UNIVERSITA' DEGLI STUDI DI ROMA

"La Sapienza"

Research Doctorate in Cell and Developmental Biology
XVIII Course

Acetylcholine modulation of neuronal differentiation: involvement of transcriptional factors

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CHAPTER 1

Neurotransmitters: early signals for nervous system development?

The morphogenesis of the nervous system is dependent on a complex genetic program, responsible for the emergence of the large number of cell types present in adulthood; through the interaction and cooperation of these different cell types the coordinated function of the nervous system as an integrated structure is made possible.

Due to this complexity, neural development and differentiation occur through a number of successive steps. Neural differentiation begins with the formation of the neural tube; cell proliferation and migration then takes place, gradually leading to the emergence of cell lineages, which then originate glial cells or neurons. A specific differentiation pathway is then selected by glial or neuronal progenitors and requires the activation of other regulatory genes, acting on the final steps of differentiation.

As far as neuron differentiation is concerned, after the emergence of neuronal precursors from neural stem cells and their proliferation, differentiation eventually occurs through the activation of several genes involved in neurite elongation, production of specific adhesion molecules, assembly of a mature neurotransmitter synthesis apparatus and expression of neuronal specific features.

Other gene products, largely still unidentified, participate in the formation of mature synaptic structures. Thus, differentiation of a specific neuronal population requires the activation of a rather large set of genes; some are shared by different neuronal types (e.g. those intervening in fibre formation and possibly in the basic neurotransmitter releasing mechanism), others are type specific.

One of the major questions related to nervous system development, and more specifically to neuron differentiation, is the identification of signals directing neuronal populations to specific phenotypes (e.g. cholinergic, adrenergic or peptidergic neurones).

A number of factors are known, which can direct neuronal or glial differentiation (Calof A. 1995). Among these, neurotrophine play a major role; however the role of cell

adhesion molecules(Cunningham 1995) and neurotransmitters (Lauder 1999; Biagioni et al., 2000) has also been demonstrated.

While growth factors have long been known for their function in development, the role of cell adhesion and neurotransmitters as modulator of gene expression, relevant for the acquisition of specific phenotypes, has only recently been recognized.

A large body of evidence, emerging from diverse experimental system 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). 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, Choline Acethyltransferase (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 able to influence various cellular events, taking place during neuron differentiation.

Developing cells are affected in a specific ways along the concentration gradient of "morphogens", developmental signals, that exert specific effects on receptive cells depending on concentration.

This concept has traditionally be applied to substances involved in pattern formation and morphogenesis, such as retinoic acid. However, it may also be appropriate to consider neurotransmitters as morphogens when they act as dose dependent morphogenetic signals in neural and non-neural tissue. Neurotransmitters are known to have these actions in primitive organisms and embryos, where they exert their effects using receptors and signal transduction mechanisms similar to those characteristic of the adult nervous system. These experimental evidence raise the possibility that the highly specialized roles played by neurotransmitters in synaptic transmission may have evolved from phylogenetically old functions, many of which are recapitulated during development. Data are available showing the action of neurotransmitters on cell motility, proliferation and survival of neural cells, as well as on neuritogenesis and expression of other neuro-specific features.

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 (ACh) stimulate activity of the cilia in sea urchin before hatching. As gastrulation starts, serotonin may control migration of primary mesenchymal cells and later acetylcholine appears to control archenteron invagination. Monoamines are involved 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).

Stimulation of proliferation has been reported on various cell populations by different neurotransmitters. Large and diverse groups of growth factors and neurotransmitters, work together to regulate cell number and identity in the developing and adult brain (Cameron et al., 1998).

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); experiment 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 is still unclear (Lauder, 1993)

Serotonin action as a positive regulator of adult granule proliferation has been demonstrated by grafting raphe explant in rats, after hippocampus serotonin input had been abolished by 5,7-dihydroxytryptamine injection (Brezun et al., 2000). Proliferation of rat cortical neural precursors can be enhanced by activation of muscarinic receptors (Ma et al., 2000). 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 oncogen". Transformation was not observed in m2 and m4 transfected cells, indicating that the action of acethylcoline on cell proliferation is mediated through the phosphatidylinositol cascade, which eventually leads to fluctuations in cellular Ca^{2+} level (Gutking at 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).

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). NMDA receptors mediate the decrease of proliferation in dentate gyrus granule cell precursors in rat post-natal development (Gould et al., 1994). Recently NMDA-mediated inhibition of proliferation has been reported *in vivo* in rat striatal neurons (Sadikot et al., 1998). Systemic injection of NMDA and AMPA receptor antagonist in adult gerbils increased BrdU labelled cells in the dentate subgranular zone (Bernabeu et al., 2000).

Some neuropeptide promote neuronal proliferation in the olfactory epithelium (Hansel et al., 2001). Conversely in cortical epithelium GABA receptor activation depolarises progenitors cell and elevates their cytosolic Ca^{2+} level, resulting in a decreased proliferation; on the other hand GABA stimulates progenitor cell motility via Ca^{2+} signalling (Barker et al., 1998). Pharmacology approaches have also suggested that monoamines increase cell proliferation in pre- and postnatal rat brain (Holson et al., 1994; Patel and Lewis, 1998). Down regulation of dopamine D3 receptors 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).

The role of neurotransmitter release on neuron survival has also been shown in mice by deletion of Munc 18-1, a neuron specific protein of the SEC 1 family involved in membrane trafficking, which leads to loss of neurotransmitter release from synaptic vesicles. The ensuing abolishment of synaptic activity during development allows normal assembly of brain structures, but impairs neuronal survival during successive developmental stages (Vehrage et al., 2000)

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, 1994; 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).

Glutamate reduces dendrite formation in embryonic mouse cortical neurons and the effect is mediated by NMDA receptors (Esquenazi et al., 2002). In developing motor

neurones dendrite arborization is dependent on glutamate receptor activation (Inglis et al., 1998); furthermore it has been shown that dendrite architecture can be remodeled, when a glutamate receptor subunit highly expressed in developing neurons is reintroduced in mature motor neurones (Inglis et al., 2002). Xenopus retinal ganglion cells express GABA receptors on their axons and growth cones; baclofen, a GABA agonist, has been observed to stimulate neurite outgrowth in cultured cells, while a GABA antagonist in vivo reduces the length of optic projections (Ferguson et al., 2002). Regulation of fibre elongation is instrumental for building the cytoarchitecture of nervous system 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 neurons in culture (Zheng et al., 1994) will turn and grow up concentration gradients of this neurotransmitter (Fig.1).

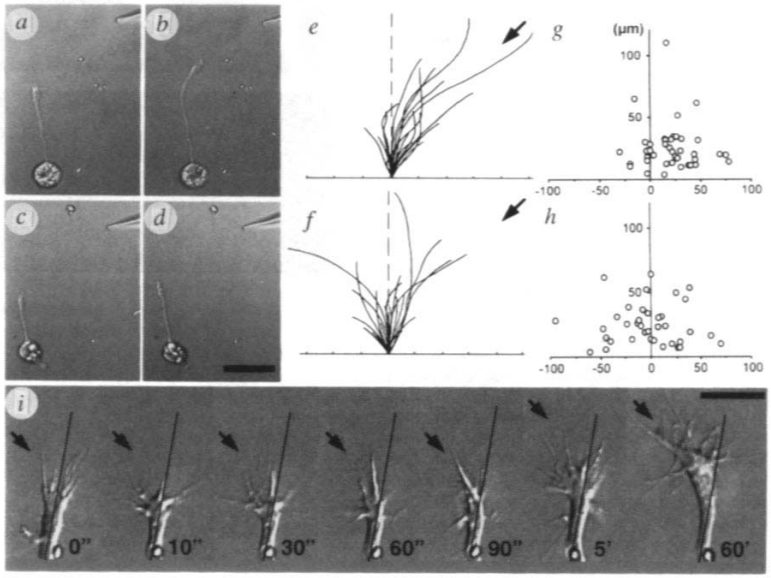

Fig.1 : Turning response of Xenopus spinal neurons in the presence of acetylcholine gradient in the absence (a,b) and presence (c,d) of d-tubocurarine. Composite drawings of the path of neurite extension for a population of neurons in the absence (e) and presence (f) of d-tubocurarine. Scatter plots of all data (g, h) corresponding to experiments illustrated in a-f. Images of a growth cone (i)subjected to the acetylcholine gradient before and at various times after the onset of the acetylcholine gradient (Zeng et al.,1994).

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 fibers (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). In this respect it is pertinent to mention that via stimulation of their receptors, both neurotransmitters and growth factors activate second messenger systems, such as cAMP, PLC-β, Ca^{2+} and βγ subunits of heterotrimeric G proteins. Utilization of common signalling pathways may provide a mechanism for interaction between neurotransmitters and growth factors (Weiss et al., 1998).

Finally as far as regulation of neurospecific gene products, noradrenaline has been shown to act early in neurogenesis, promoting the expression of proteins, as N-CAM and N-tubulin, in noggin-expressing ectodermal cells (Messenger et al., 1999). A number of data have also been obtained at later developmental stages. In slice cultures of olfactory epithelium expressing D2 receptors, dopamine enhances the expression of β-galactosidase driven by the promoter of the olfactory marker protein (OMP), a marker of mature olfactory neurones, in a dose dependent manner directly acting on epithelial cells (Feron et al., 1999). Retinal cultures of 3 day rats treated with 20 μM glutamate showed higher neuronal survival and differentiation, as judged by the higher number of MAP2 positive neurones (Govindaiah et al., 2002). Glutamate and GABA stimulate the expression of BDNF and NGF in primary cultures of hyppocampal neurones (Zafra et al., 1991); the stimulation is dependent on Ca^{2+} influx and is enhanced by activation of adenylate cyclase (Zafra et al., 1992). A similar effect is elicited by serotonin in a

glioma cell line (Meller et al., 2002). The ability of neurotransmitters to act on glial cells, stimulating growth factor expression is in favour of their role as overall regulator of the developmental program.

This large body of evidence suggests that a single neurotransmitter may exert different action on neural cells, once they have been committed to a specific differentiation pathway.

CHAPTER 2

Choline Acetyltransferase-transfected neuroblastoma clones

The presence of both ChAT and acetylcholine receptors during early embryogenesis (Role and Berg 1996) strongly suggests a role for acetylcholine during neuronal development. Several studies have shown that acetylcholine may regulate different aspects of nervous system morphogenesis at least in vitro (Zheng et al., 1994; McCobb et al., 1988; Lauder, 1993).

Mouse neuroblastoma N18TG2 cell line appears as an interesting experimental system to study ACh morphogenetic action on neuronal differentiation. They are defective for neurotransmitter production but respond to various molecules (e.g. dibutyryl-cAMP or retinoic acid) undergoing a morphological transition with formation of fibers. However they lack the ability to establish synaptic contacts. (Denis-Donini and Augusti-Tocco, 1980) and to express neurospecific trait.

In this context it's interesting to remind the properties of neuroblastoma x glioma hybrid 108CC15, 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 synthesized 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.

A construct containing the cDNA for choline acetyltransferase (ChAT), the biosynthetic enzyme for ACh, has been transfected into N18TG2 cells and its FB5 subclone, isolated to reduce the intrinsic heterogeneity of neuroblastoma lines. Clones expressing high ChAT activity have then isolated (Bignami et al., 1997); thus providing an experimental system where the role of a functional neurotransmission apparatus for the progression of differentiation can be directly analyzed.

Properties of neuroblastoma clones expressing high ChAT activity

Isolated clones expresses different ChAT levels (Fig.2A); in particular, as expected, ChAT activity was undetectable in N18TG2 cells and in its FB5 subclone, while the enzyme activity levels in the transfected clones, appeared similar or higher to that observed in the hybrid neuroblastoma-glioma 108CC15 cholinergic cell line. Differences in enzyme levels observed in the transfected clones most likely can be ascribed to a variable number of integrated constructs. Neomycin resistant clones isolated after transfection with a construct containing an antisense ChAT cDNA or with the empty vector (aCS5 and 1/2 clones respectively) do not express ChAT activity, like N18TG2 and FB5 cells (Bignami et al., 1997; De Jaco et al., 2000).

A

	ChAT Activity (nmol ACh synthetized/mg prot/min)
mouse brain	1.10±0.18
108CC15	0.21±0.01
N18TG2	n.d.
FB5	n.d.
1/2 clone	n.d.
aCS5 clone	n.d.
CS37 clone	0.23±0.04
CS42 clone	0.19±0.02
CS43 clone	0.37±0.07
2/4 clone	18.86±1.78
3/1 clone	2.27±0.47
3/2 clone	2.04±0.24

B

CLONES	*CHOLINE*	*ACh*
N18TG2	1.2 ± 0.16	nd*
2/4 clone	3.8 ± 1.03	4.81 ± 0.16
3/2 clone	1.8 ± 0.44	0.75 ± 0.10
3/3 clone	0.4 ± 0.08	0.04 ± 0.01
108CC15	25.1 ± 5.18	0.89 ± 0.22

Fig.2 . **(A)** ChAT specific activity measured in extracts of mouse brain, 108CC15 neuroblastoma-glioma hybrid cells, N18TG2 cells and in ChAT transfected N18TG2 clones. Data are expressed as mean ± SEM of at least six independent experiments run at least on triplicate cultures. **(B)**. Intracellular choline and acetylcholine levels (nmol/mg of protein) of N18TG2 cells, ChAT transfected N18TG2 clones and 108CC15 neuroblastoma-glioma hybrid cells. Values are the mean ± S.E.M. of at least five independent experiments. (* = not detectable) (Bignami et al., 1997; De Jaco et al., 2000).

In transfected N18TG2-ChAT positive clones acetylcholine was always present (Fig.2B) 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).

N18TG2 cells, as well as transfected clones not expressing ChAT, were characterised by an immature morphology, with cells bearing short processes and its ability to extend fibers remains rather poor also in the presence of differentiation agents, as retinoic acid. On the other hand, clones expressing ChAT activity (2/4, 3/1 and 3/2) display higher fiber outgrowth as compared to clone 1/2 both in the absence or presence of differentiating agents (Fig.3A-B). (Bignami et al., 1997). A morphometric analysis has shown that fiber extension was about 3-5 fold higher in transfected clones expressing ChAT activity as compared to those not expressing the enzyme (Fig. 3).

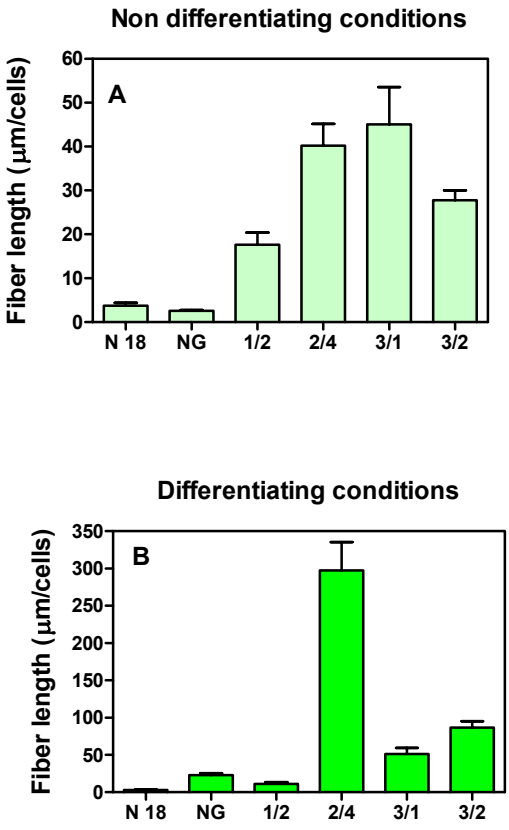

Fig. 3. Quantification of process length, normalised to cell number per microscopic field in cultures maintained in non differentiating conditions (**A**), and in the presence of 10^{-6} M retinoic acid for four days (**B)** (Bignami et al., 1997).

Moreover, the activation of ACh synthesis modified the expression of several neuronal markers; in particular increased levels of high affinity choline uptake and voltage-gated Na^+ channels were observed, while secreted AChE activity levels were reduced according to a progression in the cholinergic differentiation program (De Jaco et al., 2000). Interestingly in ChAT-transfected clones the activation of synapsin I mRNA expression, not detectable in the parental line, was also observed (Fig.4) (Bignami et al., 1997). Synapsin I belongs to a family of neuron-specific phosphoproteins and plays a key role in neurotransmitter release (Greengard et al., 1993). Synapsin I is also believed to play a role in synapse formation; the injection of synapsin I in Xenopus blastomeres accelerates synapse formation (Lu et al., 1992; Valtorta et al., 1995) while its suppression in transgenic mice results in the inhibition of synaptogenesis (Chin et al., 1995). Moreover, cultured hippocampal neurons, dissected from synapsin I-deficient mice, develop shorter and less branched axons than those observed in wild type cells (Chin et al., 1995) suggesting that synapsin I may play a major role in axonal elongation and branching.

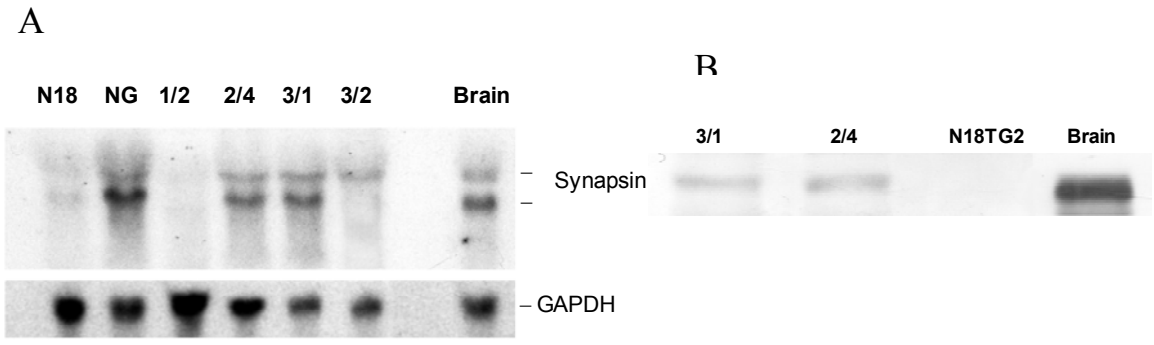

Fig.4. **A: Northern blot analysis** of total RNA from murine brain, NG108CC15, N18TG2 cells, 1/2 transfected clone not expressing ChAT activity and 2/4, 3/1, 3/2 transfected clones expressing ChAT activity. Ten micrograms of total RNA from each sample was electrophoresed and hybridised with specific probes for synapsin I and GAPDH. **B: Western Blot** analysis showing synapsin I levels in the different clones. Samples obtained from the same number of cells were loaded onto SDS-PAGE slabs (Bignami et al., 1997).

It is known that fiber elongation and other neuronal properties can be modulate by muscarinic acetylcholine receptors (mAChR) activation which induce a number of cellular responses, being coupled to several G proteins (Hulme et al., 1990; Richards,

1991). It is also known that promoters of neuronal proteins contain consensus sequences for various transcription factors among which the zinc-finger EGR-1 protein (Beckmann and Wilce, 1997; Herdegen and Leah, 1998) and that EGR family expression is increased by mAChR activation in different neuronal cells (Ebihara and Saffen, 1997; Nitsch et al., 1998; Von der Kammer et al., 1998).

QNB binding studies on homogenate of the neuroblastoma clones revealed the presence of muscarinic acetylcholine receptors in all examined clones (Fig.5) although no significant correlation between binding and ChAT activity was observed. Moreover although ChAT-positive clones showed different ability to accumulate ACh QNB binding appeared not related to the levels of ACh (De Jaco et al., 2000; De Jaco et al., 2002).

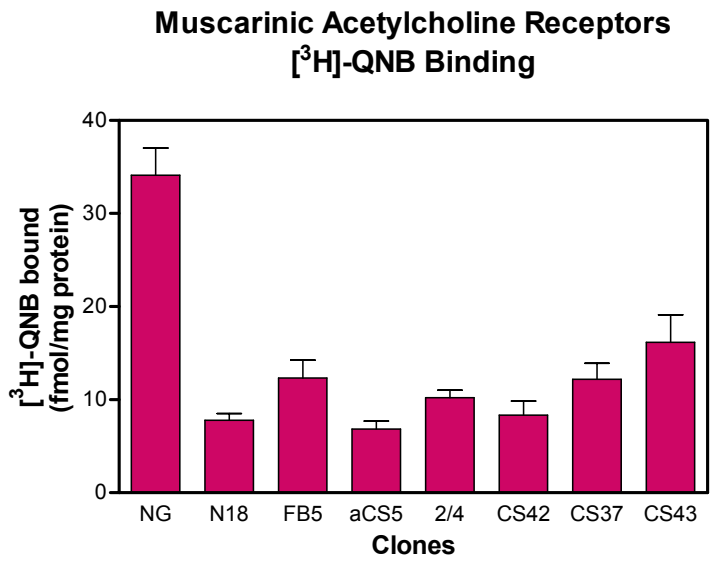

Fig.5. Presence of muscarinic acetylcholine receptors in ChAT transfected clones. [^{3}H]-QNB binding was evaluated on homogenates of 108CC15 (NG) hybrid cells, FB5 non transfected cells, CS37, CS42, CS43 ChAT positive clones and the aCS5 clone transfected with a construct containing the antisense ChAT gene. Values are the mean ± SEM of at least twelve observations (De Jaco et al., 2000; De Jaco et al., 2002).

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It has been reported that *Egr* gene family expression is increased by muscarinic receptor also in NG108CC15 cells (Katayama et al., 1993) and modulation of Egr-1 mRNA expression and EGR-1 protein synthesis was observed in cells expressing all the different muscarinic subtype receptors (Von der Kammer et al., 1998). As reported in

Fig 5, muscarinic receptors are present in the studied clones; it is thus possible to propose that in the ChAT positive clones an autocrine loop becomes active, leading to the expression of synapsin I and regulation of fibers outgrowth, through the activation of *Egr* genes.

As shown in Fig.6 EGR-1 protein was detectable only in the omogenates of the transfected clones (De Jaco et al.,2002).

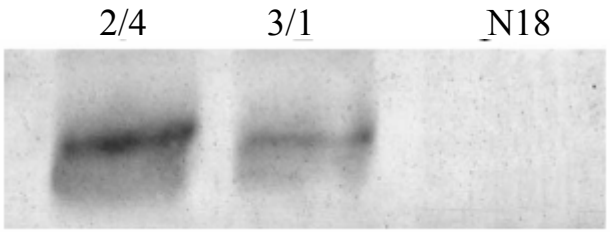


Fig. 6. Immunoblot analysis showing EGR-1 levels in different clones: clone 2/4 , clone 3/1 , N18TG2 parental clone. Cultured cells were collected in PBS, counted and then treated with solubilizing buffer. Samples obtained from the same number of cells were loaded onto SDS-PAGE slabs (De Jaco et al.,2002)..

The data summarized above support the hypothesis that the forced expression of ChAT in neurotransmitter inactive neuroblastoma cells brings about the modulation of neuron specific trait expression, and to provide evidence for the existence of a direct modulation of fiber outgrowth and neuronal marker expression by muscarinic receptor activation, which may be related to EGR-1 levels. It thus appear of interest to observe that in N18TG2 and in ChAT-transfected clones, muscarinic agonist/antagonist could modulate neurite outgrowth. Figure 7 shows the results obtained when N18TG2 cells and 2/4 ChAT-transfected clone were cultured in the presence of 10^{-6} M atropine, a mAChR antagonist. Atropine treatment reduced the average fiber length normalized to cell number only in ChAT-positive clone ($P<0.001$). Moreover the non-hydrolyzable cholinergic agonist carbachol (CCh) induced higher neurite outgrowth ($P<0.001$) in the parental non transfected N18TG2 neuroblastoma line with respect to untreated cultures; the effect of CCh was inhibited by the muscarinic receptor antagonist atropine, demonstrating that the effect of ACh on neurite outgrowth was mediated by muscarinic receptors (De Jaco et al.2002).

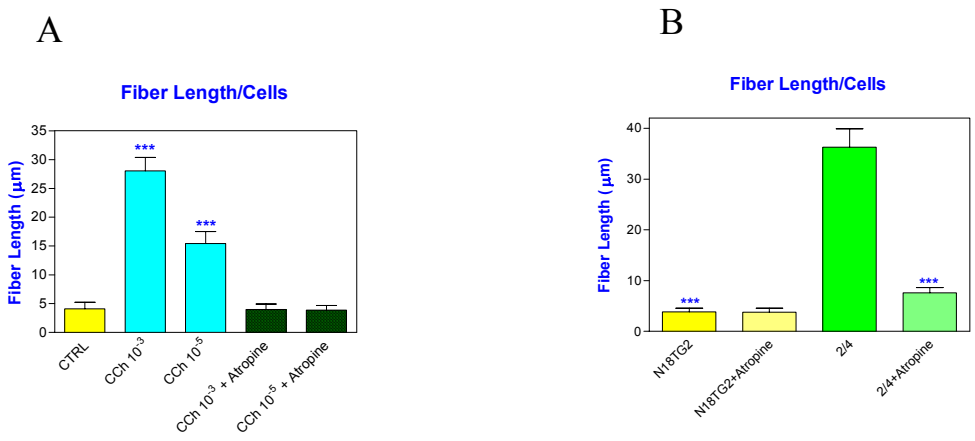


Figure 7. A Quantification of fiber outgrowth, per microscopic field in N18TG2 cultures maintained, for five days, in the presence of 10^{-3}-10^{-5} M carbachol and normalized to cell number present in each microscopic field . Carbachol induced higher neurite outgrowth which is abolished in the presence of 10^{-6} M atropine. *** P<0.001 (Mann-Whitney test). significant difference with respect to N18TG2 cultures maintained in the absence of carbachol. Micrographs of randomly selected areas of the culture dishes were taken from five independent 60-mm dishes (22 fields/ dish); values are the mean ±S.E.M. of about 110 observations for each culture condition.CTRL control condition (De Jaco et al.2002). **B**: **:** Quantification of fiber outgrowth per microscopic field in N18TG2 cultures maintained in the presence of 10^{-6} M atropine for five days and normalized to cell number present in each microscopic field. *** P<0.001 (Mann-Whitney test). significant difference with respect to 2/4 clone cultures maintained in the absence of atropine.

To evaluate the activity of transcription factors elicited by the endogenous ACh or by treatment with muscarinic agonist N18TG2 and ChAT-positive clones were transiently transfected (De Jaco et al., 2002) with the the pSyCAT-10 plasmid containing the Chloramphenicol acetyltransferase (CAT) reporter gene under the control of the human synapsin I promoter (Jungling et al., 1994). The promoter activity was about 4 fold higher in transfected clones with respect to the parental line and atropine treatment reduced its force (De Jaco et al., 2002). Furthermore the nicotinic receptor antagonist mecamylamine resulted ineffective, indicating a regulation of the synapsin I promoter mediated by muscarinic receptor activation. Moreover, as shown in Fig.8 in parental N18TG2 cells, transiently transfected with synapsin I-CAT construct, the reporter gene expression was dose dependently induced by CCh and this effect was abolished by atropine, while mecamylamine resulted ineffective (De Jaco et al., 2002).

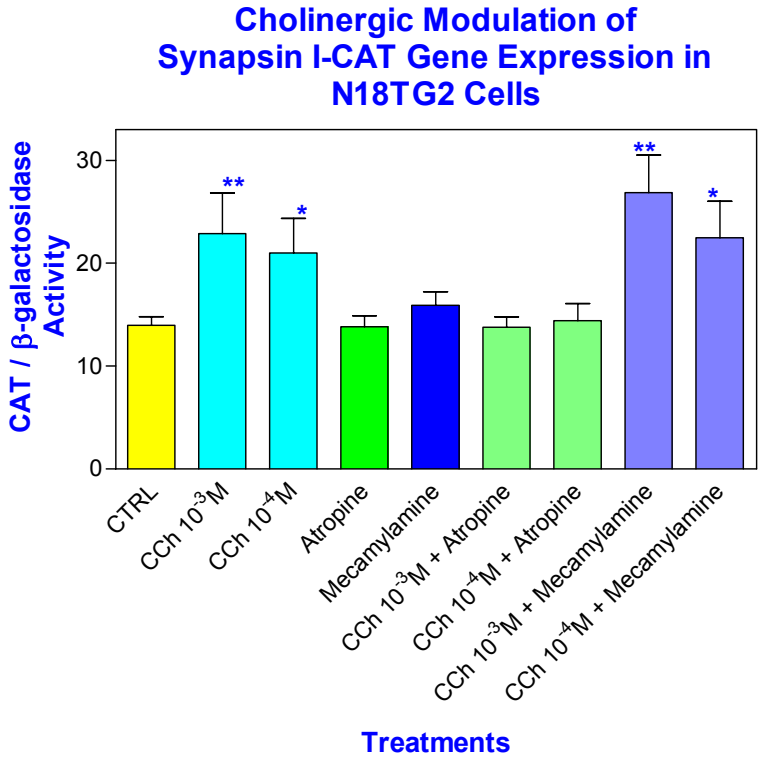


Fig.8. Muscarinic induction of synapsin I gene promoter activity in N18TG2 cells. All transfections were normalized for variations in transfection efficiency, cotrasfecting the pSyCAT-10 plasmid with a plasmid containing the β-galactosidase gene under the control of a constitutive promoter. Normalization was achieved by dividing CAT activity by β-galactosidase activity. Atropine and mecamylamine were used at 10^{-6} M final concentration. Values are the mean ± SEM of at least twelve observations. (* = P<0.05; ** = P<0.01) (De Jaco et al., 2002).

Recently a gene encoding a zinc-finger protein functioning as a master regulator of the neuronal phenotype was identified. The protein: RE1 silencing transcription factor (REST/NRSF), being expressed in non-neuronal cells but not in neurons, negatively regulates the expression of neuronal type II voltage-gated Na^+ channels (Chong et al., 1995), synapsin I (Schoch et al., 1996) and other neuron-specific proteins. Moreover

REST mRNA is expressed in yet undifferentiated neural precursors, suggesting that it prevents the precocious expression of specific neuronal proteins during neurogenesis (Schoenherr et al., 1995). Therefore the observed increase in the level of voltage-gated Na^+ channels (De Jaco et al., 2000) and synapsin I (Bignami et al., 1997; De Jaco et al., 2002) in ChAT transfected clones may be also due to a decrease in the expression of REST consequent to the progression in the neuronal developmental program observed in ChAT transfected clones. It was demonstred by RT-PCR (Fig.9).

