cultured cells, recently NMDA-mediated inhibition of proliferation has been reported in rat striatal neurons *in vivo* (Sadikot *et al.*, 1998).

GABA displays the same effect as glutamate on cortical progenitor cells; however, since GABA is ineffective in the absence of bFGF, it may act as a modulator of growth factors. As a matter of fact, since bFGF induces the expression of GABA receptors, these are proposed to mediate a feedback response to regulate the final number of cortical cells (Antonopoulos *et al.*, 1997). In a similar way, the mitogenic response of cerebellar granule progenitors to IGF-I is antagonised by depolarising concentrations of KCl (Cui and Bulleit, 1998), possibly mimicking the action of a neurotransmitter. In this context it may be relevant to notice that the GABA synthesising enzyme, GAD, is expressed in the subventricular and intermediate zones and not in the ventricular zone. This complementary localisation for GAD and GABA receptors suggests that postmitotic neurons acquire the ability to synthesise GABA, which in turn down-regulates the rate of progenitor cell proliferation in the ventricular zone (Ma and Barker, 1995).

A pharmacological approach has also suggested that monoamines conversely increase cell proliferation in pre- and postnatal rat brain (Holson *et al.*, 1994; Patel and Lewis, 1988). The down-regulation of dopamine D3 receptor expression in the adult rat brain and the receptor selective localisation in the proliferative zone of neuroepithelium support a role for dopamine in early neurogenetic events (Diaz *et al.*, 1997). It has to be mentioned that noradrenaline has been

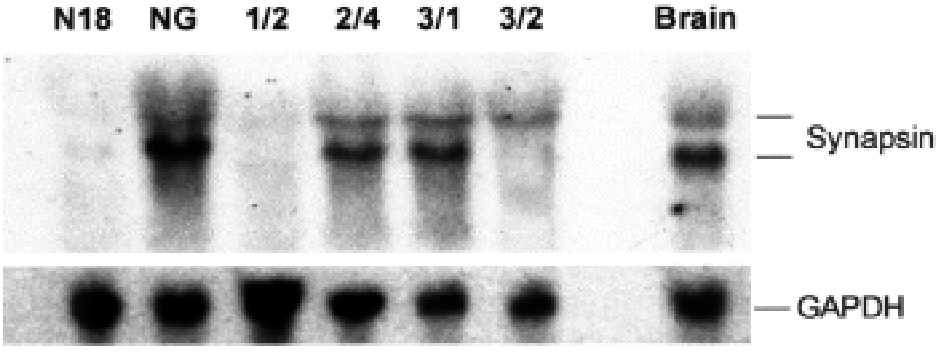

Fig. 1. Northern blot analysis of total RNA from murine brain, N18TG2 cells (N18), 108CC15 neuroblastoma-glioma hybrid cells (NG), and transfected clones of N18TG2 cells *maintained in the presence of 10^{-6} M retinoic acid for four days. Ten micrograms of total RNA from each sample was electrophoresed and hybridised with specific probes for synapsin I and GAPDH. 1/2 transfected clone of N18TG2 not expressing ChAT activity; 2/4, 3/1, 3/2 transfected clones expressing ChAT activity.*

