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TRANSCRIPTIONAL REGULATION OF HUMAN MU-OPIOID RECEPTOR GENE: FUNCTIONAL CHARACTERIZATION OF ACTIVATING AND INHIBITORY TRANSCRIPTION FACTORS

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Abstract

The organization of the nervous and immune systems is characterized by obvious differences and striking parallels. Both systems need to relay information across very short and very long distances.

The nervous system communicates over both long and short ranges primarily by means of more or less hardwired intercellular connections, consisting of axons, dendrites, and synapses. Long-range communication in the immune system occurs mainly via the ordered and guided migration of immune cells and systemically acting soluble factors such as antibodies, cytokines, and chemokines. Its short-range communication either is mediated by locally acting soluble factors or transpires during direct cell–cell contact across specialized areas called "immunological synapses" (Kirschensteiner et al., 2003). These parallels in intercellular communication are complemented by a complex array of factors that induce cell growth and differentiation: these factors in the immune system are called cytokines; in the nervous system, they are called neurotrophic factors.

Neither the cytokines nor the neurotrophic factors appear to be completely exclusive to either system (Neumann et al., 2002). In particular, mounting evidence indicates that some of the most potent members of the neurotrophin family, for example, nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), act on or are produced by immune cells (Kerschensteiner et al., 1999) There are, however, other neurotrophic factors, for example the insulin-like growth factor-1 (IGF-1), that can behave similarly (Kermer et al., 2000).

These factors may allow the two systems to "cross-talk" and eventually may provide a molecular explanation for the reports that inflammation after central nervous system (CNS) injury has beneficial effects (Moalem et al., 1999).

In order to shed some more light on such a cross-talk, therefore, transcription factors modulating mu-opioid receptor (MOPr) expression in neurons and immune cells are here investigated.

More precisely, I focused my attention on IGF-I modulation of MOPr in neurons and T-cell receptor induction of MOPr expression in T-lymphocytes.

Three different opioid receptors [mu (MOPr), delta (DOPr), and kappa (KOPr)] belonging to the G-protein coupled receptor super-family have been cloned. They are activated by structurally-related exogenous opioids or endogenous opioid peptides, and contribute to the regulation of several functions including pain transmission, respiration, cardiac and gastrointestinal functions, and immune response (Zollner and Stein 2007). MOPr is expressed

mainly in the central nervous system where it regulates morphine-induced analgesia, tolerance and dependence (Mayer and Hollt 2006).

Recently, induction of MOPr expression in different immune cells induced by cytokines has been reported (Kraus et al., 2001; Kraus et al., 2003).

The human mu-opioid receptor gene (OPRM1) promoter is of the TATA-less type and has clusters of potential binding sites for different transcription factors (Law *et al.* 2004).

Several studies, primarily focused on the upstream region of the OPRM1 promoter, have investigated transcriptional regulation of MOPr expression. Presently, however, it is still not completely clear how positive and negative transcription regulators cooperatively coordinate cell- or tissue-specific transcription of the OPRM1 gene, and how specific growth factors influence its expression.

IGF-I and its receptors are widely distributed throughout the nervous system during development, and their involvement in neurogenesis has been extensively investigated (Arsenijevic *et al.* 1998; van Golen and Feldman 2000). As previously mentioned, such neurotrophic factors can be also produced and/or act on immune cells (Kerschenseteiner et al., 2003). Most of the physiologic effects of IGF-I are mediated by the type I IGF surface receptor which, after ligand binding-induced autophosphorylation, associates with specific adaptor proteins and activates different second messengers (Bondy and Cheng 2004). These include: phosphatidylinositol 3-kinase, mitogen-activated protein kinase (Vincent and Feldman 2002; Di Toro *et al.* 2005) and members of the Janus kinase (JAK)/STAT3 signalling pathway (Zong *et al.* 2000; Yadav *et al.* 2005).

REST plays a complex role in neuronal cells by differentially repressing target gene expression (Lunyak *et al.* 2004; Coulson 2005; Ballas and Mandel 2005). REST expression decreases during neurogenesis, but has been detected in the adult rat brain (Palm *et al.* 1998) and is up-regulated in response to global ischemia (Calderone *et al.* 2003) and induction of epilepsy (Spencer *et al.* 2006). Thus, the REST concentration seems to influence its function and the expression of neuronal genes, and may have different effects in embryonic and differentiated neurons (Su *et al.* 2004; Sun *et al.* 2005). In a previous study, REST was elevated during the early stages of neural induction by IGF-I in neuroblastoma cells. REST may contribute to the down-regulation of genes not yet required by the differentiation program, but its expression decreases after five days of treatment to allow for the acquisition of neural phenotypes. Di Toro et al. proposed a model in which the extent of neurite outgrowth in differentiating neuroblastoma cells was affected by the disappearance of REST (Di Toro *et al.* 2005).

The human mu-opioid receptor gene (OPRM1) promoter contains a DNA sequence binding the repressor element 1 silencing transcription factor (REST) that is implicated in transcriptional repression. Therefore, in the fist part of this thesis, I investigated whether insulin-like growth factor I (IGF-I), which affects various aspects of neuronal induction and maturation, regulates OPRM1 transcription in neuronal cells in the context of the potential influence of REST. A series of OPRM1-luciferase promoter/reporter constructs were transfected into two neuronal cell models, neuroblastoma-derived SH-SY5Y cells and PC12 cells. In the former, endogenous levels of human mu-opioid receptor (hMOPr) mRNA were evaluated by real-time PCR. IGF-I up-regulated OPRM1 transcription in: PC12 cells lacking REST, in SH-SY5Y cells transfected with constructs deficient in the REST DNA binding element, or when REST was down-regulated in retinoic acid-differentiated cells. IGF-I activates the signal transducer and activator of transcription-3 (STAT3) signaling pathway and this transcription factor, binding to the STAT1/3 DNA element located in the promoter, increases OPRM1 transcription.

T-cell receptor (TCR) recognizes peptide antigens displayed in the context of the major histocompatibility complex (MHC) and gives rise to a potent as well as branched intracellular signalling that convert naïve T-cells in mature effectors, thus significantly contributing to the genesis of a specific immune response. In the second part of my work I exposed wild type Jurkat CD4+ T-cells to a mixture of CD3 and CD28 antigens in order to fully activate TCR and study whether its signalling influence OPRM1 expression. Results were that TCR engagement determined a significant induction of OPRM1 expression through the activation of transcription factors AP-1, NF-kB and NFAT. Eventually, I investigated MOPr turnover once it has been expressed on T-cells outer membrane. It turned out that DAMGO induced MOPr internalisation and recycling, whereas morphine did not.

Overall, from the data collected in this thesis we can conclude that that a reduction in REST is a critical switch enabling IGF-I to up-regulate human MOPr, helping these findings clarify how human MOPr expression is regulated in neuronal cells, and that TCR engagement up-regulates OPRM1 transcription in T-cells. My results that neurotrophic factors a and TCR engagement, as well as it is reported for cytokines, seem to up-regulate OPRM1 in both neurons and immune cells suggest an important role for MOPr as a molecular bridge between neurons and immune cells; therefore, MOPr could play a key role in the cross-talk between immune system and nervous system and in particular in the balance between pro-inflammatory and pro-nociceptive stimuli and analgesic and neuroprotective effects.

1. The Repressor Element 1 Silencing Transcription Factor (REST)

Nervous system development relies on a complex signalling network to engineer the orderly transitions that lead to the acquisition of a neural cell fate (Ballas and Mandel, 2005). Progression from the non neuronal pluripotent stem cell to a restricted neural lineage is characterized by distinct patterns of gene expression, particularly the restriction of neuronal gene expression to neurons (Ballas and Mandel, 2005).

The Repressor Element 1 Silencing Transcription Factor (REST), also known as the Neuron-restrictive Silencer Factor (REST), plays a pivotal role in such a context as it maintains transcriptional silencing of a range of neuronal genes in differentiated non-neuronal cells, as well as in un-differentiated neuronal cells during early lineage commitment in neurogenesis.

REST, in fact, was originally discovered as a transcriptional repressor of a large number of primarily terminal neuronal differentiation genes in non-neuronal cells and neuronal stem cells (NSCs); its transcription is generally blocked as NSCs undergo differentiation (Majumder, 2006), so that neuronal development can occur properly.

However, REST is expressed in some differentiated neurons and, when bound to a double-stranded small RNA, it is able to function also as an activator of its target gene transcription (Majumder et al., 2006), thus suggesting for REST a complex regulatory function in both embryonic and adult neuronal differentiation.

In addition, REST dysfunction has been implicated in diverse diseases ranging from Down's syndrome to cardiomyopathy and cancer, emphasising its importance also as a master regulator of normal gene expression programs (Coulson, 2005).

REST contains a DNA-binding domain and two distinct repressor domains, which can interact with several cellular repressor complexes, including huntintin protein (Htt) in the cytoplasm and mSin3A, histone deacetylases (HDACs), N-Cor, CoREST, histone H3-K9 methyltransferase G9a (HMTase), histone H3-K4 demethylase LSD1 (HDMase), DNA methyl transferase 1 (DNMT1), DNA-methyl-CpG-binding protein-2 (MeCP2) and chromatin remodelling complexes SWI/SNF in the nucleus (Fig. 1.1). Therefore REST serves as a giant communication hub for the cell, having a variety of roles in normal development as well as causing several abnormalities when deregulated (Majumder, 2006).

Normal development and abnormal development are, in fact, two sides of the same coin.

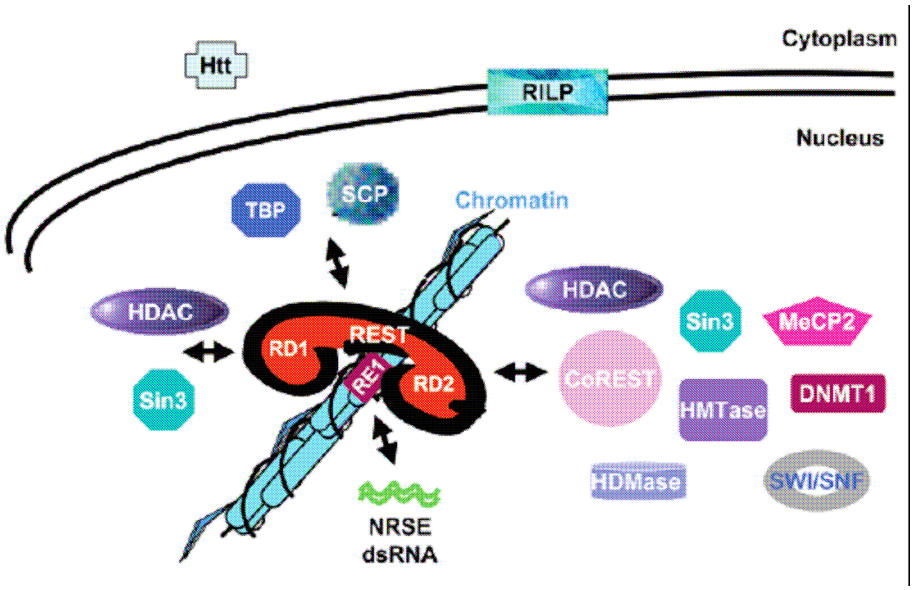


Fig. 1.1 *REST is a transcription factor that serves as a giant communication hub for the cell. In the cytoplasm, REST can interact with the huntingtin protein (Htt). REST can be translocated into the nucleus by binding to RILP, a LIM domain protein. In the nucleus, RD1 can interact with mSin3A, the histone deacetylase (HDAC) complex, and N-CoR, whereas RD2 can interact with CoREST, mSin3A/HDAC complex, histone H3-K9 methyltransferase G9a (HMTase), histone H3-K4 demethylase LSD1 (HDMase). The REST complex can also be associated with DNA methyltransferase 1 (DNMT1), DNA-methyl-CpG-binding protein-2 (MeCP2), the chromatin remodelling complex, SWI/SNF, etc. REST also binds to the TATA box binding protein (TBP) and to small RNA polymerase II carboxyl-terminal domain phosphatases (SCP), and to NRSE double-stranded RNA (dsRNA).*

1.1 Repressor Element 1 (RE1) and REST target genes

REST mediates its repressive action by binding a 21 bp specific sequence on the promoter of its target genes: the Repressor Element 1/Neuron-restrictive Silencer Element (RE1/NRSE) (Fig. 1.2).

The first two genes whose transcription REST has been shown to repress were the type-II sodium channel gene and *SCG10* (Jones and Meech, 1999); both of them bear the 21 bp RE1 sequence in their regulatory regions.

Later on, more than 30 different genes containing the RE1 element in their promoters were identified; these genes encode for a large spectrum of protein involved in neuronal development, including ion channels, neurotransmitter receptors and synthesizing enzymes, neurotrophins, synaptic vesicle proteins, adhesion molecules, cytosckeletal proteins (Paquette et al., 2000).

Since then, novel RE1 containing genes have been looked for, also taking advantage of innovative bioinformatic approaches; any specific transcription factor, in fact, can bind related yet distinct sequences and novel target genes can therefore be identified computationally by using either a consensus sequence or a position-specific scoring matrix (PSSM) (Stormo, 2000).

A consensus sequence is constructed by aligning the sequences of known binding sites in order to produce a sequence representation that includes as many of the sites as possible; RE1 consensus sequence (Fig. 1.2) is NTYAGMRCCNNRGMSAG (where N = A, C, G, T; Y = C or T; M = A or C; S = C or G) (Bruce et al., 2004). Although consensus sequences are relatively easy to construct and use in searches, they are generally a compromise between specificity and sensitivity. An alternative to using a consensus sequence is a PSSM (Fig. 1.2), which is usually derived from a collection of known binding site sequences and is a representation of the probability of each nucleotide at specific position within the transcription factor binding site (Ooi and Wood, 2007).

Fig. 1.2 *REST Position-specific scoring matrix (upper) and consensus sequence (lower).*

Taking advantage of the bioinformatic techniques above described more than 1000 putative REST target genes have been identified (Lunyak et al., 2002), the majority of which encodes for neuron specific proteins; however, this group of proteins is wide and heterogeneous, including ligands, ion channels, receptors, adhesion molecules and others.

More recently, serial analysis of chromatin occupancy (SACO) has been used to perform genome-wide identification of REST-occupied RE1 sites in a kidney cell line (Otto et al., 2007); this approach allowed for the identification of novel REST binding motifs, namely expanded RE1 and compressed RE1 (Fig. 1.3). Thus, the number of REST target genes has been found to exceed previous estimates and, unexpectedly, genes considered exclusively non-neuronal also contain an RE1 motif and are expressed in neurons (Otto et al., 2007). These findings underline the complexity of the regulatory processes mediated by REST interaction with RE1 elements, which are wide-spread in human genome.

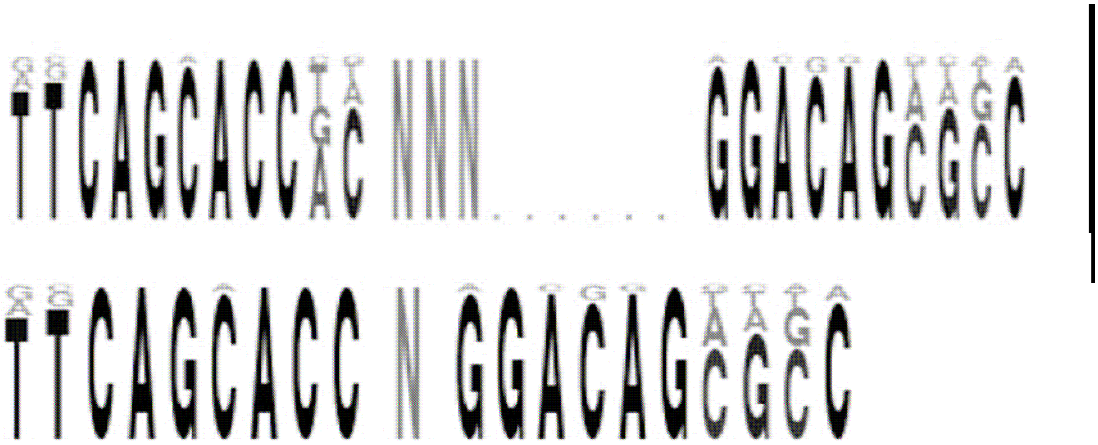


Fig. 1.3 *Position-specific scoring matrix of two novel REST binding elements: expanded (upper) and condensed (lower) RE1, respectively (Otto et al., 2007)*

1.2 REST genetic structure and mRNA alternative splicing

REST protein is encoded by a single-copy gene which is present both in rat (Palm et al., 1998) and human genome (Scholl et al., 1996); human REST is very similar to its rat counterpart, showing a 75% nucleotide homology and a 70% amino acid homology (Palm et al., 1999).

Human *REST* gene is localized on 4q12 and consists of seven exons and six introns (Fig. 1.4A): exons I, II and III encode for 5'UTR region A, B and C, respectively; one of these regions is always included in REST mature mRNA. Exons IV, V and VI contains the complete coding sequence for the REST protein, whereas exon N is included in REST splice variants just after exon V (Palm, 1999).

The modular structure of REST gene is responsible for the generation of different mRNA splice variants which encode for different REST protein isoforms, namely hREST5FΔ, hRESTN62 and hRESTN4 (Fig. 1.4B). The former is generated by the lack of exon 5 in the REST mRNA and is typical of SH-SY5Y neuroblastoma cell line; the second one arises from the incorporation of the whole exon N instead of exon VI, whilst the latter isoform originates from the incorporation of a 4 bp fragment of exon N instead of exon VI (Palm et al., 1999).

The inclusion of whole or partial exon N in the REST mRNA determines a frame shift with the subsequent generation of an early stop codon (Fig. 1.4B); therefore hRESTN62 and hRESTN4 isoforms lack of a consistent portion of the REST C-term (Palm et al., 1999), feature that confers different properties to these truncated isoforms as compared to the complete protein; because of their structural properties, in fact, hRESTN62 and hRESTN4 are supposed to be involved in REST full protein homeostasis and regulation.

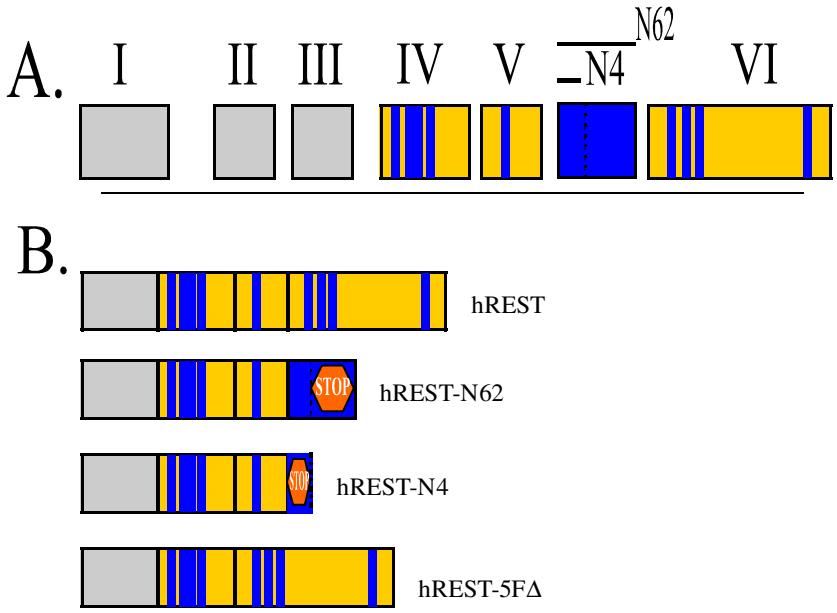


Fig. 1.4 *Schematic representation of REST encoding gene modular structure (A) and alternative splice variants of its mRNA (B).*

1.3 REST protein structure

The transcription factor REST is a 200 kDa protein (Okamoto et al., 1999), it consists of 1096 ammino acids (Palm et al., 1999) and belongs to the Gli-Krüppel transcriptional repressor family. REST bears nine C2H2 zinc-finger domains, eight of which are clustered in the N-term region of the protein, whilst the ninth is at the C-term (Chong et al., 1995) (Fig. 1.5).

Among the eight clustered zinc-finger domains, the first five mediate REST nuclear translocation, whereas the remaining three determine REST binding to DNA (Shimojo et al., 2001).

Furthermore, REST bears two repressor domains (RDs) that interact with several cellular cofactors to repress chromatin at its target promoters (Majumder, 2006): RD1 and RD2 (Fig. 1.5).

RD1 spans REST's first 83 ammino acids (Jones and Meech, 1999) and interacts with mSin3A, HDAC, N-Cor; RD2 overlaps the C-term zinc-finger domain (Chong et al., 1995) and interact with CoREST, mSin3A/HDAC complex, HMTase, HDMase, MeCP2 (Majumder et al., 2006).

hRESTN4 and hRESTN62 isoforms lack of a consistent portion of the REST C-term; therefore, they bear only the RD1 repressor domain and the first five among the eight clustered zinc-finger domains (Shimojo et al., 2001).

Because of the features mentioned above, hRESTN4 and hRESTN62 are supposed to act as REST dominant-negative regulator by interfering with REST activity (Shimojo and Hersh, 2004).