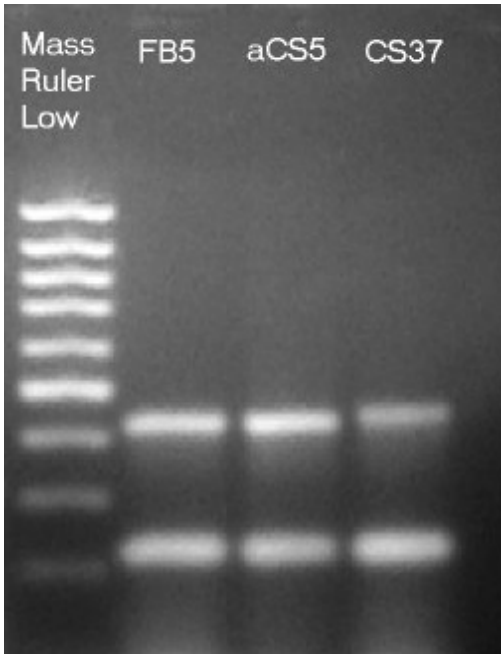


Fig.9 **A**: RT-PCR analysis of REST and L19 (ribosomial protein as positive control) expression in FB5 clone, in aCS5 ChAT-negative transfected clone, and in CS37 positive transfected clone. **B** : Densitometric analysis. The REST expression value normalized to L19, show a significant decrease in ChAT-transfected clone (Biagioni, unpublished data)

CHAPTER 3

RESEARCH AIM

The data reported above are consistent with the hypothesis that the forced expression of ChAT in neurotransmitter-inactive neuroblastoma cells trigger the activation of an autocrine loop via muscarinic receptors, which is responsible for the modulation of neuron-specific trait expression, probably through the modulation of transcription factor expression like EGR-1 or REST (Fig.10)

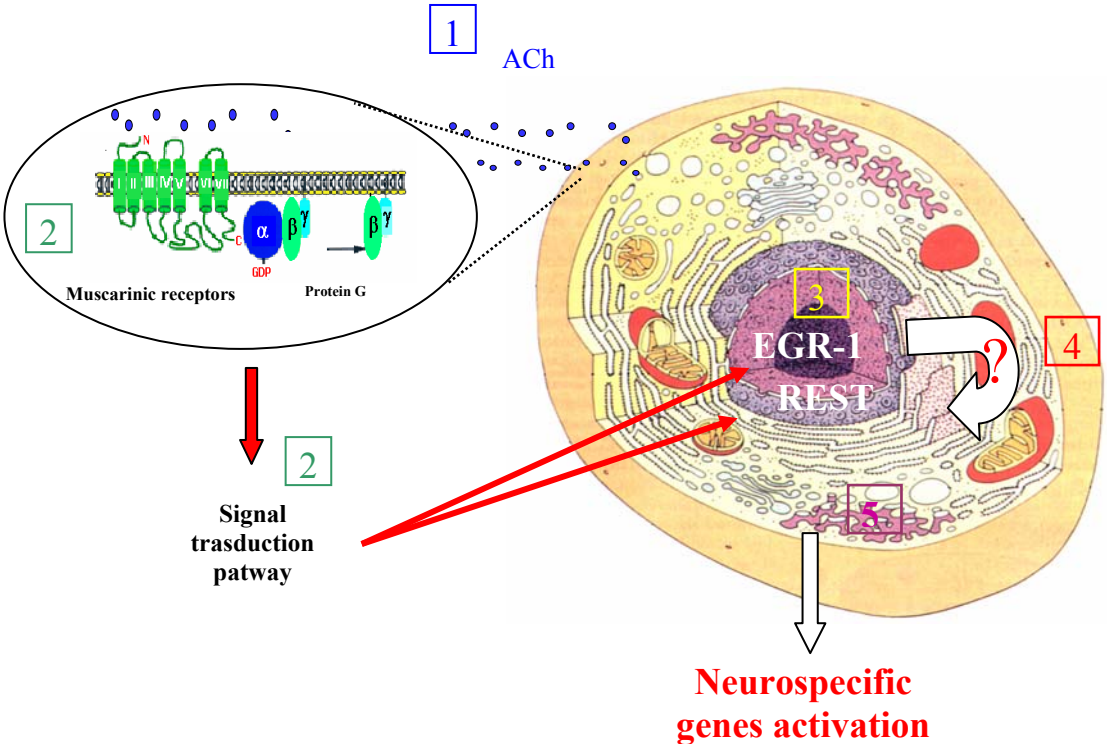


Fig.10 Model explaining neuronal features observed in ChAT-transfected clones. The forced expression of ChAT brings about the activation of an autocrine loop via muscarinic receptors, which is responsible for the modulation of neuron-specific trait expression, probably through modulation of transcription factors expression like EGR-1 or REST

The experimental work presented in this thesis was to analyze the molecular mechanism of ACh action during differentiation of neuroblastoma ChAT-transfected clones and to study, the modulation of EGR-1 and REST expression to verify the correlation between muscarinic ACh receptors activation and neuronal feature expression. Moreover, it is also interesting to analyze the possible interaction between EGR-1, which is a positive transcriptional modulator and REST, which is a transcription repressor.

Principal points dealed in this thesis:

1. Demonstration and characterization of ACh release from ChAT transfected clones to prove the hypothesis of autocrine loop existence (Fig.11).

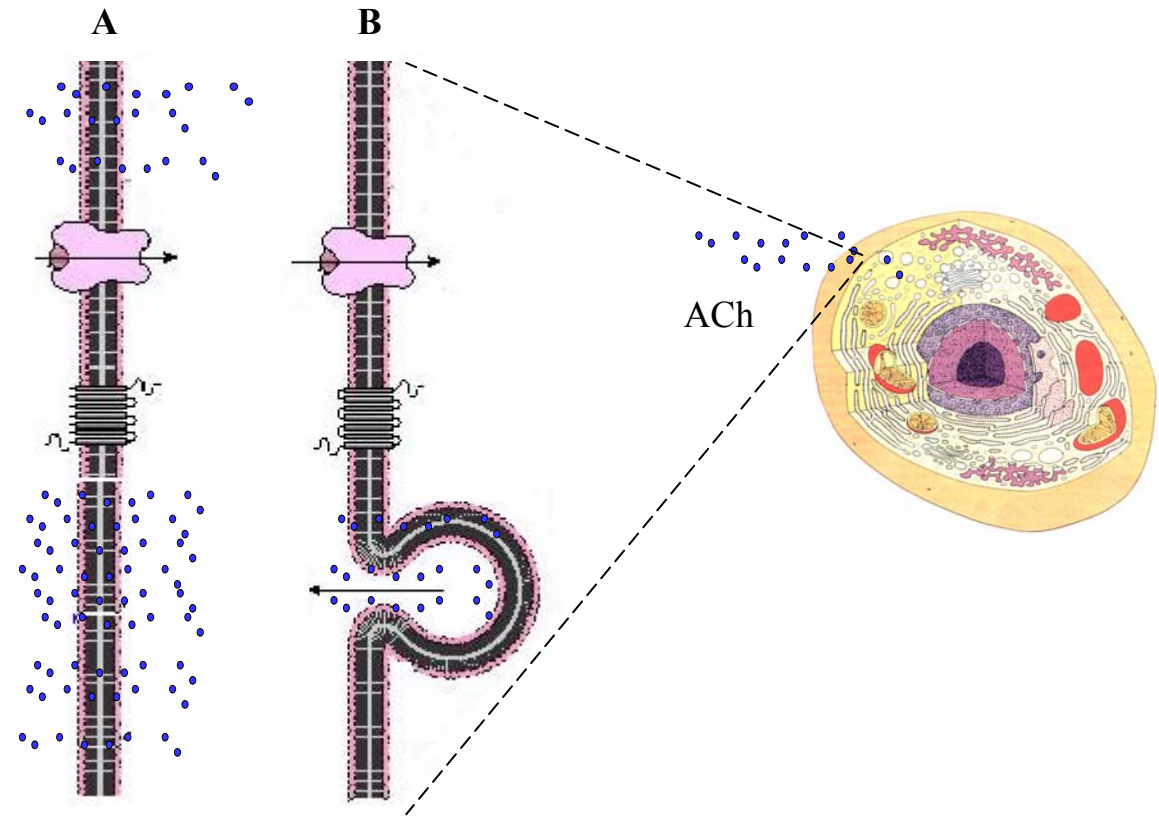


Fig.11 ACh release from ChAT-transfected clone. The release could be vesicle mediated (B) or/and also constitutive trough the membrane(A).

2. Identification and characterization of muscarinic receptor subtypes expressed in ChAT transfected clones and evaluation of their activity (Fig.12) to asses their ability to transduce ACh signal inside the cell.

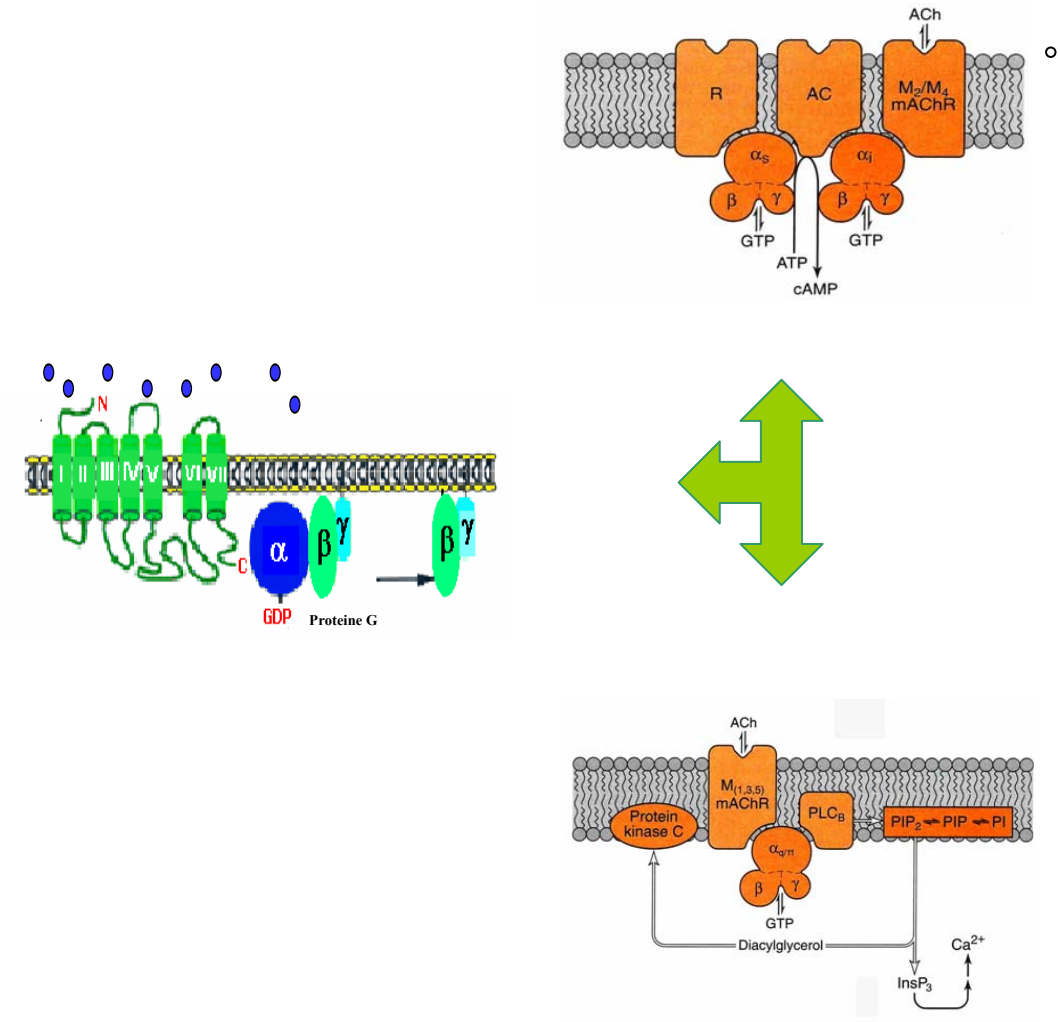


Fig.12 Muscarinic receptor subtypes. The M_2 and M_4 mAChR interact with the α– subunit of GTP-binding protein, G_i, known to inhibit adenylyl cyclase (AC). The M_1, M_3 and M_5 mAChR interact with GTP-binding proteins of the Gq family which activates phospholipase C (PLC) and modulate K+ channel conductance.

3. Study of Egr-1 expression mediated by muscarinic receptor stimulation. Stable transfection of neuroblastoma N18 with EGR-1 to analyze the transcriptional factor role in neuroblastoma differentiation (Fig.13).

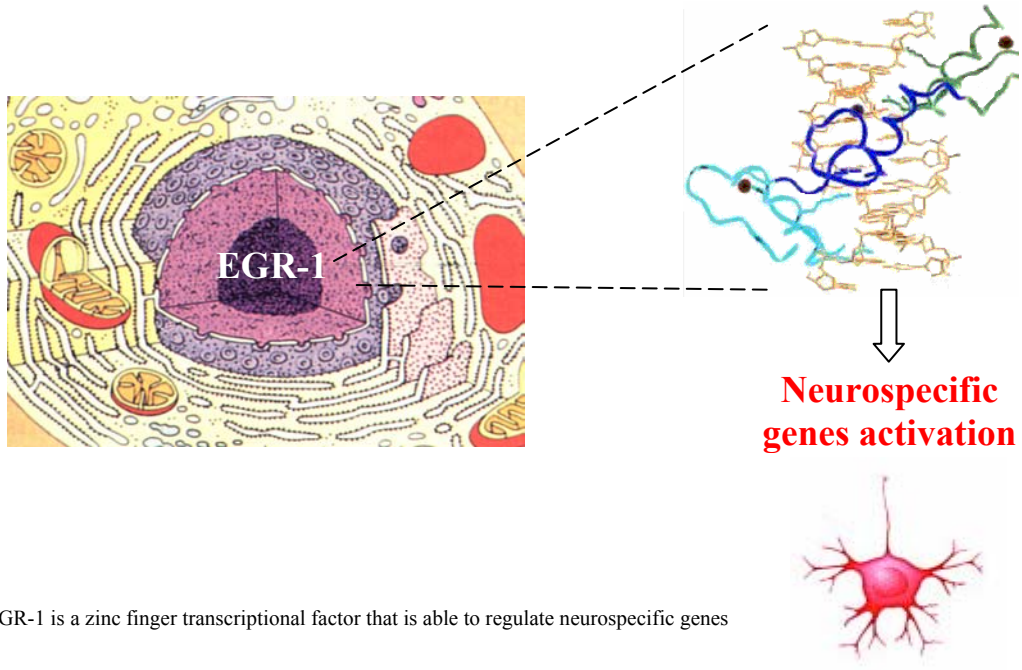


Fig.13 EGR-1 is a zinc finger transcriptional factor that is able to regulate neurospecific genes

4. Analysis of REST expression in EGR-1 transfected clone. Possible interaction between EGR-1 and REST expression (Fig.14).

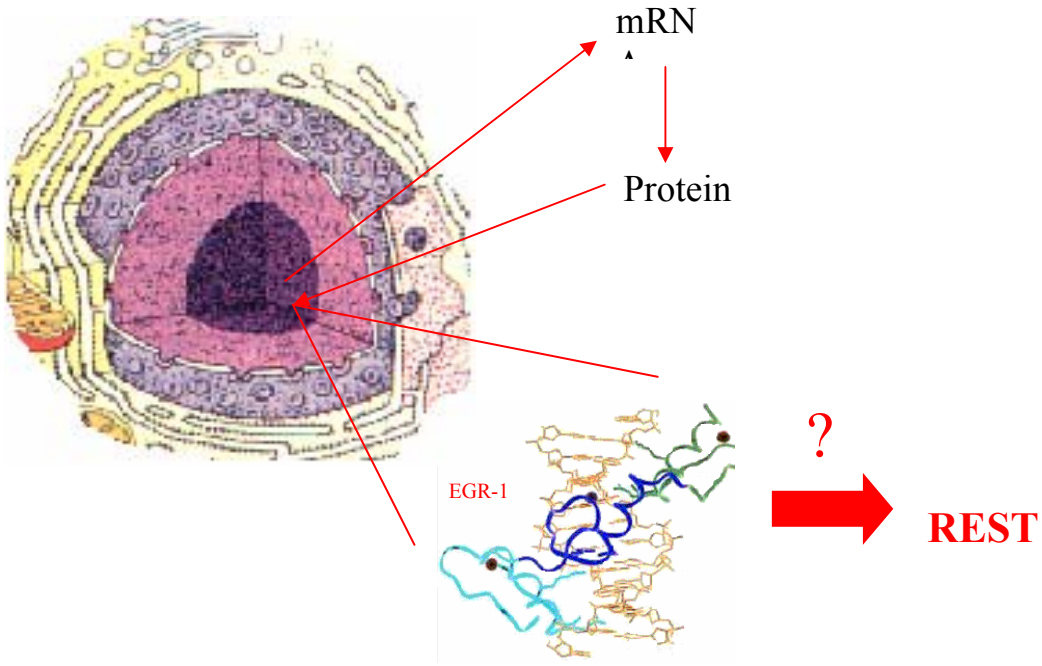


Fig.14 Possible existence of a relationship between EGR-1 and REST expression

5. Knowing that ChAT and EGR-1 transfected clones show an higher ability to growth fiber I analyzed the expression of metalloproteases. Matrix metalloproteases have in fact been recently implicated in the formation of neural connections and in axon elongation during the development of the nervous system (Fig.15).

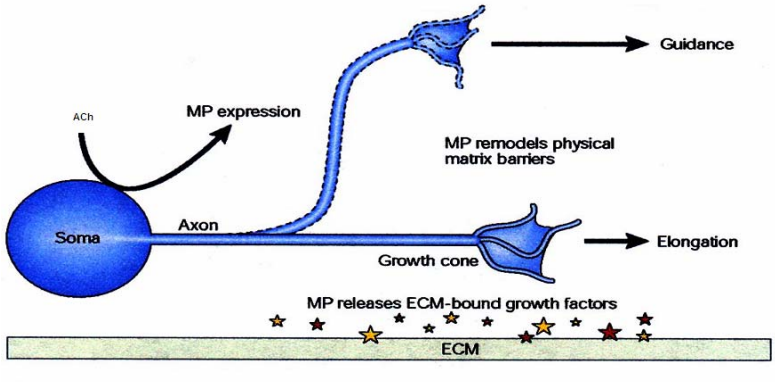


Fig.15 Role of metalloproteases in axon elongation

CHAPTER 4

ACh release

Neurotransmitter functions during early development are independent from the establishment synapses or synaptic activity, and involve processes such as cell proliferation, differentiation, migration, axon outgrowth, and axon branching (Chapter 1). In these contest, neurotransmitters are apparently released by mechanisms that are distinct from the conventional synaptic vesicular pathway (Nguyen et al., 2001; Owens et al., 2002).

Vesicular synaptic pathway is the best understood example of regulated secretion. Accumulation of neurotransmitters in synaptic vesicles at the nerve terminal is mediated by specific transporters. The fusion of synaptic vesicles with the plasma membrane at the nerve terminal is triggered by the rapid elevation of cytoplasmic Ca^{2+} during an action potential (Augustine et al., 1987; Bennett, 1997). The targeting of synaptic vesicles to the release sites and the tight excitation-secretion coupling observed at the nerve terminal are mediated by proteins specific of synaptic vesicles (Sudhof, 1995; Calacos and Scheller, 1996; Hanson et al., 1997).

A possible exception to this generally accepted model of neurotransmitter release is may represented by an integral plasmalemmal protein which can support the formation of ACh quanta. Such a protein has been isolated, characterised and called mediatophore. Mediatophore has been localized at the active zones of presynaptic nerve terminals. It is able to release ACh with the expected Ca^{2+} -dependency and quantal character, as demonstrated using mediatophore-transfected cells and other reconstituted system. Mediatophore is believed to work like a pore protein, the regulation of which is in turn likely to be dependent on the usual vesicle docking apparatus (Dunant and Israel, 2000; Falk-Vairant et al., 1996)

The constitutive (or non-regulated) secretory pathway operates in all cells and is responsible for recycling of plasma membrane components and for secretion of molecules into the extracellular environment. In contrast to synaptic vesicle exocytosis, constitutive vesicular exocytosis occurs at resting Ca^{2+} levels. Despite different sensitivities to Ca^{2+}, the pathways of synaptic vesicles at the nerve terminal and of endosomal membranes in non neuronal cells are mechanistically similar. Both are local

and do not depend on the Golgi apparatus. Retrieval of synaptic vesicles after neurotransmitter release is believed to occur through the formation of clathrin-coated vesicles, followed by their uncoating, fusion with endosomes, and sorting of synaptic vesicle proteins during budding from endosomes (Calacos and Scheller, 1996). Although the term "constitutive" implies the constant flux of secretory products to the plasma membrane, emerging evidence suggests that various trafficking steps involved in the constitutive secretion pathway may be regulated by calcium (Buys et al., 1984; Beckers and Balch, 1989; Dan and Poo, 1992; Steinhardt et al., 1994; Coorsen et al., 1996; Rodriguez et al., 1997). Moreover, molecular characterization of the secretion machinery components in both neuronal and non-neuronal cells has demonstrated that constitutive and regulated secretion pathways share homolog proteins (Schiavo et al., 1992; Bennett and Scheller, 1993; Sollner et al., 1993). These findings have prompted the suggestion that the transmitter secretion pathway at the nerve terminal has developed through addition of synaptic vesicle-specific proteins to the ubiquitous endosomal membrane recycling pathway. This idea has provided a rationale for the attempts to reconstitute the molecular machinery for neurotransmitter secretion in non neuronal cells (Cavalli et al., 1991; Alder et al., 1992; Morimoto et al., 1995). One of the functional assays for exocytosis in non-neuronal cells is based on the loading of exogenous ACh into non-neuronal cells. Surprisingly, ACh microinjection into the cytoplasm of *Xenopus* myocytes (Dan and Poo, 1992), or frog fibroblasts in culture (Girod et al., 1995) resulted in the accumulation of ACh in the membrane compartments and ACh quantal release, as detected by whole-cell patch clamp recordings. Moreover, secretion of exogenous ACh from these non neuronal cells was found to be Ca^{2+}-dependent (Girod et al., 1995). These results suggest that the rudimentary molecular machinery for the vesicular uptake of cytoplasmic ACh and quantal Ca^{2+}-dependent secretion may exist in non-neuronal cells. However, the nature of the vesicles capable of accumulating exogenous ACh, as well as the mechanism of cytoplasmic ACh penetration into the vesicles, remains unclear. Data in the literature suggest that these vesicles may be of lysosomal (Rodriguez et al., 1997), trans-Golgi (Chavez et al., 1996), or endosomal (Miyake and McNeil, 1995) origin.

In summary, it seems that the endocytic compartments in non neuronal cells are able to accumulate and secrete cytoplasmic ACh in a Ca^{2+}-dependent fashion, thus resembling

the basic functions of synaptic vesicles. On the other hand, the ubiquitous endosomal membrane recycling pathway may contribute to spontaneous quantal neurotransmitter secretion in neurons (Chang et al., 1998). Indeed, ACh is present in the cytoplasm of neuronal cells (Parsons et al., 1983), and endocytic recycling pathway operates in any type of cell, including neurons (Kraszewski et al., 1995; Dai and Peng, 1996). Constitutive exocytosis of the endosome-derived vesicles is expected to result in a detectable change of the membrane potential in the postsynaptic cell. Thus, the small amplitude of miniature endplate currents (mepcs) observed at the neuromuscular junction, which presumably reflect the exocytosis of vesicles with unusually low ACh content (Parsons et al., 1983), may reflect the exocytosis of constitutively recycling vesicles. The absence of defined quanta at the developing *Xenopus* neuromuscular synapse (Kidokoro, 1984; Evers et al., 1989), re-innervated mouse neuromuscular junction (Muniak et al., 1982) and central synapses (Bekkers et al.,1990) may reflect high proportion of "immature" synaptic vesicles, which are similar in molecular composition to constitutive recycling vesicles in that they lack some molecular components specific of synaptic vesicles.

Moreover, it has been shown that the neurotransmitters γ-aminobutyric acid and glutamate can be released in a Ca^{2+} independent manner, before synapse formation (Demarque et al., 2002). Nevertheless, there is also evidence in several preparations that Ca^{2+} is not fully required for vesicular exocytosis (Mochida et al. 1998; Tse and Tse 2000 and Zhang and Zhou 2002). Interestingly, in DRG neurons, tetanus toxin, which cleaves synaptobrevin, did not completely abolish a calcium-independent, voltage-dependent exocytosis (Zhang and Zhou, 2002). This raises the possibility that some vesicles fuse with the plasma membrane without the requirement of Ca^{2+} This hypothesis is supported by the observation that the formation of the major brain structures is not altered in Munc18-deficient mice in which vesicular release is abolished (Verhage et al., 2000). Munc18, in fact, interacts with the protein syntaxin and is supposed to influence transmitter release by controlling the formation of exocytosis-relevant protein complexes(Sudhof, 1995).

Neurotransmitters may function as chemical signals in axon pathfinding. Experiments on isolated chick embryo *Xenopus* (Young et al. 1983, Hume et al., 1983) and *Drosophila* CNS neurons (Yao et al., 2000) have indicated that the acetylcholine is

synthesized very early in neural development and is present on growing axons well before they reach their target or establish functional synapses. Experiments in culture have supported the hypothesis that acetylcholine may have a role in axon navigation; acetylcholine gradient, can cause a growth cone to change direction (Zheng 1994). The establishment of the precise fine structure of photoreceptor axon projections in the *Drosophila* visual system depends on the synthesis and release of acetylcholine. This developmental role of acetylcholine does not require the vesicular acetylcholine transporter protein, VAChT which is required for acetylcholine synaptic transmission. Spontaneous acetylcholine secretion from developing growth cones of *Drosophila* can be blocked by nicotinic receptor antagonists, but not by tetrodotoxin, which specifically blocks nerve-evoked synaptic transmission. This observation is consistent with an early role of acetylcholine in the establishment of axon projections that does not use an activity-dependent vesicle-mediated mode of delivery (Yang and Kunes .2004). These studies raise the possibility that neurotransmitters release, playing a role in development, could be dependent by mechanisms different from those which characterize synaptic transmission.

ACh RELEASE IN NEUROBLASTOMA CELLS

In the aforementioned cell model ACh is thought to have the main role in ChAT-transfected clones differentiation mediated by muscarinic receptors activation. To prove the existence of an autocrine/paracrine stimulation mechanism, it's necessary to demonstrate that ChAT-transfected clones are able to release the ACh synthesized. To analyse ACh release in ChAT transfected clones I used 2/4 clone because it shows the highest ChAT activity and ACh production. As a negative control, the release of 1/2 transfected clone, neomycin resistant, but not expressing ChAT activity, was tested. ACh release was evaluated using the choline chemioluminescent procedure.

Figure B shows representative light peak recordings of ACh standards in preliminary experiments. It is evident (Fig16 A) that a linear correlation between the light peaks measured and ACh amount exists .

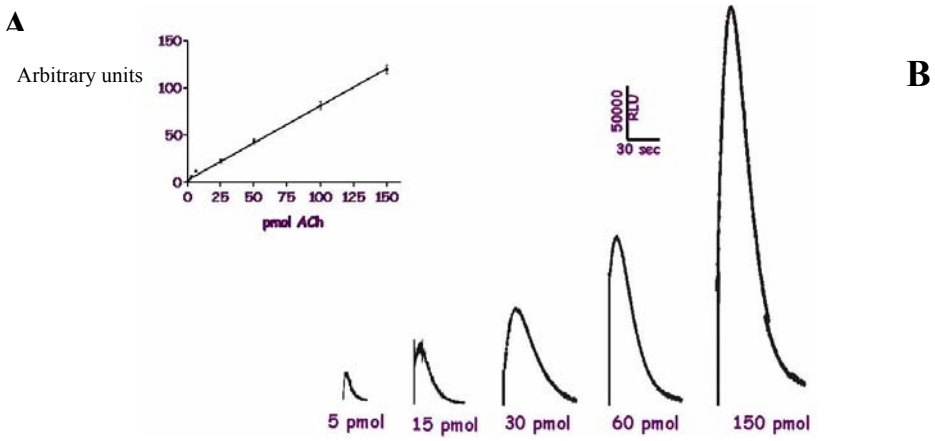


Fig.16 A linear correlation between known amount of ACh and light peak **B** recorded with choline chemioluminescent procedure.

Figure 17 shows that clone 2/4 was able to release ACh in basal conditions and that such a release was increased about two folds after potassium stimulation (KCl 80 mM). Basal ACh release showed a mean value of 18,5 ± 2.1 pmol ACh/10^5 cells, after KCl stimulation mean Ach release was 38.1 ± 4.7 pmol ACh/10^5; the difference was significant ($p < 0.001$).

The 1/2 clone recording were very low, not modified by potassium stimulation and probably they do not represent ACh secreted but a leakage of Choline in the medium by the cultured cells.

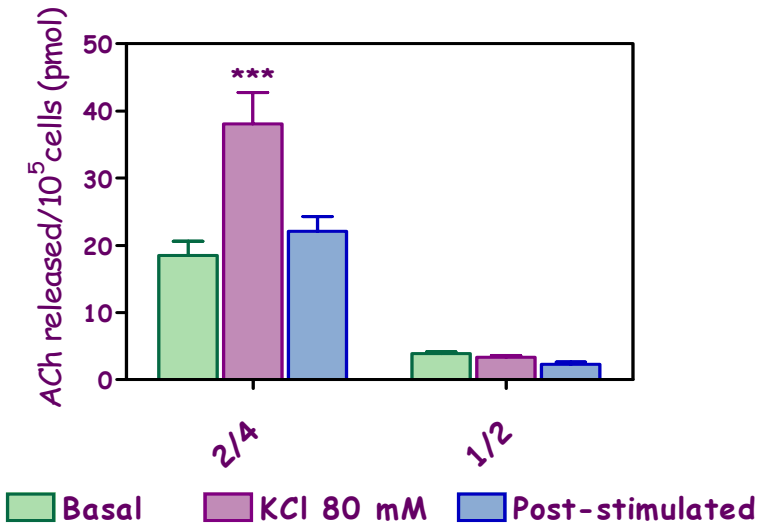


Figure 17 . Basal and KCl-stimulated ACh release in 2/4 ChAT-transfected clone and 1 /2 transfected clone, not expressing ChAT activity. ACh release was measured maintening the cell in saline solution for three consecutive periods (5' each), during the second KCl was added to a final concentration of 80mM. Asterisks indicate the a significant difference between basal and stimulated ACh release (***=p<0.001). Values are the mean±SEM of at least eight impendent experiment ;

As previously shown 2/4 ChAT-transfected clone is able to elongate fibers in differentiating conditions.

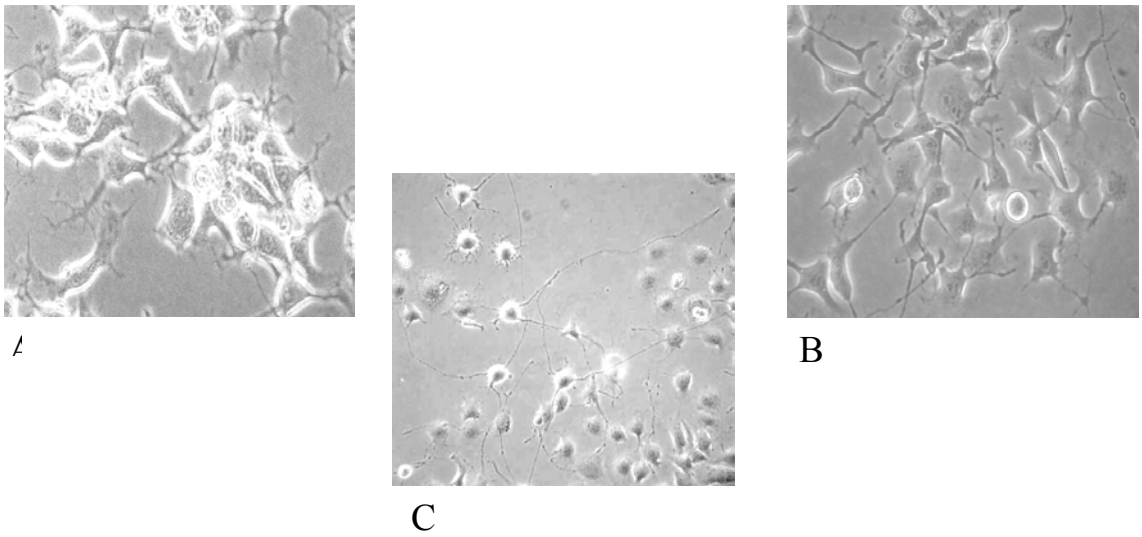


Fig. 18: 2/4 ChAT-transfected clone in basal conditions (A), after 24h (B) and after 48h (C) in differentiatimg medium with 1 mM dibutyryl cyclic AMP (dbcAMP).

Fig 18 shows that the fibers produced after 96 hours are much longer than those produced after 48 hours of treatment.To analyze the possible correlation between the levels of ACh released and fiber elongation, ACh release in 2/4 clone maintained for 48-96 hours in the presence of 1 mM dibutyryl cyclic AMP (dbcAMP) as differentiating agent, was also investigated. The cells in differentiating conditions showed a higher ability to release ACh with respect to cells maintained in non differentiating culture conditions (Fig.19). Such an increase was observed both for ACh released in basal condition and after high K^+ stimulation.