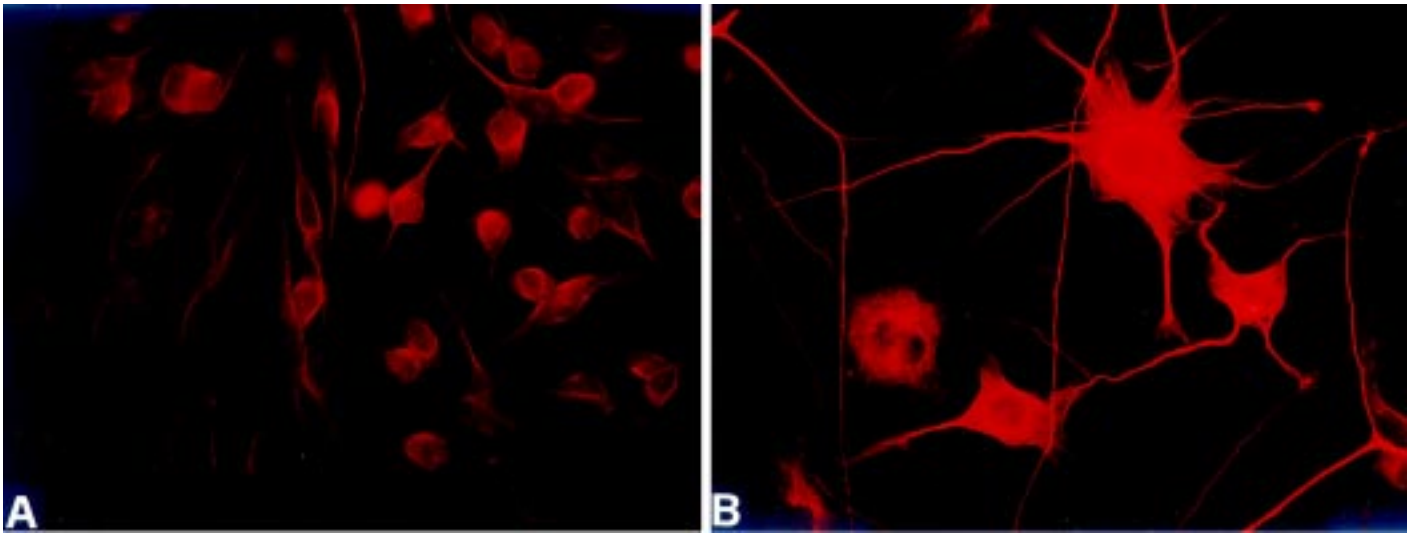

Fig. 2. Immunostaining for α–tubulin of (A) *N18TG2 cells and* **(B)** *transfected clone 2/4 expressing ChAT activity maintained in the presence of 1 mM dibutyryl-cAMP as a differentiating agent for 4 days. Magnification: 400x.*

shown to act even earlier in neurogenesis, promoting the expression of neuronal specific proteins, as N-CAM and N-tubulin, in noggin-expressing ectodermal cells (Messenger *et al.*, 1999).

A later event in neurogenesis is the formation of fibres, which is a key aspect of neuronal differentiation and is known to be regulated by growth factors (Otten, 1984; Ruitt *et al.*, 1992). Neurotransmitters come into action also as modulators of this process. Motility of growth cones is greatly reduced by dopamine in cultures of embryonic chick retina; this effect can be mimicked by forskolin, suggesting that it is receptor-mediated and operates through the cAMP intracellular signalling system (Lankford *et al.*, 1988). An inhibitory effect on fibre growth has been observed on retinal ganglion cells also for acetylcholine (Lipton *et al.*, 1988) and it has been proposed as a possible mechanism to control dendrite growth, upon reaching the appropriate target cells. A similar acetylcholine-induced retraction of growth cone has been reported for axons of Retzius neurons in leech embryos (Elsas *et al.*, 1995). On the other hand, acetylcholine counteracts the inhibitory action of serotonin on neurite elongation of B19 neuron of *Helisoma* (Mc Cobb *et al.*, 1998), showing that multiple transmitters may exert combinatorial regulation on neurite elongation as well as on electrical activity. In fact serotonin acts as an excitatory transmitter on B19 neurons, while acetylcholine elicits an inhibitory response. They have opposite effects on Ca^{2+} concentration in the growth cone as well, serotonin evoking a rapid rise and acetylcholine a decline of Ca^{2+} , which can be considered as the integrator of different (co-operative or opposite) signals regulating growth cone activity and neurite elongation (Kater *et al.*, 1988).