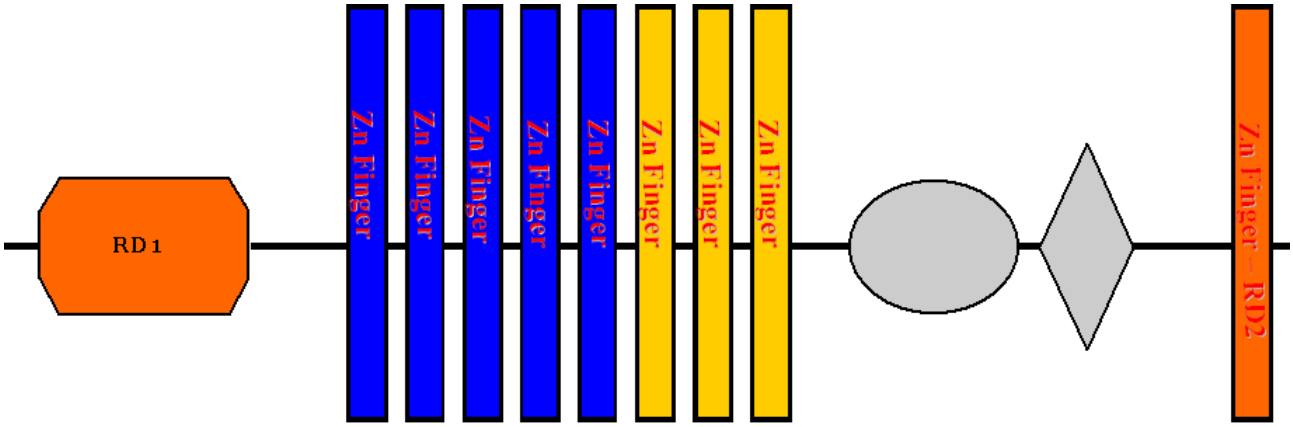


Fig. 1.5 *REST protein structure schematic representation: RD1 is responsible for interaction with mSin3; RD2, which overlaps the ninth C2H2 Zn-finger domain, interacts with CoREST, whereas the other eight clustered Zn-finger mediate DNA interaction, the first five (Blue) being important for nuclear translocation and the other three (Yellow) for DNA binding.*

1.4 REST and its co-repressor complexes: chromatin modulation and gene expression

CHROMATIN GENERAL ORGANIZATION, HISTONE POST-TRANSLATIONAL MODIFICATIONS AND GENE TRANSCRIPTION

Eucaryotic DNA is wrapped around histones to form a complex known as chromatin. The base unit of chromatin, the nucleosome, is composed of eight histones that are encircled one-and-three-quarter times by 147 bp of DNA (Ooi and Wood, 2007). Nucleosomes are arranged to form a structure that facilitates the packaging of an entire genome into a nucleus; in addition to this structural role for histones, post-translational modifications of their N-terminal tails give rise to changes in nucleosome-DNA interactions that are important for regulating transcription: such modifications, in fact, affect transcription by altering chromatin accessibility to transcription factors and/or providing specific binding sites for proteins that affect chromatin structure (Ooi and Wood, 2007).

In this form of regulation distinct patterns of gene expression are determined by chromatin modifications that do not involve changes in DNA sequence, thus making epigenetic regulation a compelling mechanism for controlling developmental events (Ballas and Mandel, 2005). For

the establishment of epigenetic modifications chromatin modifiers are recruited to specific genomic loci by DNA binding proteins, either transcriptional activators or repressors (Ballas and Mandel, 2005).

The main chromatin modifiers include histone acetylases (HATs) and HDAC, HMTases and HDMases, DNMTs. HATs induce acetylation of N-terminal histone tails, which decreases the interaction between the positively charged histone tails with the negatively charged phosphate backbone of DNA, hence resulting in a relaxation of nucleosomes (Hsieh and Gage, 2005).

HDACs catalyse the reverse reaction; in the deacetylated state histones package the DNA in a more condensed chromatin, which prevents access of transcriptional activators to their target sites, thus resulting in transcriptional repression (Hsieh and Gage, 2005). Similarly, histone methylation or demethylation influence chromatin condensation so that it becomes more or less accessible to transcription factors.

More in detail, histone H3 lysine 4 (H3K4) can be mono-, di- or trimethylated and di- or trimethylated H3K4 is a hallmark of transcriptional activity; mono- and dimethylation is removed by demethylase LSD1 (Fig. 1.6), which is a partner of REST repressor complexes.

Histone H3 lysine 9 (H3K9) can be either methylated or acetylated: acetylation of H3K9 inhibits the ability of LSD1 to demethylate H3K4 (Fig. 1.6); thus, HDAC activity, recruited as part of CoREST or mSin3 complexes, enhances LSD1 activity by removing H3K9 acetylation (Fig. 1.6). H3K9 is mono- or dimethylated by HMTase G9a, which promotes gene repression in euchromatic regions of the genome (Fig. 1.6).

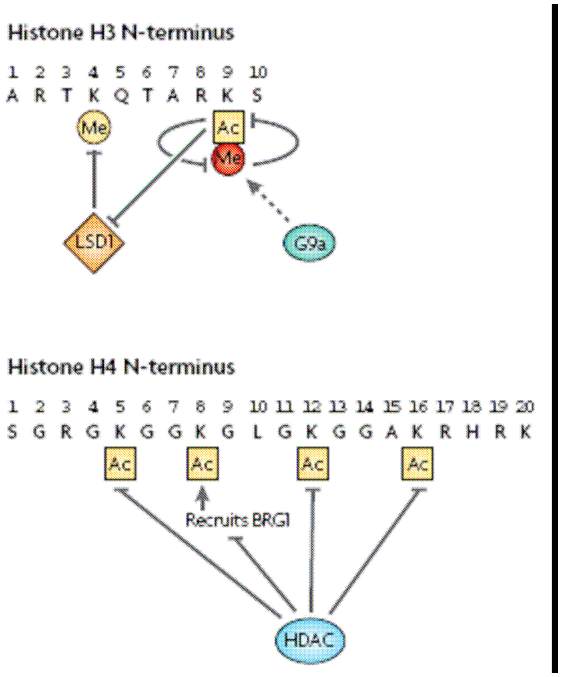


Fig. 1.6: *Histone modifications and REST complex: schematic representation of chromatin modifications involved in REST-mediated gene silencing.*

Lysine residues 5, 8, 12 and 16 of histone H4 can be acetylated and high levels of lysine acetylation have been identified in active regions of the genome, whereas low acetylation levels are found at silent loci. Acetyl groups are removed by HDACs (Fig. 1.6), which in turn are recruited as part of mSin3 and CoREST complexes.

REST INTERACTIONS WITH COREPRESSORS AND FUNCTIONAL INTERPLAY OF CHROMATIN REMODELLING ENZYMES

The initial step in REST-mediated repression is the recruitment of REST to RE1 sites. The RE1 sites are recognized by the zinc-finger domain of REST and interaction with DNA is stabilized by the ATP-dependent chromatin-remodelling enzyme, BRG1 (Fig. 1.7a). The bromodomain of BRG1 recognizes acetylated histone H4 lysine 8 (H4K8), an hallmark of active transcription. Increased H4K8 acetylation results in increased REST recruitment. When bound to chromatin, the BRG1 complex repositions nucleosomes with respect to DNA, presumably allowing REST to form a more stable interaction with the DNA (Fig. 1.7b). So, by remodelling chromatin, BRG1 seems to allow REST to gain better access to its chromatin targets (Ooi et al., 2006).

The N-terminus of REST interacts with the mSin3 complex, which contains HDAC1 and HDAC2 (Grimes et al., 2000) (Fig. 1.7c). The C-terminus of REST interacts with the CoREST complex, which contains HDAC1, HDAC2, BRG1, the H3K4 demethylase LSD1 and the H3K9 methylase G9a (Shi et al., 2004) (Fig. 1.7). Once REST is associated with DNA, HDACs that are recruited by mSin3 or CoREST remove acetyl groups from H3 and H4 lysine residues (Grimes et al., 2000). The removal of acetylation from H3K9 stimulates LSD1 activity, which removes di- and monomethylation from H3K4, thus introducing a mark of gene silencing (Lee et al., 2005) (Fig. 1.7d). Deacetylation of H3K9 can also inhibit the association of the bromodomain containing MLL complex, which contains a H3K4-specific methylase that antagonises REST function during neuronal differentiation (Wynder et al., 2005). Removal of H3K9 acetylation by HDACs also provides a substrate for G9a-mediated methylation, modification that is related to gene silencing (Fig. 1.7e). At the moment it is still unclear whether G9a is recruited directly by REST or as part of the CoREST complex. Methylated H3K9 can recruit the heterochromatic protein HP1 through its chromodomain (Fig. 1.7f), thus increasing the chromatin condensation (Ballas and Mandel, 2005).

In addition, CoREST recruits to the REST-RE1 site other silencing machinery, including MeCP2 (Fig. 1.7g); in this way REST-CoREST repression might result in the propagation of silencing across a large chromosomal interval 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 (Ballas and Mandel, 2005).

Moreover, CoREST can bind DNA directly and together with MeCP2 it forms a stable repressive complex in the absence of REST at RE1-regulated genes that contain methylated DNA (Ballas et al., 2005); the mSin3 complex as well can form stable interactions with chromatin (Hartman et al., 2005).

Collectively, these data highlight potential mechanisms by which REST could mediate long-term gene repression after only transient repressor occupancy, by initiating stable chromatin-co-repressors interactions (Ooi and Wood, 2007).

In summary, the stepwise activity of REST and its co-repressors leads to the removal of several marks that are associated with transcriptional activation, followed by their replacement with marks that are associated with transcriptional repression, and allows REST to mediate both short-term repression and long-term silencing (Ooi and Wood, 2007).

This difference between stable and transient repression of REST targets is important in several physiological and pathological processes such as neuronal differentiation, cardiac hypertrophy, vascular smooth muscle proliferation, colon cancer.

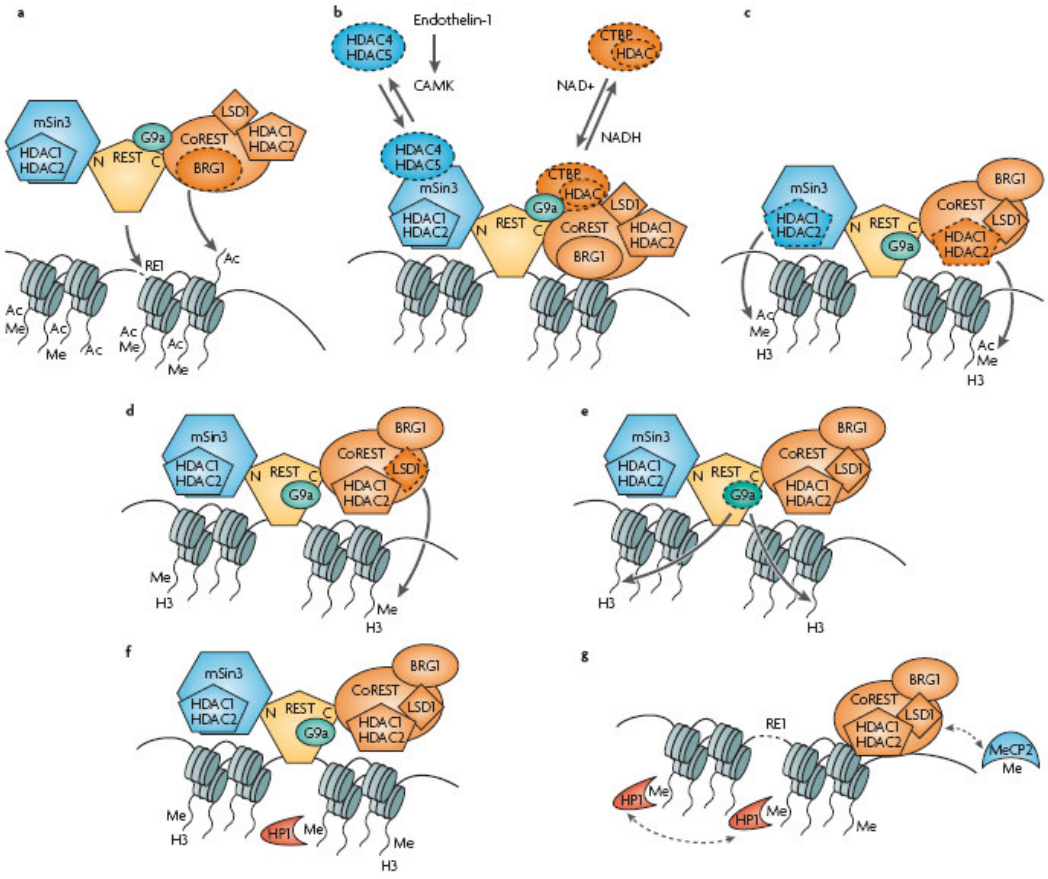


Fig. 1.7 *REST and its co-repressors mediate gene silencing in a progressive ad step-wise manner*

REST-MEDIATED DIRECT INHIBITION OF THE TRANSCRIPTIONAL MACHINERY

In addition to regulating chromatin structure, the REST complex can directly modulate transcription rates: its binding to the TATA-box-binding-proteins (TBPs) inhibits the formation of the preinitiation complex (Murai et al., 2004), whereas its interactions with RNA polymerase II small CTD phosphatases (SCPs) inhibits the activity of the polymerase (Yeo, 2005).

REST interaction with SCPs is involved in long term repression of neuronal genes in non neuronal tissues and could be part of the short term repressive process.

1.5 Neural fate determination: a very complex and tightly regulated process

REST MODULATION OF NEURONAL DIFFERENTIATION PROGRAM: RESTRICTION OF NEURONAL GENE EXPRESSION TO POST-MITOTIC NEURONS

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-neuronal cell fates outside the nervous system (Edlund and Jessel, 1999). These two events must be coordinated precisely for the 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).

REST is a key factor in chromatin epigenetic programming and its action depends on the cellular context; therefore REST is the good candidate to modulate the restriction of neuronal gene expression only to post-mitotic neurons. Moreover, REST is obligatory for the correct development of vertebrates, since perturbation of its expression or function in the developing embryo results in ectopic expression of neuronal genes in non-neuronal tissues and early embryonic lethality (Chen et al., 1998).

As epigenetic regulation of neural gene chromatin by REST is fundamental for maintaining stem cells in an undifferentiated pluripotent state as well as for preventing ectopic neuronal gene expression in non-neuronal tissues, questions arise about how REST can direct these different but related silencing programs. The silencing of neuronal genes in differentiated non-neuronal cells, in fact, is stable, inheritable and endures the lifetime, whilst embryonic stem cells, that do not express neuronal genes yet, still have the capacity of self-renewal and differentiation along all cell lineages (Ballas and Mandel, 2005).

The answer lies with REST different co-repressor complexes: the mSin3/HDAC complex is associated primarily with a dynamic mode of repression that can alternate between repression and activation, whereas CoREST complex recruits chromatin modifiers for long term silencing

of neuronal genes (Lunyak et al., 2004). Furthermore, the methylation of cytosine residues in CpG dinucleotide in the genome is also involved in the different processes that mediate long term and short term repression (Bird, 2002): RE1, in fact, contains a CpG dinucleotide which is methylated, as well as its surrounding regions, in differentiated non-neuronal cells (Ballas et al., 2005) (Fig. 1.8a).

REST has been shown to bind to RE1 regardless the methylation pattern of CpG dinucleotides (Ballas and Mandel, 2005) and this finding point to the different CpG methylation status as a candidate mark for determining long term repression instead of short term repression (Fig. 1.8). In differentiated non-neuronal cells, in which neuronal genes are silenced permanently and CpG methylation is present at RE1 sites, REST/CoREST recruits a silencing complex to neuronal genes which includes modifying enzymes such as HDAC 1 and 2, H3K4 HDMase, and H3K9 HMTases (Fig. 1.8a). As previously mentioned, methylated lysine 9 residues are binding sites for heterochromatin protein 1 (HP1), which causes further chromatin condensation (Fig. 1.8a). RE1 and adjacent regions are methylated at CpGs and associated with the methyl DNA binding protein MeCP2. MeCp2 is also associated with Sin3–HDAC complexes and DNMT1 is recruited to the methylated RE1 site, thus completing the high order chromatin condensation induced following REST interaction with its target sequence RE1 (Fig. 1.8a). Furthermore, the small CTD phosphatase (SCP) is also associated with the REST/corepressors complex and it might block RNA polymerase II activity (Fig. 1.8a). All the mentioned processes contribute to permanently repress neuronal gene expression in non-neuronal tissues.

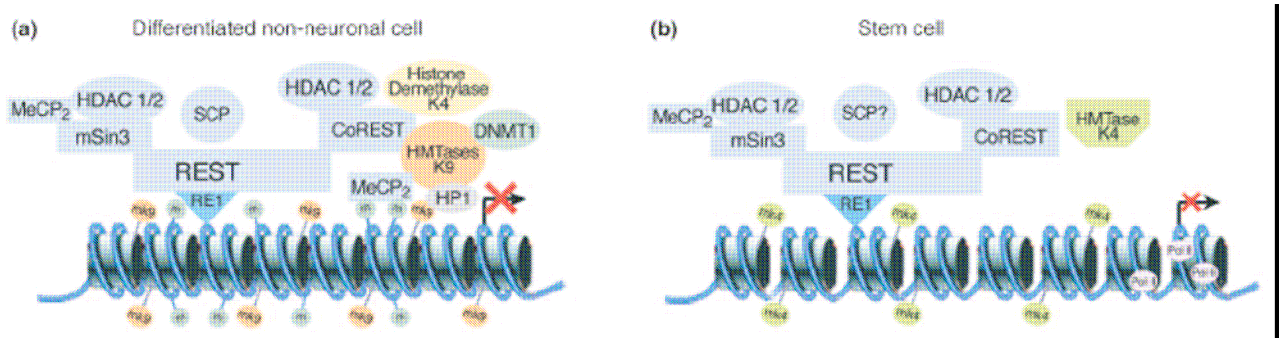


Fig. 1.8 *REST orchestrates differential epigenetic mechanisms to inactivate neuronal gene expression in non-neuronal cells:*

(a) REST-mediated long-term silencing in differentiated non-neuronal cells

(b) REST-mediated short-term repression in neuronal stem cells and progenitors

On the other hand, since neuronal gene chromatin in embryonic stem cells (ES) and neural progenitors is programmed to stay in a repressed state that is none-the-less poised for expression (Ballas et al., 2005), REST and its corepressors give rise to a more dynamic and less tight chromatin condensation (Fig. 1.8b).

HDAC 1 and 2 are the predominant modifiers recruited by REST/CoREST/mSin3 complex to transiently repress neuronal gene expression during early stages of nervous system development; furthermore, CpG dinucleotides at RE1 sites are not methylated and histones are marked by H3K4 methylation, which is typical of a transcriptionally active chromatin (Fig. 1.8b). Because of this relative low chromatin condensation, RNA pol II is associated with the 5'UTR region of neuronal gene chromatin and determines very low transcript levels (Ballas et al., 2005) (Fig. 1.8b).

Thus, the epigenetic modifications associated with the RE1 sites of neuronal genes in ES and neuronal progenitors point to an inactive, but permissive, chromatin state that is poised for subsequent activation (Ballas and Mandel, 2005). Taken together, all the above reported observations suggest that, in order to determine either long term silencing or short term repression, the core REST complex establishes a distinct set of epigenetic marks by recruiting different chromatin modifiers.

The effect of the processes previously described is to block neuronal gene expression in non neuronal tissues and in undifferentiated neural stem cells and progenitors, thus restricting neuronal gene expression only to post-mitotic neurons.

THE TRANSITION FROM EMBRYONIC STEM CELL TO MATURE NEURON: PROGRESSIVE DOWN-REGULATION OF REST AND SUBSEQUENT RELIEVING OF NEURONAL GENE EXPRESSION

The transition from stem or progenitor cell to a post-mitotic neuron requires disarming REST (Ballas and Mandel, 2005). In neuronal stem cells neuronal gene expression is silenced, since REST binds to RE1 elements and determines chromatin condensation by recruiting its co-repressor complexes; during embryonic differentiation, REST is removed at two distinct stages, first at the dividing progenitor stage and then at terminal differentiation (Ballas and Mandel, 2005) (Fig. 1.8a).

In the progenitor cells, REST is removed by proteasomal degradation (Fig. 1.8a): the subsequent reduction in REST levels relieves the expression of some neuronal gene which are required in the first steps of differentiation (Fig. 1.8b). There are, in fact, genes whose promoters have a low affinity for REST binding (Coulson, 2005).