It is interesting to notice that the amount of ACh released is correlated with the time of differentiation; in fact, the release was higher after 96 hours in culture with respect to release measured after 48 hours of culture in differentiating conditions.

Moreover it is possible to currelate ACh release levels with fibres outgrowth because this latter is, as expected, higher after 96 hours of differentiation (Fig.19).

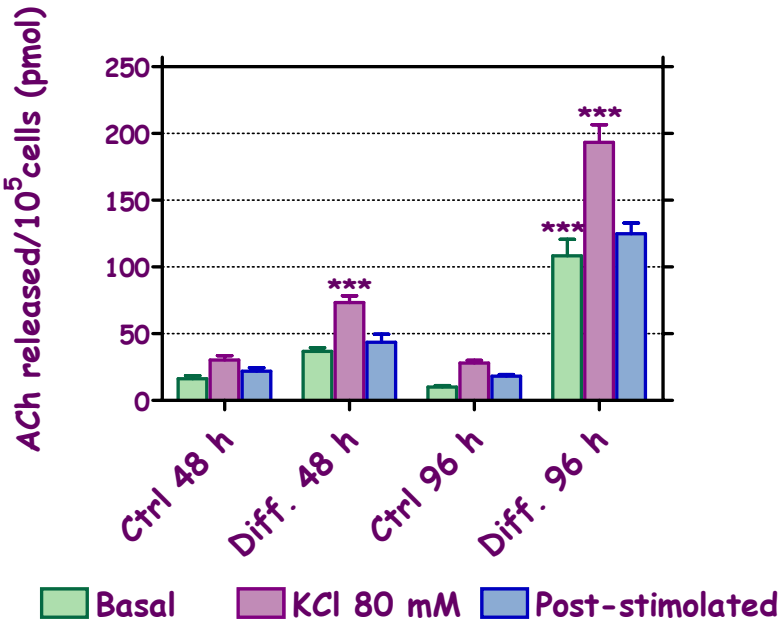


Fig.19: Basal and KCl-stimulated ACh release in 2/4 ChAT-transfected clone after 48 and 96 hours of culture in the presence of 1 mM dibutyryl cyclic AMP (dbcAMP) as differentiating agent. The ACh release increase with time in culture in differentiating conditions (***=$p<0.001$). Values are the mean±SEM of at least eight independent experiments.

In order to assess the calcium dependency of ACh release, 2/4 ChAT-transfected clone was stimulated in the absence of extracellular Ca^{2+} ions and in the presence of 1 mM EGTA.

No significant differences were observed between ACh release in cells maintained in the presence or in the absence of calcium for both basal condition and after KCL stimulation. It seems that Ach release in 2\4 cells is not depending on Ca^{2+} presence (Fig.20).

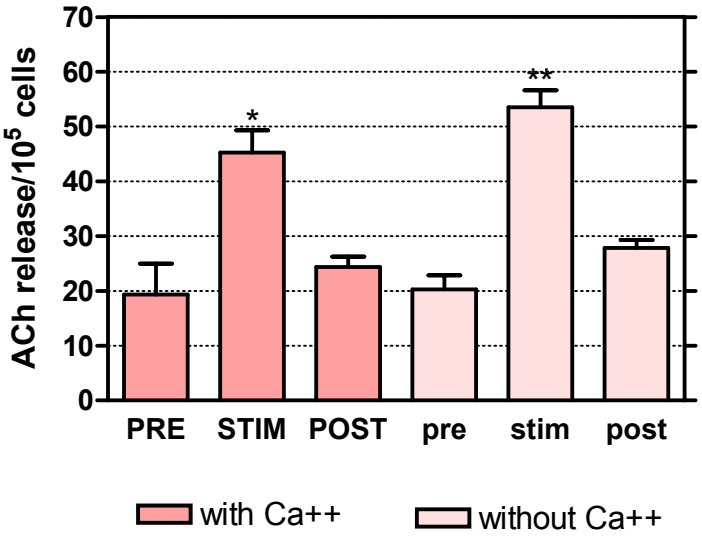


Fig.20: Basal and KCl-stimulated ACh release in 2/4 ChAT-transfected clone in the presence or in the absence of extracellular Ca^{2+} (**=p<0.01, *=p<0.05respect to pre-stimulation condition) Values are the mean ±SEM of at least five independent experiments.

Synaptic level of ACh are known to be regulated by the activity of presynaptc mAChRs mediating feedback inhibition of ACh release from Cholinergic nerve terminals (Kilinberger et al.,1984; Starke et al., 1989).

In order to asses if the ACh release in 2/4 ChAT-transfected clone is auto-modulated by muscarinic receptors I made the experiment in the presence of 10^{-6} M atropine, muscarinic antagonist.

Atropine was maintained in medium before, during and after the KCl stimulation. Fig 21 shows that a significant difference between ACh release in basal condition and after KCL stimulation is observed in cells maintained in the presence of atropine, as in control conditions.

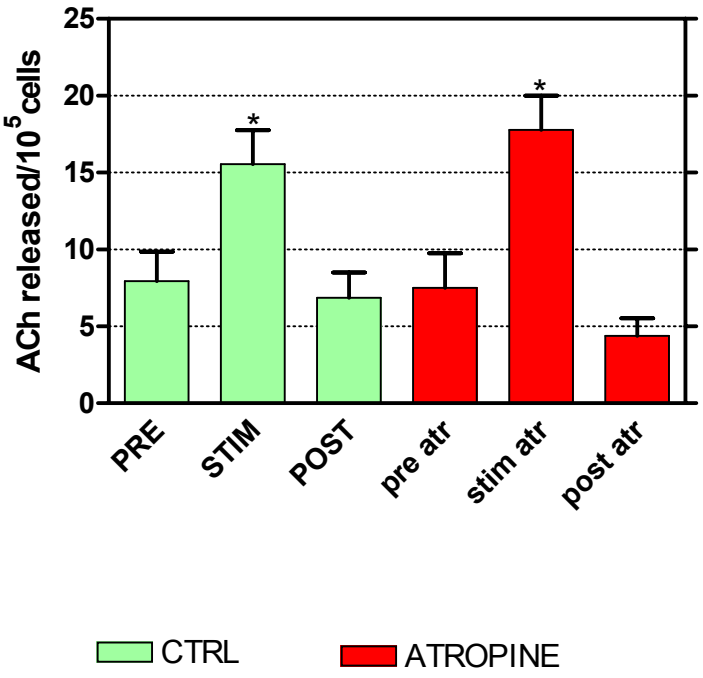


Fig. 21: Basal and KCl-stimulated ACh release in 2/4 ChAT-transfected clone in the presence or in the absence (CTRL) of 10^{-6} M atropine. (*=$p<0.05$) Values are the mean±SEM of at least five independent experiments.

These experiments prove that 2/4 ChAT-transfected clone is able to release Ach and that this release is increased by high K^+ 80mM . Moreover it was shown that the release is not regulated by Ca^{2+}, and not modulated by muscarinic receptors. It is concevaible to suppose that 2/4 ChAT-transfected clone lack some molecular components specific of synaptic vesicle release pathway and use an "immature" system to release ACh.

CHAPTER 5

Muscarinic receptors

It has been 90 years since Dale (Dale 1914) divided the action of Acetylcholine into two component: nicotinic and muscarinic. These effects are presently known to be mediated by two quite distinct classes of receptors which are characterized by different structure and molecular action mechanism and share only their ability to bind acetylcholine. The Muscarinic class of acetylcholine receptors (mAChR) are members of the super family of G protein coupled receptors (GPCRs). They are relatively abundant and mediate the diverse actions of acetylcholine in the CNS, as well as throughout non-nervous tissues innervated by the parasympathetic nervous system. Since the early 1950s pharmacological studies demonstrated the heterogeneity of muscarinic receptors that was unequivocally prove using molecular biological techniques.

In 1986 Numa and his colleagues (Kubo et al., 1986; Kubo et al.,1986b) cloned the m1 and m2 subtypes of muscarinic receptors by screening cDNA library prepared from porcine cerebrum and heart, respectively. Three more receptor subtypes (m3-m5) were then cloned by screening both cDNA and genomic libraries under low-stringent conditions using oligonucleotide probes corresponding to regions of high homology between the m1 and m2 sequences (Liao et al., 1989; Bonner et al., 1987; Peralta et al., 1987).

This family of receptors is characterized by the presence of seven hydrophobic regions in their sequence which are thought to form alpha helixes which span the membrane.

The transmembrane segment (TM) of the muscarinic receptor represents the region of highest homology among the different subtypes and across other members of this large family of G-protein –linked receptors. Major differences between sequences of the five muscarinic receptor subtypes lenght are present in: the extracellular amino terminus, the cytoplasmic carboxy terminus, and the third intracellular loop (i3). The greatest divergence arises from i3 loop which varies in length from 156(M1) to 239 (M3) residues in the five human subtypes. A comparision of the sequences shows that the

M1, M3, and M5 subtypes share the maximum homology, whereas the M2 and M4 subtype constitute a separate homologous group.

The seven TM segments are thought to form the staves of a barrel-like structure having a central pore. Hulme et al. (1990) have predicted that most of the conserved residues in the TM segments form the inner lining of the central pore, whereas the few non conserved residues are on the outside. Acetylcholine and other muscarinic ligands are thought to bind at a site within this pore, and Hulme et al. (1990) have pointed to the highly conserved nature of the central pore as the explanation for the present lack of highly selective muscarinic agonist and antagonist (Fig.22).

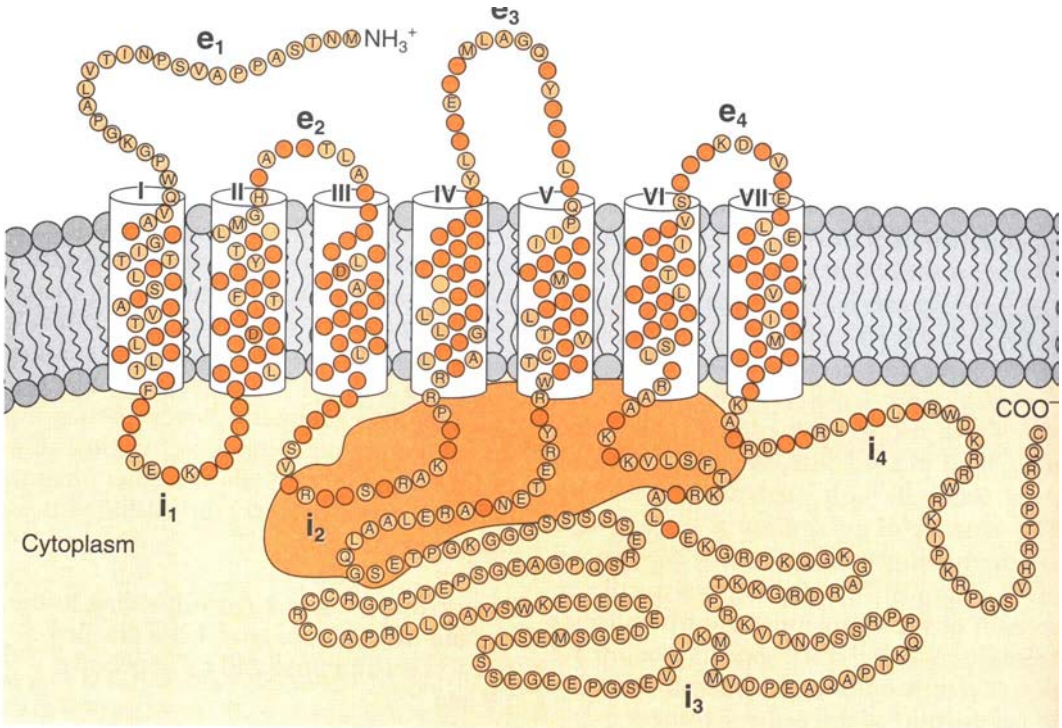


Fig. 22: Predicted amino acid sequence and transmembrane domain structure of the human M1 muscarinic receptor. Amino acid that are identical among the M1, M2, M3 and M4 receptors are darkened. The shaded cloud represent the approximate region that determines receptor-G-protein coupling.

The five muscarinic receptor subtypes are referred to as M1-M5. The subtype differ in their capability to couple to different G proteins depending on the nature of the G-protein, the receptor-G protein interaction can initiate any of several early biochemical events seen with muscarinic receptor occupation: inhibition of adenylyl cyclase, stimulation of phosphoinositide hydrolysis, or regulation of an ion channel.

The M_2 and M_4 mAChR interact with the α–subunit of GTP-binding protein, G_i, known to inhibit adenylyl cyclase (AC). The M_1, M_3 and M_5 mAChR interact with GTP-binding proteins in the Gq family to activate phospholipase C (PLC) and K+ channels. Mediators formed within the cell include cAMP, inositol trisphosphate ($InsP_3$), and diacylglycerol (DAG). The inositol phosphates are generated from phosphatidylinositol bisphosphate (PIP_2), phosphatidylinositol monophosphate (PIP), and phosphatidylinositol (PI) (Fig.23-24).

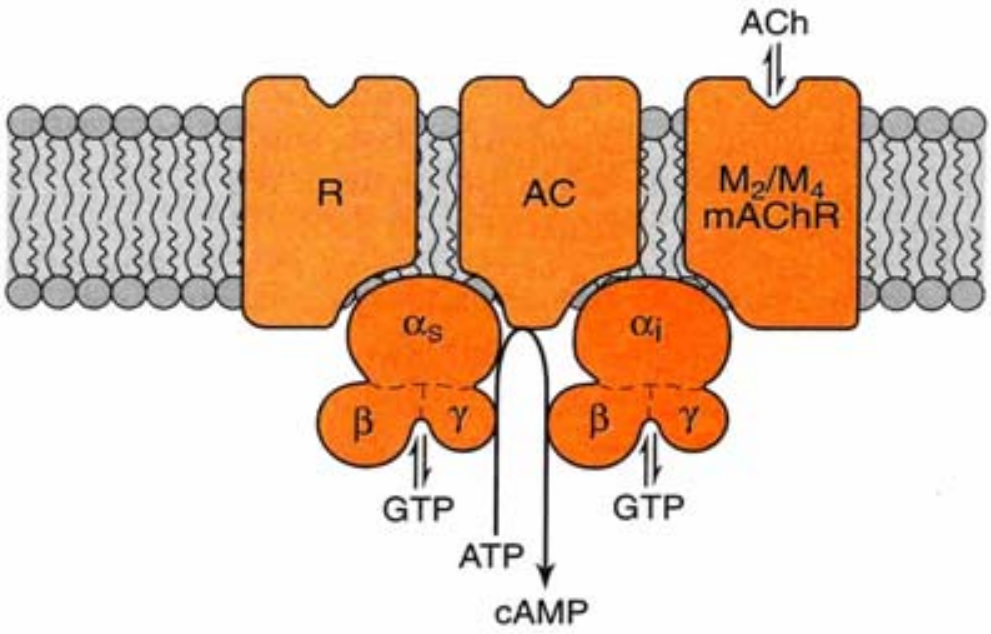


Fig23: Primary biochemical response mediated by M2 and M4 mAChR. They interact with the α– subunit of GTP-binding protein, G_i, known to inhibit adenylyl cyclase (AC)..

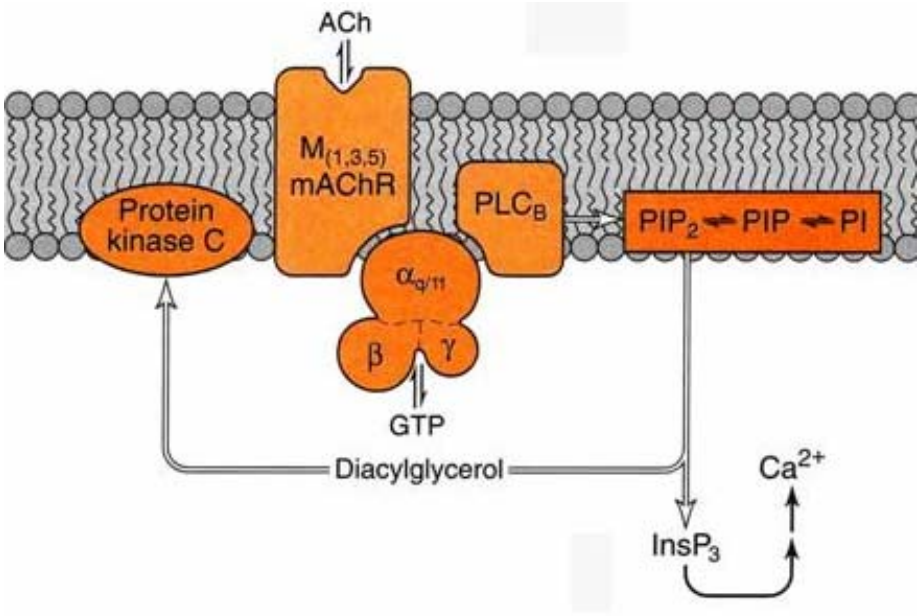


Fig.24 Primary biochemical response mediated by M1,M3, M5 and mAChR. They interact with GTP-binding proteins in the Gq family to activate phospholipase C (PLC). Mediators formed within the cell include inositol trisphosphate ($InsP_3$), and diacylglycerol (DAG). The inositol phosphates are generated from phosphatidylinositol bisphosphate (PIP_2), phosphatidylinositol monophosphate (PIP), and phosphatidylinositol (PI)

Beside their coupling to the well-established phosphatidylinositol and cyclic AMP effector systems, evidences are now available suggesting that muscarinic receptors also couple to, or intersect with, signalling pathways which involve sequential activation of serine/threonine protein kinases, thus leading to modulation of gene expression. For example muscarinic receptors can activate certain MAP-kinase pathways (Wotta et al., 1998; Berkeley and Levey.2000). Some components of the phosphokinase pathways that could conceivably be modulated by muscarinic receptors *in vivo* have the potential to enhance cell survival by means of up-regulation of certain protection systems and/or blockade of apoptosis (Rosemblum, K. et al.2000).

Cellular response of mAChRs includes the activation of neurite outgrowth, the fine tuning of membrane potential, and the regulation of mitogenic growth response in cells that are not terminally differentiated (Conklin et al., 1988).

In brain, mAChRs are involved in long term potentiation, synaptic plasticity, and higher cognitive functions, including learning and memory.

mAChRs are known to increase the expression of immediate early-genes like c-fos, c-jun, fos-b, fra-1, nurr-77, egr-1, egr-2, egr-3. Considering that these transcription factors regulate the expression of a broad variety of target genes, it is conceivable that muscarinic receptor activity is involved in the activation of plastic response in postsynaptic element or in the activation of differentiating response in neuronal progenitor (Coso et al., 1995; Huges et al. 1992; Katayama et al.,1993).

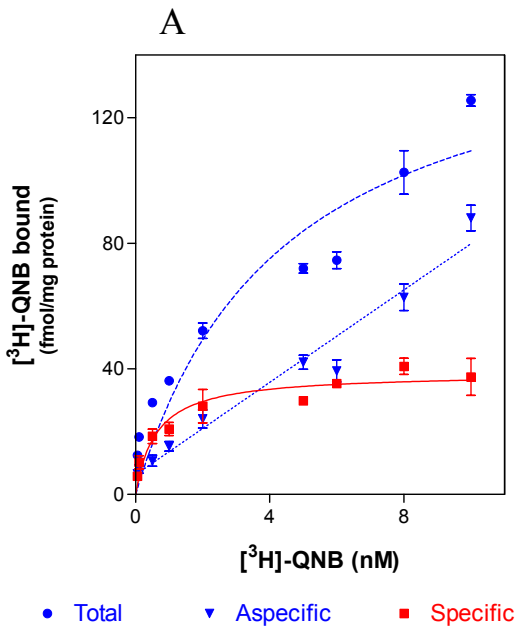
Muscarinic receptors expression in ChAT-transfected clones

QNB binding studies demonstrated the presence of muscarinic receptors in neuroblastoma N18TG2 and in ChAT-transfected clones (Fig.5)

In the present work I have analysed, by pharmacology competition, the expression of muscarinic receptor subtypes in one of the most representative ChAT-transfected clones, the 2/4 clone.

Moreover we have investigated the activation of the transduction pathway to demonstrate the functionality of muscarinic receptors in order to show that ChAT transfected clones are potential responsive to Ach .

Homogenates of ChAt-transfected cells specifically bind [^{3}H]-QNB. The binding was concentration dependent (Fig.18). The Scatchard plot of the saturation isotherms indicate that the binding sites belonging to a single class of high affinity sites and reveal a dissociation costant (*Kd*) value of 0.54± 0.16 nM. Saturation of the specific binding occurred at [^{3}H]-QNB concentration of 37.18 ±2.19 fmol/mg protein (Fig.25B).



B

$[B]/[L] = (-1/K_d)\,[B] + (B_{max}/K_d)$

$B_{max} = 37.18 \pm 2.19$ fmoli/mg prot

$K_d = 0.54 \pm 0.16$ nM

Fig.25A [^{3}H]-QNB saturation curve in 2/4 Chat positive clone. Aspecific isotherm is obtained in presence of antagonist atropine [10^{-6}M]. Specific isotherm is obtained subtracted the aspecific isotherm value from total amount of [^{3}H]-QNB binding obtained in absence of antagonist. Values are the mean ± SEM of at least five observations.

B Scathard plot analysis of data in (A).

Competition binding experiments was also performed using selective drugs for different muscarinic receptor subtypes.

Table I reports various agonist and antagonists for the different types of muscarinic receptors.

Acetylcholine Receptors (Muscarinic)

Currently Accepted Name	M_1	M_2	M_3	M_4	M_5
Molecular Biology Classification	m1	m2	m3	m4	m5
Structural Information	460 aa (human)	466 aa (human)	590 aa (human)	479 aa (human)	532 aa (human)
Subtype Selective Agonists [a]	McN-A-343 (ganglion) Pilocarpine (relative to M_3 and M_5) L-689,660 Xanomeline CDD-0097	Bethanechol (relative to M_4)	L-689,660	McN-A-343 (relative to M_2)	None known
Subtype Selective Antagonists [a]	Pirenzepine Telenzepine	Methoctramine AF-DX 116 AF-DX 384 Gallamine (non-competitive) Himbacine Tripitramine	Hexahydro-sila-difenidol *p*-Fluorohexahydro-sila-difenidol 4-DAMP	Tropicamide Himbacine AF-DX 384	None known
Receptor Selective Agonists	Bethanechol Metoclopramide Muscarine Pilocarpine Oxotremorine M	Bethanechol Metoclopramide Muscarine Pilocarpine Oxotremorine M	Bethanechol Metoclopramide Muscarine Pilocarpine Oxotremorine M	Bethanechol Metoclopramide Muscarine Pilocarpine Oxotremorine M	Bethanechol Metoclopramide Muscarine Pilocarpine Oxotremorine M
Receptor Selective Antagonists	Scopolamine QNB, (±)- QNB, R(–)- Atropine	Scopolamine QNB, (±)- QNB, R(–)- Atropine	Scopolamine QNB, (±)- QNB, R(–)- Atropine	Scopolamine QNB, (±)- QNB, R(–)- Atropine	Scopolamine QNB, (±)- QNB, R(–)- Atropine
Signal Transduction Mechanisms	$G_{q/11}$ (increase IP_3/DAG) NO	G_i (cAMP modulation) TK^+ (G)	$G_{q/11}$ (increase IP_3/DAG) NO	G_i (cAMP modulation) TK^+ (G)	$G_{q/11}$ (increase IP_3/DAG) NO
Radioligands of Choice	[^{3}H]-Pirenzepine [^{3}H]-Telenzepine [^{3}H]-QNB	[^{3}H]-AF-DX 384 [^{3}H]-QNB	[^{3}H]-4-DAMP [^{3}H]-QNB	[^{3}H]-AF-DX 384 [^{3}H]-QNB	[^{3}H]-QNB [^{3}H]-NMS

Abbreviations
AF-DX 116: 11-[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6-pyrido[2,3-b][1,4]benzodiazepin-6-one
AF-DX 384: 5,11-Dihydro-11-[2-[2-[(N,N-dipropylamino)methyl]piperidin-1-yl]ethylamino]-carbonyl]6H-pyrido[2,3-b][1,4]benzodiazepin-6-one
CDD-0097: 5-Propargyloxycarbonyl-1,4,5,6-tetrahydropyrimidine
4-DAMP: 4-Diphenylacetoxy-N-methylpiperidine methiodide
L-689,660: 1-Azabicyclo[2,2,2]octane,3-(6-chloropyrazinyl)maleate
McN-A-343: 4-(N-[3-Chlorophenyl]carbamoyloxy)-2-butynyltrimethylammonium chloride
NMS: N-Methylscopolamine
QNB: Quinuclidinyl-α-hydroxydiphenylacetate; Quinuclidinylbenzylate

Tab.I: Characteristic of the different muscarinic receptors

In particular I used **pirenzepine** for M1, **gallamine** for M2, **4-DAMP** for M3 and **tropicamide** for M4 subtype (Giraldo et al, 1988; Michel et al, 1989; Lazareno et al, 1993; Tata et al, 2000) (Fig.26) at present no selective drug for M5 subtype is avalaible.

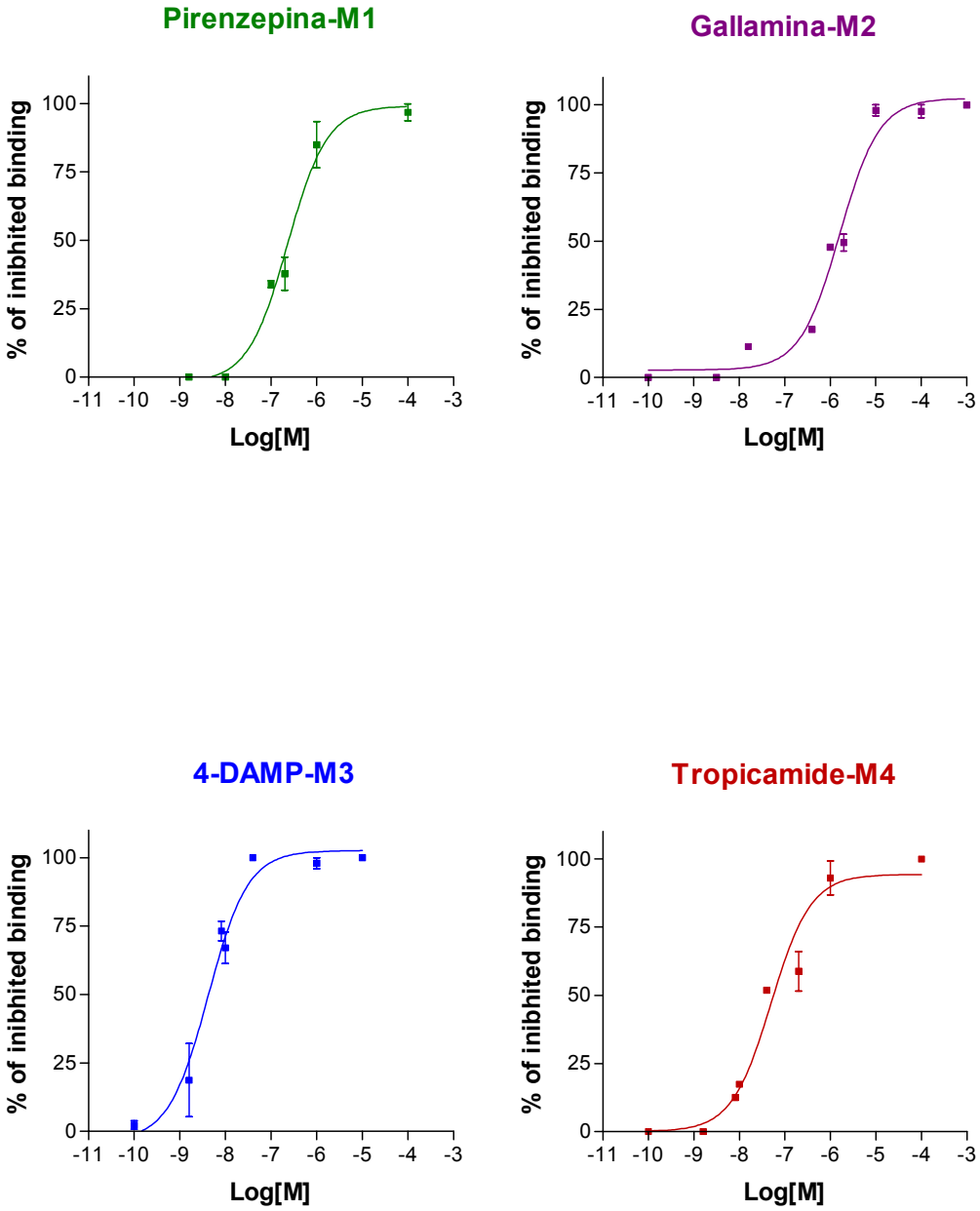


Fig.26 [^{3}H]-QNB binding inhibition by different selective antagonist treatment. Values are the mean ± SEM of at least six observations.

The IC_{50} and *Ki* values obtained for the different ligands were reported in Tab II The data obtained in these experiments compared with the *Ki* reported for other tissues (Bronzetti et al, 1996; Tata et al. 1995; 2000)

show that 2/4 ChAT-transfected neuroblastoma clone express M1, M2, M3 and M4 muscarinic subtypes.

DRUG	SELECTIVITY	Ki (nM)	EC50
PIRENZEPINE	**M1**	**$1.2x10^{-7}M$**	**$2.3x10^{-7}M$**
GALLAMINE	**M2**	**$8.4x10^{-7}M$**	**$1.6x10^{-6}M$**
4-DAMP	**M3**	**$2.3x10^{-9}M$**	**$4.3x10^{-9}M$**
TROPICAMIDE	**M4**	**$2.6x10^{-8}M$**	**$5.0x10^{-8}M$**

Tab.II Values of Ki and EC50 obtained with pharmacological competition experiments.

mAChRs and activation of signal trasduction pathways

With the aim to establish if the mAChRs expressed in ChAT-transfected neuroblastoma cells are able to activate specific signal trasduction pathways, I analyzed the modulation of the cAMP and IP3 levels after treatment with cholinergic agonists. Fig.27 shows the effect of 10^{-4} M muscarine treatment on IP3 levels both in ChAT-transfected clone and in N18TG2 parental cells.