Regulation of fibre elongation is instrumental for building the cytoarchitecture of nervous system specific structures and allowing the establishment and stabilisation of correct functional circuits. Thus it is not surprising that different neurotransmitters have opposite effects on growth cone motility and that a single neurotransmitter may have opposite effects on different neurons. As an example, neurite extension by mouse retinal ganglion neurons in culture was inhibited by acetylcholine (Lipton *et al.*, 1988) while growth cones of Rana Pipiens dorsal root ganglia (Kuffler, 1996) or *Xenopus* spinal cord (Zheng *et al.*, 1994) neurons in culture will turn and grow up concentration gradients of this neurotransmitter. However if neurotransmitters exert such morphogenetic function in early neurogenesis, a major question is the source and mechanism of their release. As a matter of fact, a spontaneous release of low levels of acetylcholine has been detected in rat dissociated retinal cell cultures (Lipton, 1988). This finding is consistent with an acetylcholine release by amacrine cells, modulating fibre elongation of ganglion cells. Moreover neuron-glia interactions could also mediate trophic functions exerted by neurotransmitters during development. Astrocytes, for example, express a large number of neurotransmitter receptors on their surface, and they also produce trophic factors that are region-specific for ingrowing fibres (Lauder, 1993). Synthesis and release of these growth factors could be modulated by neuronal activity and by neurotransmitters. If this were the case, temporally regulated expression of glial receptors and selective responses of neurons to different growth factors could result in a highly specific control by neurotransmitters of the development of neuronal circuitry (Lauder, 1993).

This large body of evidence suggests that a single neurotransmitter may exert different actions on neural cells, once they have been committed to a specific differentiation pathway. We have focused our studies on two systems: a) neuroblastoma cell lines, as a model of cholinergic neuron differentiation and b) sensory neurons, which use a peptidergic or aminoacidergic neurotransmitter apparatus; however they also activate the expression of a cholinergic system early in development. The two systems will be reviewed in the following chapters.

Neuroblastoma differentiation

The onset of acetylcholine synthesis occurs in an early phase of motor neuron development and its possible role in directing successive steps of neuron differentiation and its interaction with target cells has been considered (Vaca, 1988). In spite of data pointing to this direction, such as the presence of ChAT immunoreactivity in pre- or early migratory neurons of rat spinal cord (Phelps *et al.*, 1990) and the ability of cultured neurons to release acetylcholine at the growth cone during neurite elongation (Lipton

and Kater, 1989), clear evidence of such role is however still lacking. Neuroblastoma cells appear as an interesting experimental system to tackle this problem. Many neuroblastoma clones have been isolated from mouse and human tumours (Denis-Donini and Augusti-Tocco, 1980; Israel and Thiele, 1994). They respond to various molecules (e.g. dibutyryl-cAMP or retinoic acid) undergoing a morphological transition with extensive formation of fibres. However they lack the ability to establish synaptic contacts. (Denis-Donini and Augusti-Tocco, 1980). In this context the properties of the neuroblastoma x glioma hybrid 108CC15 provide an interesting system to analyse the role of neurotransmitter synthesis in the progression of neuronal differentiation. In fact, the inability of the parental mouse neuroblastoma N18TG2 clone to establish synaptic contacts is overcome in the hybrid line; the acquisition of the ability to attain a more advanced state of differentiation is accompanied by a marked difference in the production of acetylcholine, which is actively synthesised in the hybrid (Hamprecht, 1977), while not detectable in the parental line (Amano *et al.*, 1972). These observations pose the question whether the inability of N18TG2 cells to progress in the neuronal developmental program is related to the block in neurotransmitter production. cDNA clones for specific neuronal markers can be produced and their use in transfection experiments allows to analyse the role of specific gene products in neuron differentiation; to reproduce in artificial systems specific events of the developmental program and possibly their sequence. Along this line a cDNA construct for ChAT gene has been transfected into N18TG2 cells and clones expressing high ChAT activity have been isolated (Bignami *et al.*, 1997); this provides an experimental system where the role of a functional neurotransmission apparatus for the progression of differentiation can be directly analysed.