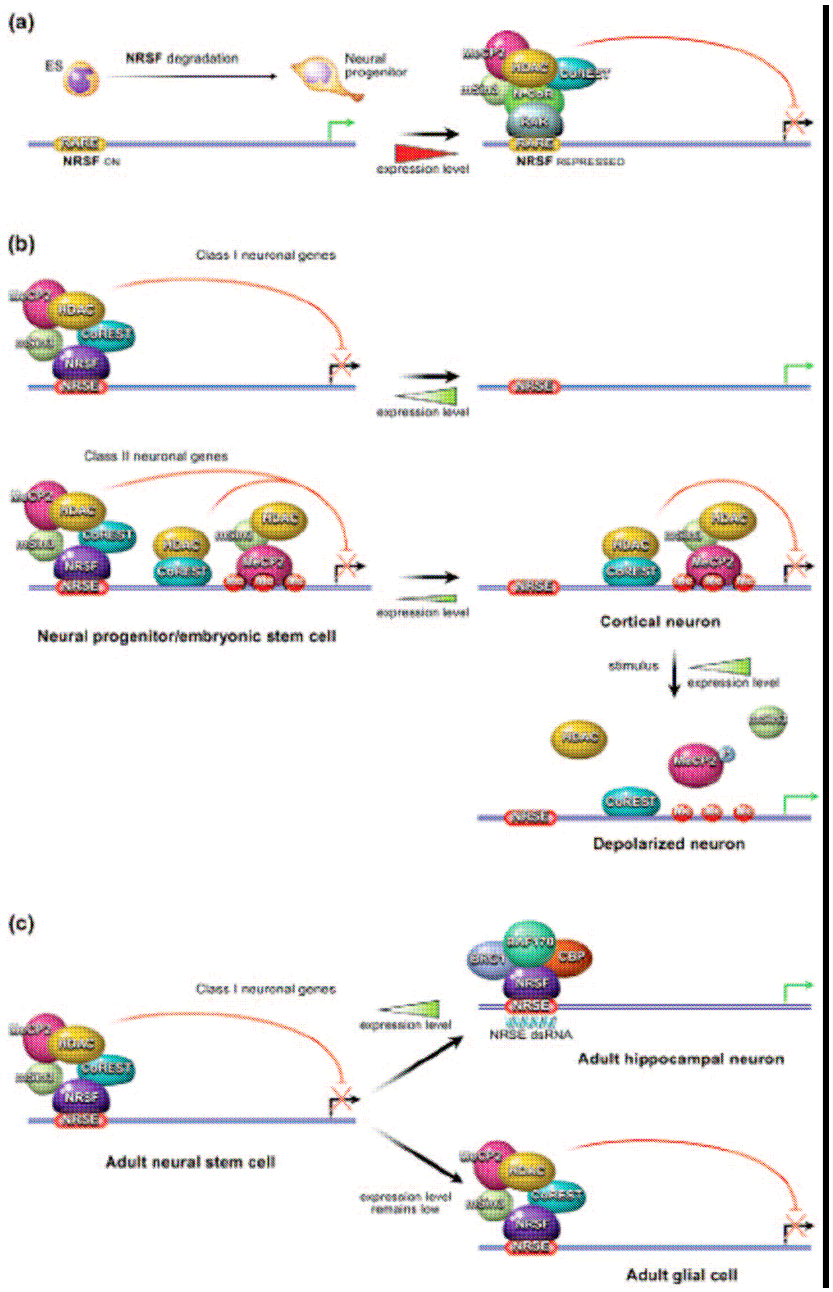


Fig. 1.9: *Context-dependent gene regulation by the REST complex:*
(a) Neuronal gene expression is low in embryonic stem (ES) cells as a result of maximal levels of REST mRNA. REST post-translational degradation (dark purple nuclei to light purple nuclei) allows neuronal gene expression to be'primed' for activation. Unliganded RAR receptor (RAR) complex binds to the retinoic acid receptor element (RARE), thus determining REST transcriptional down-regulation (large red arrow).
(b) Two classes of neuronal genes mediated by two different mechanisms of REST-mediated repression. Class I neuronal genes are repressed by the REST repressor complex in neural progenitor cells. Upon neuronal differentiation, displacement of REST complex occurs, allowing de-repression of only Class I neuronal genes. When neurons are depolarised upon stimulation, MeCP2 is phosphorylated and released from CoREST, allowing neuronal gene activation (larger green arrow).
(c) REST represses neuronal genes in adult neural stem cells. Neuronal induction triggers the production of RE1 double-stranded RNA (RE1 dsRNA) and REST is converted from a repressor to an activator of transcription.

In the mature neurons, REST is removed by chromatin and transcriptional repression, so that neuron-specific genes are fully expressed and terminal differentiation is achieved: REST gene transcription is blocked by the binding of the unliganded retinoic acid receptor-repressor complex to the retinoic acid receptor element present on the REST gene promoter (Fig. 1.8a); thus, REST corepressors are dissociated from RE1 but still present, chromatin is relaxed and therefore accessible to transcription factors.

This differential timing of neuronal gene expression in a complex process such as neuronal differentiation, therefore, depends on different REST co-repressor complexes, but also on the two distinct mechanisms that REST employs to mediate gene repression in developing neurons (Ballas et al., 2005).

In fact, neuronal genes can be divided into class I and class II genes on the base of REST repression mechanisms (Fig. 1.8b): in class I neuronal genes, RE1 sites are occupied by REST co-repressor complex, which represses chromatin so that the removal of REST from the chromatin is enough to determine their expression (Ballas and Mandel, 2005) (Fig. 1.8b). In class II neuronal genes RE1 sites are occupied by REST co-repressor complex and distinct but adjacent methylated CpG sites are occupied by CoREST/HDAC and MeCP2/mSin3/HDAC complexes (Fig. 1.8b); in this scenario, removal of REST from chromatin alone does not activate the expression of these genes because of the additional repressor complexes located on the methylated CpG sites. The class II genes, however, can be further activated upon application of a specific stimulus, such as membrane depolarisation, which relieves the additional repression and provides neuronal plasticity (Ballas and Mandel, 2005) (Fig. 1.8b).

Furthermore, neuronal differentiation could rely on the progressive action of positive transcription factors. Presently, although the identity of transcriptional activators that might function after REST departures is not known, a novel neuronal protein, named inhibitor of BRAF35 (iBRAF), has been proposed as an intriguing candidate. iBRAF expression, in fact, increases during neuronal differentiation and this event contributes to abrogate REST mediated repression of neuronal target genes (Shiekhattar, personal communications).

REST AND THE CHROMATIN AT TERMINAL DIFFERENTIATION: REST RE-EXPRESSION IN ADULT NEURONS AND ADULT NEUROGENESIS

Active neuronal chromatin of mature neurons is in a relaxed conformation and marked by an increased amount of trimethylated H3K4 (Fig. 1.10a). Re-expression of REST might result in reprogramming of neuronal genes to a repressed state by reduction of trymethylated H3K4 and compaction of chromatin, probably by histone deacetylation (Ballas and Mandel, 2005) (Fig. 1.10b).

Alternatively, REST recruits corepressors but is unable to reprogram the chromatin to a repressed state and, therefore, neuronal genes are still transcribed (Ballas and Mandel, 2005) (Fig. 1.10c).

Postnatal and adult neurogenesis can be divided into three stages: first, self renewal, fate specification (into neurons and glia) and survival of neural precursor genes; second, migration and connection of new-born neurons with pre-existing neurons; third, reorganization in the synaptic connectivity between new-born and pre-existing neurons driven by sensory experience (Hsieh and Gege, 2005); epigenetic alterations leading to chromatin remodelling could provide a coordinated system of regulating gene expression at each stage of neurogenesis.

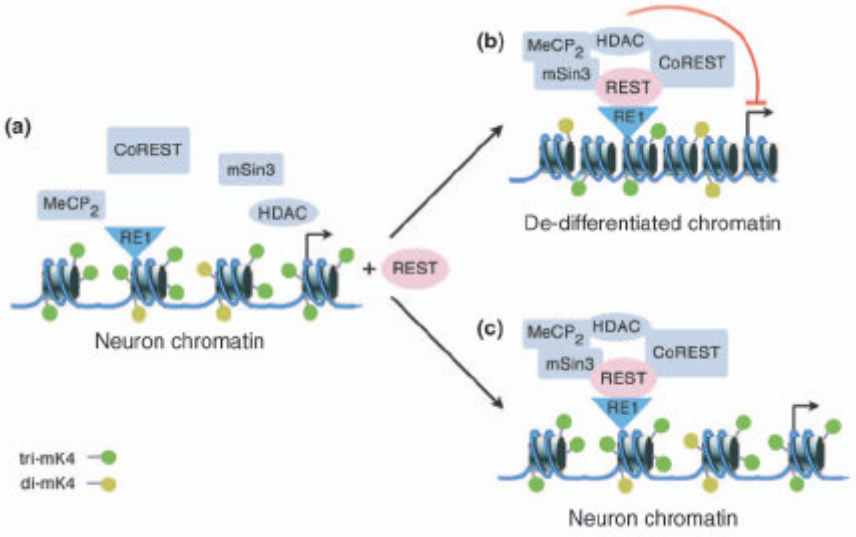


Fig. 1.10 *Different possible outcomes of REST re-expression in mature neurons:*
(a) Chromatin status in mature neurons in the absence of REST.
(b) Re-expression of REST might result in reprogramming of neuronal genes to a repressed state.
(c) REST recruits corepressors but is unable to reprogram chromatin to a repressed state.

In contrast to differentiation during embryo genesis, which is mainly driven by the progressive removal of REST, the differentiation of adult hippocampal stem cells into neurons occurs via a small non-coding double-stranded RNA (dsRNA) containing RE1 motif, that converts REST from a repressor to an activator of neuronal gene transcription (Kuwabara et al., 2004) (Fig. 1.8c). The RE1 dsRNA is about 20 bp long and, by interacting with REST transcriptional machinery in mature neurons, it prevents REST functions and determines neuronal differentiation. One model of how dsRNA acts is to directly associate with REST, altering its activity (perhaps through the induction of a conformational change) and preventing the interaction with co-repressor proteins (Hsieh and Gage, 2005). Alternatively, dsRNA could convert REST from a repressor to an activator of transcription through the action of an unknown protein(s) (Hsieh and Gage, 2005).

The implications of dsRNA in neuronal differentiation are enormous and suggest a therapeutic potential in neuroregeneration research (Majumder, 2006). It would be interesting to determine whether neurogenic stimulation such as exercise up-regulates the expression of RE1 dsRNA in adults.

NEURONAL DIFFERENTIATION AND THE TIGHTLY REGULATED BALANCE BETWEEN PRO- AND ANTI-NEURAL FACTORS

The nervous system relies on a complex network of signalling molecules and regulators to orchestrate a robust gene expression program that leads to the orderly acquisition and maintenance of neuronal identity (Wu and Xie, 2006). In the developing central nervous system, dividing neural progenitors in the ventricular zone begin to express neuronal genes as they migrate laterally. Pos-mitotic neurons eventually settle in the outer layer. This process of neurogenesis requires the coordinated up-regulation of neuronal genes and down-regulation of non-neuronal genes to ensure the proper timing of differentiation and number of neurons (Visvanathan et al., 2007).

REST and co-repressor complexes involvement in determining neural fate has been above extensively discussed; however, genome-wide analysis of RE1 sites revealed that some REST-binding sites are adjacent to non-coding micro RNAs (miRNAs) encoding genes (Ooi and Wood, 2007). These small RNAs negatively regulate target protein levels by reducing translation efficiency and/or increasing mRNA turnover, and have significant roles in cell-fate determination and tissue development (Hornstein and Shomron, 2006); since miRNAs down-regulate multiple target genes simultaneously (Lewis et al., 2005), they can serve as global regulators of gene expression.

MicroRNA genes are transcribed as a primary miRNA transcripts and processed to a 22-nucleotide mature miRNA; the seed region (2-7 nucleotides) of miRNAs is critical for target recognition and silencing (Lewis et al., 2005).

During development, many miRNAs are expressed in neurons or specific areas of the CNS, and their role in CNS development and direct target genes have just begun to be identified; by the way, the hypothesis that REST regulates miRNA expression has been experimentally validated. REST, in fact, has been shown to repress the expression of miRNA genes *mir-9*, *mir-124* and *mir-132* (Conaco et al., 2006), all of which promote neuronal differentiation. REST itself might be a target of miRNAs such as *mir-153*, the expression of which it also regulates (Mortazavi et al., 2006).

Such reciprocal repression mechanisms are widespread in biology and lead to the formation of a bistable switch which would be predicted to result in high or low, but not intermediate, levels of REST (Ooi and Wood, 2007). Other regulatory loops involving REST corepressors have been postulated, and at least one REST co-repressor, SCP1, has been shown to be a target of a REST-regulated miRNA, *mir-124*, in the developing mouse brain (Visvanathan et al., 2007).

In particular, miR-124, whose mature sequences are conserved from *C. Elegans* to humans, is the most abundant miRNA in adult and embryonic CNS (Kloosterman et al., 2006). In non-neuronal HeLa cells, mis-expressed miR-124 down-regulates 174 genes expressed at low levels in the brain (Lim et al., 2005); these results suggest that miR-124 may contribute to maintaining neuronal identity by suppressing non-neuronal genes in neurons. (Visvanathan et al., 2007).

In non-neuronal cells, including neural progenitors, the REST/co-repressor complex transcriptionally represses the expression of miR-124 and other neuronal genes; as the REST level decreases along neurogenesis, miR-124 expression is derepressed and, subsequently, miR-124 post-transcriptionally suppresses multiple anti-neural factors including SCP, CoREST, MeCP2 (Visvanathan et al., 2007).

This regulatory loop may represent a key mechanism to sense the intricate balance between pro-neural and anti-neural cues during development, to coordinate robust neuronal gene expression and to confer neuronal identity in a timely manner (Visvanathan et al., 2007). Thus, the negative feedback loop between anti-neural SCP, CoREST and MeCP2 and pro-neural miR-124 pathways may represent an evolutionary conserved developmental strategy that plays key roles in vertebrate CNS maturation (Fig. 1.11).

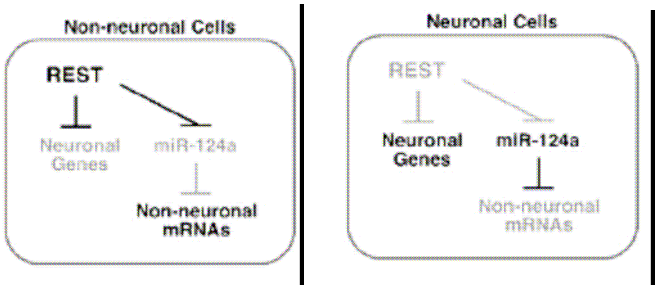


Fig. 1.11 *A hierarchy of two global negative regulators, REST and miR-124a, promotes a neuronal phenotype. (Left) REST transcriptionally represses neuronal genes and miR-124a in non-neuronal cells and neural progenitors.*
(Right) The dismissal of REST from chromatin during neurogenesis results in en masse expression of neuronal genes and down-regulation of competing non-neuronal transcripts through miR-124a function.

Given that both REST and miRNAs act as repressors and that there is a double-negative feedback between them in stabilizing and maintaining neuronal gene expression (Fig. 1.11), it has been recently demonstrated that the brain-related miRNA genes are highly enriched with

evolutionary conserved cAMP response elements (CRE) in their regulatory regions (Wu and Xie, 2006); these findings implicate the role of cAMP response element binding proteins (CREBs) in the positive regulation of these miRNAs. CREB is an important transcription factor regulating a wide-range of neuronal functions including neural survival, neuronal proliferation and differentiation and synaptic plasticity (Carlezon et al., 2005); furthermore, it can be activated via phosphorylation by multiple extracellular stimuli such as neurotrophins, cytokines and calcium, as well as a variety of cellular stresses.

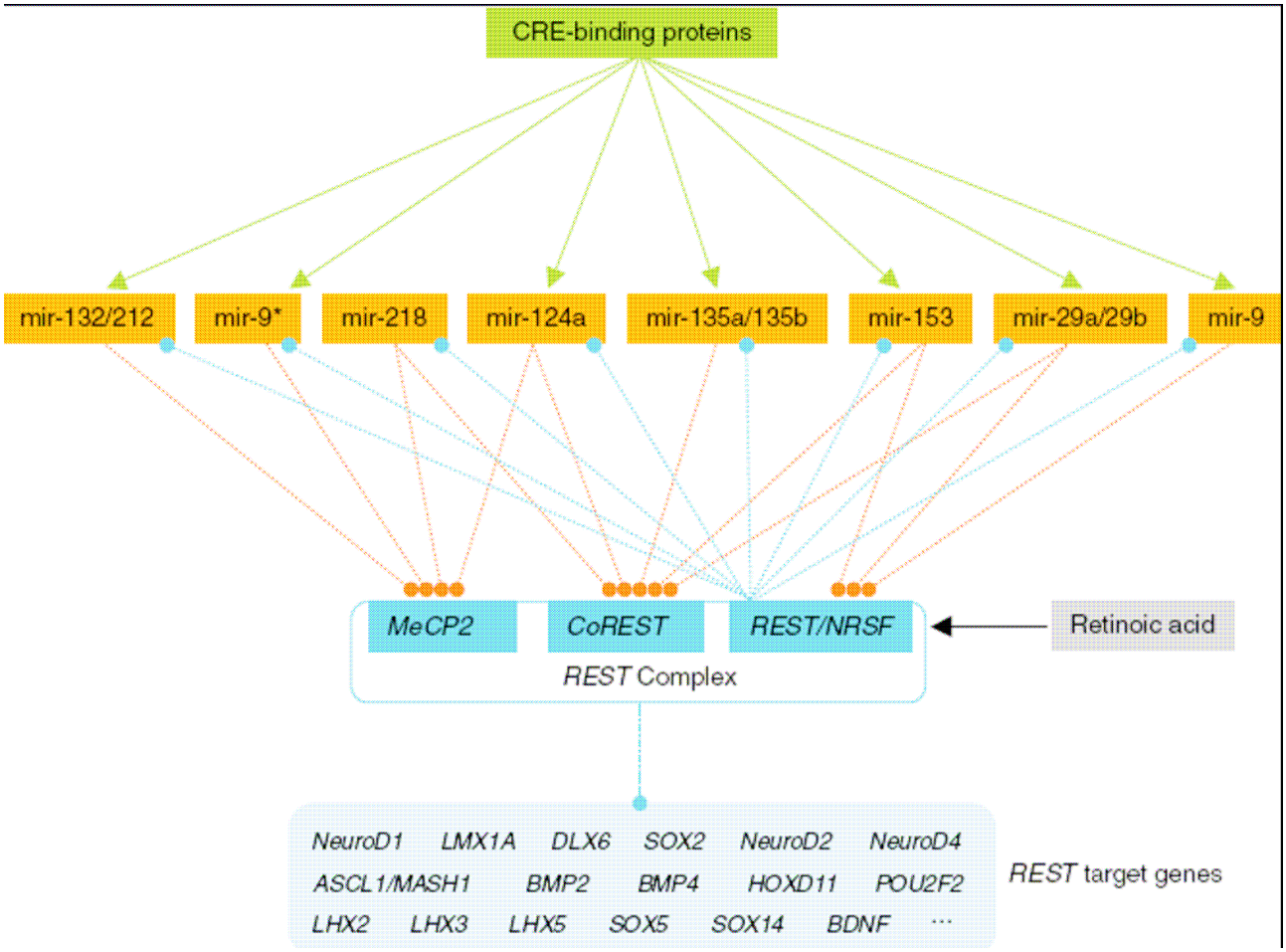


Fig. 1.12 *Schematic diagram of the interactions among REST, CREB and miRNAs.*
The three classes of regulators are represented by different colours, with the REST complex shown in blue, miRNAs shown in orange, and CREB family proteins shown in green. A list of REST target genes is shown in light blue. Positive interactions are indicated with solid lines with arrows, while negative interactions are denoted with dotted lines with filled circles.

The discovery of regulation of multiple miRNAs by CREB indicates that these miRNAs are potentially expressed in an activity-dependent manner, thus suggesting the existence of an intricate network of transcription activators and repressors acting together with miRNAs in coordinating neuronal gene expression and promoting neuronal identity (Wu and Xie, 2006) (Fig. 1.12).

1.6 REST deregulation and its function in determining diseases

Apart form its pivotal role in neurogenesis, neuronal differentiation and neuroregeneration, which has been previously extensively described, REST has been implicated in diverse diseases ranging from Down's syndrome, cardiomyopathy and cancer, thus highlighting the importance of REST-mediated regulation to the integrity of the cell (Ooi and Wood, 2007).

Transcription factors, in fact, coordinate complex gene expression programs during development and their deregulation in cancer often activates aberrant foetal-like transcription patterns (Coulson, 2005); REST has been recently found to have a seemingly paradoxical role showing both tumour suppressor activity and oncogenic activity (Majumder, 2006). Current evidence suggests that the diverse cellular context generated by intrinsic factors in the cell, the amount f REST protein present in the cell, the affinity of the REST protein for its specific target genes, and the cellular niche dictate this behaviour (Majumder, 2006).