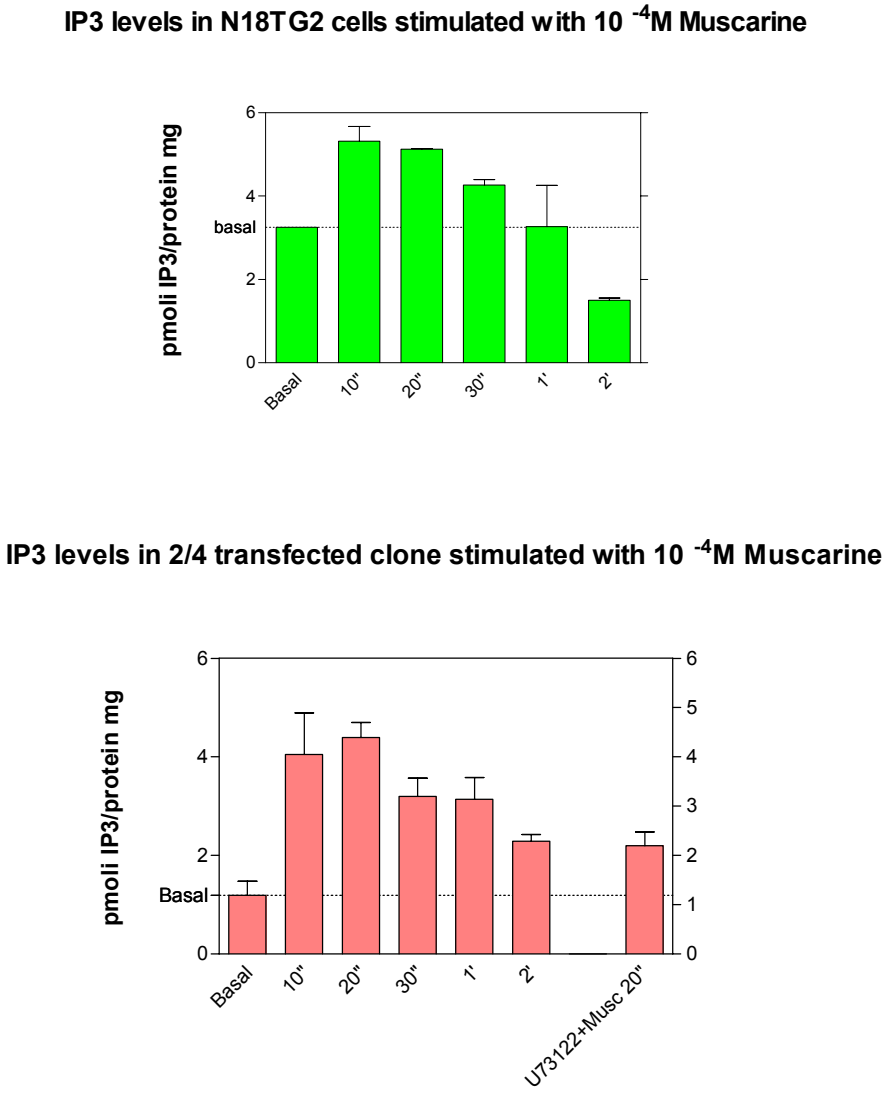


Fig.27: IP3 levels in N18TG2 parental cells and in 2/4 ChAT-positive clone stimulated with 10^{-4}M muscarine. Mus: muscarine; U73122: phospholipase C inhibitor.Values are the mean ± SEM of at least four observations.

In both cases a fast increase of IP3 was evident 10'' after the agonist addition. The increase observed in the two cell types was comparable and significant ($p<0.001$) with respect to the basal condition. Moreover in both cases IP3 level decrease 30'' after agonist addition. Finally the muscarine effects were significantly reduced ($p<0.01$) in the presence of U73122, indicating the IP3 level increase induced by muscarine is dependent on PLC activation.

Fig. 28 shows the effect of 10^{-4}M muscarine treatment on cAMP levels.

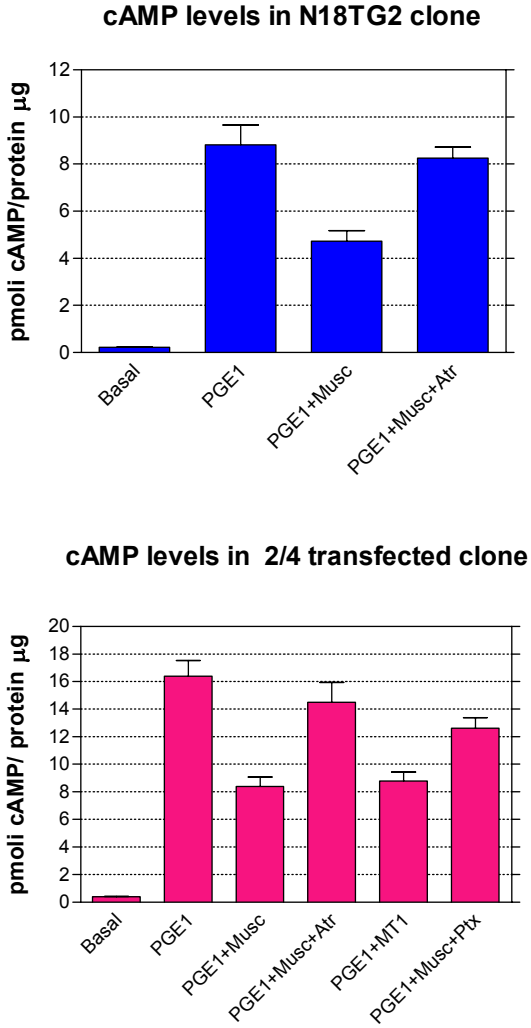


Fig.28: cAMP levels in N18TG2 parental cells and in 2/4 ChAT-positive clone. PGE1: prostaglandine E1; Musc: muscarine, Atr: atropine; MT1: mamba toxin 1, Ptx: pertussus toxin. Values are the mean ± SEM of at least four observations.

It is well known that M2 and M4 receptor are coupled to Gi and that their activation inhibit of adenylate cyclase activity resulting in a decrease of cAMP level. Therefore I analyzed if muscarine treatment of parental and ChAT-transfected clone is able to produce a decrease of cAMP level induced by PGE1 stimolation. Although the cAMP levels induced by PGE1 was different in ChAT-transfected clone and N18TG2 parental cells, the ability of muscarine to inhibit PGE-1 effect on cAMP level was similar in the two types of cells. Moreover the agonist effect was abolished by the pre-treatment with the antagonist atropine, confirming that the decrease of cAMP level is dependent on muscarinic receptor activation. Moreover the treatment of ChAT-transfected clone with the Mamba Toxin 1 (MT1), a selective agonist of M1 and M4 receptors (Potter, 2001), also reduce the cAMP level at the same extent observed after muscarine treatment, suggesting a direct involvement of M4 subtype. These observation were confirmed by the use of pertussis toxin (PTX). In fact the pre-treatment of the cells with PTX reduced the muscarine effect indicating that PTX-sensitive G-protein, likely belonging to the Gi family, was involved. As reported above this type of G-protein is usually coupled to M2/M4 receptors, therefore these data strongly suggest an involvement of M2/M4 receptor in the modulation of cAMP levels.

All together these data demonstrate that mAChRs expressed both in ChAT-transfected clone and N18TG2 cells are functional and able to activate different signal transduction pathways.

CHAPTER 6

Egr-1

The neurotransmitters can lead to long lasting changes in cell phenotype and function by altered gene expression. Mechanism must exist to couple short term, cell surface events to the long term and coordinated changes in gene expression that give rise to the required alteration in cell function. Immediate early-genes (IEG) are the first targets activated by the diverse intracellular messenger systems linking membrane events and the nucleus. These genes are defined by rapid, and often transient, induction independent of the novo protein synthesis. A subclass of immediate early genes encodes inducible transcription factors which act as third messengers coupling neurotransmission to a cascade of altered expression (Curran, 1988); egr-1 belongs to this subclass of early genes.

egr-1 is the prototypical member of a family of closely related genes including egr-2, egr-3 and egr-4.

The gene named egr-1 (early growth response gene 1) is also called zinc finger binding protein clone 268 (zif268), nerve growth factor-induced gene A (NGFI-A), gene containing sequences homologous to the Drosophila Kruppel finger probe (Krox-24) and tetradecanoyl phorbol acetate-induced sequence 8 (TIS8). The ambiguity in the nomenclature results from the fact that this gene has been independently identified in different laboratories and animal species. Thus, a term ZENK (the acronym of the previous four names) has been coined, and it is used nowadays in parallel with all the other names. It was initially identified by Lau and Nathans (1987) in mouse fibroblasts, where it was induced by serum and growth factors. At the same time Milbrandt (1987) identified NGFI-A in a screening strategy that aimed at detecting genes induced by NGF (nerve growth factor) in rat PC12 cells (Rat pheochromocytoma cells). These discoveries were quickly followed by several independent descriptions of similar gene sequences from mice, rats and humans (Almendral et al., 1988; Christy et al., 1988; Lemaire et al., 1988; Sukhatme et al., 1988; Tsai-Morris et al., 1988; Arenander et al., 1989; Cao et al., 1990; Lemaire et al., 1990; Suggs et al., 1990).

In this thesis I will use egr-1 to indicate the gene and Egr-1 to indicate the protein.

In the brain, egr-1 has a distinct basal expression, i.e. the one maintained by normal ongoing synaptic or neurohormonal/neurotrophic activity (Worley et al., 1991; Herdegen and Leah, 1998; Beckmann and Wilce, 1997). This feature is probably crucial for egr-1 functions since it allows for either increasing or decreasing the level of its expression.

egr-1 mRNA expression is detectable in the mouse cortex and hippocampus by in situ hybridization (Christy et al., 1988). A more detailed study shows basal egr-1 mRNA expression in the rat neocortex, primary olfactory, amygdaloid nuclei, nucleus accumbens, striatum, cerebellar cortex and hippocampus (Schlingensiepen et al., 1991).

The spatial expression patterns of egr-1 mRNA and Egr-1 protein show thigh correspondence that may indicate that the basal level of Egr-1 expression is regulated principally at transcriptional level (Worley et al., 1991) egr-1 mRNA is, in fact, expressed at low levels in the early post-natal rat cortex, midbrain, cerebellum and brain stem. Moreover egr-1 mRNA increase throughout postnatal development to adult levels (Watson and Milbrant, 1990). In particular, in-situ hybridization studies showed that egr-1 mRNA is firstly detectable in rat sensory cortex at postnatal day 10 and that after day 12, expression more prominent in this region and becomes apparent throughout the entire frontal cortex with a marked increase in the occipital cortex on day 14 (Herms et al., 1994).

The gene encoding Egr-1 is present in the mammalian genomes as a single copy genes. The coding region of the gene spans about 3.8 kb and consists of two exons and one intron. The exon at the 3'-end includes three zinc-finger DNA-binding domains (Tsai-Morris et al., 1988; Changelian et al., 1989), while the intron is positioned at the 5'end of the region encoding the zinc fingers (Tso et al., 1986).

The upstream region of the Egr-1 gene contains (Fig. 29) some serum response elements (SREs), binding sites for the Ets transcription factor family (EBS) (Sakamoto et al., 1991), Sp1 (specificity protein-1), CRE (Calcium/cAMP response element) sites, two CCAATT sequences, and AP-1- (Activator protein -1) binding sequences (Christy et al., 1988; Tsai Morris et al., 1988; Changelian et al., 1989). Interestingly, the Egr-1 protein can bind its own gene via the EBS 5'-CGCCCCCGC-3' sequence (Thiel and Cibelli 2002). Moreover the human promoter contain the ERE (Egr response element)

(Sakamoto et al., 1991). As a result, Egr-1 down regulates the transcription of its own gene (Cao et al., 1993). Although the molecular mechanism of this repressive activity is unknown, this is a further negative feedback loop, in addition to other repressive system, that allows only a transient but not a sustained synthesis of Egr-1 (Thiel and Cibelli 2002).

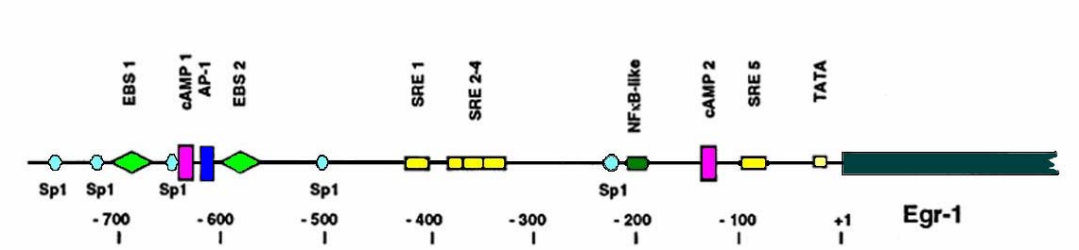


Fig.29: EGR-1 promoter. EBS: binding sites for the Ets (Ets family from sequences transduced by the E26 avian leukemia virus); cAMP:CRE-like(Calcium/cAMP response element)sequence; SRE: serum response elements; AP-1: AP-1-like (Activator protein -1) motifs binding sequences; NFkB-like binding site for nuclear factor kappa B; SP1 binding site for SP1 (specificity protein -1)

This transcriptional factor have a modular structure: different functions such as DNA-binding, activation or repression of transcription, can be attributed to distinct regions within the molecule. The modular structure of Egr-1 is depicted in figure 30.

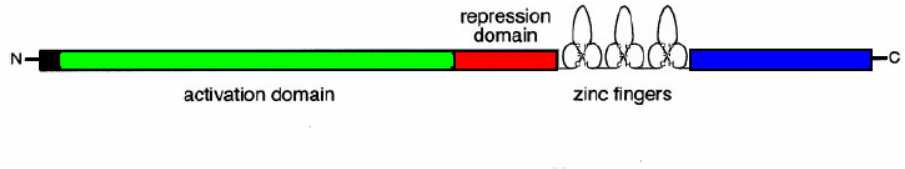


Fig.30: Modular structure of the zinc finger transcription factor Egr-1. The Egr-1 protein contains an extended transcriptional activation domain on the N-terminus and a DNA-binding domain, consisting of three zinc finger motifs. Additionally, an inhibitory domain has been mapped between the activation and DNA-binding domain that functions as a binding site for the transcriptional co-repressor proteins.

The DNA-binding domain of Egr-1 contains three zinc finger motifs and is sufficient for DNA-binding activity (Pavletich and Pabo, 1991).

The three zinc fingers domain are arranged in a semicircular structure that fits snugly into the major groove of B-DNA. Each zinc finger domain consists of anti-parallel beta sheet and an alpha-helix held together by a zinc ion coordinated by two cysteines from the beta sheet and two histidines from the alpha-helix (Pavletich and Pabo, 1991; Gashler et al., 1993).

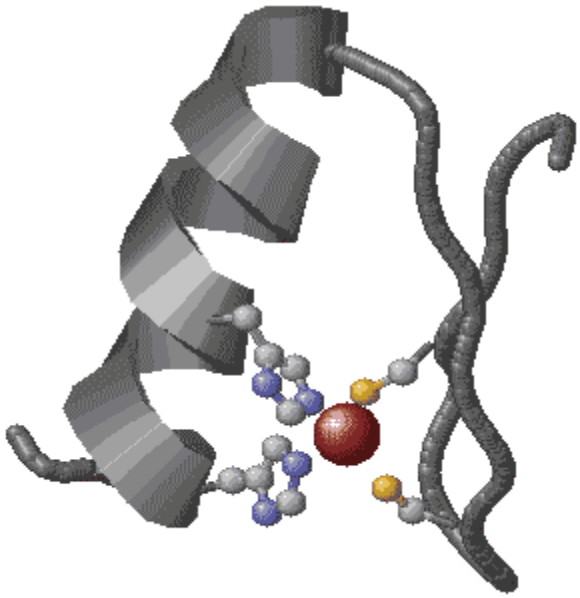


Fig.31 A zinc finger motif rappresentation. Each zinc finger domain consists of anti-parallel beta sheet and an alpha-helix held together by a zinc ion (in red) coordinated by two cysteines (sulphur in yellow) from the beta sheet and two histidines (nitrogen in blue) from the alpha-helix

Egr-1 preferentially binds to the GC-rich sequence 5'-GCGGGGGCG-3' (Christy and Nathans, 1989; Cao et al., 1993) also known as ERE (Egr-1 response element).

The activation domain of Egr-1, that is not well characterized, is made up of four regions which have the ability to stimulate transcription: A1(between amino acids 16-41), A2(89-148), A3(420-536) and A4 (226-267). (Gashler et al.1993; Thiel et al., 2000).

An inhibitor domain (R1) between the activation domain and the DNA-binding domain was identified that functions as binding site for two transcriptional co-factors termed NGFI-A binding proteins 1 and 2 (NAB1, NAB1) (Russo et al., 1995; Svaren et al., 1996). Both NAB1 and NAB2 block the biological activity of Egr-1 (Russo et al., 1995; Svaren et al., 1996; Thiel et al.,2000).

NAB-1 and NAB-2 are highly expressed in brain (Russo et al., 1995; Svaren et al., 1996). NAB proteins do not interfere with DNA binding by Egr-1, but rather repress transcriptional activity of promoters to which they are tethered (Swirnoff et al., 1998). Interestingly NAB2 is induced by nerve growth factor in PC12 cells with slightly delayed kinetics with respect to Egr-1, while NAB1 expression is unchanged. This suggests that NAB1 and in particulary NAB2 may play an important role in the regulation of the function of Egr-1 in vivo (Svaren et al., 1996).

The discovery of these two repressors produced a further level of complexity for understanding the various functions of Egr-1 because induction of egr-1 transcription gene may have no biological effect when the function of Egr-1 as a transcriptional activator is neutralized by NAB1 or NAB2. The concentration of both co-repressor in a particular cell is thus of extreme importance for Egr-1 function. Moreover, the expression of the NAB2 gene is controlled by Egr-1 (Ehrengruber et al., 2000), indicating that Egr-1 controls its biological activity in a negative feedback loop via the synthesis of NAB2. This Egr-1-induced NAB2 expression can be repressed by NAB proteins, supporting a negative feedback mechanism (Mechta-Grigoriou et al., 2000;Svaren et al.,1998) which is crucial to control a damaging overreaction in response to environmental signals. Alterations in expression of Egr-1 and Nab proteins have been associated with deficiencies in neuronal development (Mechta-Grigoriou et al., 2000; Venken et al.,2002).

Egr-1 protein was localized to the cell nucleus (Cao et al., 1990; Waters et al., 1990). The nuclear localization is provided by a bipartite signal in the DNA-binding domain (the second or third zinc finger) and in the basic flanking sequences (Gashler et al., 1993). Two protein species of molecular weights 82 and 88 KDa encoded by egr-1 are translated in vitro in a rabbit reticulocyte lysate system using the complete open reading frame of the egr-1 gene. The existence of these two proteins is due to the existence of

two start codons. Both form of Egr-1 are also found in serum or TPA-stimulated NIH3T3 fibroblast (Lemaire et al., 1990).
A doublet with apparent molecular weight of 84 kDa and a single band of 54 kDa are detected by western blotting of PC12 cell extracts after stimulation by NGF, phorbol ester or calcium ionophore (Day et al., 1990). The 54 kDa protein is a form of Egr-1 truncated prior to nucleotide 1528. This protein is exclusively found in the cell cytoplasm whereas the 84 kDa form is nuclear. The doublet at 84 kDa represents phosphorylated and unphosphorilated forms of the protein (Day et al., 1990).

Several levels of post-translation regulation have been observed for Egr-1. A consensus site for phosphorilation is present in the Egr-1 protein sequence (Milbrant,1987) and Egr-1 proteins are phosphorilated on as serine residue (Lemaire et al., 1990). Phosphorylation of Egr-1 enhances its DNA-binding activity (Huang and Adamson,1994). Phosphorylation is also proposed to mediate the interaction of Egr-1 with the NAB inhibitors (Russo et al. 1993).
Several potential N- and O- glycosylation sites are also present in the predicted Egr-1 protein sequence (Milbrant, 1987).
The DNA-binding activity of Egr-1 is dependent on the presence Zn^{2+}, Fe^{2+}or Mn^{2+} (Cao et al., 1992). The cellular redox state may control the DNA-binding activity of Egr-1 by excluding zinc from the protein and rendering the oxidised form inactive (Huang and Adamson,1994).

The transcriptional activity of Egr-1 could be regulated through the interaction with other transcription factors. DNA-binding site for Sp1 and Egr-1 proteins often overlap. In same cases, Egr-1 protein or Sp-1 binding is mutually exclusive (Akerman et al. 1991; Cao et al., 1993). Other transcriptional factors can recognize GC rich sequences (ERE-like), it is therefore possible that these transcription factors could interact with some promoters containing Egr motif within the nervous system (Beckmann and Wilce 1996).
Egr-1 protein may also interact with transcription factors bound to other sequences. A protein binding to the neural-restrictive silencer element (NRSF) (Mori et al., 1992; Schonherr and Anderson,1995), present in the human synapsin I gene promoter next to

the Egr-1 binding motif, may control Egr-1 transactivation of this gene (Thiel et al., 1994).

Egr-1 is induced in neurons after extracellular stimulation with neurotransmitters or trophic substances. Neuronal activation via all glutamate receptor subtypes can increase Egr-1 expression. For instance, in neuronal cultures, Condorelli et al. (1994) have observed that kainate was the most potent, followed by glutamate, N-methyl-D aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA), trans-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD) and quisqualate (the last two agonists activate metabotropic glutamate receptors).

Blockade of $GABA_A$ receptor by pentylenetetrazole, a channel blocker, induces Egr-1 mRNA in all subfields of the hippocampus, but has no effect on expression in the midbrain, cerebellum or brain stem (Saffen et al., 1988).

Activation of dopamine D_1 receptors by a specific agonist increases Egr-1 m-RNA expression in cultured striatal neurons, which is antagonized by the action of D_2 receptors (Simpson and Morris, 1995). Egr-1 induction is also modulated by muscarinic receptors. The non-selective muscarinic receptor agonist pilocarpine increases the neuronal expression of Egr-1 in rat cortex and hippocampus (Huges and Dragunow 1994). Pre-treatment of animals with atropine (non selective muscarinic antagonist) or pirenzepine (M1 selective antagonist) significantly reduces induction of Egr-1 in both cortex and hippocampus.

Administration of carbachol, noradrenaline, and bradykinin induced Egr-1 mRNA expression within 1h in mouse neuroblastoma x rat glioma hybrid NG105-15 cells. Egr-1 induction by carbachol was inhibited specifically by atropine but not affected by α -bungarotoxin (Katayama et al., 1993).

The neuronal stimuli describe above are coupled to diverse intracellular second messengers cascades.

Calcium ions and cyclic AMP cause the activation of immediate early gene transcription through the phosphorylation of calcium response element (CREB) which activates transcription of immediate early genes through the Calcium/cAMP response element (CRE) (Sheng at el., 1990).

The product of phospholipase C activity induces the expression of immediate early genes through the serum-response element (SRE) (Gilman, 1981). These second messenger pathways are subject to cross-talk at multiple levels (Ewards,1994).

In response to increased cytosolic calcium level, several downstream signal transduction pathways are activated and many of them have been implicated in Egr-1 activation. Sgambato et al. (1998) observed a transient activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), spatially coincident with the onset of Egr-1 expression in the lateral striatum, following in vivo stimulation of the glutamatergic corticostriatal pathway. In addition, Elk-1 (one of ternary complex factors binding to SRE) and CREB transcription factors were activated (phosphorylated) simultaneously with ERK and Egr-1 induction. Egr-1 induction as well as Elk-1 and CREB phosphorylation were abolished by the inhibition of ERK activation. Thus transient activation of the MAPK/ERK signalling pathway targeted both CREB and Elk-1 transcription factors and, as a result, induced Egr-1 expression via CRE and SRE transcriptional regulation sites. Greenwood and Dragunow (2002) observed that the treatment of the SK-N-SH neuroblastoma cells with a cholinergic agonist, carbachol, led to an increased Egr-1 expression, preceded by the phosphorylation of CREB. In PC12 cells, the activation of protein-tyrosine kinase (such as Fps, Src) led to Egr-1 expression mediated by Ras (a small Gprotein) and Raf (a serine/threonine protein kinase) (Qureshi et al., 1991; Alexandropoulos et al., 1992). In these cells treated with NGF, Egr-1 induction was also mediated by Src, Ras and Raf (D'Arcangelo and Halegoua, 1993; Wood et al., 1993) perhaps by MAPK, with a possible involvement of JNK (Jun N-terminal kinases) as well as by PI 3-kinase (phosphatidylinositol 3-kinase). Calcium influx through voltage-dependent calcium channels into PC12 cells caused Src activation, formation of an Shc/Grb2 complex (an adaptor protein/growth-factor-receptor binding protein 2), leading to Ras and MAPK activation and subsequent induction of Egr-1 (Rusanescu et al., 1995). Lerea et al. (1995) observed an NMDA induction of Egr-1 in dentate granule neurons, which was dependent on phospholipase A2 and lipoxygenase. These data suggest that the induction of Egr-1 by different factors may be mediated through different MAP kinases. The induction of Egr-1 also depends on the activation of protein kinase A (PKA) and protein kinase C (PKC) (Mechta et al., 1989; Ginty et al., 1991; Vaccarino et al., 1992; Simpson and Morris,

1995). Possible signal transduction pathways involved in controlling Egr-1 expression are depicted in Fig. 32.

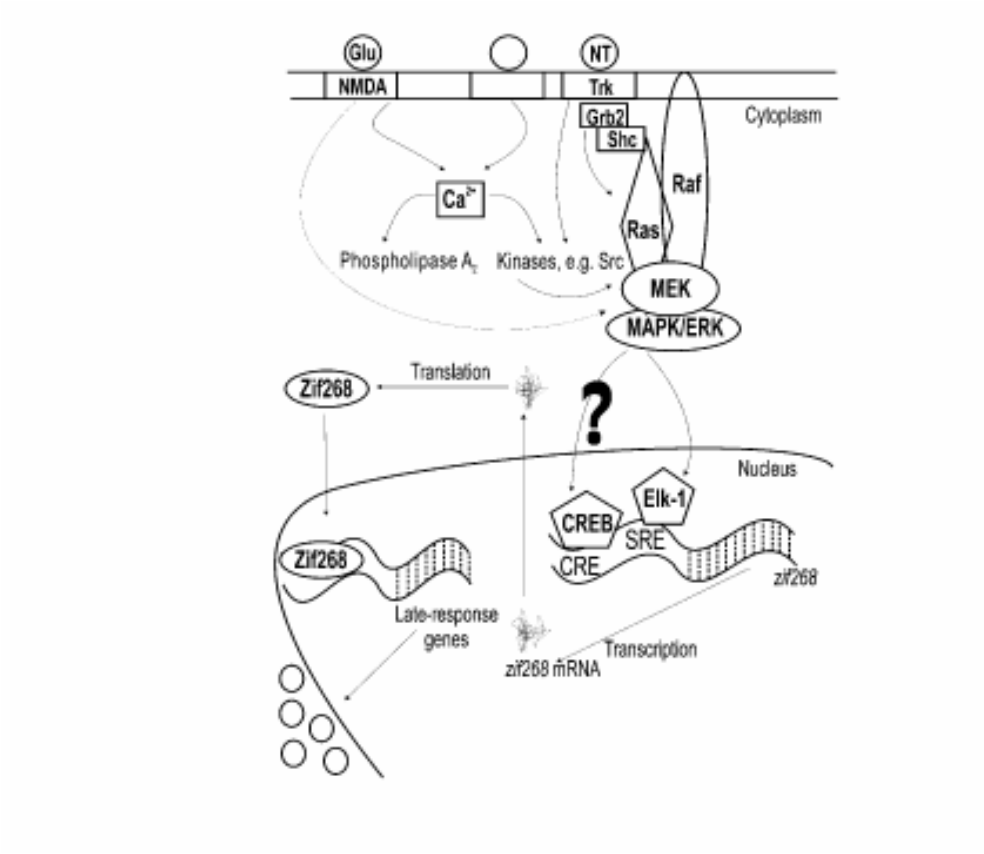


Fig.32 . Schematic representation of possible signal transduction pathways involved in controlling Egr-1 induction.

Egr-1, whose expression is linked to neural activity, is one of the inducible transcription factors (ITF) in the neuronal cells. Egr-1 can act in concert with other transcription factors to modulate (activate or repress) the expression of the target genes by binding to their promoters. The details of these mechanisms are yet to be clearly identified. The regulatory sequence for Egr-1 binding is present within the promoters of a host of different late-response genes, thereby enabling Egr-1 to exert a commanding influence on long-term cellular homeostasis by regulating the expression of such genes (Tab.III). Although several genes, whose expression in the brain can be controlled by Egr-1 , have been identified, most of them probably still remain unknown. Owing to the great

diversity of possible target genes, different potential functions of Egr-1 can be expected. It may be engaged in cell growth and differentiation, as well as in structural and metabolic changes in the mature neural cell. Even though discovering the target genes of Egr-1 is a valuable effort, it will be difficult to uncover its role without the knowledge of the mechanisms by which Egr-1 interacts with other transcription factors. Given many possibilities of such interactions in the cell, it is obvious that these mechanisms create a very efficient and sensitive, but potentially very complicated, regulatory system.

Genes identified as regulated by Zif268
Gene name
Synapsin I and II
Glutamate dehydrogenase
Neurofilament light chain
Nicotinic acetylocholine receptor (α7 subunit)
Adenosine deaminase
Glutamic acid decarboxylase
Thymidine kinase
Acetylocholinesterase
Phenylethanolamine *N*-methyltransferase
Apolipoprotein A1
Desmin
p75 nerve growth factor (p75 NGF) receptor
Transforming growth factor α1 (TGF-α1)
Platelet-derived growth factor A
Neuropeptide Y
Neuroserpin
Human low density lipoprotein receptor
Thymus-expressed chemokine (TECK)
IP-30
TNFα-related apoptosis inducing ligand (TRAIL)

Transcription factors interacting with Zif268
Transcription factor
cAMP responsive element binding protein binding protein (CBP/p300)
c-Fos
JunD
Nerve growth factor-induced gene B, nur77 (NGFI-B)
NF-κB
c/EBPβ

Tab III Genes regulated by Egr-1 and transcription factors interacting with Zif268

EGR-1 EXPRESSION IN ChAT-TRANSFECTED NEUROBLASTOMA

Egr-1 has been implicated in the differentiation of various cell types. In human myeloblastic leukemia HL60 cells, Egr-1 is transcriptionally silent but is activated when these cells are induced to differentiate along either the macrophage or the granulocyte lineage (Krishnaraju et al., 1995). Additionally, in other cell lines the expression of this gene is induced by molecule, such as retinoic acid, known to promote differentiation. Following retinoic acid treatment Egr-1 protein levels remain at high constitutive levels in differentiated P19 cells, (Mouse embryonic teratocarcinoma cell line) indicating a distinct role for this transcription factor in the induction and maintenance of differentiated state (Darland et al., 1991). In rat brain the levels of Egr-1 transcripts are induced by neuronal activation (Bhat et al., 1992**;** Lam et al., 1997) and thyroid hormone (Mellstrom et al., 1994; Pipaòn et al., 21-23), which is known to be required for adequate development of the dendritic arbour of different neuronal type (Lam et al., 1997). The morphological differentiation induced by serum withdrawal in N2A neuroblastoma cells can be blocked by the presence of Egr-1 antisense oligonucleotides in the culture medium. Stable transfection of N2A cells, overexpressing the Egr-1 protein, extend very long neurites much higher then in parental cell line (Pignatelli et al., 1999). The PC12 cell line respond to NGF by undergoing growth arrest and proceeding to differentiate towards a neuronal phenotype. NGF stimulation induce expression of Egr-1. The overexpression of NAB2, a corepressor of Egr-1, blocks the ability of NGF to induce differentiation of PC12 (Qu et al., 1998). The same result could be obtained with the overexpression of Egr-1 binding domain, because it acts like a selective antagonist of Egr (Levkovitz et al., 2001).

These data are very suggestive of an in vivo role for Egr-1 in the late stages of neuronal differentiation, when neuronal processes begin to develop and connections among cells begin to be established. In support of this idea is also the presence of Egr-1 binding site in the promoters of several neuronal genes (Tab III), such as synapsin I, Synapsin II, Synaptobrevin II and neurofilament.

In the present work, I analysed the role of Egr-1 in neuroblastoma differentiation induced by ACh.

It was previously demonstrated that Egr-1 protein is expressed in ChAT positive clones but not in N18TG2 parental cells (Fig.6) and it was shown that EGR family expression is increased by mAChR activation in different neuronal cells (Ebihara and Saffen, 1997; Nitsch et al., 1998; Von der Kammer et al., 1998).

In order to demonstrate that Egr-1 expression, in ChAT transfected clones, is due to activation of muscarinic receptors, I have analyzed by western blotting the protein levels after stimulation of N18TG2 and 2/4 ChAT positive clone with muscarinic agonist and antagonist.