Properties of neuroblastoma clones expressing high ChAT activity

In transfected N18TG2-ChAT positive clones acetylcholine was always present and its level appeared to be related to the levels of enzyme activity, although a linear relation could not be observed (Bignami *et al.*, 1997). ChAT activity was undetectable in N18TG2 cells and in neomycin resistant clones transfected with the antisense ChAT construct.

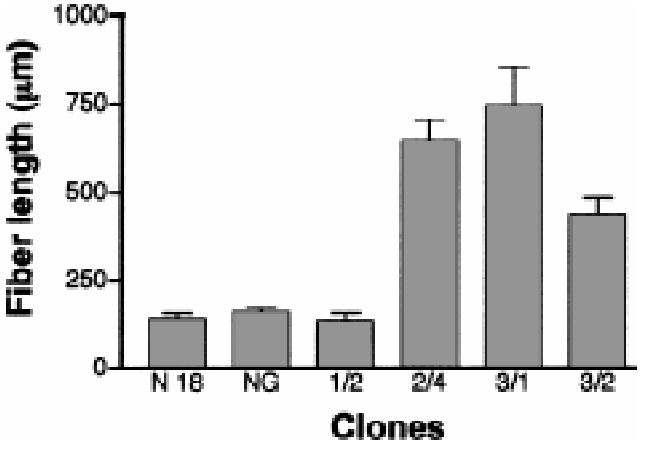

Fig. 3. Quantification of process length per microscopic field in cultures. *N18: N18TG2 cells; NG: 108CC15 hybrid cells; 1/2: transfected clone not expressing ChAT activity; 2/4, 3/1 and 3/2: transfected clones expressing ChAT activity.*

In order to determine whether the forced expression of the neurotransmitter biosynthetic enzyme in N18TG2-ChAT positive clones could modify the expression of mRNA for neuronal proteins, related to exocytosis of synaptic vesicles in the nerve terminals, the expression of synapsin I mRNA was analysed. Synapsin I mRNA was not detectable in N18TG2 cells and in the neomycin resistant clone negative for ChAT activity; on the other hand it became evident in all the ChAT positive clones (Bignami *et al.*, 1997), showing mRNA levels similar to that observed in the 108CC15 hybrid cells (Fig. 1).

N18TG2 cells, as many neuroblastoma clones, display little fibre outgrowth in basal culture condition; this can be significantly enhanced in response to various differentiating agents (Denis-Donini and Augusti-Tocco, 1980). Thus, it appeared of interest to establish whether in ChAT transfected clones, outgrowth of processes was modified and whether there was any relation between ChAT expression and morphological differentiation. Cell morphology was also analysed in the presence of retinoic acid or dibutyryl-cAMP, as differentiating agents. N18TG2 cells as well as transfected clones not expressing ChAT were characterised by an immature morphology, with cells bearing short processes. On the other hand, clones expressing ChAT activity showed a higher ability to respond to differentiating agents with the emission of a larger number of longer fibres (Bignami *et al.*, 1997); in all cases the majority of the cells extended processes which appeared as a prominent feature of the culture, both for their length and branching (Fig. 2). A morphometric analysis has shown that fibre extension per microscopic field was about 3-5 fold higher in transfected clones expressing ChAT activity as compared to those not expressing the enzyme (Fig. 3). The ability to grow fibres was very high in N18TG2-ChAT positive clones; lower values were observed for the hybrid 108CC15 clone, while ChAT-negative transfected clones showed values very near to those shown by N18TG2. As far as the presence of branching points on the elongating fibres, all the ChAT positive transfected clones showed considerably higher values as compared to N18TG2, 108CC15 cells and clones not expressing ChAT activity (Bignami *et al.*, 1997).