REST TUMOR-SUPPRESSION FUNCTION

REST was first linked with specific cancers some years ago from studies in medulloblastoma (Lawinger et al., 2000), neuroblastoma (Palm et al., 1999) and small cell lung cancer (Coulson et al., 2000). In fact, although normal human bronchial epithelial cells, similar to other non-neuronal cells, primarily express epithelial markers and not neuronal markers, human small cell lung cancer (SCLC) primary samples as well as cell lines express both epithelial and neuronal markers, a hallmark of neuroendocrine tumours. It is known that normal bronchial epithelial cells express REST, apparently to block the spurious expression of neuronal genes. An excellent work from Judy Coulson's laboratory showed that several human SCLC primary samples, along with established SCLC cell lines, and not normal bronchial epithelial cells or human brain tissue, expressed enormous levels of a truncated isoform of REST(Coulson et al., 2000) (Fig. 1.13d). This isoform, similar to hRESTN4 or hRESTN62, is likely to function as a dominant-negative inhibitor of REST activity in these cells, causing the expression of neuroendocrine markers such as arginine vasopressin. Other laboratories showed that the reduced REST activity seen in some other SCLC cell lines was due to the lack of REST protein expression rather than to the expression of the dominant-negative isoform, leading to the concomitant abnormal expression of REST target genes (Coulson et al., 2005).

Furthermore, one laboratory showed that several human non SCLC cell lines expressed the REST mRNA and protein but did not express the SWI/SNF complex, a cellular cofactor required for REST activity (Watanabe et al., 2006) Thus, the abnormal blockade of REST activity in these tumour cells, resulting from the expression of a dominant-negative isoform of

REST, inhibition of the REST gene expression itself, or inhibition of a cofactor required for REST function, might lead to the expression of neuroendocrine genes and correlate with tumour phenotype.

Presently, a role for REST in tumorigenesis seems more likely to be widely recognised, given that it has emerged as one of the first tumour suppressors to be predicted from an unbiased RNA interference (RNAi) library screen in a recent study (Westbrook et al., 2005).

In this work, RNAi-mediated epithelial cell transformation was screened using an in vitro breast cancer precursor model, and REST was one of only five candidate genes that survived validation (Fig. 1.13a). Anchorage independent growth was promoted by REST knockdown in this model, but suppressed by REST over-expression in a colon cancer cell line. Interestingly, the genomic region around at least one REST allele was deleted in a third of colon cancers supporting a role for this transcriptional repressor as a tumour suppressor (Westbrook et al., 2005) (Fig. 1.13b). Both breast and colon cancer can display some neuroendocrine features and reduced transcription of REST, as it is well documented also in small cell lung cancer (Coulson et al., 2000), as previously mentioned, whereas many neuroendocrine genes that would normally be restricted through RE1 motifs in non-neuronal cells are aberrantly expressed (Neumann et al., 2004). Involvement of REST in neuroendocrine gene expression was also found in a prostatic LNCaP cell line (Fig. 1.13c), where the loss of REST function accompanied the acquisition of the neuroendocrine phenotype in the LNCaP prostate cancer cells (Tawadros et al., 2005). This trans-differentiation is associated with androgen independent progression, suggesting that REST dysfunction could contribute to worsening of disease.

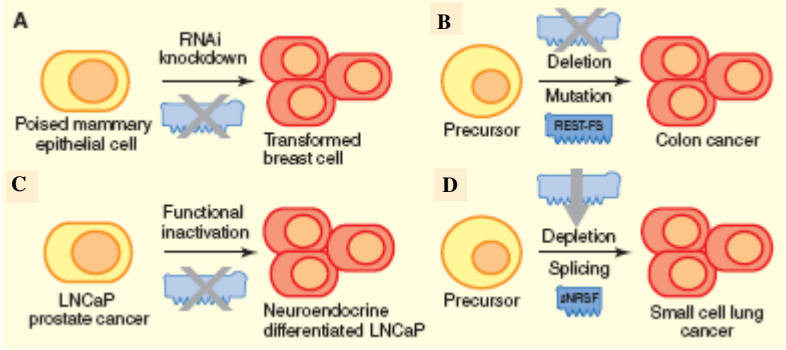


Fig. 1.13 *Experimental models and possible routes for REST/NRSF dysfunction in cancer: REST knock-down, deletion and mutation are involved in the transformation of poised mammary epithelial cell into transformed breast cancer and in development of colon cancer (A and B, respectively).*

REST functional inactivation is involved in neuro-endocrine differentiation of prostatic LNCaP cell line (C). REST depletion and alternative splicing is responsible for small cells lung cancer (D)

REST ONCOGENIC FUNCTION

In contrast to the tumour-suppressor function of REST, it has been found that REST has an oncogenic function in medulloblastomas (Majumder et al., 2006). Medulloblastoma, one of the most malignant brain tumours in children, is believed to arise from undifferentiated neural stem/progenitor cells present in the cerebellum. Pathways regulating cerebellar development, such as Hedgehog and Wnt, have been found to be activated by genetic alterations during medulloblastoma tumorigenesis (McMahon, 2000). Both Hedgehog and Wnt are thought to regulate proliferation and differentiation of neural stem cells and may play a similar role in medulloblastoma.

However, mutations activating these pathways have been documented in only a modest percentage of human medulloblastoma tumours.

At this regard, it has been shown that many human medulloblastoma samples and established human medulloblastoma cell lines over-expresses REST, as compared to neuronal cell lines or normal brain cells (Lawinger et al., 2000) As expected, the REST-positive human medulloblastoma tumour cells did not express the REST target genes, such as synapsin, indicating that it is the REST repressor function that is important in tumorigenesis. In support of this view, the expression of the dominant positive variant REST-VP16 in REST-expressing tumour cells has been shown to cause the activation of REST target genes and the subsequent blockade of tumorigenicity (Su et al., 2006) However, further work showed that REST alone was not sufficient to cause tumorigenesis. David Anderson's group found that neuronal cells constitutively expressing REST do not form tumours and appear to acquire a normal neuronal morphology, except that they manifest axon pathfinding errors (Paquette et al., 2000). Similarly, it has been found that transgenic mice expressing REST in neuronal cells appear to develop normally without tumour formation (Majumder, 2006). The observation that several medulloblastomas over-express Myc suggests that also this transcription factor could be involved in medulloblastoma tumorigenesis. In fact, when neuronal stem cells that over-express activated Myc and full-length REST are grown under conditions that favour proliferation in vitro, the cells looked similar and showed a similar proliferation rate as compared to cells expressing only Myc (Su et al., 2006). However, when the same cells are grown under conditions that favour differentiation, only the REST plus Myc-expressing cells are blocked from differentiation, thus showing a proliferation advantage for them over the control cells.

These findings clearly indicate that contemporary expression of REST and Myc is responsible for medulloblastoma onset (Fig. 1.14a). In addition, Myc plus REST-expressing neuronal stem cells, and not the control cells, give rise to tumours in the mouse cerebellum, the site of human medulloblastoma formation, if implanted *in vivo*. Furthermore, these tumours were blocked in neuronal differentiation and were morphologically similar to human medulloblastoma, whereas the same cells that produce tumours in the cerebellum do not produce tumours when transplanted in the cortex, thus indicating the critical role of the local brain environment in the formation of tumours (Su et al., 2006).

Moreover, it has also been found that countering the effects of REST by REST-VP16 in Myc plus REST-overexpressing neuronal stem cells determines apoptosis and counteracts the tumorigenic potential of the cells, similar to what it has been observed in human medulloblastoma cell lines. Thus, efficient medulloblastoma tumorigenesis occurs when two conditions are met: neuronal stem cells are forced to undergo neuronal differentiation and express both Myc, causing overall increased proliferation, and REST, the repressor function of which causes blockade of differentiation (maintenance of "stemness").

The oncogenic role played by the overexpression of REST in neuronal cells is further supported by studies from other laboratories, which found an overexpression of REST in several neuroblastoma cells with concomitant repression of neuronal differentiation genes (Higashino et al., 2003) (Fig. 1.14b). Furthermore, when the neuroblastoma cells are forced to differentiate, REST expression decreases and neuronal markers increase in the same cells.

Therefore, these studies suggest that the abnormal overexpression of REST in neuronal cells, in which REST is normally not present, blocks these cells from terminal neuronal differentiation and produces the cancerous phenotype, perhaps by forcing the cells to persist in a stem/progenitor state.

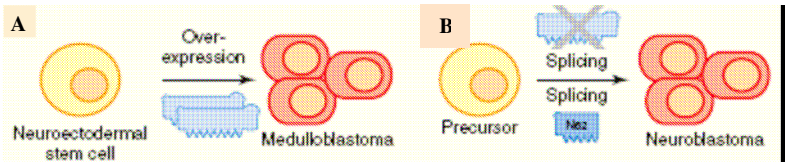


Fig. 1.14 *Experimental models and possible routes for REST/NRSF dysfunction in cancer: REST over-expression is involved in medulloblastoma tumorigenesis (A), whereas REST alternative splice variants generation determines neuroblastoma onset (B).*

REST INVOLVEMENT IN DISEASES OTHER THAN TUMORS

Apart from its suppressor-suppressor and oncogenetic functions, REST has also been found to regulate several genes that may impact other important biological processes. For example, ischemic insults were found to derepress REST mRNA and protein in dying neurons, and this has been suggested to be a critical mechanism of insult-induced neuronal death (Calderone et al., 2003). In addition, REST and huntingtin protein were found to form a complex that forces REST to localize in the cytoplasm in normal neuronal cells. On the contrary, in patients with Huntington's disease this interaction is ablated by the huntingtin mutation, resulting in the translocation of REST in the nucleus and causing blockade of neuronal gene expression (Zuccato et al., 2003), thus suggesting a role of REST in Huntington's disease. REST was also found to repress the mu-opioid receptor in neuronal cells, and thus it may have a role in opium addiction (Kim et al., 2004). Similarly, REST was found to repress the serotonin 1A receptor, which is implicated in depression and anxiety (Lemonde et al., 2004). Among non-neuronal tissues, REST has been found to be present in normal ventricular myocytes and to repress the expression of multiple foetal cardiac genes (Kuwahara et al., 2004). In cardiac dysfunction and arhythmogenesis, REST expression has been found to be inhibited, resulting in the expression of foetal cardiac genes. These results suggest that REST has a role in maintaining normal cardiac structure and function and that its deregulation may cause cardiac dysfunction. A very attractive study performed by Ian Wood and Noel Buckley's groups showed that REST was expressed in vascular smooth muscle cells, where it repressed the expression of a critical potassium channel gene and thereby regulated the normal and diseased state of the cell (Cheong et al., 2005).

1.7 Summary and conclusion

In order to mediate neuronal gene repression REST assembles repressor cofactors on target genes via its two repressor domains, thus determining chromatin condensation and presiding to long-term silencing of neuronal genes in non-neuronal tissues and to short term repression of neuronal genes in neuronal stem cells and progenitors.

REST exerts two different repressor mechanisms (Fig. 1.15A): one is relieved just by REST down-regulation along neuronal differentiation, the other needs further stimuli to be overcome.

REST expression in neurons is down-regulated during differentiation at first by proteasomal degradation ad thereafter by transcriptional repression (Fig. 1.15B).

REST deregulation determines different effects according to the cell context: in non neuronal cells REST deregulation contributes to the tumorigenesis of breast, colon, prostatic and lung

cancer (Fig 13), whereas in neuronal cell REST mis-expression is involved in the tumorigenesis of moedulloblastoma and neuroblastoma (Fig 14).

Considering the reported observations, therefore, it can be concluded that REST is not only a key factor in determining the proper development of central nervous system, but it is also important to preside important cellular processes, the deregulation of which can contribute to the onset of serious diseases such as Huntington disease, anxiety, depression, cardiac dysfunction and cancer.

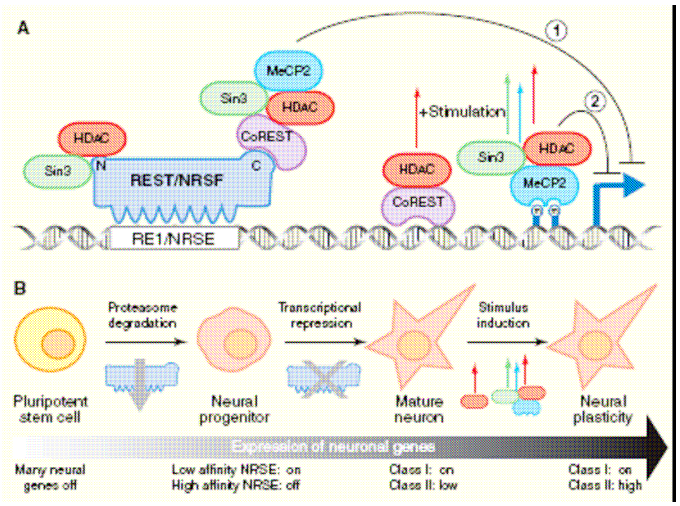


Fig. 1.15 *The availability of REST/NRSF and dynamic co-factor complexes regulates neuronal gene expression in development and plasticity:*

(A) REST assembles cofactors on target genes determining both short-term (1) and long-term (2) silencing.

(B) Titration of REST levels occurs as neurogenesis proceeds to release repression of individual subset of neuronal genes.

2. Insulin-like Growth Factor I: peptides, receptors, binding proteins, biological functions

The insulin-like growth factors and their receptors are implicated in the regulation of protein turnover and exert potent mitogenic and differentiating effects on several cell types (Laviola et al., 2007). During development, regulation of proliferation and neural cell fate in the Central Nervous System (CNS) is determined by complex interactions between several growth factors and neurotransmitters. One factor that appears to be important for normal brain development as well as having a plethora of effects in the adult animal is Insulin-like Growth Factor I (IGF-I) (Anderson et al., 2002), an endogenous peptide with both endocrine and autocirne/paracrine action; IGF-I modulates neuronal cell signaling and determines neurotrophic and neuroprotective effects (Russo et al., 2005). Most of the physiologic effects of IGF-I are mediated by the type-I IGF surface receptor: following ligand binding-induced autophosphorylation, this receptor associates with specific adaptor proteins and activates different second messengers (Bondy and Cheng 2004), including phosphatidylinositol 3-kinase (PI3-K), mitogen-activated protein kinase (MAPK) (Vincent and Feldman 2002; Di Toro *et al.* 2005) and the Janus kinase (Jak)/Signal Transductor and Activator of Transription 3 (STAT3) signalling pathway (Zong *et al.* 2000; Yadav *et al.* 2005).

IGF-I and its receptors are widely distributed throughout the nervous system during development, and their involvement in neurogenesis has been extensively investigated (Arsenijevic *et al.* 1998; van Golen and Feldman 2000).

2.1 Historical Overview

In 1957 Salmon and Daughaday reported that serum factors, produced by hepatic cells after exposure to growth hormone (GH), were able to mediate cartilage sulfatation and longitudinal bone growth activity. In conjunction, Dulak and Temin found out cell proliferative circulating factors in serum which showed insulin-like activity not suppressed by anti-insulin antibodies. Those factors were defined "sulfatation factors" and "non suppressible insulin-like activity I and II" respectively; these small molecular mass peptides were later renamed as somatomedines, thus replacing the previous terminology, because of their modulation of pituitary-derived GH action.

Shortly thereafter, the two mammalian somatomedines were defined as IGF-I and IGF-II according to their structural homology with proinsulin. In the middle 1990s, Sara and co-

workers identified a brain-specific variant of IGF-I, des(1-3) IGF-I or "truncated" IGF-I, which lacks the first three amino-acids and is more potent than intact IGF-I (Sara et al., 1994).

In the last decade, the increasing availability of animal models for brain injury and neuronal degeneration has allowed the investigation of the role of IGF-I in prevention and rescue of damaged neuronal cells, thus pointing to the potential therapeutic use of IGF-I in the treatment of nervous system diseases (Russo et al., 2005).

2.2 IGF system overview: peptides, receptors and binding proteins

IGF-I exerts its pleiotropic functions in an endocrine, autoctrine and paracrine fashion (Russo et al., 2005). Its main endocrine action is to mediate the growth-promoting effects of pituitary GH (Laviola et al., 2007); in fact, GH is known to induce the synthesis and release of IGF-I by the liver, thus controlling the growth and differentiation of several tissues in the body. Circulating IGF-I is mostly bound to high affinity IGF-I binding proteins (IGFBP) (Duan, 2002), which protect the hormone from proteolysis and modulates its interaction with IGF-I receptor.

A significant component of IGF-I function is also due to its autocrine and paracrine actions: Toellefsen and associates observed that, after removal of serum-containing growth medium, spontaneous differentiation of C2 muscle cells was accompanied by increased expression of the IGFs, thus indicating IGF-I as an essential autocrine growth factor for muscle cells (Laviola et al., 2007). Moreover, IGF-I and its receptor are up-regulated during neurogenesis and neuronal cell differentiation as well as after neuronal injury, thereby confirming its important role as autocrine/paracrine growth factor (Russo et al., 2005). Furthermore, systemic IGF-I is not readily transported through the blood-brain-barrier, and therefore local production of IGF-I is considered the primary source of the ligand for brain cells (Russo et al., 2005).

THE IGF PEPTIDES

IGF-I and -II are growth promoting peptides, members of a superfamily of related insulin-like hormones that includes insulin and relaxin in vertebrates and bombyxin, locust insulin-related peptide and molluscan insulin-like peptide in invertebrates. IGFs are major growth factors, whereas insulin predominantly regulates glucose uptake and cellular metabolism.

The gene encoding for IGF-I is highly conserved among mammals, birds and amphibians and its expression is affected at many levels, including gene transcription, splicing, translation and secretion (Russo et al., 2005). IGF-I mRNA expression is detected in many brain regions during embryogenesis, with its expression being particularly high in the spinal cord, midbrain, cerebral cortex, hippocampus and olfactory bulb (Rotwein et al., 1988); more precisely IGF-I is

expressed in neuronal cells with large soma and complex dendritic formations, including sensory and projecting neurons (Bondy and Lee, 1993). In most neurons, IGF-I expression decreases significantly postnatally, along with cell maturation, albeit exceptions in mitral and tufted cells of olfactory bulb (Siddle et al., 1994).

IGF-II mRNA is highly expressed in the embryonic rat central nervous system (CNS), although data on its expression in neuroepithelial cells are conflicting (Bondy et al., 1990); IGF-II is the most abundantly expressed IGF in the adult CNS, with the highest expression levels found in myelin sheaths, leptomeninges, microvasculature and choroid plexus, all non neuronal structures that enable diffusion of growth factors to their sites of activity (Bondy et al., 1990).

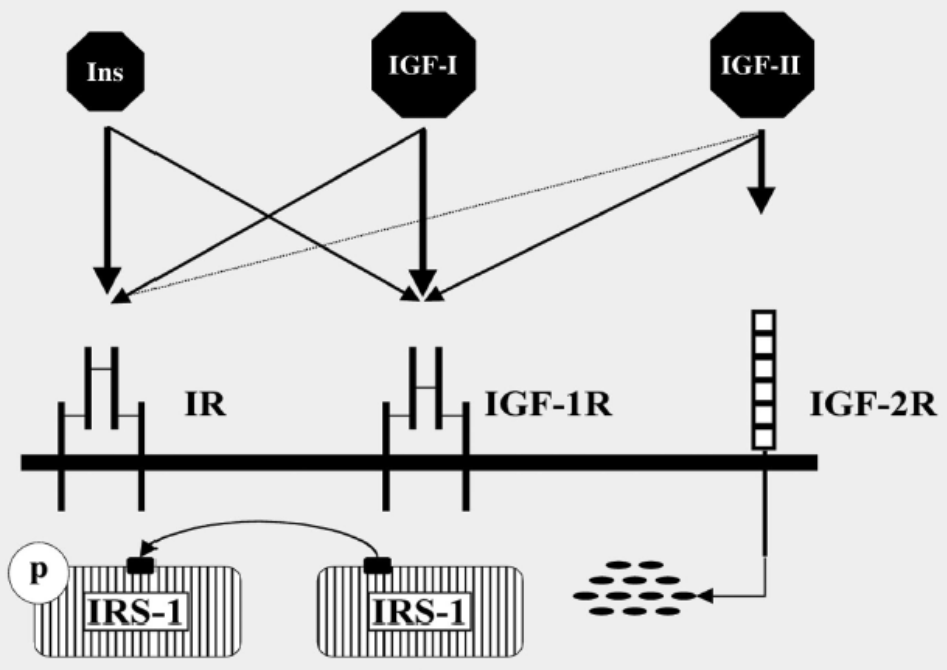


Fig. 2.1 *Schematic representation of peptides and receptors belonging to IGF-I system*

THE IGF RECEPTORS AND THEIR FUNCTIONS

The biological effects of the IGFs are mediated by different receptors (Fig. 2.1): the main one is the type-I IGF receptor (IGF-IR), a glycoprotein on the cell surface that transmits IGF binding to a highly integrated intracellular signalling system via intrinsic tyrosine kinase activity that phosphporylates the insulin receptor substrates (IRS-1 to IRS-4) (Fig. 2.1), thus activating different downstream signalling cascades such as MAPK and PI3-K (Russo et al., 2005). Various IGF-I receptor subtypes with distinct structure or binding properties have been described; atypical IGF receptors, for example, interacts with insulin as well as with IGFs with relatively high affinity (Siddle et al., 1994). Moreover, hybrid insulin/IGF receptors have been identified, although their physiological significance is still unclear (Soos et al., 1990).