As shown in Figure 33 Egr-1 protein was not detectable in N18TG2 nuclear extract of untreated cells but when the cells are stimulated with CCh, which is a muscarinic receptor agonist, a band is recognized at the expected molecular weight. The CCh effect is strongly reduce by the pre-treatment with atropine, a muscarinic receptor antagonist. On the other hand 2/4 ChAT-positive clone costitutively expresses Egr-1 and CCh stimulation does not further increase the signal. Egr-1 band almost disappears when the cells are pre-treated with atropine.

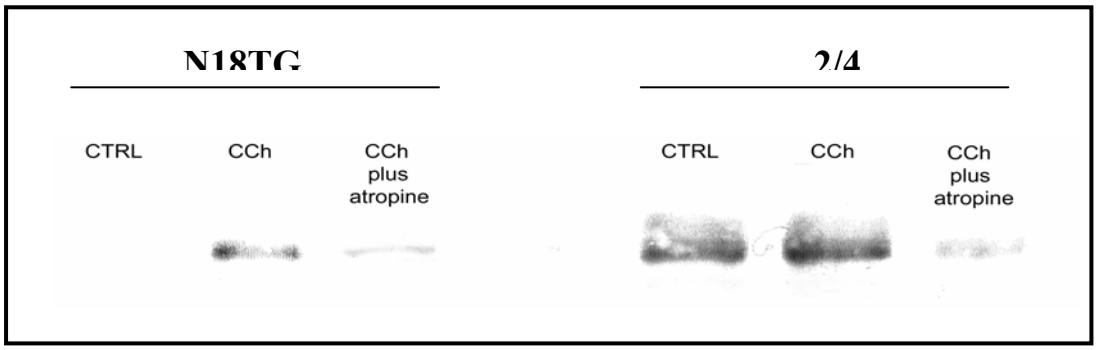


Fig.33 Western Blotting on nuclear extract samples. The cells are treated with CCh 10^{-4}M and/or atropine 10^{-6}M. N18TG expresses Egr-1 only after CCh stimulation, this effect is abolished by pre-treatment with atropine. 2/4 ChAT positive clone shows Egr-1 band at basal condition, CCh stimulation does not increase the expression level. Level of Egr-1 is lower after pre-treatment with muscarine. Samples obtained from the same number of cells were loaded onto SDS-PAGE slabs.

To analyze the effect of colinergic stimulation on the Egr-1 mRNA expression, I stimulated, after six hours of starvation, N18TG2 cells with CCh (10^{-4}M) for different times: 0 (CTRL), 30', 60', 90',120'. RT-PCR (reverse transcriptase PCR) analysis (Fig 34) shows that, after the addiction of carbachol the level of Egr-1 mRNA expression increases to reach a maximum after 60' and then decreases, whereas there's no significative change in GAPDH mRNA level, the endogenous amplicone used to normalize the signal.

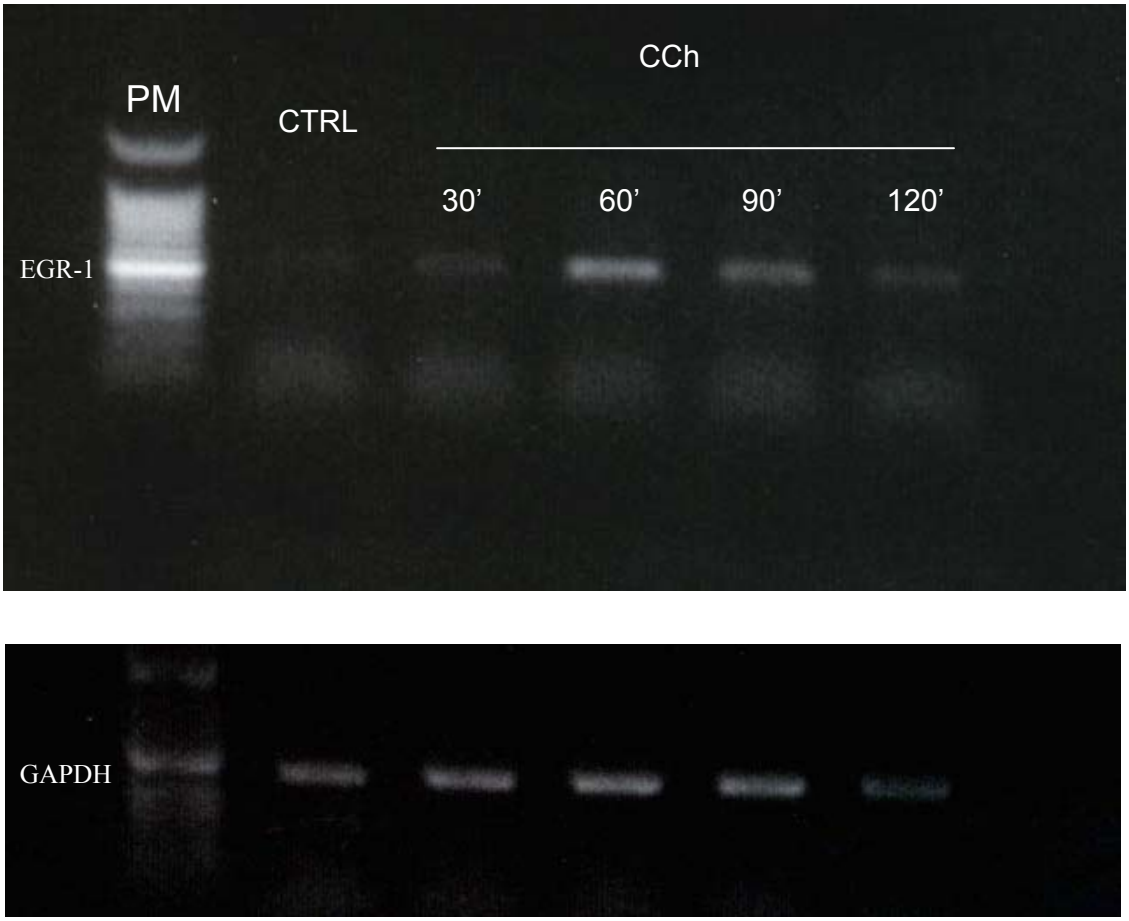


Fig.34 RT-PCR using mouse Egr-1 and mouse GAPDH primers in N18 TG2 cells after CCh stimulation for different time

Considering RT-PCR performed with a defined number of cycle is only a semi-quantitative technique, to confirm the time course and to obtain a more quantitative evaluation of Egr-1 mRNA expression, I analyzed the samples using a real-time RT-PCR.

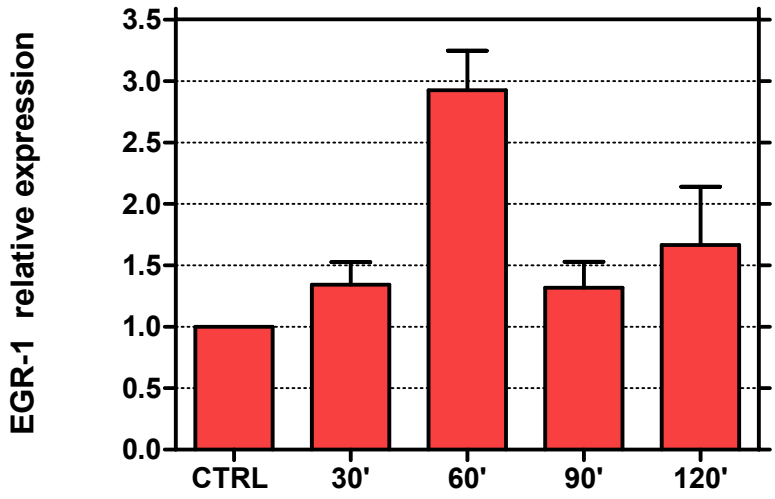


Fig.35: Real time RT-PCR analysis. Egr-1 mRNA expression in N18TG2 cells stimulated with 10^{-4}M CCh for different times. The expression is referred to the level in untreated cells. (CTRL). Values are the mean ± S.E.M. of about six independent experiments.

Data presented in Figure 35 confirm that, in N18TG2 cells, mRNA expression induced by CCh stimulation is depending on treatment time. The maximum is reached after 60' of stimulation but the expression level decreases after 90' or 120' of stimulation. This data are consistent with results obtained in other cell types where muscarinic stimulation induces a strong but temporary induction of Egr-1 mRNA (Von der Kammer et al., 1998; Katayama et al., 1993).

To analyze the specific involvement of muscarinic receptors subtypes on Egr-1 mRNA induction, a pre-treatment N18TG2 cells with different muscarinic antagonist for 30'

followed by stimulation with CCh for an hour, was performed. mRNA levels were evaluated by real-time RT-PCR.

In this experiment I used Pirenzepine as antagonist for M1, Gallamine for M2, 4-DAMP for M3,Tropicamide for M4 and atropine as antagonist of all different muscarinic receptor subtypes.

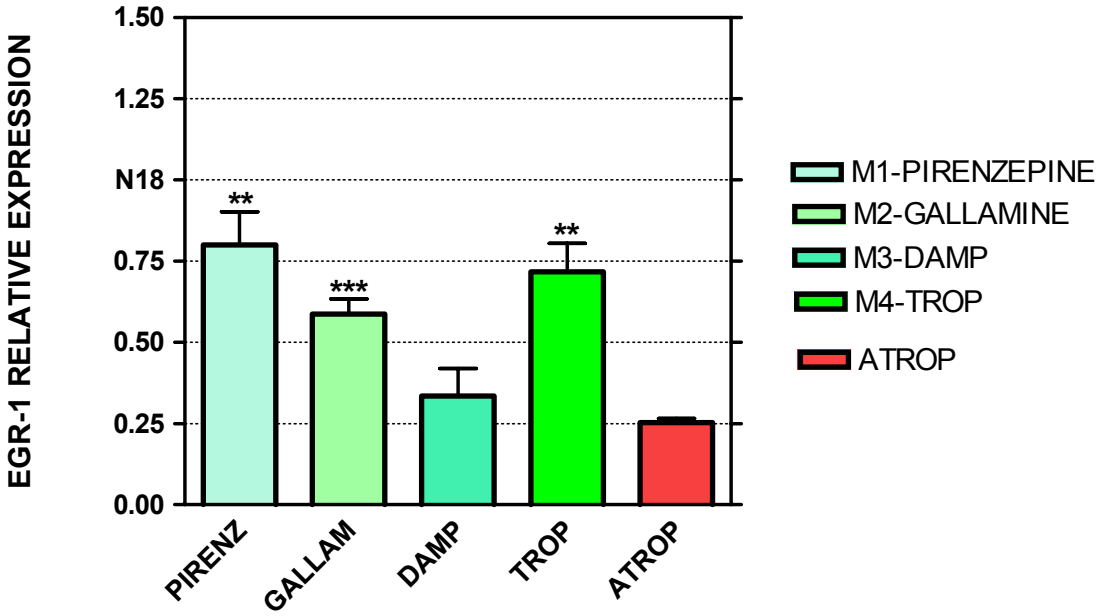


Fig.36: Real time RT-PCR analysis. Egr-1 mRNA expression in N18TG2 cells stimulated with 10^{-4}M CCh after pre-treatment with muscarinic antagonist. The expression is referred to the level in untreated N18TG2 cells. Values are the mean ± S.E.M. of at least six independent experiments. **P < 0.001 *** P< 0,0001

Analysis of mRNA expression (Fig.36) shows that 4-DAMP is able to reduce egr-1 expression at levels similar to those obtained by atropine pre-treatment, while the other antagonists were less efficient suggesting that the most important effect on egr-1 trascription is due to signal transduction pathway activated by M4 receptors.

It has been reported that Egr-1 is implicated in cellular differentiation (above mentioned references) and I have demonstrated that muscarinic receptors activation modulate egr-1 transcription also in ChAT-transfected neuroblastoma cells.

In order to study the relationship between, differentiation induced in ChAT transfected clones by ACh mucarinic stimulation and egr-1 expression, I transfected N18TG2 cells with a construct containing the cDNA for mouse egr-1. egr-1 stable transfected clones were selected for neomycin resistance and were characterized for their ability to express egr-1. The stable expression of egr-1 gene was detected by RT-PCR. The expression of egr-1 gene in transfected clones appeared higher than in N18 TG2 cells and in 2/4 ChAT-transfected clone (Fig.37).

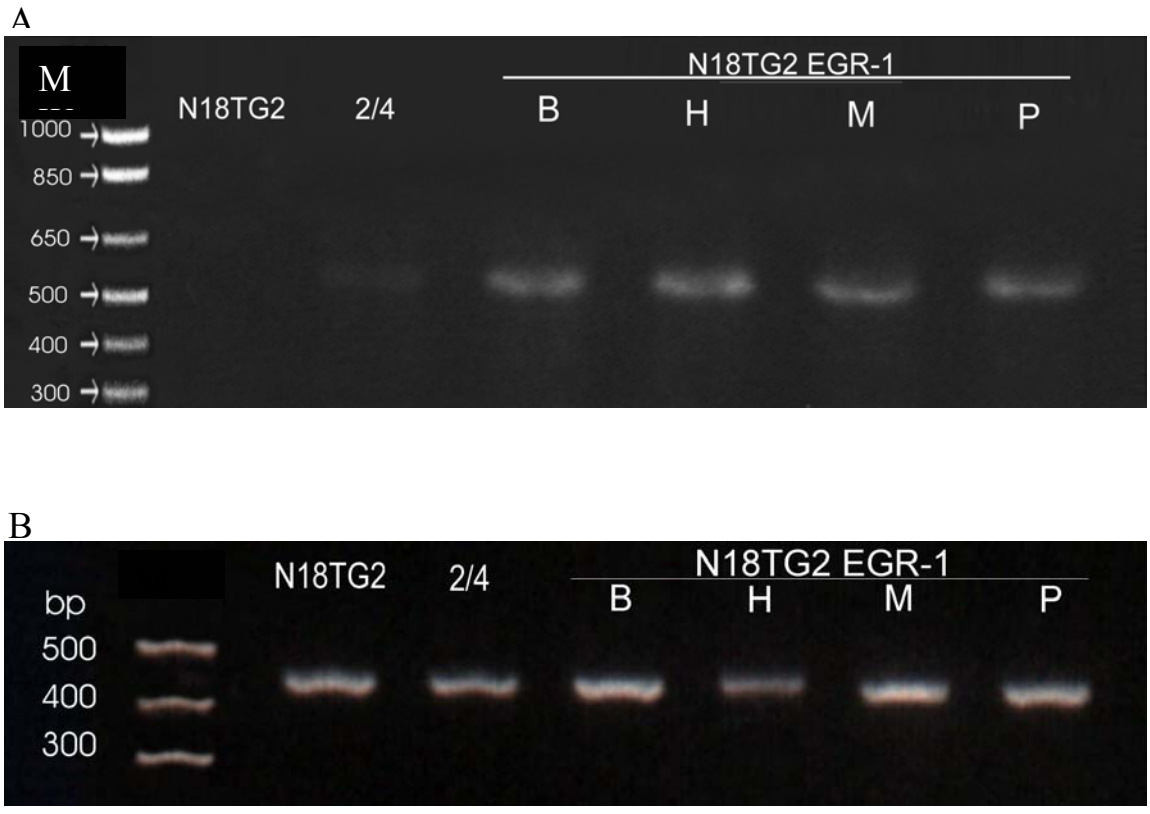


Fig.37: RT-PCR using mouse Egr-1 (A) and mouse GAPDH primers (B) in N18 TG2 cells, in 2/4 ChAT transfecte clone, and Egr-1 transfected clones.

The Egr-1 transfected clones have a morphology more similar to ChAT transfected clones than to N18TG2 parental cells (Fig. 38).

N18TG2 neuroblastoma cells is characterized by a roundish immature morphology, with cells bearing short processes (Fig.38 A). EGR-1 transfected clones (Fig B-C) shows a significantly different morphology. They in fact display longer and more branched fibers, while the cell body appears flattened on the culture dish. They appear more similar to 2/4 transfected clone (Fig.38 D).

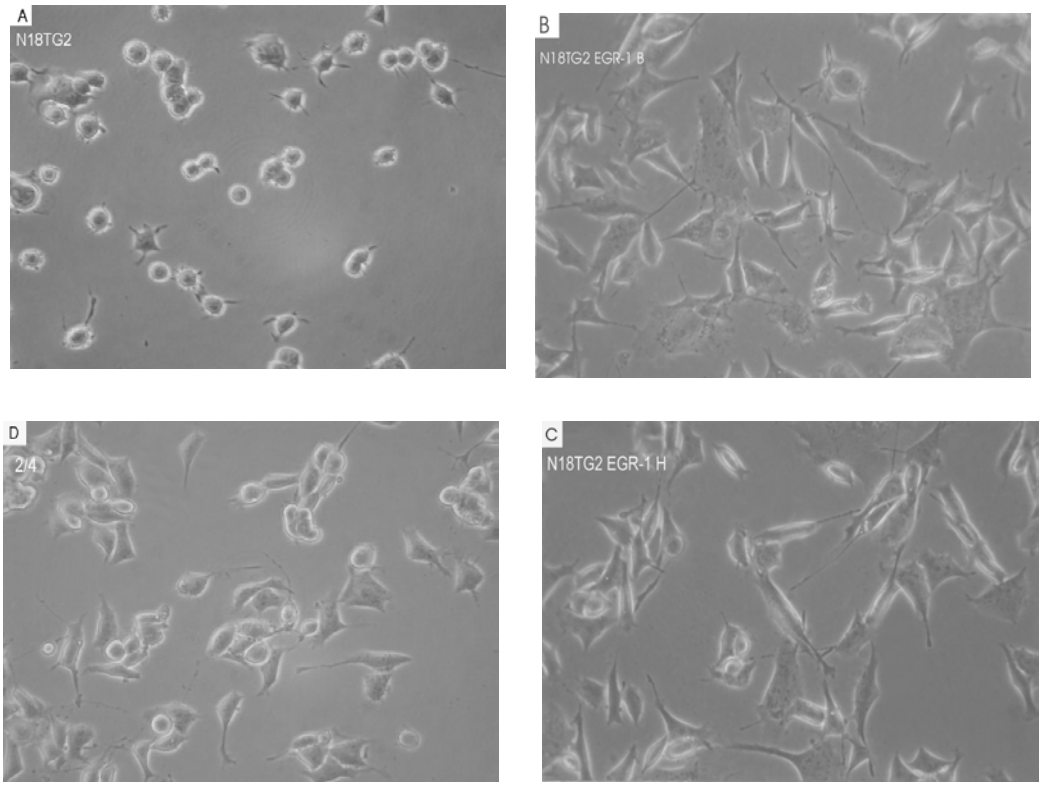


Fig.38: Phase-contrast micrographs of N18TG2, 2/4 ChAT-transfected clone and Egr-1 transfected clones cultured in non differentiating condition. Magnification 40X

It is possible to point out this difference by immunostaining with an anti βIII-tubulin monoclonal antibody.

βIII-tubulin belongs to a specialized class of tubulins specific for neurons, is considered to be one of the earliest neuron-associated cytoskeletal marker proteins [Moody et al., 1989; Lee et al., 1990b; Easter et al., 1993; Katsetos et al., 1993]. 1993]. Its expression, either immediately before, or during terminal mitosis, (Moody et al., 1989; Easter et al., 1993; Katsetos et al., 1993; Haendel et al., 1996), suggests that βIII-tubulin may be regulated by transcription factors necessary for neuronal lineage commitment and early morphological differentiation [Dennis et al., 2002].

The existence of three critical steps along the pathway of cytoskeletal specialization in differentiating neurons was recently demonstrated: initially, when neuroepitelial cells activate microtubular reorganization towards neurons, upregulating class βIII-tubulin expression; next, a stage when βIII-tubulin is citoplasmically accumulate in neuroblasts; and finally, a stage when most βIII-tubulin is incorporated into microtubules, and thus, indicating, when microtubular reorganization starts in mature neuron (Fanarraga et al. 1999).

Fig. 39 shows representative microscopic fields of N18TG2 cells, 2/4 ChAT transfected clone and one of the Egr-1 transfected clone, cultured in non differentiating condition and immunostained with an anti- βIII - tubulin monoclonal antibody .

In N18TG2 cells, which are representative of an immature stage of development, βIII-tubulin has a cytoplasmic and perinuclear localization. In transfected clones, both ChAT and Egr-1 transfected clones, βIII-tubulin seems to be peripherally localized with stronger spots along fibers, this is clear at higher magnifications (Fig.39 panels B,C).

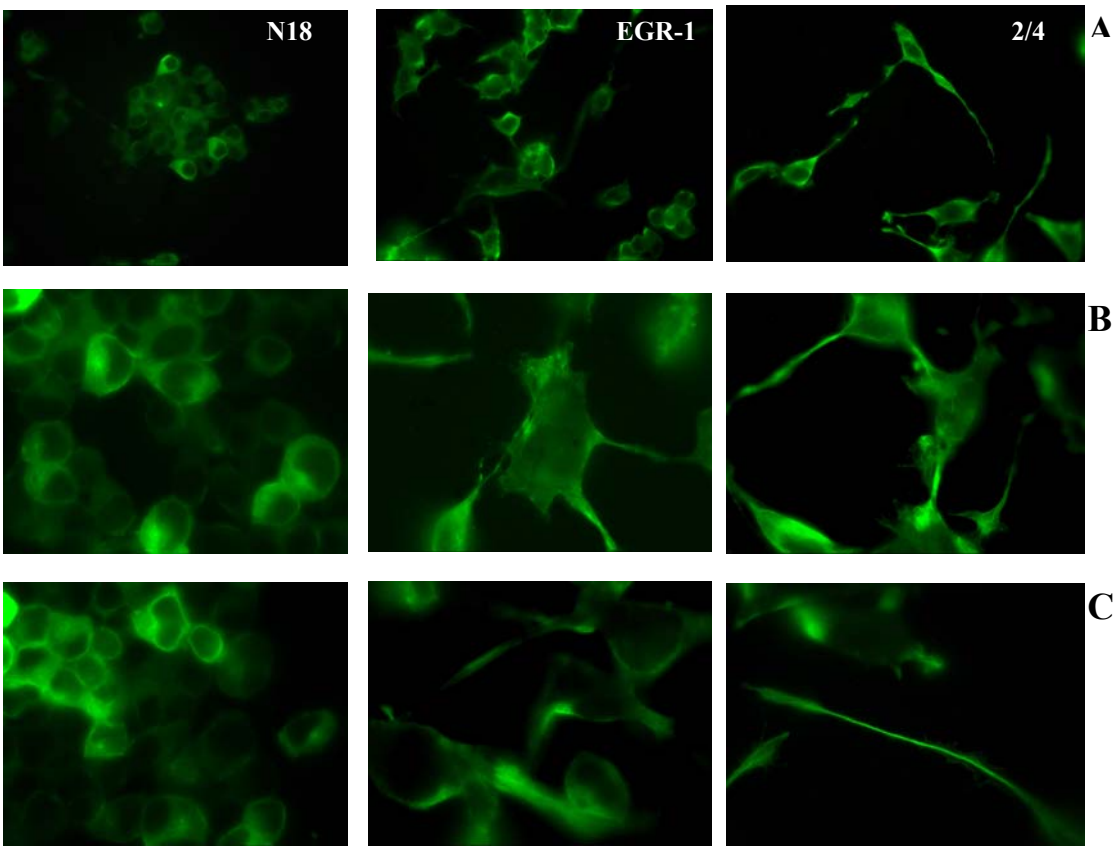


Fig. 39: : Immunostaining for βIII - tubulin in N18TG2 cells, 2/4 ChAT transfected clone and one of the Egr-1 transfected clone cultured in non differentiating condition. Panels A magnification: 40X; Panels B and C: magnification 100X.

One of the most important characteristic of ChAT transfected clones is the higher ability to extend fibers compared to N18TG2 parental cells; and it was also demonstrated that it is depending on muscarinic receptors activity (chapter 2). To test the possible Egr-1 involvement in neurite outgrowth I cultured the Egr-1 transfected clones for 4 days in the presence of 1 mM dibutyryl cyclic AMP (dbcAMP), as differentiating agent. Immunostaining for βIII - tubulin (Fig 40) shows that Egr-1 clones display an higher ability to grow longer and more branched fibers with respect to N18TG2 cells, moreover tubulin, probably incorporated into microtubules, is localized mainly in the fibers (see insect).

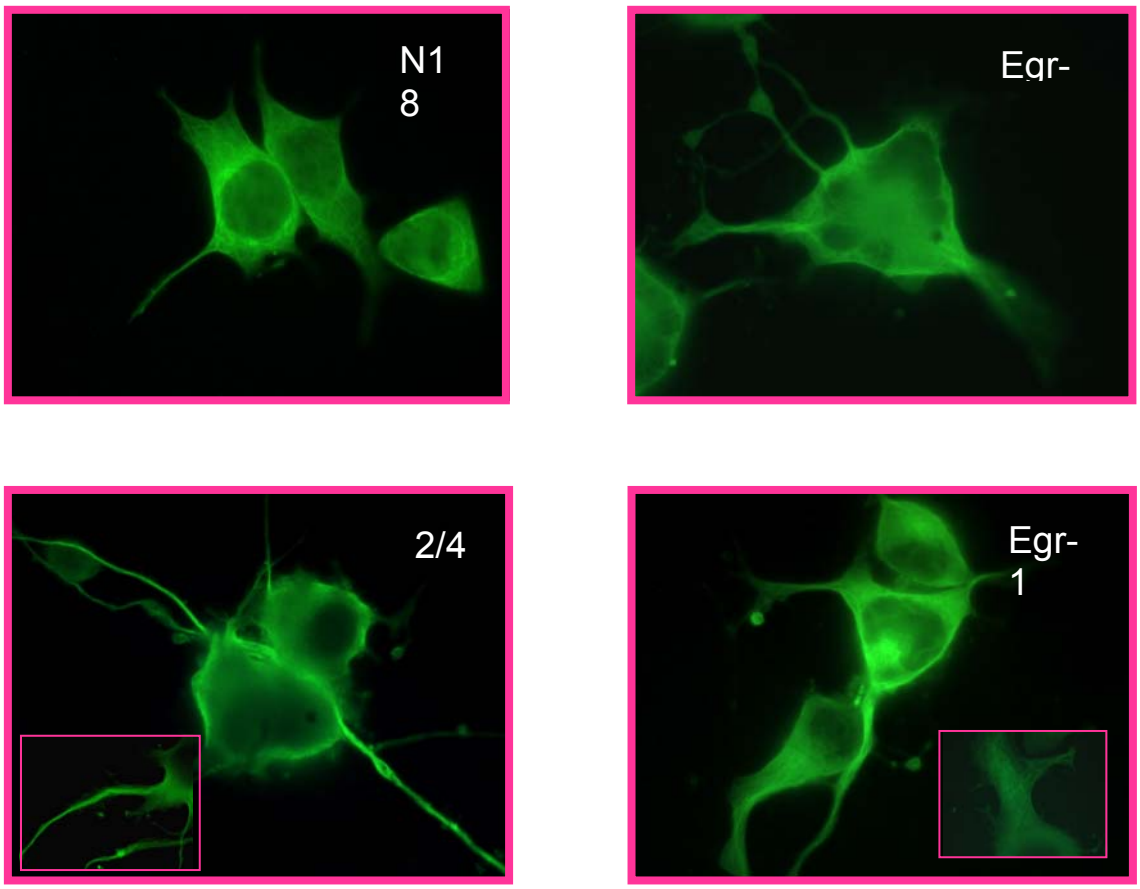


Fig.40: Immunostaining for βIII - tubulin in N18TG2 cells, 2/4 ChAT transfected clone and one of the Egr-1 transfected clone cultured for 4 days in the presence of 1 mM dibutyryl cyclic AMP (dbcAMP), as differentiating agent. Magnification 100X.

For a more accurate evaluation of morphological differentiation I performed a morphometric analysis. Figure 41 shows the results of the analysis carried out on the parental clone and on two transfected clones. Cell numbers and fiber lengths per microscopic field were evaluated and considering that cell number per microscopic field is not constant, fiber length was normalized to cell number, and fiber number per cell was also reported. A significant increase of both fiber number/cell and length/cells were observed in Egr-1 transfected clones with respect to N18TG2 cells and 2/4 ChAT-transfected clone. Differences between 2/4 clone and each of the EGR-1 transfected clones were always significant ($P < 0.001$) by nonparametric Mann-Whitney test.

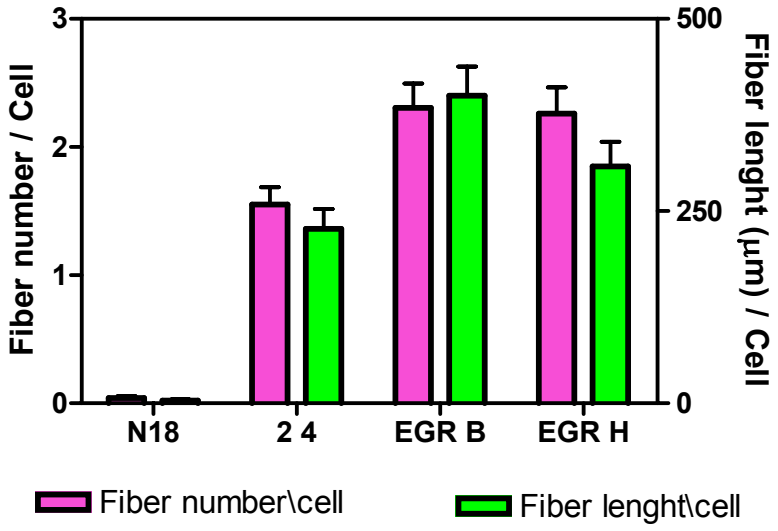


Fig.41 Quantification of fiber outgrowth, per microscopic field in N18TG2 cells, 2/4 ChAT transfected clone and one of the Egr-1 transfected clone maintained for 4 days in the presence of 1 mM dibutyryl cyclic AMP (dbcAMP), as differentiating agent and normalized to cell number present in each microscopic field. Micrographs of randomly selected areas of the culture dishes were taken from five independent 60-mm dishes (22 fields/ dish); values are the mean ±S.E.M. of at least 110 observations for each culture condition. Differences between 2/4 clone and each of the EGR-1 transfected clone were always significant ($P < 0.001$) by nonparametric Mann-Whitney test,

These experiments show that egr-1 expression in neuroblastoma cells, is modulated by muscarinic receptors, therefore the higher basal egr-1 level observed in ChAT-transfected clones could be explained by ACh constitutive autostmulation of muscarinic receptors. Furthermore the egr-1 overexpression in N18TG2 cells induce neurite outgrowth similar to those observed in ChAT-transfected clones, so it is possible to speculate that egr-1 is a key regulator differentiation induced by ACh.

CHAPTER 7

REST

Neurogenesis, a process central to vertebrate development, requires the acquisition of neural cell fates within the developing nervous system and, in parallel, maintenance of non-neural cell fates outside the nervous system (Edlund and Jessell 1999).

These two complementary events must be coordinated precisely for correct formation of the nervous system. Furthermore, neurogenesis requires that, within the developing nervous system, only post-mitotic neurons will express neuronal genes, because neural stem cells or progenitors have not yet committed to a neural lineage (Temple 2001). These requirements raise the fundamental question of how neuronal gene chromatin is epigenetically programmed in different cellular contexts.

For the establishment of epigenetic modifications representing distinct stages of differentiation, chromatin modifiers, such as DNA methyltransferases, histone methyltransferases and histone acetyltransferases, are recruited to specific genomic loci by DNA binding proteins, either repressors or activators (Peterson and Laniel 2004). A compelling candidate for orchestrating epigenetic events is the DNA binding protein, REST (RE1 silencing transcription factor; also called NRSF). REST was discovered in 1995 as a repressor of neuronal genes containing a 21 bp conserved motif, known as RE1 (repressor element 1 or NRSE) (Chong et al., 1995; Schoenherr and Anderson, 1995). The NRSE is a 21-bp DNA sequence (Figure 42) originally isolated from the promoters of the genes that express, primarily in neurons, the type II sodium channel *(*Maue et al., 1990; Kraner et al., 1992*)* and SCG10 (Mori et al., 1990; Mori et al., 1992). Numerous genes with expression restricted mainly to the nervous system contain an NRSE motif. A Blast search of the Celera mouse database identifies at least 324 gene promoter with NRSE consensus sequences, and functional analysis of cloned promoter sequences reveal that NRSE consensus sequences reside in a diverse set of genes of interest to neuroscience (Tab. IV).