In conclusion stable transfection of neuroblastoma cells with ChAT gene construct is followed by the synthesis of ACh and mRNA for synapsin I, and by enhanced neuritogenesis. Thus, in the ChAT transfected clones the expression of at least some neuronal features are promoted although it remains to be demonstrated whether they are able to assemble a fully functional neurotransmitter apparatus. Binding experiments have demonstrated the presence of different levels of muscarinic acetylcholine receptors in all examined clones (Augusti-Tocco *et al.*, 1998, De Jaco *et al.*, 2000). This observation is consistent with the existence of an autocrine loop, which may be responsible for the enhancement of expression of neuron specific traits. Although further experiments are necessary to establish a causal relation between the two events (transfection and increased expression of neuron specific traits) these observations would be in line with the proposed role for neurotransmitter as one of the differentiating signals operating in the development of nervous system.

Dorsal root ganglia development

Structure and function of DRG

Dorsal root ganglia are metameric structures of peripheral nervous system; they transduce peripheral stimuli and convey their

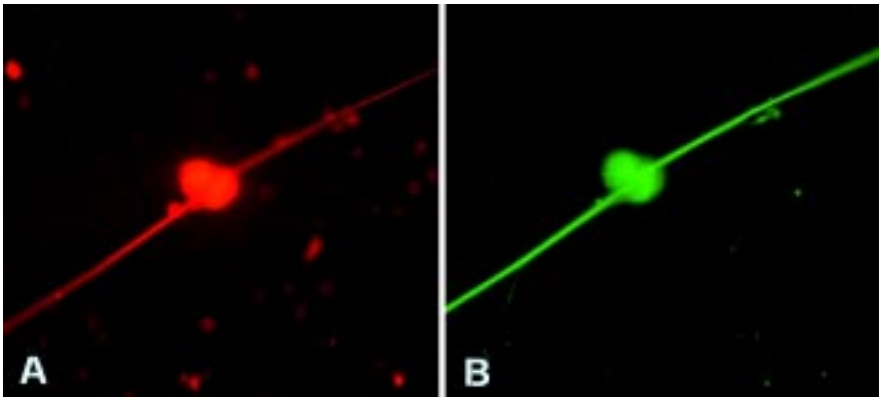

Fig. 4. Immunocytochemical localisation of (A) ChAT and (B) the 68 kDa neurofilament subunit in E7 DRG cultures. *Cultures were incubated with a rabbit polyclonal antibody against chick ChAT (obtained from Dr. Epstein, University of Wisconsin - USA) and with a mouse monoclonal antibody against the 68 kDa neurofilament subunit (Sigma) as primary antibodies and then with anti rabbit IgG TRITC and anti mouse IgG FITC as secondary antibodies. (magnification: 400x).*

signals to the central nervous system through their central fibres entering the spinal cord. They are derivatives of neural crest cells that, migrating laterally to the spinal cord, reach the rostral part of the corresponding somite and form the rudiment of sensory ganglia (Le Douarin, 1984). These will give origin to neurons and non neuronal cells (satellite, Schwann and fibroblastic cells) found in the adult ganglia (Pannese, 1974). The final number of neurons in DRG is dependent on mitotic and apoptotic events occurring during gangliogenesis. In chick embryo DRG mitotic activity starts soon after the ganglia rudiment is formed and is arrested at different times in the various cell types, which become manifest as the result of cellular differentiation. Arrest of neuron proliferation occurs between E6.5 and E7.5. (Mc Millan *et al.,*1978). Proliferation of non neuronal cells starts with some delay with respect to neurons and continues until late stages (Jacobson, 1979). As a consequence, at early stages neurons interact mainly with other neurons, while at later stages neurons are mainly surrounded by non-neuronal cells. In rat and mouse DRG development a similar sequence of proliferative events can be observed (Lawson *et al.*, 1974; Lawson and Biscoe, 1979).

Sensory neurons respond to various neurotrophic factors. Nerve growth factor has first been recognised as necessary to support development of DRG neurons. It acts as a survival and differentiation factor, promoting neurite outgrowth and showing some selectivity for one of the sensory neuron classes, the small neurons (Otten, 1984; Ruitt *et al.*, 1992). Large neurons have been shown to depend for survival on other neurotrophins, such as brain derived neurotrophic factor and neurothophin-3 (Schecterson and Bothwell,1992).