The type-II IGF receptor is a single chain polypeptide, with a short cytoplasmic domain lacking tyrosine kinase activity, which is bound with high affinity by IGF-II (Fig. 2.1). This receptor is identical to the cation-independent mannose-6 phosphate receptor and plays a multifunctional role in the mediation of lysosomal enzyme trafficking, endocytosis, lysosomal degradation of extracellular ligands (Morgan et al., 1987). The IGF receptors are widely expressed throughout the CNS, with high levels of expression found in specific cell types such as developing cerebellum, midbrain, olfactory bulb and in the ventral floorplate of the hindbrain (Bondy et al., 1990). The level of IGF-IR decreases to adult levels soon after birth, but remains relatively high in the choroid plexus, meninges and vascular sheats (Bondy et al., 1992). It is not surprising that knockout of the IGF-I receptor gene produced, in addition to *in utero* growth retardation, a characteristic strong brain phenotype, the so called small brain (Russo et al., 2005). The type-II IGF receptor is highly expressed in the pyramidal cell layers of the hippocampus, the granule layer of the dentate gyrus, olfactory bulb, choroid plexus, and in the cerebral vasculature, ependymal cells, retina, pituitary, brain stem and spinal cord (Hawkes and Kar, 2003).

THE IGFS BINDING PROTEINS (IGFBP)

A family of six high-affinity IGF-binding proteins coordinate and regulate the biological activity of IGF in several ways: transport IGF in plasma and control its diffusion end efflux from vascular space; increase the half-life and regulate clearance of the IGFs; provide specific binding sites for the IGF-s in the extracellular and pericellular space; modulate, inhibit or facilitate interaction of IGFs with their receptors (Russo et al., 2005). IGFBPs activity is tightly regulated by post-translational modifications such as phosphorylation or glycosylation; their effects are further regulated by the presence of specific proteases, which cleaves the binding proteins, thus generating fragments with reduced or no affinity for the IGFs (Jones et al., 1995). A growing body of evidence suggests an important role for IGFBP in the nervous system. mRNA expression profiles and location of the most abundant IGFBP-2, -4 and -5 in the normal developing and adult CNS are well defined (Russo et al., 1994), whereas IGFBP-3 and -6 are expressed in CNS at lower levels (Naeve et al., 2000) and IGFBP-1 is not expressed (although its expression can be induced under certain experimental conditions) (Zhou et al., 2001).

However the overexpression of IGFBP-1 mRNA in brain generates the characteristic phenotype named small brain; in fact, IGFBP-1 is known to inhibit somatic linear growth, weight gain, tissue growth and glucose metabolism (Lee et al., 1997). IGFBP-1 up-regulation, therefore, as observed in a variety of clinical situations, may itself contribute to growth retardation and

impaired foetal brain development that seem to be due to IGFBP-1 sequestration of IGF-I (Russo et al., 2005).

IGFBP-2 is expressed early in embryogenesis in neuroectoderm structures including neural tube and neuroepithelium. Later in development, IGFBP-2 mRNA is detectable throughout the brain (Lee et al., 1993), particularly in brain regions undergoing continuous remodelling as is the olfactory bulb, the cerebellum, and the hippocampus (Lee et al., 1993). IGFBP-2 associates to cell surface proteoglycans in rat brain tissue (Russo et al., 1997) and neuronal cells (Russo et al., 1999). Although the role of these cell membrane complexes is not completely understood, Russo and colleagues have recently shown that IGF-I complexes with IGFBP-2 can promote neurogenesis in adult stem cells and further demonstrated that neurogenesis was inhibited by IGFBP-2 antibody blockade, thus suggesting a pivotal role for IGFBP-2 in this process. Despite these key functions, ablation of the Igfbp-2 gene generated a phenotype less dramatic than that initially predicted, thus suggesting functional redundancy in the IGFBP family during development of the CNS.

IGFBP-3 is normally expressed at a low level in the CNS, mainly in non-neuronal structures including epithelial cells (Russo et al., 1994). In a study by Ajo and co-workers, IGFBP-3 was found to be up regulated in rat cerebral cortical cells after GH stimulation, thus promoting proliferation of neural precursors, neurogenesis, and gliogenesis mediated by locally produced IGF-I and its modulator IGFBP-3. Conversely, a study by Rensink and colleagues proposed that IGFBP-3 might contribute to neuronal degeneration in Alzheimer Desease. It is therefore possible that, as seen in other systems, IGFBP-3 might promote either enhancement or inhibition of IGF-I action in brain cells in vitro, depending on the experimental conditions.

IGFBP-4 is normally expressed at a very low level in the CNS, where its mRNA is found in a variety of brain cell types including meningeal cells, astrocytes, and foetal neuronal cells (Chernausek et al., 1993). During early brain development, IGFBP-4 expression is increased, and its mRNA is easily detectable in regions such as the choroid plexus, meninges, and the basal ganglia (LaTour et al., 1990), whereas postnatally, IGFBP-4 mRNA is found in the meningeal cell layer surrounding the developing cerebellum in the hippocampal formation and olfactory bulb (Chernausek et al., 1993).

IGFBP-5 gene expression is highly abundant during brain development, thus indicating a key role of this IGFBP during embryogenesis. In rodents, IGFBP-5 appears to be co-expressed with IGF-I in principal neurons of sensory relay systems, cerebellar cortex, hippocampal formation, and many other neuron-rich regions, including the olfactory bulb (Russo et al., 1994).

IGFBP-6 is poorly expressed in the nervous system, and information regarding its mRNA expression distribution, in both the developing and adult nervous system, is limited.

IGFBP-6's unique property of preferential binding to the IGF-II ligand (Bach, 1999), coupled with the fact that this ligand is the most abundantly expressed IGF in the adult CNS, suggests that the IGFBP-6/IGF-II complex has a unique role in modulating IGF-II function in the adult brain (Naeve et al., 2000).

2.3 IGF-I and IGF-IR: structure, function, cell signalling pathways.

IGF-I GENE AND PROTEIN STRUCTURE

IGF-I encoding gene is quite complex and gives origin to several different transcripts by alternative splicing. More precisely, mammalian IGF-I gene is about 50-80 kb long, consists of 6 exons and its transcription is modulated by pituitary-derived GH (Ishii et al., 1998); IGF-I is in fact responsible for most of GH-mediated effects.

IGF-I is a 70-amino-acid polypeptide hormone which consists of 4 structural domain in its mature form: A, B, C, D. A and B domains are structurally related to α and β chain of insulin; C domain is similar to insulin C peptide but it is not cleaved during IGF-I maturation process; the small D domain is not present in insulin, whereas the C-terminal E peptide is present only in IGF-I prohormone and it is cleaved in the Golgi apparatus during secretion (LeRoith and Roberts, 2003).

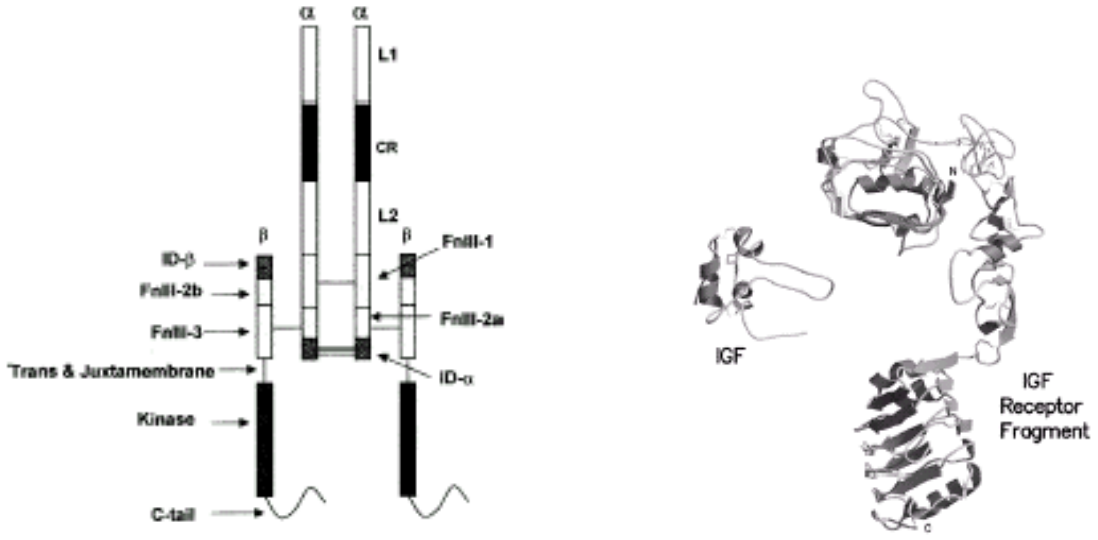


Fig. 2.2 *Schematic representation and topography of IGF-I receptor and IGF-I*

IGF-I RECEPTOR STRUCTURE AND MAIN PHOSPHORYLATION SITES

Most, if not all, of the effects of IGF-I result from its activation of the IGF-IR (LeRoith and Roberts, 2003). The IGF-I receptor is a hetero-tetrameric protein, consisting of two identical extracellular α-subunits containing a cysteine-rich IGF-binding site, and two transmembrane β-subunits, bearing an intrinsic tyrosine kinase activity (Fig. 2.2.). The IGF-I receptor is highly homologous to the insulin receptor at both DNA and protein levels, particularly in the tyrosine kinase domain (84%), but differs markedly in other regions (22-26% homology in the transmembrane domain, 45% homology in the carboxyl-terminal domain) (Pedrini et al 1994). Binding of IGF-I to its receptor causes the activation of the tyrosine kinase, leading to autophosphorylation of the intrinsic tyrosines and subsequent tyrosine phosphorylation of multiple cytoplasmic substrates. This activity depends on an ATP-binding site (lysine 1003) and on a cluster of three tyrosine residues at positions 1131, 1135, and 1136 (Laviola et al., 2007). Mutation of the ATP-binding site (Kato et al., 1993) and/or substitution of phenylalanines for tyrosines in the 3-tyrosine cluster (Gronborg et al., 1993) is associated with complete inactivation of the receptor kinase (Cheng et al., 2000). Additionally, the distal tyrosine (position 1316) in the C-terminal domain of the β-subunit appears to be important for tumour formation, but it is not essential for IGF-I-stimulated mitogenesis (Blakesley et al., 1996). Additional tyrosine residues in the C-terminal domains of the β-subunit are also important in signal transduction by the IGF-I receptor (LeRoith et al., 1995). The insulin receptor cytoplasmic domain bears 13 tyrosine residues, whereas the IGF-I receptor shows 15 tyrosines. Seven out of the 13 tyrosines in the insulin receptor are phosphorylated in response to insulin binding (Kohansky, 1993), as are eight out of the 15 in the IGF-I receptor (O' Connor 1997). These tyrosine residues are clustered in three different domains: the juxta-membrane, catalytic, and carboxyl-terminal regions. The roles of tyrosine residues in the juxta-membrane and catalytic domains appear to be conserved. However, experimental evidences suggest that intrinsic differences in the catalytic domains may explain signalling selectivity (Laviola et al., 2007). Therefore, it is possible that signalling emanating from the IGF-I receptor to either proliferation or differentiation could be determined at the receptor level by activation of specific tyrosine residues on the β-subunit, recruiting distinct signalling molecules further down the signal transduction pathway.

THE IGF-I SIGNAL TRANSDUCTION PATHWAY

The IGF-I receptor interacts with and signals to adaptor molecules, namely the insulin receptor substrate(IRS)-1/4 and the Shc (Src homology collagen) proteins (p46/p52/p66), which serve as signalling nodes for distinct intracellular pathways (Fig. 2.3). Both IRS-1 and IRS-2 interact

directly with the insulin and IGF-I receptors (White, 1997), IRS-2 being apparently a better substrate for the insulin than for the IGF-I receptor (Kim et al., 2001), supporting the view that the biological specificity of the two receptors depends, at least in part, from preferential signalling events (Urso, 1999). Phosphorylated IRSs recruit and activate class 1a phosphoinositide 3-kinase (PI 3-kinase) via the two SH2 domains of the adaptor protein p85, leading to synthesis of membrane-associated phosphorylated inositols (Fig. 2.3). These molecules, in turn, recruit and activate phosphoinositide-dependent kinases (PDKs), which then phosphorylate and activate other protein kinases, including Akt/protein kinase B, p70rsk and protein kinase Cζ (PKCζ) (Fig. 2.3) (Vanhaesebroeck and Alessi, 2000). There is substantial evidence that PI 3-kinase activity mediates a wide range of insulin/IGF effects, including stimulation of glucose transport, glycogen synthesis, protein synthesis, mitogenesis, inhibition of apoptosis, and regulation of gene transcription (Shepherd et al., 1998).

Another IGF-I-activated signalling pathway, which is coupled mainly to mitogenic and transcriptional responses, involves the recruitment to both IRS-1 and Shc of the guaninenucleotide-exchange factor Sos, via the SH2 domain of the adaptor Grb2 (Fig. 2.3). This leads to activation of the small G-protein Ras, which in turn activates the protein serine kinase Raf and the extracellular-signal-regulated kinase (ERK) cascade (Fig. 2.3). IGF-I receptor activation is also coupled to the stimulation of a family of MAP kinases, besides ERK-1/2, including Jun kinase (JNK)-1 and -2 (Derijard et al., 1994) and p38 MAP kinase (Han et al., 1994). JNKs phosphorylate the amino terminus of c-Jun, increasing its ability to activate transcription (Derijard et al., 1994). Multiple targets have been identified downstream the MAP kinases, including ribosomal S6 kinase (Rsk 90), MAPKAP, phospholipase A2, and multiple transcription factors (Davis, 1993). IGF-I has also been shown to induce tyrosine phosphorylation of the cytoplasmic protein Crk (Fig. 2.3), which shares some sequence homology with Grb2 and Nck. Crk has been found to associate with mSOS (Matsuda et al., 1994) and may be involved in IGF-I receptor-mediated modulation of the Ras-MAP kinase signaling pathway, thus enhancing IGF receptor-dependent mitogenesis (Okada and Pessin, 1997).

IGF-I-induced phosphorylation of IRS-1 results also in the recruitment of the phosphotyrosine phosphatase Syp through its two SH2 domains, leading to increased phosphatase activity and down-regulation of hormone-triggered tyrosine phosphorylation events. PTP-1B and PP-2A represent additional phosphatases involved in the fine regulation of IGF-I/insulin-induced signalling (Buckley et al., 2002).

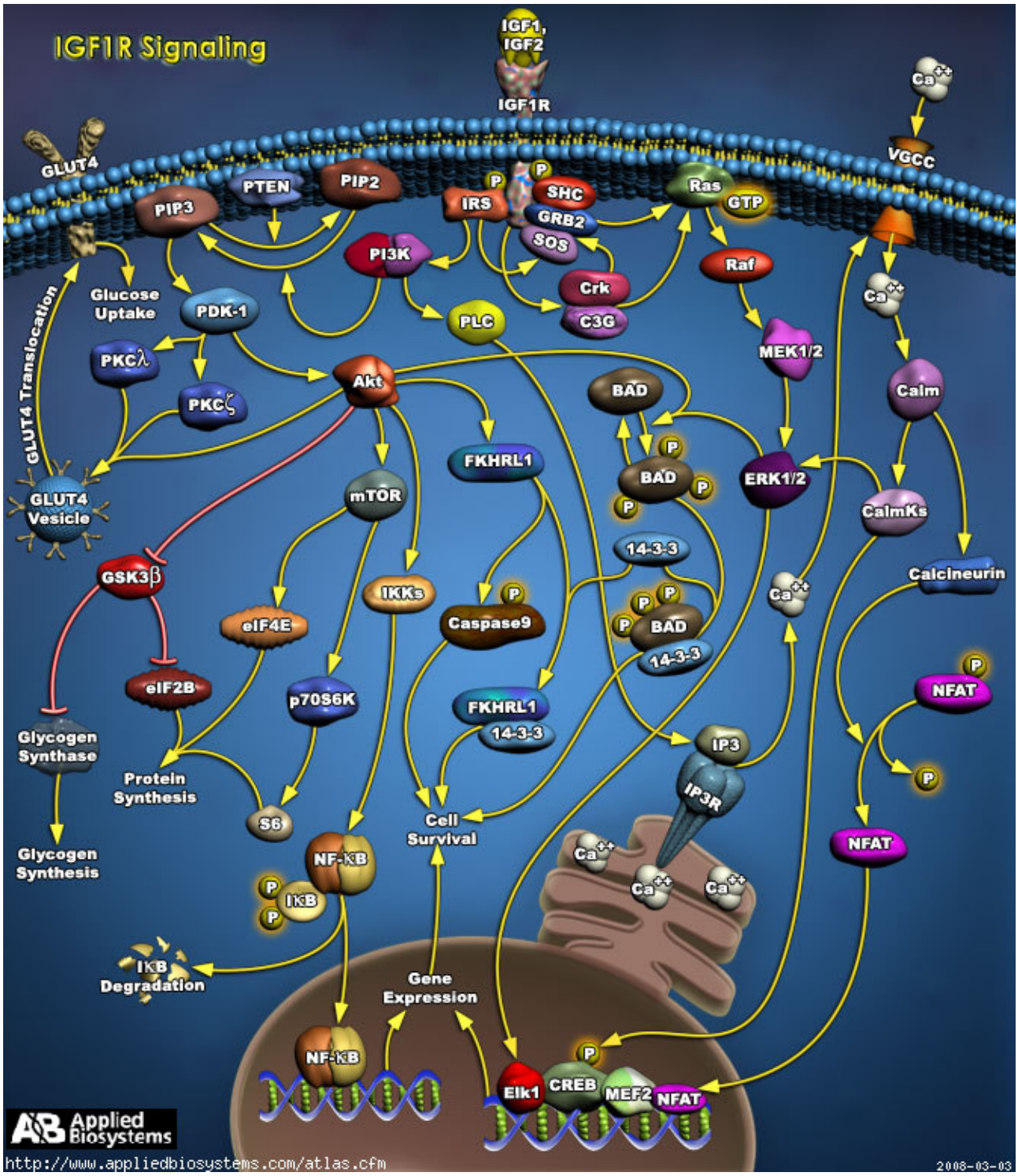


Fig. 2.2 *IGF-I/IGF-IR signalling: IGF-I interaction with its receptor determines IRS phosphorylation and subsequently triggers different transduction pathways, including MAPK and PI3-K cascades and thus regulating activation of several transcription factors and cellular responses*

Moreover, different G-proteins show a specific pattern of association with the insulin or the IGF-I receptor. Gαq has been shown to be a substrate for the insulin receptor, and it has been implicated in insulin signaling to glucose transport (Imamura et al., 1999); in contrast, IGF-I

but not insulin receptor appears to be constitutively associated with Gi, and to induce the release of Gβγ subunits following activation (Dalle et al., 2001).

Furthermore, different studies showed that also STAT proteins can be activated by receptor and non-receptor tyrosine kinases (Ihle, 1996). Upon tyrosine phosphorylation, STAT proteins form homo- or heterodimers through intermolecular interactions of their SH2 domains with the phosphorylated tyrosines and rapidly translocate into the nucleus, thus inducing gene expression (Zong et al., 2000).

So far, more than 40 different polypeptide ligands have been shown to cause STAT activation, including Epidermal Growth Factor (EGF) (Ihle, 1996), Platelet-Derived Growth Factor (PDGF) (Darnell 1997), insulin (Chen et al., 1997) and IGF-I (Zong et al., 2000; Yadav et al., 2005).

IGF-I binding to IGF-IR, in fact, determines phosphorylation and activation of JAK1 and JAK2, which in turn activate STAT3 (Zong et al., 2000). Different possibilities of STAT3 activation by IGF-IR via JAKs have been proposed: activated receptor associates with JAKs, which phosphorylate STAT3, or JAKs could provide STAT3 recruitment site in the activated receptor complex, which itself determines STAT3 phosphorylation (Zong et al., 2000).

More recently, Yadav and associates suggested another interesting model for STAT3 activation by IGF-I/IGF-IR: STAT3 was found to be associated with JAK1, thus indicating that STAT3 is constitutively associated with IGF-IR (Yadav et al., 2005). Upon stimulation with IGF-I, the majority of STAT3 dissociates very quickly from JAK1 (5 minutes), diereses and translocates to the nucleus.

In another study, RACK1 was proposed as a necessary adaptor molecule for STAT3 recruitment to IGF-IR (Zhang et al., 2006). Via JAK1/STAT3 activation, IGF-I up-regulates Suppressor Of Cytokine Signal -3 (SOCS3) which in turn blocks JAK1 phosphorylation (Yadav et al., 2005), thus providing an effective negative feed-back process to control IGF-I cell signalling.

The involvement of JAK/STAT pathway in IGF-I mediated cell signalling is very interesting, being this pathway very important for cell survival in the brain (Yadav et al., 2005).