Gene product	Species[a]	Gene product	Species[a]
Acetylcholine receptor, β2 nicotinic	RM[b]H[b]	Na+ channel PN3 (TTX resistant)	R
Acetylcholine receptor, M4 muscarinic	R[b]	Na+ channel Type II	R[b]
Arginine vasopressin	H[b]	Na+ channel type X, α subunit	R
Atrial natriuretic peptide	R[b]	Na+, K+-ATPase, α3-subunit	M[b]
BDNF	RM[b]	Neurexin IIIα	H
Ca2+ ATPase, calmodulin-sensitive	R	Neuronal proliferation differentiation	
Ca2+ channel (T-type, α1H)	M	control-1 protein (Npdc1)	M
Cadherin 13	M	Opioid receptor (mu 1)	RH[b]
Calbindin	M[b]	Oxysterol binding protein 2	H
CaM kinase	R	Pentraxin 2, neuronal	M
CaM-Kinase II inhibitor α	R	Pentraxin receptor, neuronal	M
CART protein	R	Prolactin-releasing peptide receptor	R
catenin II αN	M	Protocadherin 8	RH
Cdc42 GTPase-inhibiting protein	M	Rab3D	M
CDK5 activator	M	RAN binding protein 16	H
Choline acetyltransferase	R[b]	Rap2 interacting protein 8	H
Connexin 57	M	Rb-8 neural cell adhesion protein	M
Connexin 62	H	SCG10	R[b]
Corticotropin releasing hormone	RM[b]H	Sec 6	M
Dopamine-β-hydroxylase	H[b]	Secretogranin II	RM
Dynamin 1	M[b]	Serotonin receptor 5-HT1A	R
Dynein, heavy chain	M	Serotonin transporter	M
$GABAA_A$ receptor δ subunit	R[b]	Somatostatin	R
$GABAA_A$ receptor γ2 subunit	H[b]	Somatotropin hormone	H
Gap junction protein α10	M	Spermidine synthase	H
Gephyrin	H	STAT6	M
Glutamate receptor GluR2	R[b]M	Synapsin	H[b]
Glutamate receptor KA2	RM	Synaptophysin	R[b]
Glutamate receptor KA5	RM	Synaptotagmin IV	RMH
Glutamate receptor NMDAR1	R[b]H	Synaptotagmins 6 and 13	R
Glutamate receptor NMDAR2B	R[b]	Syntaxin binding protein 1	MH
Glutamate receptor NMDAR2C	R[b]	Taste receptor type I (T1r3)	M
Glycine receptor α1	H	TNF receptor 6b	H
Glycine receptor α3	R[b]H[b]	Toll-like receptor 8	H
Huntingtin associated protein	RMH	Transcription factor HES-3	RM
Insulin receptor	H	Transcription factor NeuroD	R
K+ channel β1 (Shaker related)	M	Transcription factors E2F and POU4F3	H
K+ channel KCNQ1	R	TRP3 receptor-activated cation channel	M
L1 neural cell adhesion molecule	MH[b]	Tyrosine kinase Ack	M
LIM-3 homeoprotein	M	Tyrosine kinase ALK	M
Melatonin receptor 1B	H	Vanniloid receptor-like protein 1	RM

[a]R, M, and H refer to rat, mouse, and human genes, respectively. [b]Functional silencing activity of RE1-like element was demonstrated.

Tab IV: Selected NRSE-containing genes of interest to neuroscience

NRSF is a member of the GLi-Krüppel family of transcriptional zinc-finger proteins, it contains a cluster of eight zinc-finger repeats near its N-terminus, followed by a region rich in basic amino acids, a cluster of six proline-rich repeats, and a single zinc finger

near the C terminus (Fig.42) (Tapia-Ramirez et al.,1997). A number of splice variants of NRSF exist, including REST4 (Figure), a neuron-specific variant lacking the C-terminal repression domain (Timmusuk et al., 1999).

Deletion and GAL-4 analyses have demonstrated two independent repressor domains of NRSF, one at each terminus (Tapia-Ramirez et al., 1997; Thiel at al., 1998).

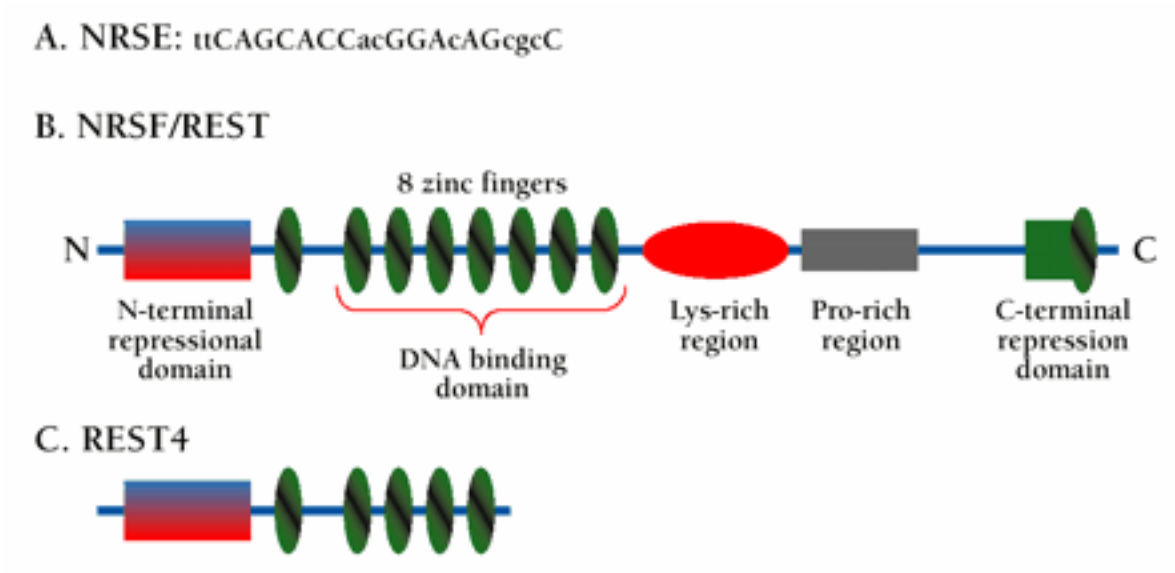


Fig.42: The NRSE–NRSF system. A. Consensus sequence of the NRSE deduced from functional analysis of silencer elements in nineteen genes (57). Nucleotides in lower case vary frequently among functional silencer elements.
B. Domains of the NRSF protein (also known as REST).**C.** REST4 is a C-terminally truncated splice variant of the NRSF protein.

The amino terminal repressor domain interacts with mSin3, a corepressor found in all eukaryotes that recruits histone deacetylases (HDACs) (Grimes et al., 2000; Huang et al., 1999). The mSin3–HDAC complex, however, is primarily associated to a dynamic

mode of repression that can alternate between repression and activation and, therefore, by itself, would probably be inadequate for long-term silencing of neuronal genes. This conundrum was solved by the discovery of the corepressor CoREST, which interacts directly with the carboxy terminal repressor domain of REST (Andres et al., 1999; Ballas et al., 2001) and, like to mSin3, estabilish complexes with HDACs (Ballas et al., 2001;You et al., 2001) . Interestingly, unlike mSin3, CoREST is present only in organisms with a nervous system (Dallman et al.,2004), pointing to CoREST as a more specialized corepressor. Several recent studies indicate that the REST–CoREST complex recruits chromatin modifiers for long-term silencing of neuronal genes (Lunyak et al., 2002; Roopra et al., 2004) (Figure 1a). Specifically, CoREST can form immuno-complexes not only with HDACs but also with the histone H3 lysine 9 (H3–K9) methyltransferase G9a (Shi et al., 2003) and with the newly discovered histone H3 lysine 4 (H3–K4) demethylase LSD1 (Shi et al., 2004) (that is also known as KIAA0601 or BHC110) (Hakimi et al., 2002) both of which mediate modifications associated with gene silencing. These histone-modifying enzymes are required for REST–CoREST silencing in non-neuronal cells (Ropra et al., 2004; Shi et al., 2003; Shi et al., 2004).

Furthermore, CoREST recruits to the REST–RE1 site other silencing machinery, including the methyl DNA-binding protein MeCP2 and the histone H3–K9 methyltransferase SUV39H1 (Lunyak et al., 2002). Heterochromatin protein 1 (HP1), which causes condensation of chromatin and is associated with histone H3–K9 methyltransferases, is also present on the neuronal gene chromatin (Lunyak et al., 2002) specifically on the RE1 region (Roopra et al., 2004). The effects of these modifications result in histone deacetylation, the absence of H3–K4 methylation, the presence of H3–K9 methylation, which creates binding sites for HP1 and condensation of the targeted chromatin (Fig.43). Additionally, the recruitment of silencing machinery by REST–CoREST might result in the propagation of silencing complex along a large chromosomal trait containing several neuronal genes that do not have their own REST

binding sites (Lunyak et al., 2002), suggesting a relationship between higher order chromatin structure and patterns of gene expression.

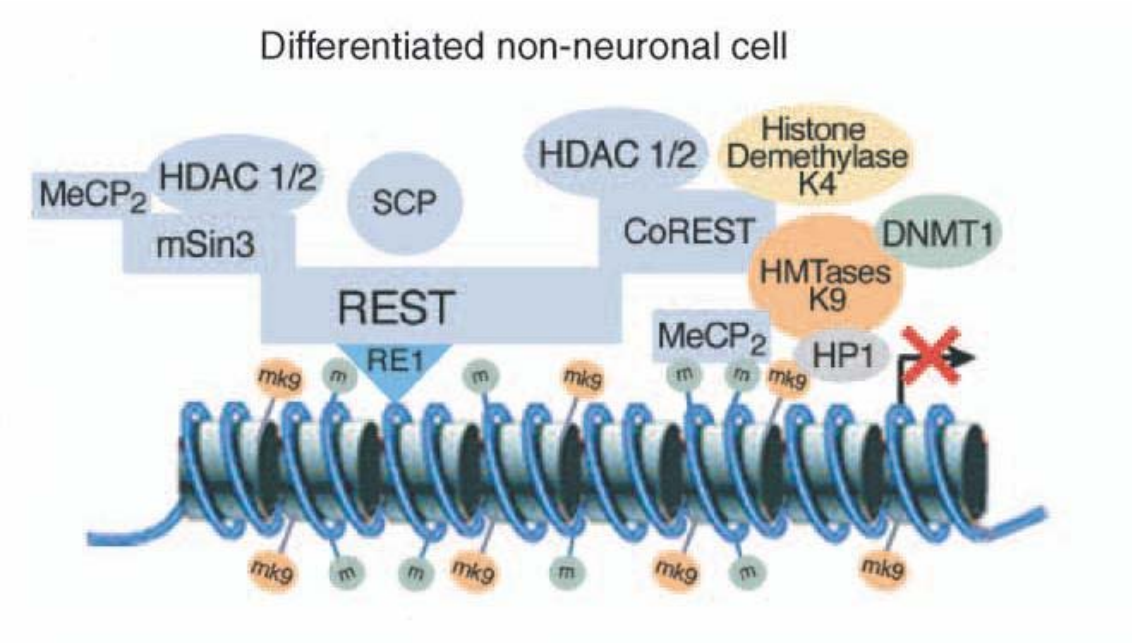


Fig.43: REST–CoREST orchestrates differential epigenetic mechanisms to inactivate neuronal genes in non-neuronal cells. REST–CoREST recruits a silencing complex to neuronal genes in terminally differentiated non-neuronal cells. Neuronal gene chromatin is a substrate for chromatin modifying enzymes including histone deacetylases (HDAC 1,2), histone H3 lysine 4 demethylase (histone demethylase K4), and histone H3–K9 methyltransferases (HMTases K9). Methylated lysine 9 residues (mK9) are binding sites for heterochromatin protein 1 (HP1), which causes chromatin condensation. The REST binding site (RE1) and adjacent region is methylated at CpGs (m) and associated with the methyl DNA binding protein MeCP2. MeCp2 is also associated with Sin3–HDAC complexes. DNA methyltransferase 1 (DNMT1) is recruited to the methylated RE1 site. The small carboxyl terminal domain (CTD) phosphatase (SCP) might block RNA polymerase II activity

The silencing of neuronal gene chromatin in differentiated non-neuronal cells is stable, inheritable and endures the lifetime of the animal. By contrast, embryonic stem cells (ES), although also non-neuronal, still have the capacity for self-renewal and differentiation along different cell lineages.

Arising the question whether these two fundamentally different non-neuronal cell types utilize similar epigenetic mechanisms to suppress the same neuronal genes. Recent studies suggest that erasure and reprogramming of chromatin does not occur and that neuronal gene chromatin in ES and progenitor cells is programmed to stay in a repressed state that is none-the-less poised for expression (Ballas et al., 2005). In this contest REST is bound to the RE1 motif and in spite of this it was demonstrated that RNA polymerase II (Pol II) is present on RE1 sites in the 5' untranslated regions of several neuronal genes, resulting in a very low transcript levels (Ballas et al., 2005). It is conceivable that epigenetic modifications, associated with RE1 sites of neuronal genes in stem cells, that point to an inactive chromatin state that is poised for subsequent activation may be exsist. Interestingly RE1 motif and surrounding sequences in neuronal genes are not methylated; whether the absence of DNA methylation prevents recruitment of specialized machinery necessary for long-term silencing typical of differentiated non-neuronal cells remains to ascertain. Moreover recent studies have shown that, in P19 embryonal carcinoma stem cells, a family of small Pol II carboxyl-terminal domain phosphatases (SCPs) are probably recruited by REST to the RE1 sites of neuronal genes (Yeo et al., 2005). Phosphatase inactive forms of SCP interfere with REST function and promote neural differentiation (Yeo et al., 2005). One of the roles of the SCPs in ES cells might be to contribute to a poised state by maintaining lower levels of Pol ll activity on neuronal genes. On the other hand SCPs were also found in REST complexes in differentiated non-neuronal cells (Yeo et al., 2005). Although in these cells Pol ll is not associated with neuronal genes, SCPs might provide additional security for the silenced state. Taken together, these findings suggest that the core REST complex establishes a distinct set of epigenetic marks by recruiting different chromatin modifying proteins in differentiated non-neuronal and ES cells.

How does the inactive yet permissive chromatin state escape being converted to an active state? Several diverse enzymatic activities might help to maintain neuronal genes in a state of suspended animation. For example, HDACs, which reduce of acetylated

histones levels; SCPs, which might reduce activity of Pol II; and the histone H3–K4 demethylase LSD1, which is present in CoREST immuno-complexes of ES cells might all contribute to maintain the poised state. Finally, microRNAs have recently been proposed as key players in the self-renewal of stem cells (Cheng et al., 2005). These small non-coding RNAs might complement the activities of chromatin modifiers, either by blocking translation of neuronal mRNAs or by selective degradation of neuronal transcripts.

There are two separate models to explain REST regulation of neuronal genes during embryonic and adult neurogenesis (Fig.44). In both embryonic and adult neural stem cell neuronal genes are actively repressed by REST repressor complexes. During embryonic differentiation (Fig.44 left), REST is removed at two distinct stages, first at the dividing progenitor stage by proteosomal degradation (broken pink oval), and then at terminal differentiation (mature neuron). In the mature neuron, REST corepressors are dissociated from RE1 but still present, chromatin is relaxed and neuronal genes are expressed. During cortical differentiation, degradation of REST protein precedes its dismissal from RE1 sites (Ballas et al., 2005). The identity of transcriptional activators that might function after REST departure is not known. During differentiation of adult neural stem cells (Fig. 44 right), REST remains on neuronal gene chromatin, and a small double stranded non-coding RNA containing RE1 (green wavy line between RE1 and REST), converts REST from a repressor to an activator by dismissal of corepressors and recruitment of coactivators. Whether this dsRNA plays a role in differentiation of neural stem and progenitor cells during development has yet to be ascertain. Epigenetic regulation of neuronal gene chromatin by REST is fundamental for maintaining stem cells in an undifferentiated pluripotent state and for proper acquisition of neural fate during neurogenesis. The disappearance of REST during cortical neurogenesis appears to be a prerequisite for normal neuronal function in the adult. These observations have led to the proposal that REST levels must be downregulated in order to permit acquisition of the terminally differentiated neuronal phenotype. The PC12 cell line provided an excellent model to test REST downregulation during neuronal differentation. By generating stable lines that express REST under the control of an inducible promoter, it was possible to induce REST prior to the treatment with a specific growth factor signal (NGF) that cause terminal differentiation. The presence of

REST completely blocked induction of sodium channel mRNA in response to NGF. Consistent with the effects of REST on sodium currents, persistent expression of REST reduced the growth of neurites in response to NGF (Ballas et al., 2001). Although the specific REST target genes for neurite growth are not known, the suppression is consistent with the large number of proteins important to neuronal physiology that are known to be regulated through the REST repressor pathway (Schoenherr et al., 1996).

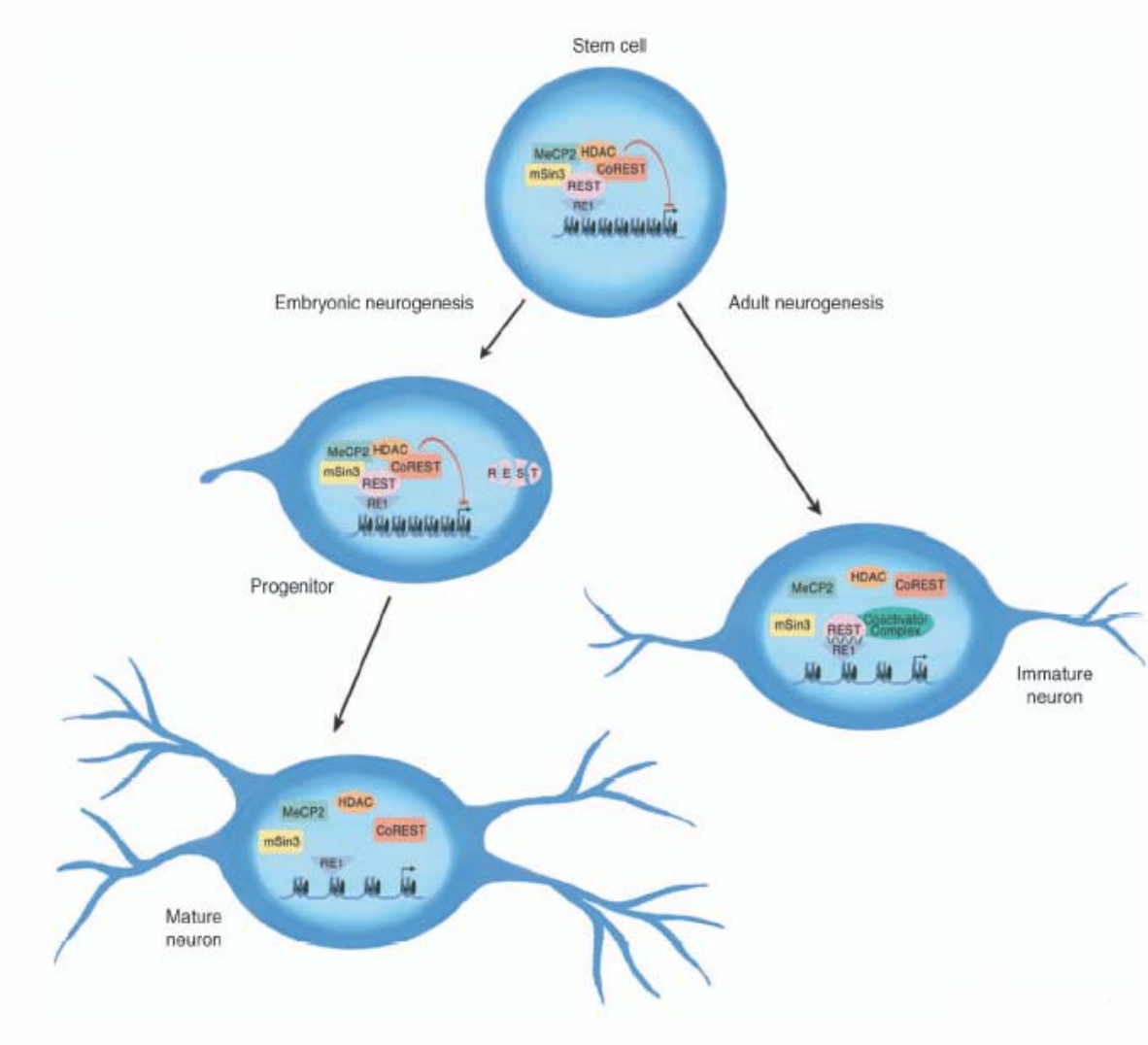


Fig.44: REST regulation of neuronal genes during embryonic and adult neurogenesis.

REST IN ChAT-TRANSFECTED NEUROBLASTOMA CELLS

Nervous system development relies on a complex signalling network to engineer the orderly transitions that lead to the acquisition of a neural cell fate. Progression from the non neuronal pluripotent stem cell to a restricted neural lineage is characterized by distinct patterns of gene expression, in particular the restriction of neuronal gene expression to neurons. REST, which is a trascriptional repressor, restrict neuronal traits to neurons by blocking their expression in non neuronal cells.

N18TG2 cells are representative of an early developmental stage and express immature neuronal phenotype, ChAT-transfected clones instead, seem to proceed toward a more specific differentiation state, in fact they express neurospecific features absent in N18TG2 cells. In ChAT-transfected clones, for example, increased levels of voltage-gated Na^{2+}channels and synapsin I were observed, and each of these genes are regulated by REST. It was shown a significant REST expression decrease in ChAT-transfected clone (De Jaco et al., 2002). In order to asses if REST levels could be modified by muscarinic receptors activation, REST expression in cells pretreated with muscarinic agonist and antagonist was analyzed by RT-PCR. Fig 45 shows that in N18TG2 cells pre-treated with atropine, a muscarinic antagonist, REST expression decrease but in ChAT-transfected clone, pretreated with carbachol, a muscarinic agonist, the expression increase.

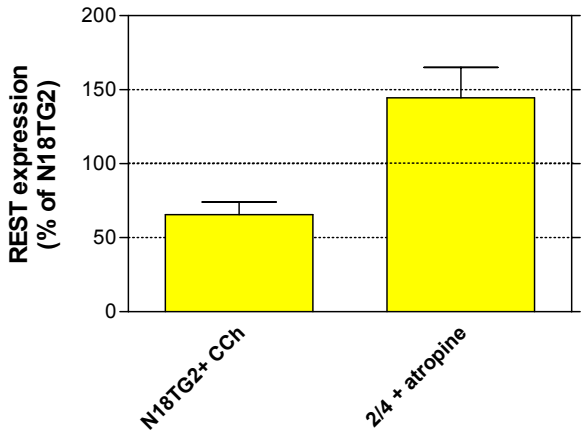


Fig.45: REST expression is referred as per cent of N18TG2 level. CCh pre-treatment in N18TG2 cells produces a decrease, instead atropine pre-treatment in 2/4 ChAT-transfected clone produces an increase of REST expression.

Studies on REST promoter have shown that in a region wich seem involved negative control of gene expression is present an ERE sequence, that could be recognized by Egr-1 (Koenigsberger et al., 1999). It should be possible to suppose that Egr-1 can regulate the expression of REST so I analyzed REST expression in Egr-1 transfected clones by RT-PCR.

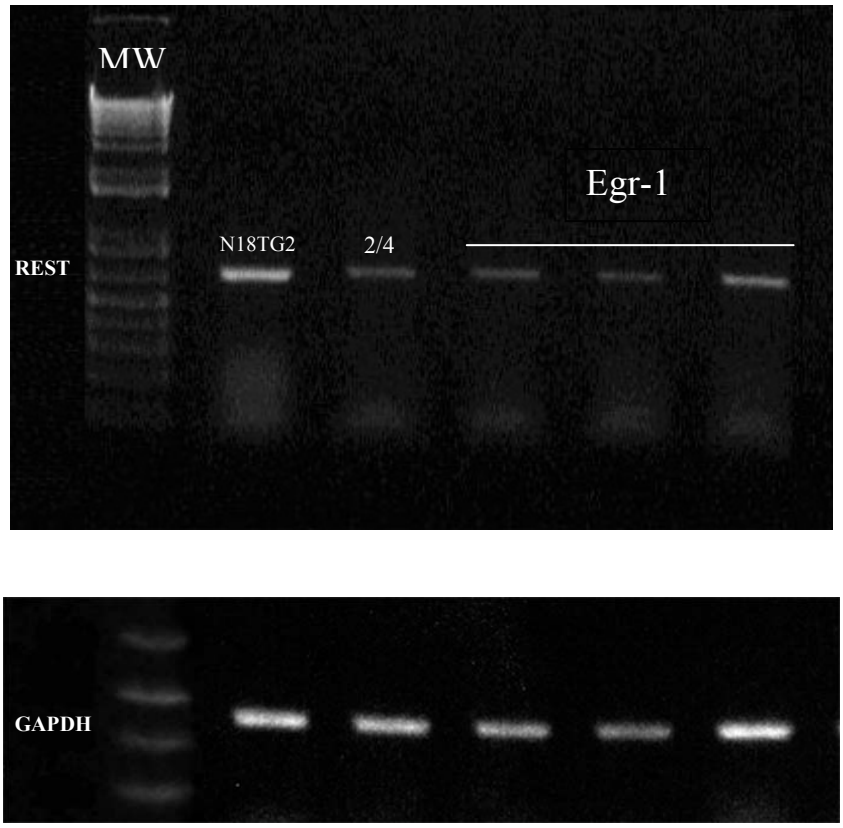


Fig.46: : RT-PCR using mouse REST and mouse GAPDH primers in N18TG2 cells, in 2/4 ChAT transfected clone and Egr-1 transfected clones

Fig 46 shows that REST expression appeared higher in N18TG2 than in 2/4 ChAT-transfected clone and Egr-1 trasfected clones.

To confirm this difference and to obtain a quantitative evaluation of REST mRNA expression, I analyzed samples using a Real-time polymerase chain reaction. Data in Fig.47 shows that, in all Egr-1 transfected clones REST is expressed at lower levels than in N18TG2 although the higher decrease is shown by 2/4 ChAT-transfected clone.

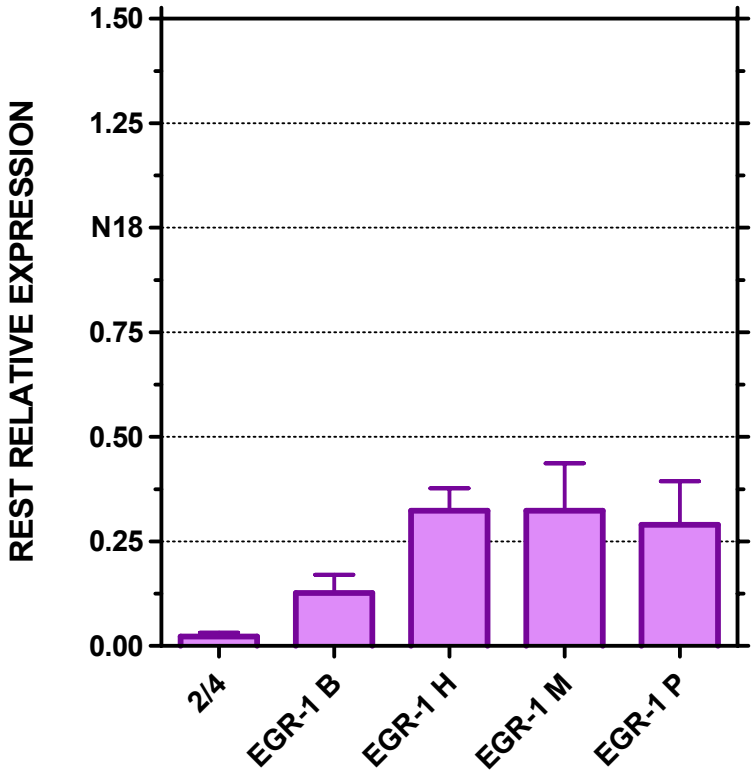


Fig.47: Real time PCR. REST mRNA expression in 2/4 ChAT-transfected clone and Egr-1 transfected clones in respect to REST expression in N18TG2. Values are the mean±SEM of at least six in pendent experiment.

These data suggest that Egr-1 could be implicated directly or indirectly in the regulation of REST transcription, reducing its levels thus contributing to the expression of neuronal features in ChAT-transfcted clones.

CHAPTER 8

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a part of a larger family of structurally related zinc-dependent metalloproteinases called metzincins. Other subfamilies of the metzincins are ADAMs, bacterial serralysins and the astacins. Metzincins use three histidine (H) residues to bind the zinc ion at their active site. These residues occur in the conserved sequence motif HExxHxxGxxHZ, where Z is a family-specific residue: serine in most MMPs, aspartate in ADAMs, proline in serralysins and glutamate in astacins. A distinct β-turn at the active site, which is delineated by a methionine residue ('met-turn') seems to be essential for the protease activity. There is about 20% similarity between metzincin subfamilies (Stocker, et al., 1995) , but identity in the catalytic domain is much higher. Extracellular proteases are crucial regulators of cell function.

The family of MMPs has classically been described in the context of extracellular matrix (ECM) remodelling, which occurs throughout life in different processes that range from tissue morphogenesis to wound healing. Recent evidence has implicated MMPs in the regulation of other functions, including survival, angiogenesis, inflammation and signalling. There are at least 25 members of the MMP family and, collectively, these proteases can degrade all constituents of the ECM. As a result of their potent proteolytic activity, abnormal MMP function can also lead to pathological conditions.

Every MMPs have an N-terminal signal sequence (or "pre" domain) that is removed after it directs their traslocation into endoplasmic reticulum. Thus most MMPs are secreted; however, six of them display transmembrane domains and are expressed as cell surface enzymes. The pre domain is followed by a propeptide ("pro" domain) that maintains enzyme latency until it is removed or disrupted, and a catalytic domain that contains the conserved zinc-binding region (reviewed in Nagase & Woessner 1999). The catalytic domain dictates cleavage site specificity through its active site cleft, through specificity sub-site pockets that bind amino acid residues immediately adjacent to the scissile peptide bond, and through secondary substrate-binding exosites located outside the active site (Overall 2001). With the exception of MMP7 (matrilysin),

MMP26 (endometase/matrilysin-2), and MMP23, all MMPs have a hemopexin/vitronectin-like domain that is connected to the catalytic domain by a hinge or linker region. The hinge region, in turn, varies in length and composition among the various MMPs and also influences substrate specificity (Knauper et al. 1997) (Fig. 48). Gelatinases A and B (MMP2 and MMP9, respectively) (Fig.48) are further distinguished by the insertion of three head-to-tail cysteine-rich repeats within their catalytic domain. These inserts resemble the collagen-binding type II repeats of fibronectin and are required to bind and cleave collagen and elastin (Murphy et al. 1994, Shipley et al. 1996). In addition, MMP9 has a unique type V collagen-like insert of unknown function at the end of its hinge region. Finally, the membrane-type (MT) MMPs have a single transmembrane domain and a short cytoplasmic C-terminal tail (MMPs 14, 15, 16, and 24) or a C-terminal hydrophobic region that acts as a glycophosphatidyl inositol (GPI) membrane-eanchoring signal (MMP17 and MMP25) (Itoh et al. 1999, Kojima et al. 2000). These domains play an essential role in the localization of several important proteolytic events to specific regions of the cell surface.

To accomplish their normal (or pathological) functions, MMPs must be expressed by a defined cell type and present in the pericellular location, at the right time, in the right amount, and they must be activated or inhibited appropriately. Thus MMPs are strictly regulated at transcriptional and post-transcriptional levels and are also controlled at the protein level via their activators, their inhibitors, and their cell surface localization (Fig.49).