DRG neurons are known to utilise neuropeptides as neurotransmitters. The main peptides observed in sensory neurons are substance P, calcitonin gene related peptide and somatostatin, but smaller amounts of cholecystokinin, vasoactive intestinal polypeptide and galanin have also been reported (Castrignanò *et al.*, 1990; Lawson, 1992); besides, the presence of aminoacids such as glutamate, aspartate (Battaglia and Rustioni, 1998) and GABA has been observed. A peculiar character of DRG is that the majority of these neurotransmitters have been localised in small dark neurons. In fact, with the exception of aspartate, calcitonin gene related peptide and glutamate also found in some large light neurons, all others are present mainly in medium small neurons. Although the relationship between the morphological diversity and functional role of the different neuronal types has not been clarified, it has been suggested that different neurotransmitters may be involved in the transmission of different stimuli (Duce and Keen, 1977).

Presence of a cholinergic system in DRG

Choline acetyltransferase and high affinity choline uptake

The presence in DRG of ChAT, the acetylcholine biosynthetic enzyme, has been reported in more than one vertebrate species (Malatova *et al.*, 1985; Bhave and Wakade, 1988), although no direct evidence was provided of its association with sensory neurons. In chick DRG the expression of ChAT has been observed at early developmental stages (Marchisio and Consolo, 1968); however, no evidence was available of synaptic terminals on sensory neurons, responsible for a possible cholinergic input, and cholinergic terminals were not found in the spinal cord dorsal horn, where DRG neurons establish synaptic contact with their central target. Thus motor fibres running in close association to the ganglia were considered as a possible source of ChAT activity found in dissected ganglia. More recently the evaluation of ChAT activity in extracts of chick DRG freshly dissected from the lumbosacral region has shown that a low level of activity (about 15 pmoles of acetylcholine synthesised/hr/ ganglion) can be detected in DRG as early as E7. As development progresses, both total and specific ChAT activities increase and the value measured at E18 is approximately two orders of magnitude higher than that found at E7. A further increase is observed at 5 days post hatching (Tata *et al.*, 1994).

The hypothesis that the enzyme activity revealed in the isolated ganglia was due to motor fibres running in close apposition to DRG, not removed in the dissection procedure, was excluded by measuring ChAT activity on DRG neurons maintained in culture, either as explants of the intact ganglia or after dissociation into single cell suspension. ChAT activity in DRG explants and dissociated cells from different developmental stages, after 72 hr in culture, shows an expression pattern very similar to that observed in freshly dissected ganglia. Furthermore ChAT activity in dissociated cells or explants in culture showed an increase over a period of 3 days, thus ruling out the hypothesis that motor fibres, still associated to the ganglia, may represent the source of the enzyme. Over the time in culture in fact motor fibre tracts would degenerate and their eventual contribution to the enzyme activity would decrease (Tata *et al.*, 1994).

On the other hand immunostaining of whole ganglia or cultured cells (Fig. 4), revealed that ChAT immunoreactivity is associated to neurons; furthermore it is not restricted to a specific neuronal

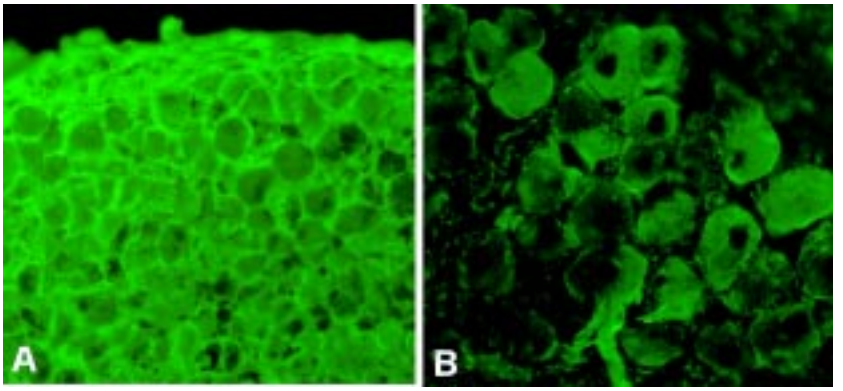

Fig. 5. Immunocytochemical localisation of mAChR *on* **(A)** *E12 and* **(B)** *E18 chick DRG cryostat sections. Sections were immunostained using the monoclonal M35 antibody (Chemunex-France) which recognises all mAChR subtypes and then incubated with a secondary antibody (anti-mouse IgM-FITC conjugated). (magnification: x400).*