2.4 Pleiotropic action of IGF-I in the Brain

Whether IGF-I is produced locally (Rotwein et al., 1988) or reach brain cells systemically (Trejo et al., 2001), it exerts potent neurotrophic, neurogenic, and neuroprotective/antiapoptotic activities (Fig 2.4). Studies using the IGF-I -/- mice models (Cheng et al., 1998), which have reduced brain size and altered brain structures (Cheng et al., 2001) and show

alteration of myelination processes (Ye et al., 2002), have clearly demonstrated that most of the IGF functions determined *in vitro* also apply to the in vivo situation, affecting a wide range of brain cells: *in vitro* studies have indeed demonstrated that IGF-I promotes mitogenesis and differentiation in glial cells (Cao et al., 2003), oligodendrocytes (McMorris and McKinnon, 1996), neuronal cells (Morrione et al., 2000), adult stem cells (Brooker et al., 2000), and brain explants (Russo et al., 1994), and regulates axon myelination (Cheng et al., 1999). Furthermore, IGF-I enhances growth cone motility and promotes neurite outgrowth (Feldman et al., 1997).
The dramatic phenotype of IGF-I null mice appears to be the result of reduced oligodendrocyte proliferation and maturation, which is also associated with reduction of axonal growth (Ye et al., 2002). However, some early studies from Bondy and co-workers have reported instead that in IGF-I null mice, myelination is reduced but proportionate to the neuronal composition.

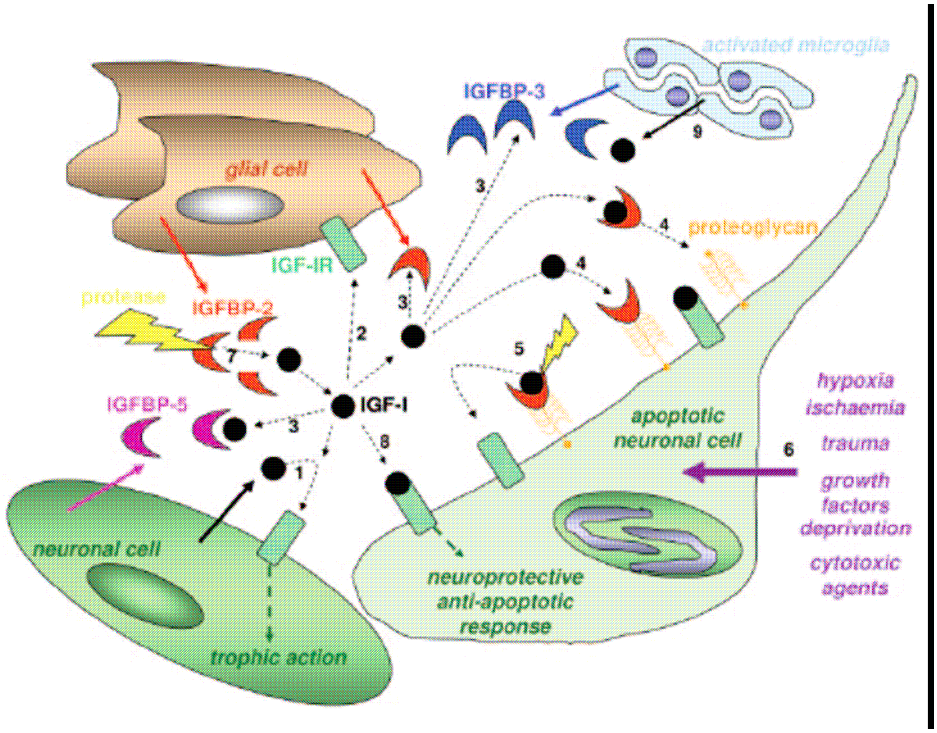


Fig 2.4 *A model for neurotrophic and neuroprotective actions of IGF-I in brain.*
Secreted IGF-I exerts local autocrine (1) or paracrine (2) trophic actions. A family of IGFBPs (3) modulates IGF-I bioavailability. IGFBP-2, the most abundant brain IGFBP, mediates pericellular storage of IGF-I via interaction with cell surface proteoglycans (PG) (4) or components of the extracellular matrix. Cell surface IGF-I/IGFBP-2/PG complexes (4) are suggested to play a role in targeting of IGF-I to its membrane receptors. IGFBP-2 mediated IGF-I receptor targeting at the cell surface, and this event might be further potentiated by the presence of a specific IGFBP-2 protease (5), which generates IGFBP-2 fragments that have reduced affinity for IGF-I. In response to a number of cerebral insults (i.e., hypoxia/ischemic brain injury) (6), IGFBP-2 proteolysis might also affect the level of pericelluar IGF-I (7), therefore augmenting its neuroprotective activity (8). Following cerebral insult, activation and recruitment of specialized brain cells (9) might further contribute to modulate the local IGF system (9).

Furthermore, ablation of the Igf-1 gene has revealed deficit in the numbers of specific neurons, oligodendrocytes in the olfactory bulb, dentate gyrus and striatum (Cheng et al., 1998) and in cochlear ganglion neurons. These anatomical differences are likely to be the consequences of alteration in proliferation, survival, or differentiation caused by the absence of IGF-I during development.

A study from Vicario-Abejon et al. suggests that reduction of stem cell proliferation/differentiation in the IGF-I null mice might be the cause of reduced plasticity/maturation normally occurring in some brain regions (i.e., olfactory bulb) postnatally (Vicario-Abejon et al., 2003). In addition to gross structural brain abnormalities, the IGF null mice also show alteration of important brain metabolic functions such as reduced glucose uptake, the major source of energy of neuronal cells (Cheng et al., 2000).

However, actions of IGF are not limited to foetal life (Bondy and Chin, 1991), but extend into postnatal (Russo et al., 1994) and adult life (Trejo et al., 2004), with effects on proliferation, neuronal differentiation, and maturation maintained in some regions of the adult brain. In fact, alterations in levels of IGF-I have been reported in the brains of aging rats (Sonntag et al., 1999), alterations that also correlate with deterioration of cognitive functions observed in elderly patients with low circulating IGF-I (Sytze and Aleman, 2004).

In support of studies on the use of IGF-I as therapeutic agent, many investigators have examined the cellular and molecular mechanisms of IGF action in nervous system diseases. In this regard, the IGF-I has been shown to promote the survival and differentiation of neuronal cells, including sensory (Oorschot and McLennan, 1998), sympathetic, and motor neurons (MNs) (Sendtner, 1995). In fact, the IGFs are the only known growth factors that support both sensory and motor nerve regeneration in adult animals (Arsenijevic et al., 2001).

IGF-I is involved in brain plasticity processes (Vicario-Abejon et al., 2003), and it specifically modulates synaptic efficacy by regulating synapse formation, neurotransmitter release, and neuronal exicitability (Torres-Aleman, 1999).

IGF-I also provides constant trophic support to neuronal cells in the brain and in this way maintains appropriate neuronal function (Torres-Aleman, 1999). Alteration of this trophic input may lead to brain disease as seen in neurodegenerative disorders such as Alzheimer's disease (AD) (Dore et al., 1997), Ataxia telangiectasia (Peretz et al., 2001), Huntington's disease (Humbert et al., 2002), and Parkinson's disease (Offen et al., 2001). A recent study by Lichtenwalner et al. showed that neurogenesis declines in brains of aged mice, but it is efficiently restored after IGF-I administration via intracerebroventricular infusion.

The ability of IGF-I to promote neuronal survival is associated with the ability of this factor to prevent apoptosis, and IGF-I appears to be a potent agent for rescuing neurons from apoptosis. For example, IGF-I prevents Nmethyl-d-aspartate (NMDA)- and nitric oxide-induced apoptosis in hippocampal and cortical neurons (Zheng et al., 2002).

Moreover, IGF-I has been shown to enhance the survival of rat embryo cerebellar granule cells and stimulate their terminal differentiation into cerebellar granule neurons (Linseman et al., 2002). Additionally, IGF-I promotes the survival of rat hypothalamic and hippocampal neurons in vitro (Zhang et al., 2003).

A role for IGF-I in the regulation of Schwann cell survival has also been reported, as has the ability of IGF-I to prevent apoptosis in sympathetic neurons exposed to high glucose (Russel and Feldman, 1999). Rat hippocampal neurons are also rescued by IGF-I from the induction of apoptosis by amyloid-derived peptides (Dore et al., 1999), and a similar antiapoptotic effect of IGF-I is observed in rat cerebellar neurons subjected to serum or potassium withdrawal or high KCl levels (D'Mello et al., 1997), okadaic acid treatment (Fernandez-Sanchez et al., 1996), and in MNs during normal development or (D'Costa et al., 1998) after axotomy or spinal transection (Kermer et al., 2000).

Also neuroblastoma cells are rescued from hyperosmotic shock-induced apoptosis by IGF-I (Van Golen and Feldman, 2000) or from metabolic stress including exposure to low glucose (Russo et al., 2004). Finally, IGF-I inhibits mature oligodendrocyte apoptosis during primary demyelination and prevents apoptosis in neurons after nerve growth factor withdrawal (Russel et al., 1998) or serum withdrawal in brain explants and neuronal cells (Russo et al., 2004).

2.5 Neuroendocrine cross-talk between IGF-I and other growth factors in the nervous system

Growth factors such as IGF-I do not exists in isolation *in vivo* (Russo et al., 2005). Hence, the presence of other growth factors may further modulate IGF-I biological activity and cellular responses (Fig. 2.5). For example, Fibroblast Growth Factor -2 (FGF-2) has been shown to act synergistically with IGF-I in increasing the number of neuritis in primary neuronal cell culture (Torres-Aleman et al., 1990) as well as to promote chromaffin cell proliferation (Drago et al., 1991) and to differentiate neuroblastoma cells (Russo et al., 2004) (Fig. 2.5).

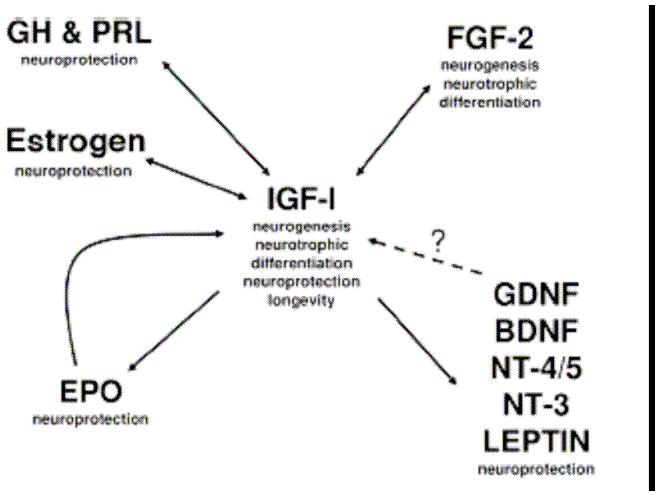


Fig. 2.5 *Neuroendocrine cross-talk. In vivo, growth factors such as IGF-I do not exist in isolation. Hence, the presence of other growth factors may further modulate the biological activity and cellular responses of IGFs.*

Erythropoietin (EPO) is traditionally known as a hematopoietic cytokine produced by the foetal liver and adult kidney in response to hypoxia. However, the expression of EPO and EPO-receptors in the CNS and the up-regulation of EPO by hypoxic-ischemic insult suggest that this cytokine is an important mediator of the brain's response to injury. In fact, in vivo EPO administration protects hippocampal CA1 neurons and retinal neurons from ischemic damage and prevents brain injury after a number of "insults" (Grasso et al., 2004) by mechanisms involving both inhibition of apoptosis and neurotrophic actions (Wen et al., 2002).

Recent work by Wang et al. demonstrated that IGF-I protection of primary neuronal cells exposed to low oxygen concentration correlates with activation of HIF-1α expression (Wen et al., 2002). In the same studies using an in vivo model of hypoxia/ischemic brain injury, they also show that IGF-I transcriptional activation correlates with that of HIF-1α, suggesting that HIF-1α might mediate some of the IGF-I responses.

In another study by Chavez and LaManna, it was shown that IGF-I induces HIF-1α transcriptional activity in rat cerebral cortex and neuronal cells (PC12) and that this induction is abolished by a selective IGF-I receptor antagonist (JB-1).

Among the numerous endocrine systems regulating brain physiology, sex steroids play an important role, being part of their effects on the brain mediated by neurotrophic factors including IGF-I (Quesada and Micevych, 2004) (Fig. 2.5).

Studies from the Garcia-Segura group have demonstrated that estradiol and IGF-I increase survival and differentiation of developing foetal rat hypothalamic neurons.

They have demonstrated that estrogen-induced activation of the estrogen receptor requires the presence of IGF-I and that both estradiol and IGF-I use the estrogen receptor to mediate their

trophic effects on hypothalamic cells (Fig 2.5). In vivo sex steroids affect IGF-I levels in the endocrine hypothalamus, arcuate nucleus, and median eminence (Garcia-Segura et al., 1996). Furthermore, increased clinical and basic evidence suggests that gonadal steroids affect the onset and progression of several neurodegenerative diseases and the recovery from traumatic neurological injury. In the brain, similarly to the IGF system, both estrogen synthesis and estrogen receptor expression are up-regulated at sites of injury. Once again, it is suggested that the neuroprotective effects of estrogen may be exerted independently of the classical nuclear estrogen receptors involving modulation of the IGF-I signalling (Azcoita et al., 2002).

2.6 IGF-I altered expression in Central Nervous System and related diseases

IGF-I UP-REGULATION FOLLOWING CNS INJURY.

A role for endogenous IGF-I in the injured brain is suggested by a number of studies showing the induction of components of the IGF system after transient unilateral hypoxic/ischemic injury and stroke in the rat model, where IGF-I mRNA induction is seen within infracted regions by 3 d after hypoxia (Gluckman et al., 1992). In addition, also IGFBP genes such as IGFBP-2, IGFBP-3, and IGFBP-5, are differentially induced in specific regions after hypoxic/ischemic injury in the same model, suggesting that they may modulate the actions of IGF-I in a spatiotemporal-specific manner. The induced expression of IGF-I and IGFBP-2, -3, -4, and -5 in reactive microglia, oligodendrocytes, astrocytes, and surviving neurons (Klempt et al., 1994) of the peri-infarcted area, including areas distant from the region of cell loss, suggests a role for the local IGFBPs in transporting IGF-I from its sites of production to the sites of action. These findings might point to a role for cell-associated IGFBP-2 (134) to modulate local IGF-I bioavailability.

However, in the early stages of the injury response, also IGFBP-3 expression increases rapidly in vascular endothelial cells throughout the affected hemisphere, with the maximal expression levels at 24 h and a subsequent down-regulation 48 h later (Hammarberg et al., 1998). This early transient induction of IGFBP-3 in brain vascular endothelial cells is a likely mechanism by which brain vascular endothelial cells potentially regulate up-take of circulating IGF-I after the hypoxic-ischemic insult (Lee et al., 1999). However, the precise mechanism involved remains to be determined. Nevertheless, these data might point to a potential role for IGFBP-3 as carrier/transporter of vascular IGF-I into the brain tissue in the early phases of the injury response, when local IGF-I availability and expression might not be sufficient to trigger the neuroprotective mechanisms after hypoxic-ischemic insult. This is also true for IGFBP-5, which is up-regulated after severe hypoxic-ischemic injury in the infant rat brain (Beilharz et al., 1993),

and in cerebellar granule cells during apoptosis (Roschier et al., 2001). In both cases, the increase of IGFBP-5 expression is believed to be required to maximize the availability of IGF and thus it potentiates the IGF-triggered survival signaling.

After peripheral nerve injury (lumbar motoneurons), also IGFBP-6 mRNA and protein expression are strongly up-regulated in the spinal motoneurons. This increased expression of IGFBP-6 in the damaged nerve is spatiotemporally associated with a local increased expression of the IGFs (Hammarberg et al., 1998). It is therefore likely that IGFBP-6 might play a specific role in controlling IGF availability to lesioned motoneurons and thus regulates axonal regeneration (Hammarberg et al., 1998).

These findings all suggest that the IGF-I and its binding proteins, after injury, may act as endogenous neuroprotective agents and limit the degree of neuronal and glial loss in damaged brain.

IGF-I SYSTEM DEREGULATION AND MALIGNANCIES IN THE CENTRAL NERVOUS SYSTEM

IGF-IR plays a crucial role in the induction and maintenance of the malignant phenotype. Increased expression of IGF-I, IGF-II, and IGF-IR is present in a wide range of human cancers (Adams et al., 2000), including lung, breast, thyroid, and prostate carcinomas, rhabdomyosarcomas, leukemias, and the peripheral nervous system tumour neuroblastoma (Lenninger et al., 2002), as well as they are overexpressed to varying degrees in numerous CNS tumours (Zumkeller and Westphal, 2001), including low-grade gliomas, glioblastomas, medulloblastomas, astrocytomas, ependymomas, and meningiomas.

Furthermore, IGF-IR overexpression leads to cellular transformation (Baserga, 2000), tumor cell proliferation (Singleton et al., 1996), and growth (Butler et al., 1998), whereas disruption of IGF-IR expression reverses the transformed phenotype (Baserga, 2000).

Expression of the IGF family in CNS malignancy has been most thoroughly investigated in glioblastoma. IGF-IR expression is higher in glioblastomas than in normal brain, and glioblastoma cell lines exhibit a dose-dependent IGF-I stimulation of both receptor autophosphorylation and thymidine incorporation, indicating a full receptor functionality (Merril and Edwards, 1990).

Much of the *in vitro* work on the role of IGF-IR in transformation has been performed in rat glioblastoma cell lines, where researchers have found through mutational analyses that IGF-I stimulates the migration (Brockmann et al., 2003) and invasion of glioblastoma cell lines in culture, prerequisites for tumor cell metastasis, and that it acts as a potent survival factor, even overcoming proapoptotic stimuli (Chakravarti et al., 2002). Glioblastoma cell treatment with IGF-I triple helix-forming DNA (Trojan et al., 2003), antisense IGF-IR (Resnicoff, 1998),

kinase-defective IGF-IR (Seely et al., 2002), or additional IGF-IR mutant dominant-negative constructs (Burgaud et al., 1995) all induce growth suppression and/or apoptosis, resulting in decreased tumor growth.

In infiltrating astrocytomas, IGF-IR expression increases early in tumor development, appearing in stage II tumors and continuing into stage IV. IGF-IR protein levels are increased in the majority of medulloblastoma cases, with concomitant increases in phosphorylated forms of the downstream signalling molecules Erk-1, Erk-2, and Akt/PKB (Del Valle et al., 2002).

The IGF system is also important in neuroblastomas, which typically occur peripherally but are occasionally found intracranially. IGF expression is present in all stages of primary neuroblastoma tumors (Sullivan et al., 1995), while IGF-I coupled to IGF-IR promotes both autocrine and paracrine growth and survival of human neuroblastoma cell lines (Martin and Feldman, 1993). Furthermore, IGF-IR overexpression protects neuroblastoma from apoptosis, whereas inhibition of IGF-IR using antisense strategies blocks tumor growth and induces regression of neuroblastoma tumors in mice (Liu et al., 1998). Therefore, since neuroblastoma is also thought to arise through improper differentiation, these studies coupled with the CNS tumor reports suggest a critical role for the IGF-I system in neuronal tumor development and growth.

2.7 IGF-I potential therapeutic applications

IGF-I THERAPY IN ACUTE CENTRAL NERVOUS SYSTEM DISEASE

After traumatic brain injury, bioassayable neurotrophic activity in cortical wounds is not induced until 6 d after injury in adult rats and 3 d after injury in infant rats. These observations of a temporal mismatch between the induction of cell death and the expression of neurotrophic activity led to the hypothesis that earlier administration of potential neurotrophins such as IGF-I might be neuroprotective (Russo et al., 2005).

The neuroprotective action of IGF-I is likely to be dependent on the capacity of IGFBPs to translocate IGF-I from the CSF to the site of injury, as IGF-II administered in conjunction with IGF-I attenuates the neuroprotective effect of IGF-I, possibly by competing for IGFBPs binding(Guan et al., 1996).

IGF-I THERAPY IN CHRONIC CENTRAL NERVOUS SYSTEM DISEASE

IGF-I has also been explored as a potential therapeutic agent in chronic neurological disease, such as MN disease (Mitchell et al., 2002), which is a heterogeneous group of neurodegenerative disorders that selectively affect upper and/or lower MN leading to muscle atrophy and weakness. This disease of late middle age is relentlessly progressive and almost

invariably lethal within 5 yr of onset of symptoms. Amytrophic lateral sclerosis (ALS) accounts for approximately 80% of all cases of MN disease and has an estimated incidence of three to five cases per 100,000. Accumulating data support the therapeutic use of IGF-I in the treatment of ALS. As discussed earlier, IGF-I is essential for normal development and is the only known neurotrophic factor capable of supporting both sensory and motor nerve regeneration in adult animals (Anlar et al., 1999): IGF-I prevents apoptosis in MN, glial cells, and muscle cells, all cell types affected by ALS (Cleveland and Rothstein, 2001). Moreover, IGF-I serum levels are decreased in ALS patients and could contribute to the development of disease (Torres-Aleman et al., 1998). However, available data are conflicting since two placebo-controlled trials of IGF-I in ALS patients have produced mixed results.

More recently, it has been shown that if IGF-I is given before motor signs of encephalomyelitis are obvious, it appears to delay disease onset. Once disease takes hold, however, IGF-I shows neither beneficial effect on remyelination (Cannella et al., 2000) nor worsens disease outcome.

Similarly, it has been suggested that chronic IGF-I administration may have some therapeutic role in AD, as IGF-I *in vitro* inhibits amyloid induced neuronal death (Dore et al., 1997), induces choline acetyl-transferase (Dore et al., 2000), and affects CNS amyloid-β levels.