Considerating that MMP substrate specificities mostly overlap, the biological function of individual MMPs is largely dictated by their differential patterns of expression. Indeed, differences in the temporal, spatial, and inducible expression of the MMPs are often indicative of their unique roles. Accordingly, most MMPs are closely regulated at the level of transcription, with the notable exception of MMP2, which is often constitutively expressed and controlled through a unique mechanism of enzyme activation (Strongin et al. 1995) including post-transcriptional mRNA stabilization (Overall et al. 1991). Nevertheless, data in the literature indicate that the basal expression of MMP2, MMP14 (MT1-MMP), and TIMP2 is co-regulated, which is consistent with their cooperation during MMP2 activation and with specific similarities

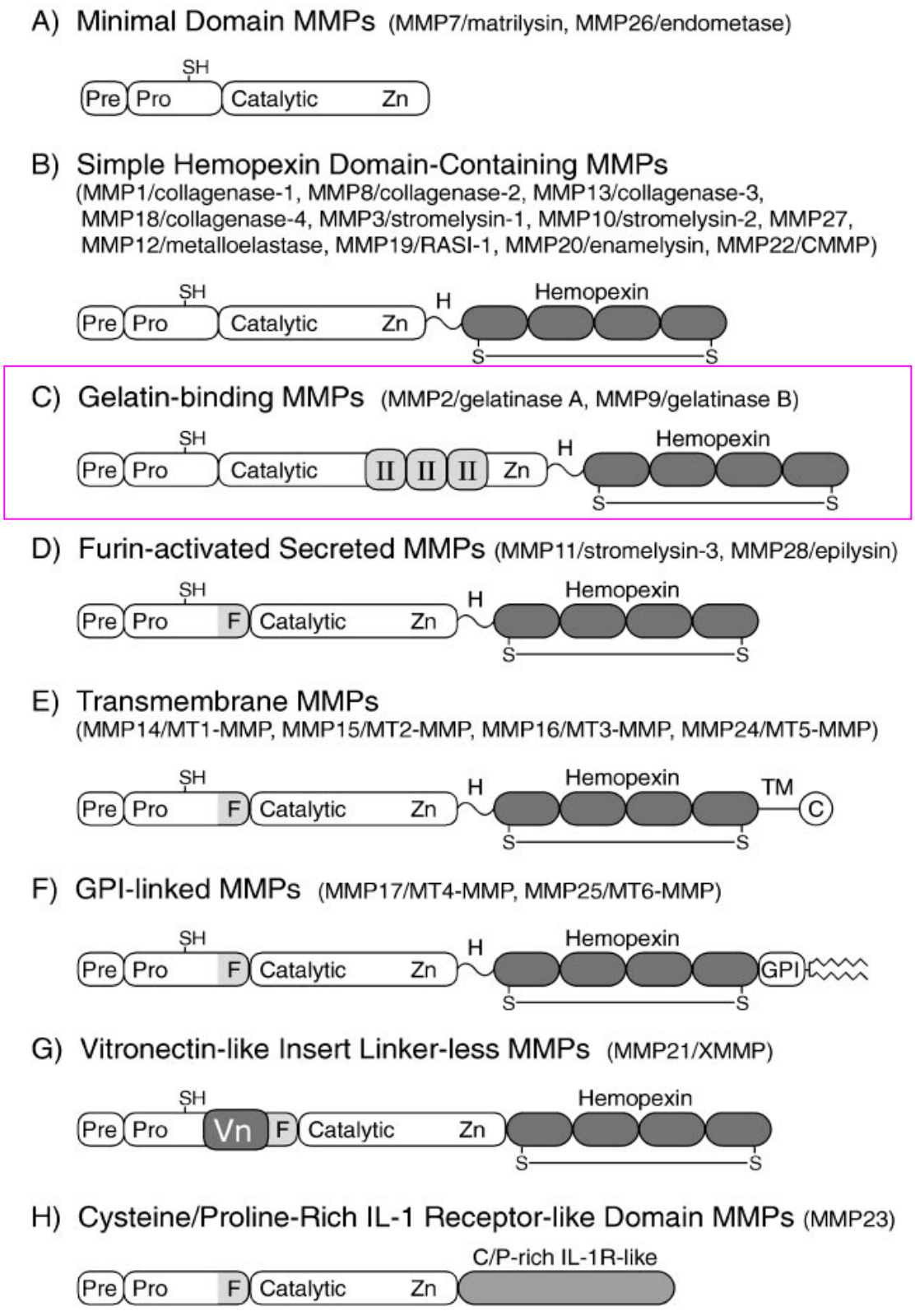


Fig.48: Domain structure of the MMPs. Pre, signal sequence; Pro, propeptide with a free zinc-ligating thiol (SH) group; F, furin-susceptible site; Zn, zinc-binding site; II, collagen-binding fibronectin type II inserts; H, hinge region; TM, transmembrane domain; C, cytoplasmic tail; GPI, glycophosphatidyl inositol-anchoring domain; C/P, cysteine/proline; IL-1R, interleukin-1 receptor. The hemopexin/vitronectin-like domain contains four repeats with the first and last linked by a disulfide bond.

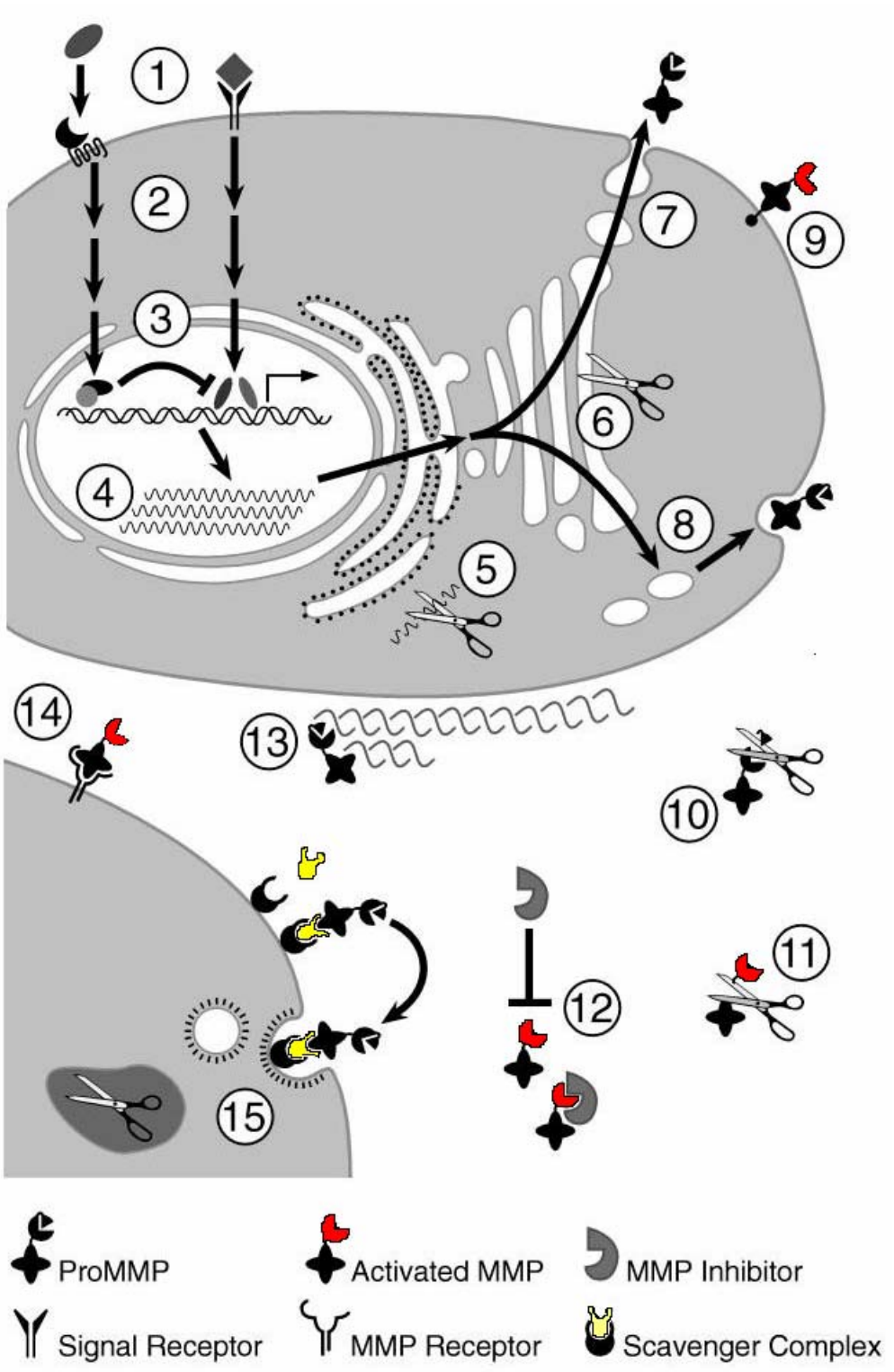


Fig.49: Regulation of the MMPs. MMP regulatory mechanisms include inductive and suppressive signaling (1), intracellular signal transduction (2), transcriptional activation and repression (3), post-transcriptional mRNA processing (4), mRNA degradation (5), intracellular activation of furin-susceptible MMPs (6), constitutive secretion (7), regulated secretion (8), cell surface expression (9), proteolytic activation (10), proteolytic processing and inactivation (11), protein inhibition (12), ECM localization (13), cell surface localization (14), and endocytosis and intracellular degradation (15).

in their gene promoters (Lohi et al. 2000). Otherwise, MMP gene expression is regulated by numerous stimulatory and suppressive factors that influence multiple signaling pathways (Fini et al. 1998).
For example, the expression of various MMPs can be up- or downregulated by phorbol esters, integrin-mediated signals, extracellular matrix proteins, cell stress and changes in cell shape (Kheradmand et al. 1998; reviewed in Sternlicht & Werb 1999). In some cases, one signal may coordinately regulate some MMP genes and differentially regulate others (Uria et al. 1998). Post-trascriptional mechanism can also influence MMP expression. For example mRNA transcripts can be stabilized by different factors (Delany et al. 1995, Vincenti 2001) or multiple transcripts can be generated by alternative mRNA splicing (Matsumoto et al. 1997) or polyadenilation (reviewed in Sternlicht & Werb 1999).

Like other proteolytic enzymes, MMPs are synthesized as inactive proenzymes or zymogens. Their latency is maintained by an unpaired cysteine sulfhydryl group near the C-terminal end of the pro-peptide domain. This sulfhydryl residue acts as a fourth ligand for the active site zinc ion, and activation requires the removal of this bound by the hydrolisis of the propeptide domain or by ectopic perturbation of the cysteine-zinc interaction (VanWart&Birkedal-Hansen 1990). The extracellular activation of most MMPs can be initiated by other already activated MMPs or by several serine proteinases that can cleave peptide bonds within MMP pro domains (Woessner & Nagase 2000). However, MMP2 is refractory to activation by serine proteinases and is instead activated at the cell surface through a unique multistep pathway involving MT-MMPs and TIMP2 (Fig.50) (Strongin et al. 1995). Tissue inhibitors of metalloproteinases (TIMPs) are specific inhibitors of MMPs that participate in controlling of the local activities of MMPs in tissues. First, a cell surface MT-MMP binds and is inhibited by the N-terminal domain of TIMP2, and the C-terminal domain of the bound TIMP2 acts as a receptor for the hemopexin domain of ProMMP2. Then, an adjacent, active MT-MMP cleaves and activates the tethered ProMMP2. Following the initial cleavage of ProMMP2 by MT1-MMP, a residual portion of the MMP2 propeptide is removed by another MMP2 molecule to yield a fully active, mature form of MMP2 (Deryugina et al. 2001). While the C-terminal domain of TIMP2 participates

in the cell surface docking and activation of MMP2, its N-terminal domain acts as MMP inhibitor. Not surprisingly, low-to-moderate levels of TIMP2 promote the activation of MMP2, whereas higher levels block its activation by saturating free MT-MMPs that are needed to remove the MMP2 prodomain (Strongin et al. 1995).

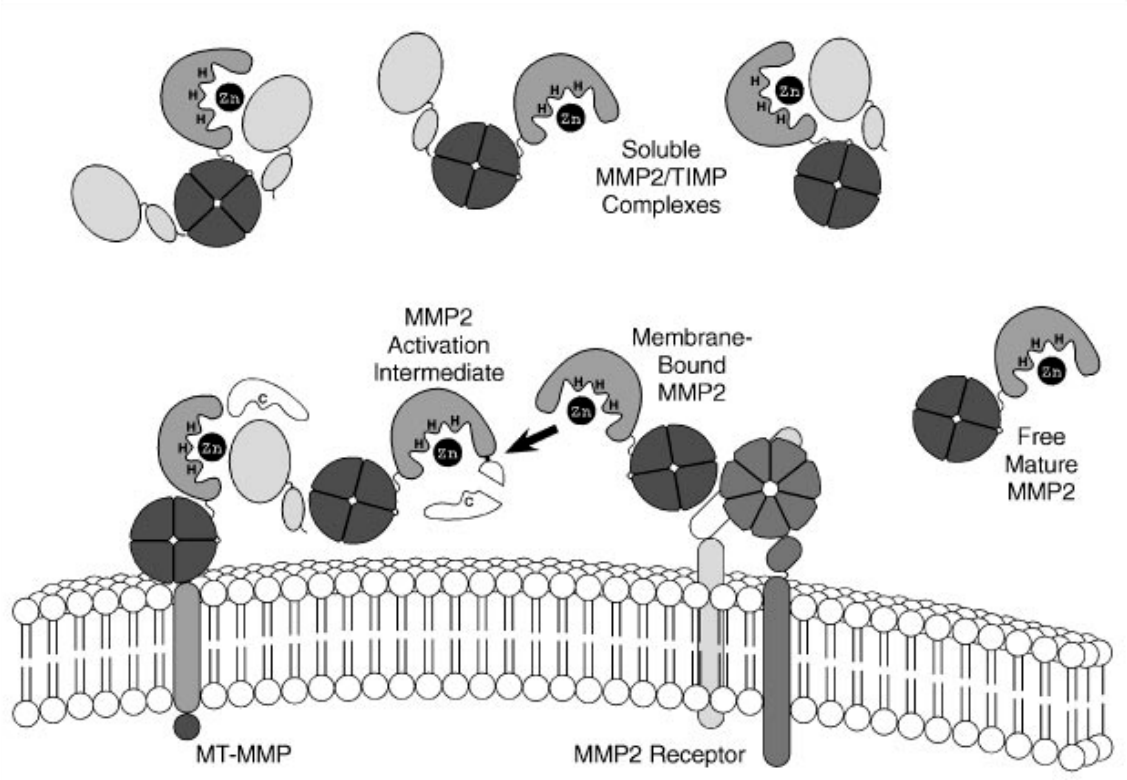


Fig.50: Cell surface activation of MMP2. A ProMT-MMP is activated during transport to the cell surface by an intracellular furin-like serine proteinase, at the cell surface by plasmin, or by non-proteolytic conformational changes. The activated MT-MMP is then inhibited by TIMP2 and the hemopexin domain of ProMMP2 binds to the C-terminal portion of TIMP2 to form a trimolecular complex. An uninhibited MT-MMP then partially activates the ProMMP2 by removing most of the MMP2 propeptide. The remaining portion of the propeptide is removed by a separate MMP2 molecule at the cell surface to yield fully active mature MMP2. Mature MMP2 can then be released from the cell surface or bound by another cell surface MMP2-docking protein. It can also be inhibited by another TIMP molecule or left in an active state depending on local MMP:TIMP molar ratios.

The TIMPs represent a family of at least four 20–29 kDa secreted proteins (TIMPs 1–4) that reversibly inhibit the MMPs in a 1:1 stoichiometric fashion (reviewed in Edwards 2001, Sternlicht & Werb 1999, Gomez et al. 1997). Four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified in vertebrates, and their expression is regulated during development and tissue remodelling. Under pathological conditions associated with unbalanced MMP activities, changes of TIMP levels are considered to be important because they directly affect the level of MMP activity. They share 37–51%

overall sequence identity, a conserved gene structure, and 12 similarly separated cysteine residues. These invariant cysteines form six intra-chain disulfide bridges to yield a conserved structure. Although some studies indicate that the inhibitory activity of the TIMPs resides almost entirely in the N-terminal domain (O'Shea et al. 1992,Willenbrock&Murphy 1994, Huang et al. 1997;Bodden et al. 1994), both C- and N-terminal domains influence enzyme-inhibitor binding (Willenbrock & Murphy 1994). Individual TIMPs differ in their ability to inhibit various MMPs (reviewed in Woessner & Nagase 2000). In addition, the TIMPs differ in terms of their gene regulation and tissue-specific patterns of gene expression (Edwards 2001).

In according to the classic role of MMPs in modulating the motility of cells across tissue matrices, metalloproteinases might regulate the migration of precursor cells to their destinations during neural development. Neural stem cells express MMP2 and all four TIMPs (Frolichsthal-Schoeller,. et al. 1999), and the migration of an oligodendrocyte progenitor requires MMP activity *in vitro*. Another role for MMPs in CNS development might lie in myelinogenesis, the process whereby oligodendrocytes extend several processes from their soma that reach and enwrap axons to form myelin. The initial expansion of oligodendroglial processes is massive and could require remodelling of the brain matrix by MMPs. This hypothesis has been tested and oligodendrocytes were found to express MMP9 during the period of myelinogenesis. Furthermore, the inhibition of MMP activity *in vitro* prevented the extension of oligo-dendroglial processes (Oh,. et al.1999). In parallel with myelinogenesis, metalloproteinases also participate in axon elongation (Fig.51). Early studies showed that the presence of proteolytic activity at neuronal growth cones during attachment and reattachment events (Pittman, 1985); some of the activity is probably contributed by metalloproteinases, as interference with MMP activity inhibited growth-cone motility (Karkkainen et al 2000).. Inducers of neuronal differentiation and axonal outgrowth, such as nerve growth factor, laminin or retinoic acid, enhanced the expression of MMP2,-3 and -9 by dorsal root ganglion (DRG) neurons (Sheffield et al., 1994), PC12 and neuroblastoma cells (Machida et al., 1989; Chambaut-Guerin et al., 2000). Furthermore, growth cones of PC12 cells that stably expressed MMP3 had a reduced capacity to penetrate a reconstituted basement membrane (Machida et al., 1989). In a study in which neurite outgrowth of DRG neurons that grow on top of normal adult

nerves was evaluated, the slow neurite elongation was further reduced by treatment with metalloproteinase inhibitors (Zuo et al., 1998). By contrast, pre-treating the nerves with recombinant MMP2 accelerated neurite growth (Zuo et al., 1998). Further studies led to the conclusion that DRG neurons expressed MMP2 that degraded and inactivated the neurite-inhibiting activity of chondroitin sulphate proteoglycans present on nerves, leading to the exposure of permissive laminin for neurite outgrowth (Zuo et al., 1998). Indeed, cleavage of a specific peptide bond in an ECM molecule leads to profound functional changes in other systems. For example, the cleavage of the Ala586–Leu587 bond in the α2 chain of laminin-5 by MMP2 induced migration of breast epithelial cells by exposing a cryptic pro-migratory site on laminin-5 (Giannelli et al., 1997).

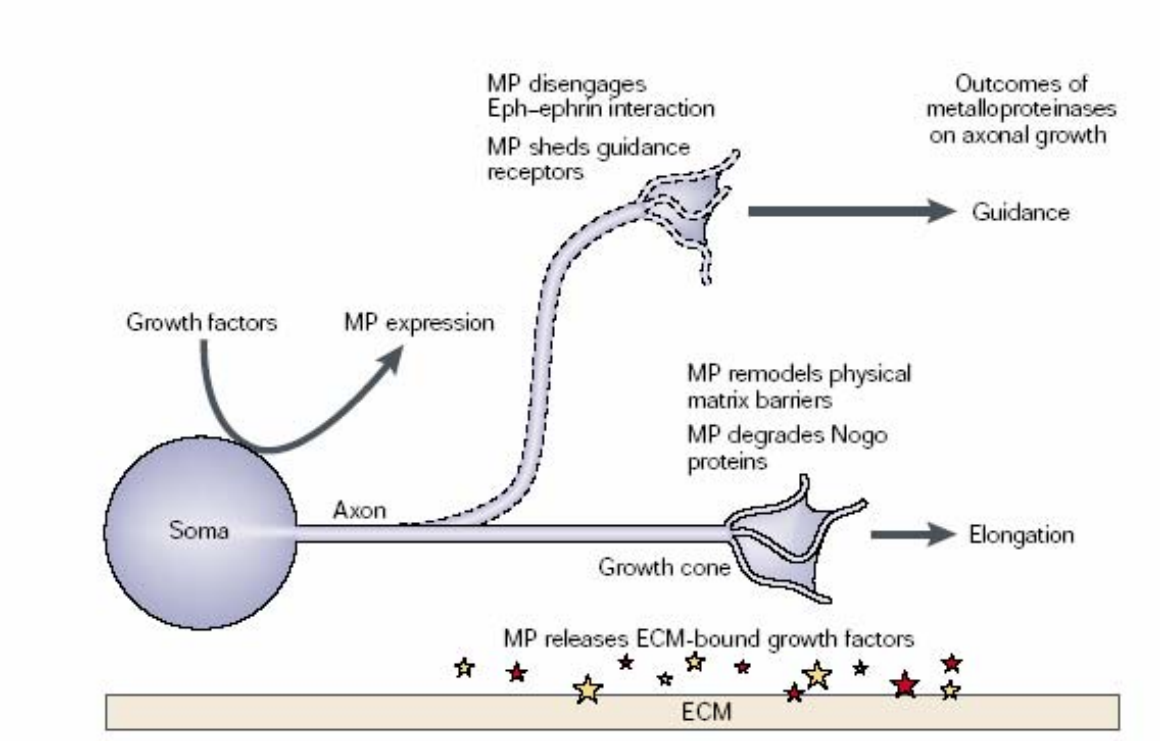


Fig.51: Metalloproteinases regulate axonal growth. Several growth factors, including nerve growth factor (NGF), increase the expression of metalloproteinases (MPs) by neurons. MPs, in turn, regulate neurotrophic factor activity. One mechanism involves the release of growth factors that are anchored to the extracellular matrix (ECM). MPs that are located in the vicinity of growth cones can promote the elongation of axons owing to their ability to remodel the ECM and degrade inhibitory molecules such as the Nogo proteins. By interacting with molecules implicated in axonal guidance

Finally, in concordance with the activity of MMP2 on proteoglycans described above, metalloproteinases might be used in the CNS to destroy other inhibitory proteins. C6 glioma cells and fibroblasts transfected with MT1-MMP could digest NI250 (Belien et

al., 1999), a Nogo protein identified as one of the most potent inhibitors of axonal elongation. In this way, some MMPs might act by neutralizing inhibitory proteins for axonal outgrowth. Although these data implicate metalloproteinases in the creation of penetrable paths for axonal elongation (Fig.51), metalloproteinases can also regulate guidance cues for growth cones. Ephrins are guidance molecules that bind to receptor tyrosine kinases of the Eph family. When the growth cone of a neuron that expresses Eph receptors encounters ephrin ligands on the surface of another cell, this facilitates the adherence of the cells to each other and bidirectional signalling to occur. The growth cone then overcomes these adhesive forces and breaks away from the ephrin surface. Hattori et al. 1993 showed that the adhesive ephrin–Eph interaction is broken *in vitro* by ADAM10, which becomes activated after engagement of the Eph receptor, another guidance molecule is netrin-1,which binds a receptor known as DCC (deleted in colorectal carcinoma). When axon outgrowth from embryonic dorsal spinal explants was evaluated *in vitro*, the facilitatory activity of netrin 1 was potentiated by IC3 and GM6001, hydroxamate metalloproteinase inhibitors (Galko et al., 2000). It was found that DCC was shed from the cell surface by the activity of an unidentified MMP; preventing the ectodomain shedding of DCC with a metalloproteinase inhibitor resulted in responsiveness to netrin 1 (Galko et al., 2000). The recent report of a phenotype-based GENE-TRAP screen to identify genes that control wiring patterns in the mouse CNS further implicates metalloproteinases in axonal guidance. ADAM23 was one of the genes identified in this screening; its inactivation *in vivo* led to neurological defects, tremor and ataxia (Leighton et al., 2001).

MMP IN NEUROBLASTOMA CELLS

ChAT-transfected clones show an higher ability to grow fibers than N18TG2 parental cells, and I have demonstrated that stable transfection of N18TG2 with Egr-1 increase neurite outgrowth.

It was shown that Egr-1 regulate the expression of MT-MMP1, which is necessary for MMP-2activation (Haas et al., 1999), one of the MMP expressed during nervous system development.

For this reason it seems interesting to study MMP2 activity in neuroblastoma cells and transfected clones to analyze the possible correlation between fibers outgrowth and MMP activity.

The activity of MMP2 in N18TG2 cells and ChAT-transfected clones were studied by zymography. Zymography involves the electrophoretic separation of proteins in the presence of SDS and in the absence of reducing agents on a polyacrylamide gel containing gelatine, which is the substrate of some MMPs, for example MMP2 and MMP9. Gelatin therefore represent an in situ protease substrate for MMPs. I analyzed the culture medium of cells maintained in different conditions. In order to asses if the MMP2 activity is dependent on muscarinic activation, I analyzed N18TG2 and ChAT-transfected cells treated with muscarinic agonist and antagonist.

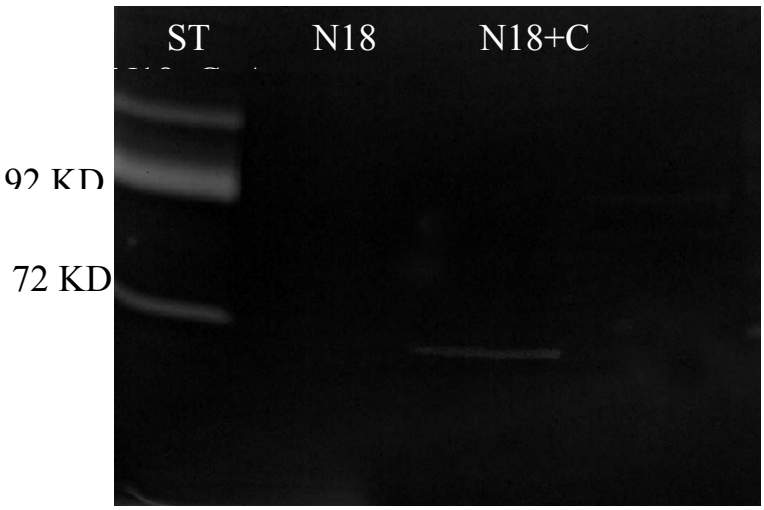


Fig.52: Zymography comprising 10% polyacrylamide, 1 mg/mL gelatine. Culture media of N18TG2cells, N18TG2 treated with CCh 10^{-4}M (N18TG2+C), and N18TG2 treated with CCh 10^{-4}M and atropine 10^{-6}M (N18TG2 + C + A) were analyzed. A band of

proteolitic activity is recognized only in N18TG2+ C corresponding to 62 KD MMP2 activated form. Standard is obtained with sample of periferic blood treated with sample buffer.

As shown in Figure 52 a proteolitic band is detectable only in N18TG2 cells cultered in the presence of Charbacol 10^{-4} M. As expected, the observed band has an experimental MW of 62 KD, corresponding to MMP2 actived. If the cells are treated with CCh and atropine 10^{-6} M the effect of CCh is abolished.

Then I analyzed the culture media of two different ChAT-transfected clones (2/4 and 3/1) before and after atropine treatment. Proteolitic bands of MMP2 activated form is present in 2/4 and 3/1 clone but disappear when the cells are treated with atropine. Two bands corresponding to active form of MMP9 (88KD) and its proenzyme form (92 KD) are also observed, but they seem to be constitutively expressed and not change after atropine treatment (Fig.53).

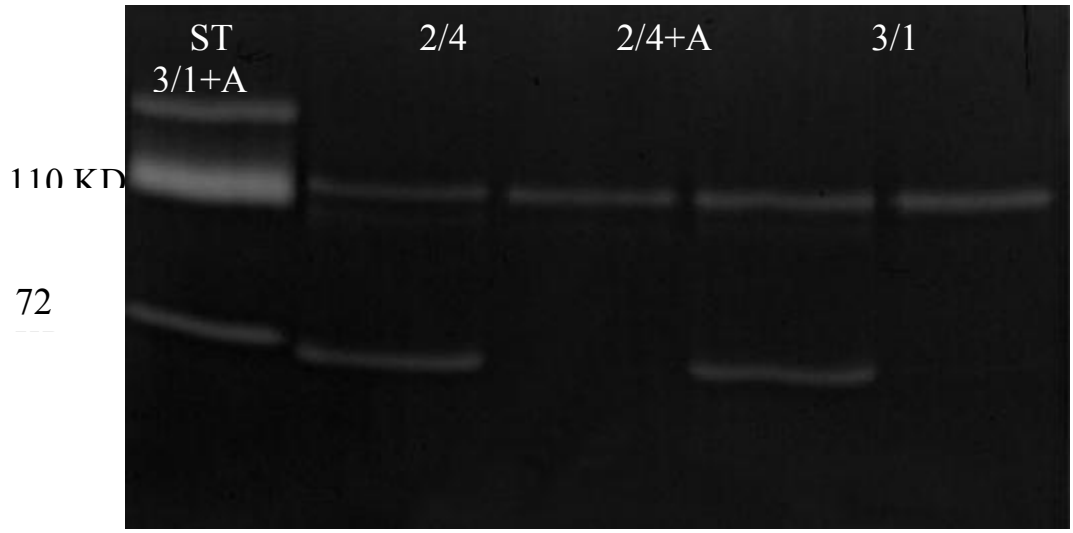


Fig.53: Zymography comprising 10% polyacrylamide, 1 mg/mL gelatine. Culture media by ChAT-transfected clones (2/4 and 3/1) abd ChAT-transfected clones treated with atropine 10^{-6}M (2/4 +A, 3/1 +A) were analyzed. Bands of MMP2 activated form (62 KD), present in ChAT-transfected clones, disappear after atropine treatment.

In order to demonstrate if these bands are produced by MMP activity I analized proteolitic activity in absence of Zn^{2+} and Ca^{2+} ions, considering that MMPs are Zn^{2+} and Ca^{2+} dependent enzyme. Furthermore I used a specific MMP synthetic inhibitor (SB-3CT 5μM) to abolish the proteolitic activity, as shown in Fig.54 and Fig.55 each

of these treatment prevence the diction of the specific bands, confirming that proteolitic activity is due to MMP.