subpopulation but appears as a common marker of all sensory neurons; in ganglia dissected 5 days after hatching, when development is completed, in fact, ChAT staining has spread over the whole ganglion and its intensity appears homogeneous (Tata *et al.*, 1994). Similar results were observed in rat DRG where a majority of neurons resulted ChAT positive (Sann *et al.*, 1995; Biagioni *et al.*, 1999).

Cholinergic neurons are provided with a high affinity uptake system for choline, which ensures the necessary substrate for ChAT activity; the HACU is blocked by hemicholinium and is typically present at the pre-synaptic terminals of cholinergic neurons. Its distribution in brain closely parallels that of ChAT and a functional coupling between ChAT activity and HACU have been suggested (Manaker *et al.*, 1986). Observations on cultured neurons from E7 and E18 dorsal root ganglia indicate that HACU is present in sensory neurons and that it is not a transient property of immature neurons (Tata *et al.*, 1994). In conclusion DRG neurons express the necessary proteins to synthesise acetylcholine starting at a rather early developmental stage.

Acetylcholine receptors

The coexistence of cholinergic markers with specific sensory neurotransmitters, such as neuropeptides (Castrignanò *et al.*, 1990) and glutamic acid, together with the early onset of their expression, poses the question of the possible function for acetylcholine in sensory neurons. In order to gain some insight on the role of acetylcholine in this instance it appeared of interest to look for the presence and localisation of acetylcholine receptors in DRG. Nicotinic receptors have been demonstrated on the soma of subpopulations of chick (Boyd *et al.*, 1991), amphibia (Morita and Katayama, 1989) and rat (Sucher *et al.*, 1990) sensory neurons; mAChRs have also been identified in amphibia (Morita and Katayama, 1984) rat (Wanke *et al.*, 1994; Bauer *et al.*, 1994; Bernardini *et al.*, 1999, Tata *et al.*, 1999) and chick (Tata *et al.*, 1995, 1997; Bernardini *et al.*, 1998). mAChRs appear particularly interesting, since they are capable to activate multiple signal transduction pathways, depending on the specific subtype(s) present.

The use of the non-selective mAChR radioligand [^{3}H]-quinuclidinyl benzilate allowed to establish the presence of mAChR in chick DRG (Tata *et al.*, 1995). Moreover distribution of mAChR was investigated in chick DRG during development (E12 and E18) and after hatching by light and electron microscopy, using a monoclonal antibody that recognises the acetylcholine binding site, shared by all five mAChR subtypes (Bernardini *et al.*, 1998). At E12 immunostaining is found in a large number of neurons differing in size, location in the ganglion and degree of immunolabelling (Fig. 5). Conversely, at E18 and after hatching the immunopositive neurons are characteristically located in the medio-dorsal region of the ganglion, and can be ascribed to the large class of small and medium size neurons. At the electron microscope, the immunoreaction product in sensory neurons appears as vesicle-like elements; unmyelinated nerve fibres, both in central and peripheral branches, are also immunopositive, suggesting that at least part of such receptors are transported into the fibres. Finally a large number of perineuronal satellite cells, and both myelinating and unmyelinating Schwann cells are intensely labelled (Fig. 5) (Bernardini *et al.*, 1998). The consistent presence of mAChR immunoreactivity along the soma plasma membrane at the earlier stage analysed suggests an important role for mAChRs during neuronal differentiation, when exchange of information between adjacent neurons, as well as between neurons and satellite cells, may be required. However, the declining association of mAChRs with soma plasma membrane, late in development and in adults, together with their transport along the fibres (Bernardini *et al.*, 1998) would exclude a prominent role of these receptors in mediating cellular interactions inside the ganglia, when development has been completed. A similar picture emerges from a successive study, using specific antibodies for different receptor subtypes (Bernardini *et al.*, 1999). Immunostaining as well as PCR and Northern blot analysis have shown that M_2 is the predominant subtype in rat and chick (Bernardini *et al.*, 1999; Tata *et al.*, 1999). *In situ* hybridisation studies (Tata *et al.*, 1999) in rat have also revealed that mAChRs are expressed in both neuronal and glial cells, confirming the existence of cell signalling pathways between neuronal and non-neuronal cells mediated by acetylcholine (Coles and Abbott, 1996). Analysis of the second messenger pathway operated by selective muscarinic agonists has demonstrated that both cAMP, IP_3 and Ca^{2+} signalling systems can be activated by muscarinic receptors (Tata *et al.*, 1997).