Evidence suggests that altered levels of growth factors and/or their receptors, which exert trophic and survival function in the nervous system, may underlie neuronal degeneration as seen in AD brain. AD patients show changes in circulating levels of both insulin and IGF-I and decreased circulating insulin and IGF-I might influence the development of neurofibrillary tangles, another AD "marker." Therefore, insulin and IGF-I signalling could be a potential therapeutic target in AD.

NEUROPATHY: A POTENTIAL TARGET FOR IGF-I THERAPY

The study of the IGF system in peripheral nerve injury has focused on both crush and transection of the sciatic nerve. Sciatic nerve crush in the rat increases IGF-I mRNA distal to the crush site, whereas IGF-I expression at the crush site is not increased until 4 d after injury (Pu et al., 1995). IGF-I and IGF-IR up-regulation are observed after sciatic nerve transection and most of the evidence for IGF-promoted neurite extension comes from studies of transected peripheral nerves. For example, rats treated with IGF-I after sciatic nerve transection exhibit increased MN survival and re-innervation of muscle, showing that IGF-I mediates functional neurite regeneration *in vivo* (Vergani et al., 1998). Moreover, functional sciatic nerve regeneration is also promoted by IGF-I treatment in mice after sciatic nerve crush and the

spatiotemporal regulation patterns of the IGFs and IGF-IR suggest a functional endogenous role of the IGF system in neurite regeneration after injury (Russo et al., 2005).

Because of these neurite-promoting effects, IGFs may have a role in the treatment of neuropathy, particularly in the treatment of diabetic neuropathy.

TARGETING THE IGF/IGF-IR SIGNALING IN CANCER THERAPY

Targeting IGF-IR expression and signalling is a new therapeutic approach to cancer treatment in numerous tumor types (Werner, 1998). A truncated IGF-IR that acts as a dominant negative receptor or antisense RNA to IGF-IR prevents metastatic breast carcinoma (Dunn et al., 1998). A dominant negative IGF-IR introduced using retroviral infection decreases pancreatic tumor burden, particularly when used in combination with chemotherapeutic drugs (Min et al., 2003). Antisense IGF-IR strategies also enhance the susceptibility of Ewing's sarcoma to doxorubicin-induced apoptosis (Scotlandi et al., 2002), implying a use for IGF-IR disruption in combinatorial drug therapy(Li et al., 1994). Other cancers for which IGF-IR interference is effective as a treatment strategy include melanoma, lung carcinoma, ovarian carcinoma, and rhabdomyosarcoma (Baserga, 1996). One problem with these treatment strategies in the CNS is delivery; however, gene therapy approaches will likely be more advantageous and less traumatic for IGF-I inhibition treatment paradigms in CNS tumors.

THE POTENTIAL THERAPEUTIC USE OF N-TERMINAL PEPTIDE DERIVED FROM IGF-I

The hypothesis that IGF-I might be degraded by removal of an N-terminal peptide to leave des-N-(1-3) IGF-I, which would have lower affinity for the IGFBPs but maintain affinity for the receptor, was first suggested by Sara and colleagues. However, des-IGF-I has subsequently been shown to be markedly less neuroprotective than IGF-I.

This suggested the possibility that the N-terminal tripeptide, GPE, might itself have biological function and therefore its effect in models of brain disease has been extensively investigated. GPE given centrally or as an i.v. infusion has marked neuroprotective effects in rodent models of hypoxic-ischemic injury, thus protecting a wide variety of neuronal phenotypes (Guan et al., 1999). The actions of GPE include neuronal protection and inhibition of both caspase-3-dependent and caspase-3-independent neuronal cell death and protection of astrocytes. The tripeptide is now in phase 1 clinical studies for use as an acute neuroprotectant.

2.8 IGF-I and neurogenesis in the adult brain

In most regions of highly developed mammals the majority of neurogenesis is terminated soon after birth. However, new neurons are continually generated throughout life in the subventricular zone and the dentate gyrus of the hippocampus (Anderson et al., 2002). During

development, regulation of proliferation and neural cell fate in the CNS is determined by complex interactions between a number of growth factors and neurotransmitters (Anlar et al., 1999). Neurogenic neurons in the adult brain must retain some of these features of early development to enable the proliferation, differentiation and migration of progenitor cells. The influence of IGF-I on the developing nervous system is well described, with demonstrated effects on many stages of brain development including cell proliferation, differentiation and survival. The influence of IGF-I on neurogenesis on adult nervous system is only now just emerging: research to date indicates that IGF-I has many of the same effects on neuronal progenitor cells in the adult CNS as it does in the developing nervous system.

Indeed, the effects of IGF-I on proliferation and neuronal differentiation appear to be conserved in neurogenic regions during adulthood, as IGF-I induces adult-derived hippocampal cell proliferation (Anderson et al., 2002); moreover, adult rat hippocampal progenitor incubated with IGF-I not only proliferate faster, but also exhibit a greater rate of neurogenesis than control cultures (Aberg et al., unpublished data). However, the total amount of new neurons in the adult dentate gyrus at any one time is determined by the balance between neurogenesis and apoptosis (Biebl et al., 2000); therefore, since IGF-I induces proliferation and differentiation but it has also potent antiapoptotic effects, it is still difficult to understand whether the positive effect of IGF-I on adult neurogenesis is due to a pure neurogenetic effect rather than an antiapoptotic one or to a combination of both (Anderson et al., 2002).

Consequences of neurogenesis in adult brain have yet to be determined; interestingly, the rate neurogenesis in adult brain is affected by aging, stress and injury, all conditions that are associated with changes in IGF-I levels (Anderson et al., 2002).

The decline of memory with increasing age, for example, may in part be explained by age-related reduction in hippocampal neurogenesis (Cameron and McKay, 1999), which in turn is related to IGF-I and IGF-IR down-regulation in the same brain regions (Sonntag et al., 1999) and to reduced IGF-I serum levels (Ghigo et al., 1996).

Furthermore, intracerebroventricular administration of IGF-I attenuates both the reduction in neurogenesis and the cognitive decline observed in aging rats (Lichtenwalner et al., 2001), thus underlining the importance of IGF-I mediated neurogenesis in preserving adult brain from aging-induced damages.

Also depression, a common and life-threatening illness often brought about by chronic stress, correlates with decreased hippocampal volume in patients with stress-related neuropsychiatric disorders (McEwen, 2000). Chronic stress, in fact, induces serotonin down-regulation and glucocorticoids up-regulation; high glucocorticoid levels may inhibit neurogenesis by somehow

interfering with IGF-I action (Andreson et al., 2002), whereas serotonin is important for IGF-I secretion (Shaeffer and Sirotkin, 1997).

IGF-I-mediated neurogenesis in adult brain may also play a significant role in the functional recovery observed following adult brain injury (Anderson et al., 2002), as IGF-I and IGFBPs accumulation and increase in the number of IGF-I receptors are observed in the brain parenchyma after the damage.

Further studies are needed to elucidate to which extent IGF-I is able to rescue damaged neurons and to induce neurogenesis in adult brain disorders; nevertheless, stimulating neurogenesis via the IGF-I system may represent an attractive and challenging therapeutic strategy.

3. Mu-opioid receptor and its transcriptional regulation

Opioids are the main class of analgesics used in the management of moderate to severe pain (Rivera-Gines et al., 2006). Three major types of opioid receptors, μ, δ and κ, have been identified: they share an high homology both at nucleotide and at amino acid level, they are activated by structurally-related exogenous opioids or endogenous opioid peptides, and contribute to the regulation of several functions including pain transmission, respiration, cardiac and gastrointestinal functions, and immune response (Zollner and Stein 2007).

Pharmacological studies demonstrated that mu-opioid receptor (MOPr) is the main site of interaction for morphine-induced analgesia, tolerance and physical dependence (Rivera-Gines et al., 2006).

MOPr belongs to the super-family of G-protein coupled receptors (Kieffer, 1995) and is temporally and spatially expressed in the central as well as in the peripheral nervous system, with receptors varying in densities in different regions and playing different roles (Mansour et al., 1995).

MOPr in endogenous neuronal setting is coupled, via GTP-binding proteins, to inhibition of adenylyl cyclase (Herz, 1993), activation of receptor-operated K^+ currents and suppression of voltage-gated Ca^{2+} currents (Duggan and North, 1983).

These effects on ion conductance are important in opioid-mediated analgesia as they result in depression of neuronal firing. Presinaptically, in fact, opioids inhibit Ca^{2+} influx and the subsequent release of glutamate and neuropeptides from primary afferent terminals, whereas, postsynaptically, opioids hyperpolarize ascending projection neurones by increasing K^+ conductance (Zollner and Stein, 2007).

In this way MOPr agonists interfere with pain transmission, thus playing their analgesic effect.

As opioid receptors share high structural homology but show distinct expression patterns and functional profiles, their transcriptional modulation is likely to be important to determine such differences.

The human mu-opioid receptor gene (OPRM1) promoter is of the TATA-less type and has clusters of potential binding sites for different transcription factors (Law et al. 2004); several studies, primarily focused on the upstream region of the OPRM1 promoter, have been carried out to better characterize transcriptional regulation of OPRM1.

Different endogenous mediators have been shown to up-regulate OPRM1 expression in neuronal cell lines as well as in neural primary culture, including IL-4 (Kraus et al., 2001), IL-6 (Börner et

al., 2004), TNF-α (Kraus et al., 2003) and IGF-I (Bedini et al., 2008). Similarly, pro-inflammatory cytokines induce OPRM1 transcription in T-lymphocytes, thus suggesting a role for opioids in immune system. This aspect is intriguing, since morphine and other MOPr agonists have been shown to exert immuno-modulator effects, thus suggesting the existence of a balance between inflammation and pain on one side and immunomodulation and analgesia on the other.

Therefore, MOPr could be a link between nervous system and immune system, thus giving a pivotal contribution to the cross-talk between them.

3.1 Structural features of MOPr

EXTRACELLULAR LOOPS

Opioid receptors have a high similarity in transmembrane domain (TM) 2,3,5,6 and 7 and three intracellular loops; on the contrary, almost no homology is found in the extracellular loops (ECL) and in the N-terminal tail as well as in the C-terminus (Zollner and Stein, 2007) (Fig. 3.1).

This variability in ECLs seems to be important for binding to specific ligands by modulating their ability to interact with the binding pocket (Massotte and Kieffer, 2005).

Furthermore, in addition to guiding ligands on their way to the binding pocket, the ECL may regulate the "on-off" transition in the absence of ligands (Klco et al., 2005).

Moreover, an intact disulfide bond between a highly conserved cysteine residue in the second ECL and an equally well-conserved cysteine residue in the third ECL is an important regulator of MOPr function.

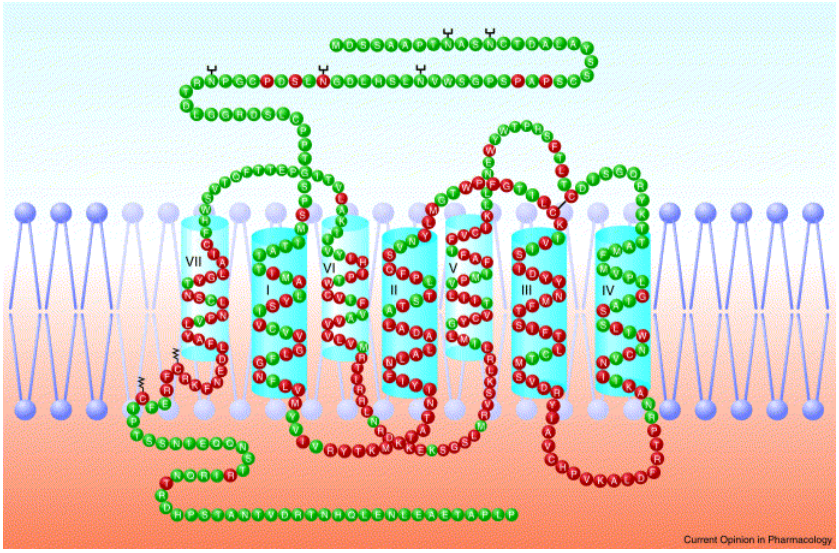


Fig. 3.1 *Schematic representation of MOPr structure*
Transmebrane domains are highlighted as light blue cylinders; amino acids shared with other opioid receptors are depicted as red cyrcles, typical MOPr amino acids as red cyrcles

TRANSMEMBRANE DOMAINS

TM residues within the lipophilic environment of the cell membrane are key in ligand recognition and/or signal transduction (Surratt et al., 1994). Ligand binding with hydrophilic and aromatic residues within the helical core, in fact, triggers outward movements of TM helices 3, 6 and 7 to promote the formation of the active receptor state that results in G-protein coupling and signal transduction (Hulme et al., 1999). In particular, for MOPr, histidine, asparagines and tyrosine residues within TM 3, 6 and 7 are critical for receptor activation (Mansour et al., 1997).

C-TERMINAL TAILS

The C-terminal portion of the opioid receptor determines coupling to second messengers and is important for receptor trafficking: MOPr point mutation of any of the serine/threonine residues within the C-terminal tail, in fact, result in significant reductions in the rate of receptor internalisation in HEK cell lines (Koch et al., 2001); similarly, in CHO-K1 cells expressing a mutated MOPr with C-terminal serine/threonine changed to alanine, reductions in receptor signalling and trafficking were also observed (Wang, 2000).

3.2 MOPr signal transduction

Mu-opioid receptors are prototypical G_i/G_0-coupled receptors (Zollner and Stein, 2007). After opioid agonists bind to the receptor, dissociation of the trimeric G-protein complex into Gα- and Gβγ-subunits can subsequently lead to the inhibition of cyclic 3'5' adenylyl cyclase and/or to direct interaction with K^+, Ca^{2+} and other ion channels in the membrane (Fig. 3.2).

Adenylyl cyclase inhibition is mediated by Gα-subunits and decreases cAMP levels, thus reducing PKA activity and the subsequent activation of CREB transcription factors (Fig. 3.2); as a consequence, genes bearing CRE elements in their promoters are down-regulated.

The ion channel modulation by opioids is mainly mediated by Gβγ-subunits (Herlitze et al., 1996); the inhibition of calcium channels blocks pro-nociceptive substance P release from primary afferent sensory neurons in the spinal cord (Kondo et al., 2005).

At the post-synaptic membrane, MOPr mediates hyperpolarization by activating K^+ channels, thereby preventing excitation and propagation of action potentials.

Furthermore, MOPr may regulate the function of N-methyl-D-aspartate (NMDA) channels (Mao, 1999) as well as of the transient receptor potential vanilloid type one (TPRV1) (Endres-Becker et al., submitted for publication)

Interestingly, Gβγ-subunits seem to activate phospholipase Cβ (PLCβ) (Chen et al., 1995) (Fig. 3.2); as a consequence, PKC is activated and opens calcium channels in the plasma membrane.

The entry of Ca^{2+} into the cells stimulates calcium-activated adenylyl cyclase to produce cAMP. Therefore, MOPr agonists determine a bi-directional regulation of intracellular cAMP whose physiological relevance is yet to be fully understood. However, most of the present data indicate that opioids mainly inhibit cAMP production (Zollner and Stein, 2007).

MOPr engagement by opioid agonists has been noted to activate also MAPK signalling cascade (Belcheva et al., 1998) (Fig. 3.2); a suggested mechanism includes the activation of PLC, generating diacylglycerol that binds to and induces PKCε, that in turn signal to matrix metalloproteinases which cleave membrane-anchored epidermal growth factor (EGF)-type ligands, thereby initiating EGF receptor transactivation and ultimately activation of the MAPK phosphorylation cascade (Belcheva et al., 2005). Although the significance of this latter signalling pathway is still not fully unravelled, MAPK cascade might be involved in MOPr homologous desensitisation (Schmidt et al., 2000).

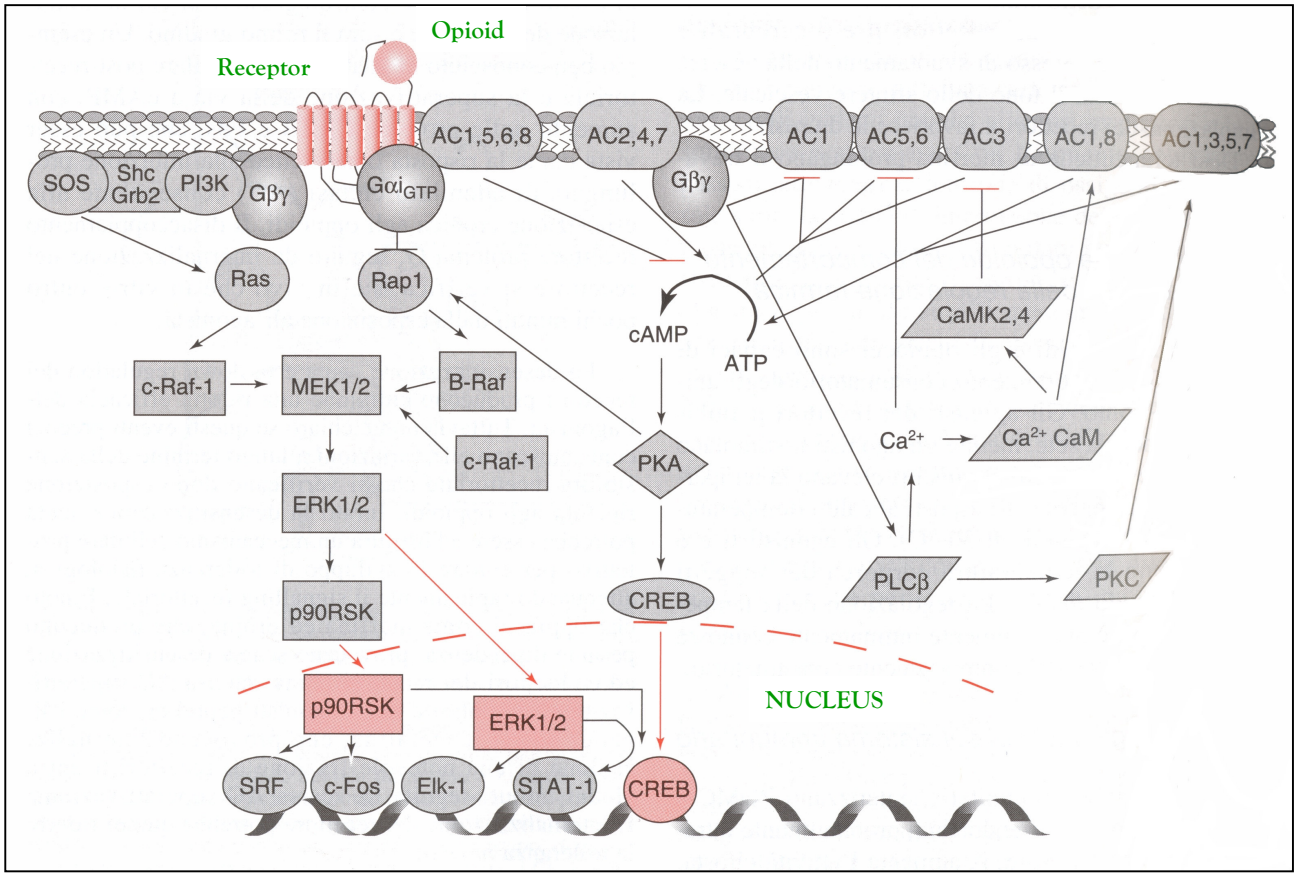


Fig. 3.2 *MOPr signal transduction pathway*

3.3 MOPr ligands and sites of action

ENDOGENOUS LIGAND

The first opioid peptides were isolated from brain, spinal cord, pituitary gland, adrenals, immune cells and other tissues; such peptides share the common amino-terminal sequence Tyr-Gly-Gly-Phe-[Met/Leu] and originate from proteolytic processing of precursors.

Later on, a novel family of endogenous opioid peptide have been identified: endomorphins (Zadina et al., 1993). Endomorphin-1 (Tyr-Pro-Trp-Phe) and Endomorphin-2 (Tyr-Pro-Phe-Phe) bind selectively to MOPr with high affinity (Horvath, 2000).

Endogenous opioid peptides can be released from neurons and axon terminals by depolarisation and can exert pre- and post-synaptic effects. In addition, endogenous opioids are produced in many non neuronal tissues, including lymphocytes, monocytes and granulocytes in inflamed tissues (Rittner et al., 2001). Furthermore, in models of inflammatory pain, opioid peptide-containing immune cells migrate from blood stream to the injured tissue (Machelska et al., 1998) as well as endogenous opioids are released locally upon stressful stimuli (cold water swim test, postoperative pain) or releasing agents (catecholamines). In this way endogenous opioids can bind to MOPr on sensory neurons, thus inhibiting their excitability and increasing nociceptive threshold (Stein et al., 1993).

EXOGENOUS LIGANDS

Exogenous opioid ligands can be classified into three groups: full agonists, partial agonists/antagonists and full antagonists. The standard to which all other opioid analgesics are compared is morphine, a derivative of poppy flowers that bind preferentially to MOPr.