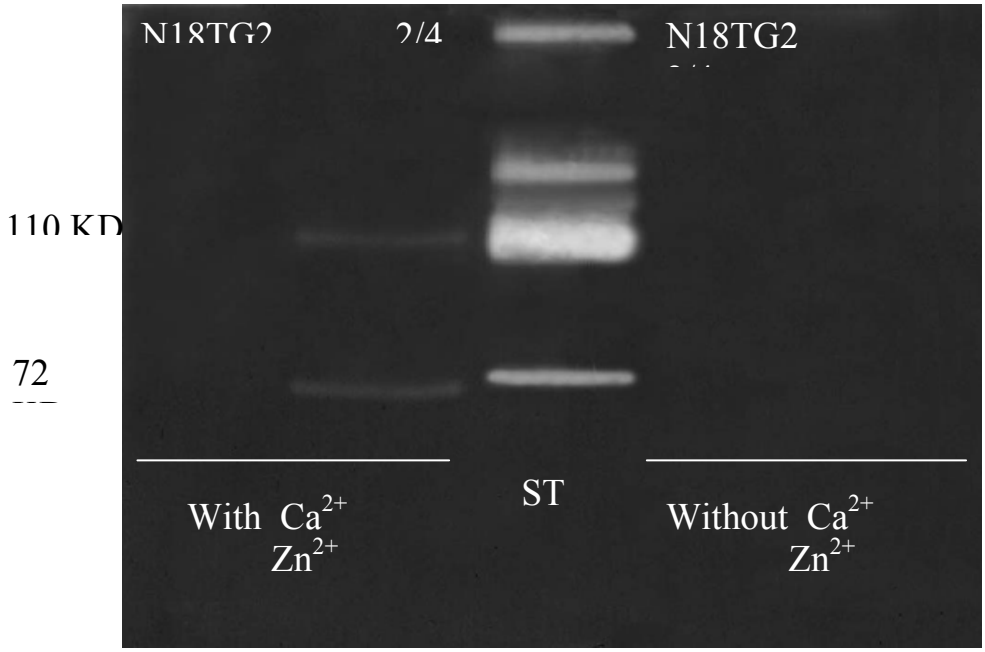


Fig.54: Zymography comprising 10% polyacrylamide, 1 mg/mL gelatine. Culture media by N18TG2 and 2/4 ChAT-transfected clones in the presence and in the absence of Zn^{2+} and Ca^{2+} were analyzed. In absence of ions proteolitic band of MMPs disappears

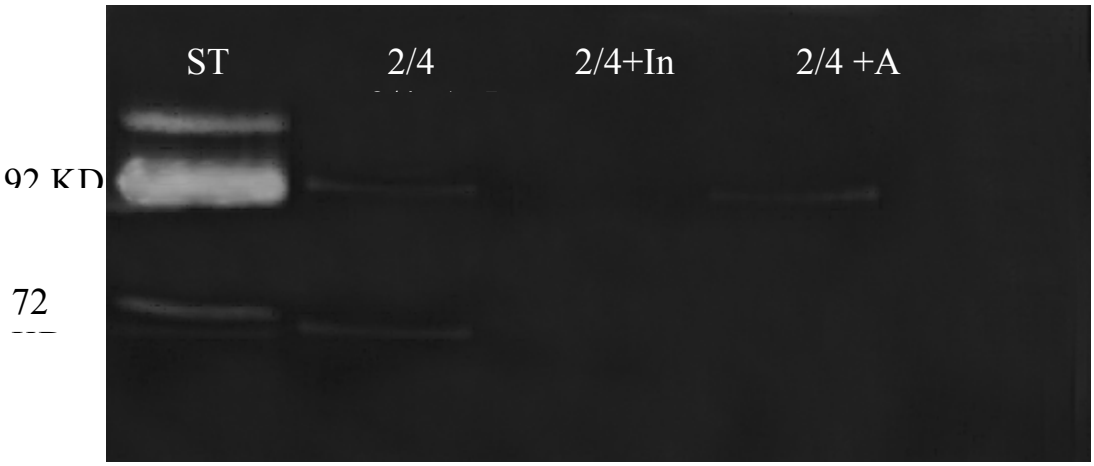


Fig.55: Zymography comprising 10% polyacrylamide, 1 mg/mL gelatine. Culture media by 2/4 ChAT-transfected clones maintened in basal condition (2/4) or treated with atropine (2/4 + A). Samples treated with the specific inhibitor SB-3CT 5μM (2/4 + In, 2/4+A+In) were also loaded on the gel.

Zymography analysis on Egr-1 transfected clones was also performed. As shown in Figure 56 proteolitic activity of MMP2, like in 2/4 ChAT-transfected clone, is present in Egr-1 transfected clone. This observation suggests a possible involvement of Egr-1 in the regulation of MMP2 activity.

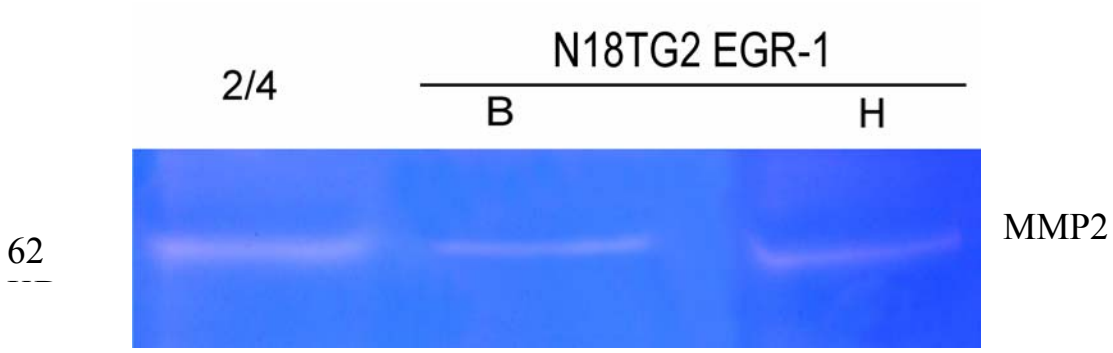


Fig.56 Zymography comprising 10% polyacrylamide, 1 mg/mL gelatine. Culture media conditionated 2/4 ChAT-transfected clone and Egr-1 trasfected clones were analyzed.

DISCUSSION

A large body of evidence, emerging from diverse experimental system 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 which can direct neuronal differentiation playing alternative roles in the development of nervous system as regulators able to influence various cellular events, taking place during neuron differentiation before synapses formation.

As far as the cholinergic system, Choline Acethyltransferase (ChAT) immunoreactivity has been demonstrated in dividing cells of mouse ventricular germinal zones, (Schambra et al., 1989) in pre- or early migratory neurons of rat spinal cord (Phelps et al., 1990) and muscarinic receptors have been revealed in rat central nervous system by autoradiography as early as day 14 of embryonic life (Schlumpf et al.,1991).

ACh acts on proliferation of rat cortical neural precursors by activation of muscarinic receptors. It exherts inhibitory effect on fibre growth on retinal ganglion cells (Lipton et al., 1988) and it has been proposed as a possible mechanism to control dendrite growth, upon reaching the appropriate target cells. An 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).

It was shown that neuroblastoma N18TG2 cell line appears as an interesting experimental system to study ACh morphogenetic action on neuronal differentiation because they are defective for neurotransmitter production and when they are forced to express ChAT they acquire new neuronal features (Chapter 2).

The aim of this thesis is the elucidation of the molecular mechanism by which ACh exherts a morphogetic activity on ChAT - transfected neuroblastoma cells.

Neurotransmitter functions during early development are independent from the establishment synapses or synaptic activity, in these contest, neurotransmitters are apparently released by mechanisms that are distinct from the conventional synaptic vesicular pathway (Nguyen et al., 2001; Owens et al., 2002). Molecular characterization of the secretion machinery components in both neuronal and non-neuronal cells has demonstrated that constitutive and regulated synaptic secretion pathways share homolog

proteins (Schiavo et al., 1992; Bennett and Scheller, 1993; Sollner et al., 1993). These findings have prompted the suggestion that the transmitter secretion pathway at the nerve terminal has developed through addition of synaptic vesicle-specific proteins to the ubiquitous endosomal membrane recycling pathway.

It seems that the endocytic compartments in non neuronal cells are able to accumulate and secrete cytoplasmic ACh in a Ca^{2+}-dependent fashion, (Cavalli et al., 1991; Alder et al., 1992; Morimoto et al., 1995; Dan and Poo, 1992; Girod et al., 1995) thus resembling the basic functions of synaptic vesicles. On the other hand, the ubiquitous endosomal membrane recycling pathway may contribute to spontaneous quantal neurotransmitter secretion in neurons (Chang et al., 1998). Indeed, ACh is present in the cytoplasm of neuronal cells (Parsons et al., 1983), and endocytic recycling pathway operates in any type of cell, including neurons (Kraszewski et al., 1995; Dai and Peng, 1996).

Acetylcholine measurements demonstrate that ChAT-positive cells can synthesize and release acetylcholine in the culture medium, and that such a release can be increased, e.g. when cells are depolarized by high potassium.

These data support the hypothesis that acetylcholine may act on ChAT-positive cells by establishing an autocrine/paracrine loop; muscarinic acetylcholine receptors would be activated on those cells releasing acetylcholine.

In addition, the data demonstrate that ChAT-positive cells increase neurotransmitter release after treatment with differentiating agents, which induce an increase in both length and number of neurites. The increase in ACh release could be explained by the increase in the surface available for release, due to neurite extension. In vivo, neurotransmitter release during an intensive neurite outgrowth could play a role in the mechanisms of axon guidance to its target.

As ACh release in ChAT-N18TG2 cells is induced by high potassium (potassium-dependent) it could be mediated either by vesicles or by a pore system similar to that of the mediatophore. Our data demonstrate that the release is not dependent on extracellular calcium.

It has been shown that the neurotransmitters γ-aminobutyric acid and glutamate can be released in a Ca^{2+} independent manner, before synapse formation (Demarque et al.,

2002). Nevertheless, there is also evidence in several preparations that Ca^{2+} is not fully required for vesicular exocytosis (Mochida et al. 1998; Tse and Tse 2000 and Zhang and Zhou 2002). Interestingly, in DRG neurons, tetanus toxin, which cleaves synaptobrevin, did not completely abolish a calcium-independent, voltage-dependent exocytosis (Zhang and Zhou, 2002). This raises the possibility that some vesicles fuse with the plasma membrane without the requirement of Ca^{2+}

Anyway it still has to be demonstrated, in this experimental system, whether the release is dependent on intracellular calcium stores. The hypothesis is that these cells release acetylcholine through still immature vesicles, in which calcium-dependent release mechanism is not yet fully developed. A possible further development of these experiments would be the electron microscopic analysis of transfected cells, to study the presence and localization of synaptic vesicles.

Pharmacological experiments on ChAT-transfected clones show that M1, M2, M3 and M4 muscarinic receptors are expressed on ChAT-positive cells, while second messenger analysis indicates that they are functionally capable of activating their specific signal transduction pathways.

All these data suggest that the progression along their differentiation pathway of ChAT-positive cells can be triggered by the acetylcholine released in the culture medium.

A further step in the thesis is the identification of those transcription factors involved in the appearance of neurospecific markers following the activation of muscarinic receptor-dependent intracellular pathways.

Egr-1 is a transcription factor strongly involved in differentiation and in neurite extension in several cell types.

The expression of this gene seems induced by molecule, such as retinoic acid, known to promote differentiation. Following retinoic acid treatment Egr-1 protein levels remain at high constitutive levels in differentiated P19 cells, (Mouse embryonic teratocarcinoma cell line) indicating a distinct role for this transcription factor in the induction and maintenance of differentiated state (Darland et al., 1991). In rat brain the levels of Egr-1 transcripts are induced by neuronal activation (Bhat et al., 1992**;** Lam et al., 1997) and thyroid hormone (Mellstrom et al., 1994; Pipaòn et al., 21-23), which is known to be required for adequate development of the dendritic arbour of different neuronal type (Lam et al., 1997). The morphological differentiation induced by serum

withdrawal in N2A neuroblastoma cells can be blocked by the presence of Egr-1 antisense oligonucleotides in the culture medium. Stable transfection of N2A cells, overexpressing the Egr-1 protein, extend very long neurites much higher then in parental cell line (Pignatelli et al., 1999). The PC12 cell line respond to NGF by undergoing growth arrest and proceeding to differentiate towards a neuronal phenotype. NGF stimulation induce expression of Egr-1. These data are very suggestive of an in vivo role for Egr-1 in the late stages of neuronal differentiation, when neuronal processes begin to develop and connections among cells begin to be established. In support of this idea is also the presence of Egr-1 binding site in the promoters of several neuronal genes (Tab III), such as synapsin I, Synapsin II, Synaptobrevin II and neurofilament.

The data here reported by western blot show that Egr-1 is expressed in transfected cells and it is not expressed in parental N18TG2 cells. Egr-1 expression can be induced in parental N18TG2 treated with charbachol and can be repressed in ChAT-positive cells by atropin.

When N18TG2 cells are treated with acetylcholine Egr-1 mRNA expression increases to reach a maximum after 1 hour treatment. Considering this time as that necessary for the maximum activation of the transcription factor, it has been possible with pharmacological studies, to find out that regulation of Egr-1 is mediated by M3 receptors. Therefore acetylcholine, through M3 muscarinic receptors, can activate Egr-1 in neuroblastoma cells.

N18TG2 neuroblastoma has also been stably transfected with Egr-1 and neurite extension has been measured in these cells; morphometric analysis shows that Egr-1 transfected cells exhibit a neurite extension ability similar to that of ChAT-positive cells, and much greater than parental cells.

Egr-1 is a finely regulated transcription factor, which undergoes several inhibition mechanisms that keep its levels under constant control; it is therefore of interest to analyze the expression in our cells of inhibitors such as NAB1 and NAB2 in fact it was shown that the overexpression of NAB2, blocks the ability of NGF to induce differentiation of PC12 (Qu et al., 1998).

In addition, in order to better correlate Egr-1 expression with neurite outgrowth it would be appropriate to identify the target genes of Egr-1 in ChAT- and Egr-1 transfected cells, and which are the molecules involved in nerve fiber extension.

During the development of the nervous system neural precursors travel within the extracellular matrix extending fibers toward their target, and this phenomenon requires the activation of matrix metalloproteinases (MMPs) to hydrolize some of the components of the surrounding extracellular matrix.

Early studies showed that the presence of proteolytic activity at neuronal growth cones during attachment and reattachment events (Pittman, 1985); some of the activity is probably contributed by metalloproteinases, as interference with MMP activity inhibited growth-cone motility (Karkkainen et al 2000). Inducers of neuronal differentiation and axonal outgrowth, such as nerve growth factor, laminin or retinoic acid, enhanced the expression of MMP2,-3 and -9 by dorsal root ganglion (DRG) neurons (Sheffield et al., 1994), PC12 and neuroblastoma cells (Machida et al., 1989; Chambaut-Guerin et al., 2000). Furthermore, growth cones of PC12 cells that stably expressed MMP3 had a reduced capacity to penetrate a reconstituted basement membrane (Machida et al., 1989). In a study in which neurite outgrowth of DRG neurons that grow on top of normal adult nerves was evaluated, the slow neurite elongation was further reduced by treatment with metalloproteinase inhibitors (Zuo et al., 1998). By contrast, pre-treating the nerves with recombinant MMP2 accelerated neurite growth (Zuo et al., 1998).

In this thesis it was demonstrated that muscarinic receptors can modulate the activity of MMP2, which in turn could be at least in part involved in the neurite outgrowth mechanism. To support this hypothesis, in the literature it is reported that Egr-1 is involved in the expression of MT-MMP1, which is necessary for MMP2 activation.

Accordingly, in our experiments Egr-1 positive clones, as well as ChAT-positive clones, express MMP2, unlike their parental counterparts.

With the aim of better defining the transcriptional mechanism downstream the self activation of muscarinic receptors in ChAT-positive clones, I also studied the expression of REST transcription factor. This is a repressor of neurospecific genes, among which are synapsin I and voltage-activated sodium channels, both genes upregolated in ChAT-positive clones.

REST levels must be downregulated in order to permit acquisition of the terminally differentiated neuronal phenotype. The PC12 cell was used as model to test REST downregulation during neuronal differentation. By generating stable lines that express REST under the control of an inducible promoter, it was possible to induce REST prior to the treatment with NGF that cause terminal differentiation. The presence of REST completely blocked induction of sodium channel mRNA in response to NGF. Consistent with the effects of REST on sodium currents, persistent expression of REST reduced the growth of neurites in response to NGF (Ballas et al., 2001). Although the specific REST target genes for neurite growth are not known, the suppression is consistent with the large number of proteins important to neuronal physiology that are known to be regulated through the REST repressor pathway (Schoenherr et al., 1996).

PCR experiments indicate that REST expression is decreased in ChAT-positive clones, and that it can be modulated by muscarinic receptors stimulation.
REST expression is also decreased in Egr-1 positive clones, and therefore Egr-1 is a potential regulator of REST expression, either directly or indirectly.
However, as it is clearly pointed out by studies on the regulation of REST expression, the mechanism is likely to involve other molecules, in fact the decrease of REST expression is more evident in ChAT-positive clones than it is in Egr-1 transfected cells.

In conclusion in this thesis it has been demonstrated that acetylcholine can induce differentiation of ChAT-positive cells, because it is released by the cells that synthesize it and it acts on muscarinic receptors of the M3 type, which are present on the surface of the same cells. Muscarinic receptors activate mechanisms that increase the levels of Egr-1 and decrease the levels of REST. The combined action of these two transcription factors, possibly with the cooperation of other still unidentified components, enables the transfected cells to enhance neurite outgrowth and to increase MMP2 activity.

CHAPTER 10

MATERIALS AND METHODS

Neuroblastoma cell cultures

N18TG2 cells, derived from mouse C-1300 neural crest tumor (Nelson et al., 1976), were cultured in Dulbecco's modifed Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). ChAT-transfected clones were obtained as previously described (Bignami et al., 1997) using the pcDL1-ChAT expression plasmid (Ishii et al., 1990) which contains a 2.1-kb insert for the complete coding sequence of rat ChAT. The transfected clones were cultured in DMEM supplemented with 10% FCS and 400 mg/ml geneticin. The cells were seeded in 90-mm tissue culture dishes at a density of $5X10^5$ cells and maintained at 37°C in a 10% CO2 atmosphere: for subculturing they were removed from the culture dish with 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS) for 5 min at room temperature. The doubling time of transfected clones ranged between 21 and 23 h; no signifcant differences were observed with respect to N18TG2 cells.

ACh release

To evaluate ACh release we used the choline oxidase chemiluminescent procedure previously described by Israel and Lesbats (1981). The transfected clone 2\4 was seeded in 35 mm tissue culture dishes at a density of $2x\ 10^5$ cells and maintained in culture for two days. At this time, each dish was repeatedly washed with a saline solution (136 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 6 mM $CaCl_2$ in 10 mM Tris buffer, pH 8.6) in order to avoid interference by choline present in the medium. The dishes were then incubated at 37°C for three consecutive periods (5 min each) in 750 μL of the saline solution; during the second incubation period, 20 μL of KCl (final concentration 80 mM) were added to trigger the release.

ACh release by 2\4 clone was also measured in the presence of 10^{-6} M atropine as muscarinic receptor antagonists.

Culture medium was added to a reaction mixture containing 10 μL luminol (1 mM stock solution), 5 μL horseradish peroxidase (2 mg/mL stock solution) and 5 μL AChE (1000

U/mL stock solution purified on a Sephadex G-50 column). Chemiluminescence was recorded using a Lumat LB9507 (EG & G Berthold, Bad Wilbad, Germany) and when the light emission reached a stable baseline, 50 μL choline oxidase (50 U/mL stock solution) were added. Control experiments showed that the ACh assay was linear over a concentration range between 0.1 and 150 pM (the slope of linear regression was 0.78 ± 0.1 and the F-test for the slope showed a significant difference from zero at $p < 0.0001$). The calcium dependency of the release mechanism was tested by incubating dishes with a Ca^{2+} -free saline solution containing 1 mM EGTA.

Statistical analysis

Non-parametric statistical comparisons within different groups were performed using Mann–Whitney U-test. Difference were considered statistically significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Binding and competition experiments

Binding and competition experiments were performed according to the procedure of Tata et al, (1995; 2000). Briefly, the cells grown on 90-mm-diameter culture dishes were harvested and then collected by centrifugation. Cells were then resuspended in Tris-HCl 0.01M pH 7.4 containing 40 μg/ml leupeptin and 20 μg/ml pepstatin as protease inhibitors and homogenized with quick freezing in dry ice and thawing for three times. Aliquots of cellular homogenate were added to an incubation medium containing different concentration (0.05-10 nM) of [^{3}H]-quinuclidinyl benzilate (^{3}H-QNB) (specific activity, 49 Ci/mmol- Amersham) in Tris–HCl (0.01M, pH7.4) and maintained for 1 h at room temperature. Non-specific binding was evaluate in the presence of 1 μM atropine.

For competition experiments the cellular homogenates were pre-incubated for 20 min at room temperature in the presence of different muscarinic ligands at concentration ranging from 10^{-10}M to 10^{-4}M. The ligands used were pirenzepine for M1, gallamine for M2, 4-DAMP for M3 and tropicamide for M4 (Giraldo et al, 1988; Michel et al, 1989; Tata et al, 2000). [^{3}H]-QNB was subsequently added at a final concentration of 0.5 nM and the samples were incubated for additional 40 min at room temperature. The optimal

incubation time and temperature were established in previous experiments (Tata et al, 2000).

The binding reactions in both types of experiments were stopped adding Tris–HCl (0.01M, pH7.4) at 4°C. The incubation mixture was filtered through glass microfiber filters Whatman GF/C. After three washes with Tris–HCl (0.01M, pH7.4), filters were put into 5 ml of Insta gel (Packard) liquid scintillator and counted for radioactivity in a Tri-Carb 2100 TS (Packard). The *Kd* and Bmax values were calculated using a Scatchard plot analysis, IC_{50} values and competitor dissociation constant (*Ki*) values were obtained according the method of Cheng and Prusoff (1973).

Values obtained both in binding and competition experiments are the average of at least three independent experiments performed in duplicate.

Measurement of cyclic-AMP and d-myo-inositol 1.4.5 triphosphate levels

The cells (neuroblastoma N18TG2 cells and 2/4 ChAT-transfected clone) were plated onto 60-mm-diameter dishes. After 72 h the medium was removed and the dishes were washed with Krebs-Henseleit-Hepes (KHH) buffer (140.7mM Na^+, 5.3mM K^+, 132.4 Cl^-, 0.98 mM PO^{2-}, 1.25 nM Ca^{2+}, 0.81mM Mg^{2+}, 5.5 mM glucose, 20.3 mM Hepes) pH 7.4.

For cAMP assay the samples were previously incubated for 10 min at 37°C in KHH containing 1mM 3-isobutyl-1-metylxantine (IBMIX) as phosphodiesterase inhibitor.

Some samples were then stimulated for 30 min at 37°C with only $5x10^{-6}$M PGE1 to increase the cAMP levels while other samples were stimulated in the same time with $5x10^{-6}$M PGE1 and muscarinic agonists such as muscarine (10^{-4}M) or mamba toxin –1 (MT1, 10^{-7}M) (Potter, 2001). 200 ng/ml of PTX was also used when required to block the Gi/G_0 proteins. After stimulation the incubation medium was removed and the cells were harvested in 10% trichloroacetic acid (TCA) and incubated for 10 min in ice. The samples were then centrifuged at 5000 rpm for 10 min at 4°C and the supernatant was collected. TCA was then removed by washing of the extract 5 times with 2 volumes of water-saturated diethyl ether.

For IP_3 assay the cells were pre-incubated with 20mM LiCl and then with 10^{-4}M muscarine at 37°C. The IP_3 levels were measured over a period of 10 s to 2 min. The

inhibitor U73122 (10^{-5}M; Calbiochem) was used when required to block the phospholipase C (PLC) acticity. After stimulation the pH of the samples was adjusted to 7.5 with 0.25M $NaHPO_3$ and then they were treated as above reported for cAMP (Aducci and Marra, 1990). Finally the cAMP and IP_3 levels were measured using a radioimmunoassay (Amersham).
Snake toxins that bind specifically to individual subtypes of muscarinic receptors (Life Sci. 68: 2541-2547)

Stable transfection of mouse Egr-1 cDNA

N18TG2 cultures (5 x 10^5 cells) were cotransfected with 0,6 μg of pIBW3 and 5,4 μg of pCMVegr-1-SPORT6 plasmids (ATCC), by the polyethylenimine (PEI) procedure (Boussif et al., 1995). The pCMVegr-1-SPORT6 plasmid contains the sequence of mouse EGR-1. Plasmid pIBW3, containing the neomycin resistance gene, and was obtained from S. Pellegrini (Institut Pasteur, Paris, France).
Briefly 2x10^5 cells were seeded on 35 mm plastic dishes; after 2 days in culture they were treated with 6 μg of DNA mixture in the presence of 5x10^{-5} M PEI (final concentration) for one hour.
Selection of stably transfected clones was performed with geneticin (600 mg/ml) for 15 days.

Morphometric analysis

For morphometric analysis cells were grown in 60-mm plastic dishes. Five days after seeding the cultures were washed with PBS, fixed for 20 min in 4% paraformaldehyde in PBS at room temperature and stained with 0.5% Coomassie Brilliant Blue R-250 in 1% formic acid, 50% isopropanol and 49% H_2O. Micrographs of randomly selected areas of the cultures were taken from five 60-mm dishes (21 fields /dish, a total of about 105 observations for a single clone). Measurements of the chosen cellular parameters (cell number, fiber number and length) were carried out manually on each photographic field.

Immunocytochemical localisation of βIII tubulin

Cells, cultured on cover slides, were fixed for 10 min. in 4% paraformaldehyde in PBS pH 7.4 at room temperature and then washed in PBS pH 7.4 containing 1% bovine serum albumin. The slides were pre-incubated first in 0.1 M glycine in PBS and then in PBS containing 10% normal goat serum, 1% bovine serum albumin, 0.2% Triton X-100 for 40 min. at room temperature. Samples were incubated with primary antibody (monoclonal anti mouse βIII Tubulin, Promega) diluted (1:1000) in PBS containing 1% normal goat serum, 1% bovine serum albumin, 0.2% Triton X-100 overnight at 4°C. The cultures were then washed and incubated with the secondary antibodies (anti-mouse IgG FITC diluted 1:70) for 90 min. at room temperature. After washing samples were mounted in Glycine/PBS 3:1 (v/v). No staining was observed in controls omitting primary antibodies

Nuclear Extraction

Cells were plated on 90 mm plastic dishes and after 72 hours, were washed twice, with PBS, and then maintained in medium without serum for six hours. Cultures were treated with 10^{-4}M Charbacol for 1 hour, and when necessary a pre-treatment with 10^{-6}M atropine for 30 min was performed. Cells were then washed twice with PBS, scraped from the plates in hypotonic buffer, swollen on ice, and lysed with 1% Nonidet P-40. Nuclei were pelleted and extracted in 200µl of ice-cold 10 mM HEPES (pH 7.9), 400mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM dithiothreitol, 1% Nonidet P-40 in the presence of pepstatin and aprotinin. Protein concentrations of nuclear extracts were determined by the Bradford protein assay

Western blot analysis

SDS-polyacrylamide gel electrophoresis was performed on 8% slab gels according to Laemmli (1970) and proteins then transferred to nitrocellulose membranes. The blots were incubated overnight at 4°C with an affinity purified rabbit polyclonal anti-EGR-1

antibody (Santa Cruz Biotechnology) at a diluition of 1:500. Secondary antibody used to reveal immunocomplexes was anti-rabbit IgG alkaline phosphatase conjugated. The bands were stained with nitro blue tetrazolium in the presence of 5-bromo-4-chloro-3-indolyl-phosphate.
The same protein amount of sample obtained from nuclear extraction was loaded on each lane. No difference in transferred protein was observed on nitrocellulose membranes stained with 0.3% Ponceau S in 3% TCA.

RNA Extraction and RT-PCR

Total RNA was prepared using the TRI Reagent (Sigma) according to the manufacturer's instructions for the isolation of RNA for RT-PCR. Purified RNA was treated with RNase-free DNase I (Ambion).
RT-PCR analysis was performed with the Promega RT-PCR kit using Go Taq polymerase (Promega). The first strand cDNA was synthesized by reverse transcription of total RNA (2μg) from cells using random hexamers. Controls, omitting reverse transcriptase, were included in the reactions.
Quantitative PCR was performed in a final volume of 25 μL with 1X Go Taq reaction buffer, 0.2 mM dNTP, 1 μM forward primer, 1μM reverse primer, 1.25 U Go Taq Polimerase and 200ng template cDNA.
Specific primers designed for mouse **Egr-**1 were:
forward: 5'-GGT TTG ATA ATG ATG AAG GGG ACCA-3'
reverse: 5'-CAT CAC GTT CCC GTG TTA AAG TATC-3'
The two oligonucleotides were paired to amplify a 532 bp product . 22 PCR cycles were performed on cDNA using the following profile: 95°C for 40 sec, 58°C for 40 sec, 72°C for 40sec.

Specific primers designed for mouse **REST** were:
forward: 5'- CTACATGGCACACCTGAAG -3'
reverse: 5'- GGGATGCTTAGATTTGAAATGG -3'

The two oligonucleotides were paired to amplify a 550 bp product . 26 PCR cycles were performed on cDNA using the following profile: 95°C for 40 sec, 55° C for 40 sec, 72°C for 45sec.

GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as standard. Specific primers designed for mouse **GAPDH** were:

Forward: 5'- ACCACAGTCCATGCCATCAC-3'

Reverse: 5'- TCCACCACCCTGTTGCTGTA-3'

The two oligonucleotides were paired to amplify a 452 bp product . 18 PCR cycles were performed on cDNA using the following profile: 95°C for 40 sec, 66° C for 40 sec, 72°C for 40sec.

REAL TIME PCR

Real-time PCR was performed on the reverse transcription (RT) products with the SYBR Green JumpStart Taq ReadyMix (Qiagen) in a Lightcycler apparatus (Biorad), following the manufacturer's instructions.

Thermal cycling conditions comprised an initial denaturation step at 95°C for 3 min followed by 45 cycles at 95°C for 30 sec. Annealing temperature was 55°C for egr-1, and 50°C for REST. As final steps, we included two cycles: one at 95°C and the other at the corresponding annealing temperature of each tested gene, both for 1 min. All samples were run in duplicate, and each well of PCR contained 25 μL as a final volume, including 2.5 μL of cDNA, 0.2 μM forward primer, 0.2μM reverse primer, and 12.5 μL SYBR Green JumpStart Taq ReadyMix and 0.2 internal reference dye.

The threshold cycle (CT), defined as the fractional PCR cycle number at which the fluorescence reaches 10 times the baseline standard deviation, was compared for the expression.

Delta-Delta ct method was used to evaluate the relative expression ratio for all genes compared with HPRT (hypoxanthine-guanine phosphoribosyltransferase),used as internal control gene.

Specific primers designed for mouse **egr-1** were:

Forward 5'- TCCGACCTCTTCATCCTC-3'

Reverse 5'- ATGTCAGTGTTGGGAGTAG-3'
for mouse **REST** were:
Forward: 5'- GTGACTACAAAACAGCAGATAG-3'
Reverse: 5'- TGGGATGCTTAGATTTGAAATG-3'
And for mouse **HPRT** were
Forward: 5'-AGTCCCAGCGTCGTGATTAG-3'
Reverse: 5'-CCATCTCCTTCATGACATCTCG-3'

ZYMOGRAPHY

Cells used for zymography were cultured until semiconflence condition, with or without atropine 10^{-6}M or/and Charbacol 10^{-4}M, and after two wash, the cultures were maintained in DMEM with or without atropine 10^{-6}M or/and Charbacol 10^{-4}M in the absence of serum. After 24h cells were collected and counted.

Gelatin zymography was used for detection of MMP-2 and MMP-9 activity.

The proteins present in medium conditioned by cells were concentrated with salting out technique. To obtain maximum protein precipitation 56% of ammonium sulphate salt was used, then samples were centrifuged at 10000X g for 10 minutes. Supernatant was decanted and precipitates were resuspend (40-45 μL for 10^6cells) in activation buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM $CaCl_2$). The gel consisted of 7.5% acrylamide containing 1 mg/ml gelatin (Gelatin, type A, from pork skin, electrophoresis reagent, Sigma). Sample of periferic human blood was loaded on the gelatin gel as standard. For gelatin zymography, after electrophoresis, gels were soaked in 2.5% Triton X-100 (in 50 mM Tris, pH 7.6) with gentle shaking at room temperature for 60 min with one change. Gels were rinsed three times in activation buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 5 mM CaCl2) and then incubated at 37°C for 16–20 h. The gels were stained with Coomassie blue for 1 h and destained in a solution of 45% methanol and 10% acetic acid. Gelatinase activities appeared as clear bands against a blue background.

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