The distribution of AChRs suggests that acetylcholine in DRG may be involved in two main processes: 1) during development, in the control of both neuronal and non neuronal cell differentiation; and 2) in adults, in the modulation of the release of classical sensory neurotransmitters or in the conduction of peripheral stimuli. These two processes may be regulated via activation of different mAChR subtypes.

As far as the first process, it is relevant to recall that the existence of a reciprocal communication between neurons and glial cells is well known (Chiu and Kriegler, 1994). Glial cells synthesise and release different neuroactive substances, including neuropeptides and growth factors, involved in neuronal differentiation and survival (Martin, 1992; Coles and Abbott, 1996). Conversely, neurons control the proliferation and differentiation of glial cells (Mirsky and Jessen, 1996). In particular, the relationship between Schwann cells and axons has been extensively investigated, since their interaction is necessary for the maintenance of the axon structural and functional integrity, as well as for the

modulation of their activity (Chiu and Kriegler, 1994). The mechanisms that regulate these interactions are not well understood, although the ability of glial cells to respond to different ligands, such as glutamate, GABA, histamine and ACh has been demonstrated (Coles and Abbott, 1996, Verkhratsky and Kettenmann, 1996). In particular, applications of muscarine increase calcium levels in Schwann cells, confirming the presence of mAChRs (Jahromi *et al.*, 1992). In line with these observations, it is possible that, particularly at early developmental stages, the mAChRs expressed by both satellite and Schwann cells, are necessary to control differentiation of sensory neurons and the growth of their axons. Since multiple mAChR subtypes are expressed in DRG (Tata *et al.*, 1997; Bernardini *et al.* 1999) it is conceivable that their distribution may be specifically associated to single cell types and/or different cellular compartments, as indicated by their immunolocalisation (Bernardini *et al.*, 1999).

Conclusions

The data described in this review demonstrate that the expression of several markers, distinctive of the cholinergic phenotype, appears as a common feature of DRG in different species. The early activation of ChAT in development and the presence of measurable levels of acetylcholine, as well the presence and localisation of mAChRs suggest that activation of acetylcholine synthesis in DRG can be related to its ability to modulate neurite elongation, possibly in cooperation with neurotrophic factors. The predominant expression of M_2 receptors in DRG indicates that acetylcholine can modulate Ca^{2+} fluxes, which are known to influence growth cone motility and fibre elongation. The presence of muscarinic receptors also on non-neuronal cells, encircling neurons also indicates that acetylcholine can mediate glia/neuron interactions during development.

The role of acetylcholine as modulator of fibre outgrowth is also strongly suggested by the ChAT transfected neuroblastoma clones. This latter system is particularly interesting, since it can be subjected to exclusive experimental manipulations. Modification of culture conditions and activation or suppression of specific genes will allow to investigate in more details the action of acetylcholine in controlling terminal differentiation of different neuronal populations.

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