Among MOPr ligands morphine exerts the strongest analgesic effect, although it produces strong physical and psychological dependence; for this reason, many pharmacologists and chemists have been trying to synthesise morphine analogues exerting more potent analgesia but determining less addiction.

Recently, several newer compounds with restricted access to the central nervous system (CNS) have been synthesised, with the aim to activate peripheral opioid receptors exclusively, thus limiting the occurrence of central side effects (Binder et al., 2001).

SUPREASPINAL AND SPINAL SITES OF ACTION

Opioid receptors and peptides have been localized in many brain areas, including the periaqueductal gray, the locus coeruleus, and the rostral ventral medulla (Heinricher ad Morgan 1999). MOP receptors are also present in the dorsal horn of the spinal cord, which is another area important for opioid-induced analgesia.

As previously mentioned, MOPr at this level inhibits pre-synaptic calcium channels and increases post-synaptic K^+ conductance, thus resulting in depression of neuronal firing and pain transmission

PERIPHERAL SITES OF ACTION

In the late 1980s MOPr and its ligands were also localized in peripheral nervous system, including primary afferent neurons and dorsal root ganglia (DRG) (Stein et al., 2003). In particular, MOP receptors have been found mainly on small- to medium-diameter neuronal cell bodies of sensory neurons (Mousa et al., 2000).

Opioid receptors are also expressed by neuroendocrine, immune and ectodermal tissues (Slominski et al., 2000).

Recently, G-protein-coupled inwardly rectifying potassium channels (GIRK2) and MOP receptors have been co-localized on sensory nerve endings in epidermis, and it has been proposed that endothelin-B receptors trigger the release of endorphin from keratinocytes, thus suppressing pain via MOPr coupled to GIRK channels (Khodorova et al., 2003).

PERIPHERAL OPIOID RECEPTORS AND INFLAMMATION

In animal experiments local application of opioid receptor agonists elicits a more pronounced antinociceptive effect under painful inflammatory conditions than in non inflamed tissues; this happens because subcutaneous inflammation can induce an up-regulation of MOP receptor mRNA within the lumbar spinal cord (Maekawa et al., 1996) and DRG (Puehler et al., 2004). In addition, the expression of MOP receptor in sensory neurons increases time-dependently during inflammation (Zollner et al., 2003). This increase could be determined by cytokines such as IL-4, IL-6 and TNF-α, which have been shown to up-regulate OPRM1 transcription in neuronal cell lines as well as in neural primary cultures (Kraus et al., 2001; Börner et al., 2004; Kraus et al., 2003).

Other potential mechanisms contributing to enhanced antinociceptive efficacy include an increase in the number of MOP receptor bearing peripheral sensory nerve terminals (Stein et al., 2003), an increase in G-protein coupling (Zollner et al., 2003) and an enhanced opioid receptor trafficking to the neuronal membrane (Patwardhan et al., 2005).

3.4 Effects of MOPr agonists

Opioids are the most broadly effective analgesics and are used in both acute and chronic pain

The opioid effect is selective on nociception; therefore, touch, pressure, and other sensory modalities are generally unaffected.

Whereas acute pain is generally amenable to drug therapy, chronic pain is a complex disease in its own right and needs to be differentiated into malignant (cancer-related) and non malignant (neuropathic, inflammatory) pain.

Cancer-related pain is commonly responsive to opioids and therefore morphine administration to neoplastic patients represent a gold standard therapy for this kind of chronic pain.

On the contrary, opioid efficacy in muscoloskeletal and neuropathic pain is still controversial.

Apart from their analgesic effects, opioids induces several other responses following either acute or chronic administration. Most of such responses represent side effects associated with opioid therapy.

High doses of MOPr agonists induce respiratory depression through the direct inhibition of rhythm-generating respiratory neurons in the pre-Boetzinger complex (PBC) of the brain stem (Manzke et al., 2003).

Other side effects induced by opioids are sedation, nausea, vomiting and pupil constriction; the miotic effect of opioid occurs through a direct action on the autonomic nucleus of the oculomotor nerve to increase parasympathetic tone.

Opioids can also depress cough by direct effect on medullary cough centers (Schug et al., 1992); this aspect is interesting as morphine congeners with bulky substitutions at the 3 position, such as codeine, display a prevalent anti-cough effects rather than an analgesic one, thus providing an effective therapy for cough suppression.

The main side effects associated to chronic opioid administration are tolerance and addiction.

Tolerance in vivo describes the phenomenon that the magnitude of a given opioid effect decreases with repeated administration of the same opioid dose or that increasing doses of an opioid are needed to produce the same effect. Tolerance usually develops most rapidly to opioid depressant effects like analgesia and respiratory depression and very slowly to stimulant effects like constipation or miosis.

On the cellular level long-term opioid treatment-induced tolerance can result in the eventual loss of opioid receptor-activated function (desensitisation); three general mechanisms are associated with these molecular events: receptor phosphorylation, receptor internalisation and/or sequestration, receptor down-regulation.

As with many other drugs, the continuous administration of opioids over of longer periods of time produces physical as well as psychological dependence, so that stopping the drug abruptly causes the stereotypical withdrawal syndrome, whose symptoms are restlessness, mydriasis, gooseflesh, diarrhoea, shaking chills, drug seeking (Heit, 2003).

3.5 Modulation of OPRM1 transcription by genetic and epigenetic mechanisms

As previously mentioned, the three classes of opioid receptors are very similar in amino-acid sequence and structure, albeit having distinct expression patterns and functional profiles; furthermore, MOPr is temporally and spatially expressed, with differences in the receptor densities (Mansour et al., 1995).

Thus, the pharmacological properties of analgesic drugs such as morphine depends on how MOPr expression is regulated in the central nervous system (Kim et al., 2004). Hence, the transcriptional control regions within the OPRM1 gene that result in this tightly controlled expression could determine the tissue-specific responses to opioid drugs (Kim et al., 2004).

Modulation of OPRM1 transcription, therefore, plays a pivotal role in regulating MOPr expression.

OPRM1 PROMOTER AND ITS BINDING SITES FOR TRANSCRIPTION FACTORS

Several studies have been addressed so far to find binding sites for transcription factors both in OPRM1 and in mouse mu-opioid receptor gene (Oprm1) promoter region; more precisely, Oprm1 has been shown to bear two promoters, namely distal promoter (DP) and proximal promoter (PP) (Ko et al., 1997).

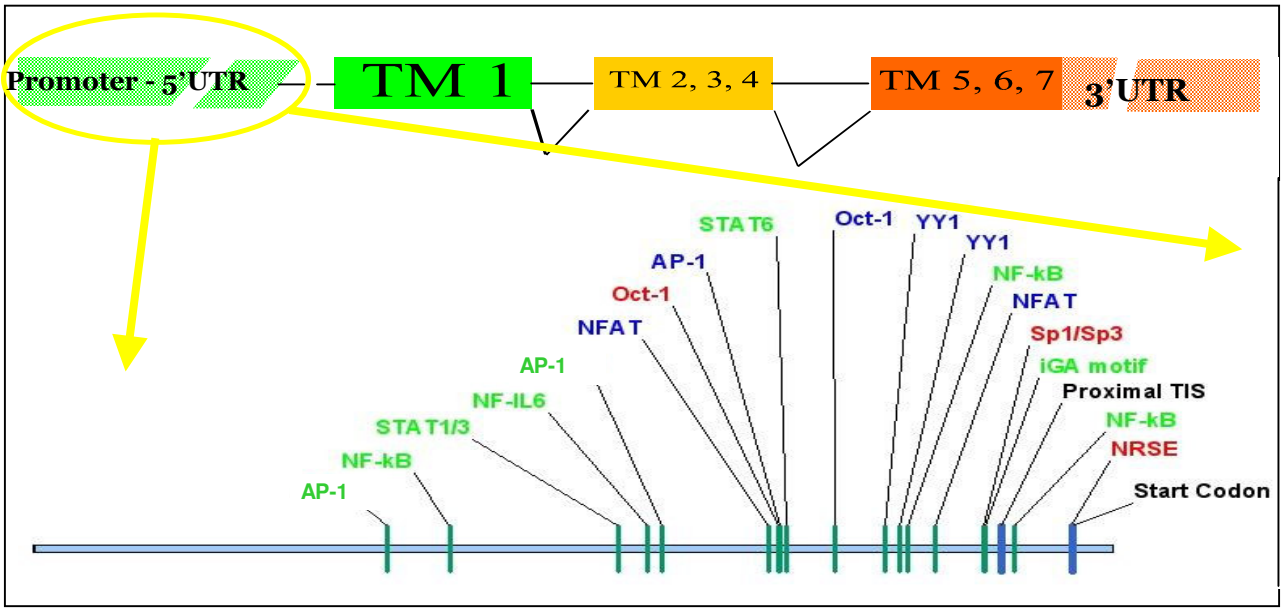


Fig. 3.3 *OPRM1 modular genomic structure (upper panel) and human OPRM1 promoter representation (lower panel). Main binding sites for transcription factors are indicated (transcriptional repressors in red, transcriptional activators in green, transcriptional factor whose action on OPRM1 transcriptional regulation is still unclear in blue).*

Extensive studies of Oprm1 regulatory regions have been carried out by Horace Loh and co-workers, who identified the DP in the region encompassing nucleotide from -994 to -784 and the PP in the region from nucleotide -450 to -249 (Law et al., 2004).

PP is preferentially used, accounting for approximately 95% of the Oprm1 gene activity in animal brain. Regulatory elements for PP include canonical Sp1 binding sites (Ko et al., 1998), AP2/Sp1 (Ko et al., 2003), single-stranded DNA-binding protein binding site (Ko and Loh, 2003). DP can be activated by Oct1 (Liang and Carr, 1996), IL-4 (Kraus et al., 2001), Sox21 (Hwang et al., 2003) and Sp1 (Xu and Carr, 2001). Other regulatory sequences were found in the 5'-untranslated region, including NF-kB (Kraus et al., 2003) and Repressor Element 1 (RE1) (Andria and Simon, 2001).

OPRM1 promoter seems not to be divided into a distal and a proximal regulatory region; similarly to Oprm1, however, also OPRM1 promoter is a TATA-less one therefore having multiple transcriptional initiation sites (TIS).

OPRM1 analysis allowed the identification of several binding sites for transcription factors (Fig. 3.3), thus enabling a better understanding of its transcriptional regulation.

An NF-IL6 site has been found at nucleotide -1481 (Im et al., 1999), suggesting a possible positive modulation of OPRM1 transcription by IL-1α and IL-1β; other positive element(s) were identified in the promoter region from -563 to -292 (Xu and Carr, 2001), whereas negative regulatory elements have been found from -776 to -564 (Xu and Carr, 2001). Binding sites for Oct-1 (from -1027 to -1020) (Fig. 3.3), YY1 (from -657 to -641) and NFAT (from -1062 to -1056 and from -484 to -478) have been proposed (Xu and Carr, 2001).

Furthermore, other binding sites for transcription factor have been recognized and experimentally verified to be bound by the predicted transcription factor (Fig. 3.3): a STAT6 binding element at nucleotide -997 (Kraus et al., 2001), two AP-1 binding sites at nucleotides -2388 and -1434 (Börner et al., 2002), three NF-kB binding sites at -2174, -577 and -207 (Kraus et al., 2003), a STAT1/3 element at -1583 (Börner et al., 2004).

Interestingly, a functional RE1 site was found also in OPRM1 at nucleotides from -9 to + 12 (Fig 3.3), just overlapping the start codon (Kim et al., 2004).

CHROMATIN REMODELLING AND EPIGENETIC MODULATION OF OPRM1 TRANSCRIPTION

MOPr is actively involved in the development of mammalian nervous system (Kim et al., 2004), as its expression has been reported to increase during neuronal differentiation both *in vitro* and *in vivo* (Jenab and Inturrisi, 2001).

OPRM1 transcript levels, in fact, gradually increase throughout embryonic and post-natal stages, reaching a plateau at adulthood (Ko et al., 2002). To achieve its unique expression pattern spatially and temporally, OPRM1 expression must be therefore tightly modulated both at genetic and at epigenetic level (Hwang et al., 2007).

In mammals, DNA methylation and histone modifications represent the major epigenetic mechanisms implicated in the regulation of gene transcription; DNA methylation, in fact, is essential for mammalian development (Li et al., 1992) and is associated with gene silencing in conjunction with histone core modifications through chromatin remodelling (Berger, 2001).

DNA hypermethylation and histone deacetylation lead to a more compact chromatin structure, thus determining transcriptional repression, whereas a more relaxed chromatin allows transcription factor to access to their binding elements in the promoter regions.

Many transcription factors, in fact, bind to sequences containing CpG nucleotide that can be target of DNA methylation; when such nucleotides are methylated transcription factors are unable to bind DNA (Wenger et al., 1998); alternatively, methyl-CpG-binding proteins, such as MeCP2, bind preferentially to methylated DNA and directly repress transcription, inhibit the binding of other transcription factors, structurally modify the DNA, or recruit co-repressor complexes (Fuks et al., 2003).

OPRM1 expression in developing neurons has been found to be tightly modulate at the epigenetic level (Fig 3.4): in undifferentiated mouse P19 pluripotent embryonic cells, in fact, Oprm1 transcription is silenced by hypermethylation of CpG dinucleotides and histone modifications; following differentiation, on the contrary, Oprm1 expression is significantly induced and this increase is accompanied to chromatin decondensation (Fig. 3.4) due to DNA de-methylation and histone acetylation (Hwang et al., 2007).

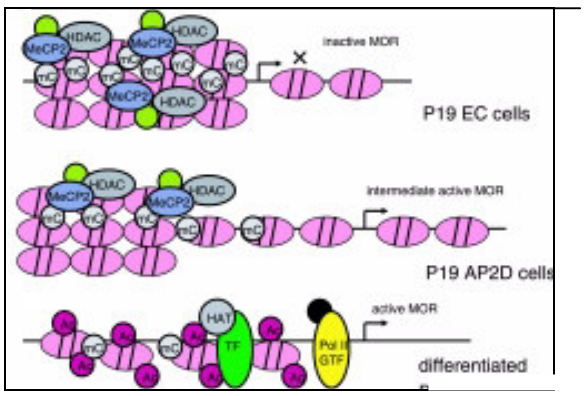


Fig. 3.4 *Epigenetic modulation of Oprm1 transcription along RA-induced differentiation of P19 mouse embryonic stem cells*

Interestingly, RE1 silencing transcription factor (REST), a transcriptional repressor that binds to the RE1 element within Oprm1 and OPRM1 promoters, determines its negative function by inducing chromatin condensation through histone deacetylation and DNA methylation (as extensively discussed in chapter 1).

Since REST has been proved to actively repress OPRM1 transcription in neuronal cells (Kim et al., 2004; Bedini et al., 2008), and is a key regulator of neuronal differentiation, it is becoming evident the role of epigenetic modulation of MOPr expression in neuronal development, thus suggesting a pivotal functions for opioids in mature neurons.

Furthermore, another transcriptional repressor that take part to epigenetic modulation of transcription has been shown to bind to the OPRM1 promoter: Sp3.

An Sp3 binding element has been recently identified at nucleotides from -219 to -189 (Choi et al., 2005); this site can be recognized by Sp3 isoform M1 and M2, which are known to exert a repressor effect on gene transcription(Choi et al., 2005).

Interestingly, another Sp3 binding element has been discovered immediately down-stream of RE1 and it has been demonstrated that Sp3 specifically binds to this site and interacts with REST to synergistically repress MOPr expression (Kim et al., 2006).

Such an interplay with a key neuronal differentiation modulator, together with the observation that MOPr expression increases along the differentiative process, confirms that epigenetic modulation of OPRM1 transcription is crucial to allow the proper temporal and spatial MOPr expression pattern in mature neurons.

3.6 OPRM1 transcription is influenced by several endogenous as well as exogenous compounds

Several different substances have been shown to differently modulate OPRM1 transcription in neuronal cells as well as in immune cells. A deeper comprehension of such processes is very important since MOPr down-regulation is involved in opioid drugs-associated side effects such as tolerance as well as it is supposed to play an important role in neuronal development and it seems to be involved in he cross-talk between neuronal system and immune system.

OPIOID LIGANDS EFFECTS ON OPRM1 TRANSCRIPTION

That MOPr ligands modulate the expression of their own receptor is an intriguing perspective, suggesting interesting feed-back loops between MOPr engagement and OPRM1 transcription; for example, DAMGO interaction with MOPr has been shown to enhance Sp1/Sp3 binding to the

OPRM1 promoter (Xu and Carr, 2001) and this event may be relevant in the regulation of OPRM1 expression.

Furthermore, morphine and endomorphins have been found to differentially modulate OPRM1 transcription in SH-SY5Y human neuroblastoma cells (Yu et al., 2003): morphine determines OPRM1 down-regulation both in undifferentiated and differentiated SH-SY5Y cells, whereas endomorphin-1 and -2 up-regulate OPRM1 transcription, albeit at different extent, in the same cell model (Yu et al., 2003).

In some preliminary experiments we reported different effects on OPRM1 transcription following SH-SY5Y exposure to DAMGO or endmorphin-1 analogues as well.

In our laboratory we have also observed that Nociceptine administration to SH-SY5Y cells determines a significant decrease in OPRM1 transcript levels, thus confirming the hypothesis of feed-back regulations between MOP and related receptors in neurons.

OPRM1 TRANSCRIPTION AND NEURONAL DIFFERENTIATION

It is very interesting that OPRM1 expression progressively increases along neuronal differentiation and that this event is related, as previously discussed, to REST action and epigenetic modulation of gene expression.

Furthermore, neuro-differentiating compounds such as retinoic acid (RA) or phorbol 12-myristate 13-acetate (PMA) have been shown to precisely modulate OPRM1 transcription and MOPr expression in neuronal cell lines like SH-SY5Y cells (Zadina et al., 1994).

More precisely, RA exerts a bi-modal action on OPRM1 transcription during neuronal differentiation *in vitro*: at the beginning of differentiative process, in fact, OPRM1 is down-regulated by RA, but it is subsequently significantly up-regulated following 96 h- to 168 h-long exposure to RA (Jenab and Inturrisi, 2001). RA-mediated differentiation of mouse embryonic P19 stem cells releases OPRM1 repression by inducing chromatin decondensation (Hwang et al., 2007).

Similarly, PMA has been shown to initially decrease OPRM1 transcription (Gies et al., 1997) and to up-regulate it following longer exposure to PMA (Zadina et al., 1994); PMA is also able to induce transcriptional activity of an OPRM1 promoter fragment lacking the region from -165 to the start codon (Börner et al., 2002).

The comprehension of the different modulation of OPRM1 transcription in undifferentiated as well as in differentiated neurons is of particular interest, as the possibility of inducing MOPr expression in selective areas of mature nervous system could be very useful in counteracting tolerance development to opioid pain therapy as well as in preventing neurodegeneration.

OPRM1 UP-REGULATION BY CYTOKINES IN BOTH NERVOUS AND IMMUNE SYSTEM

Several cytokines have been shown to up-regulate OPRM1 transcription in neuronal cell lines and primary culture as well as in immune cells; this aspect f OPRM1 transcriptional regulation is of particular interest since it suggests a possible involvement of MOPr in the cross-talk between nervous and immune system.

Pro-inflammatory cytokines such as IL-4 and IL-6, in fact, have been found to significantly up-regulate OPRM1 transcription in SH-SY5Y cells, through the activation of STAT6 (Kraus et al., 2001) and STAT3 (Börner et al., 2004), respectively.

Interestingly, IL-4 as well as other pro-inflammatory mediators up-regulate OPRM1 transcription in different types of immune cells; furthermore, interferon-γ, another cytokine important in immune responses, have been shown to shut down MOPr expression, thus suggesting that distinct differentiative programs in immune cells (namely TH1 vs. TH2 differentiation) imply an opposite modulation of OPRM1 transcription.

Other cytokines such as TNF-α (Kraus et al., 2003) and IL-1 (Vidal et al., 2001) are able to induce OPRM1 transcription in neurons and endothelial cells respectively; TNF-α up-regulates OPRM1 transcription also in several different immune cells, thus confirming the tight connection between nervous system and immune system.

Therefore, a branched network of transcriptional regulation emerges for OPRM1 modulation by cytokines. This is of particular interest considering that morphine and other MOPr agonists are potent immuno-modulators; morphine main effects on immune system are summarized in table 3.1. A deep understanding of all the processes involved in pain transmission and inflammation, immuno-modulation and analgesia will allow to better tailor analgesic as well as anti-inflammatory therapies and to find novel potential therapeutic targets.

TABLE 3.1 - Main immuno-modulator effects of morphine
Lymphoid Organ Atrophy
Reduced Natural Killer (NK) Cell Activity
Reduced Macrophage (MΦ) Activity
Reduced MΦ Secretion of IL-1
Reduced MΦ Secretion of IL-6
Reduced MΦ Secretion of TNF-α
Decrease in CD4/CD8 double positive Cells
Increase in CD4 and CD8 single positive Cells
Increase in IL-4 Expression
Decrease in IFN-γ Expression
Reduced T-Cell Secretion of IL-2
Phosphorylation of MAPK in Lymphocytes

4. Immune System and T-lymphocyte activation

4.1 Innate and adaptive immunity: the components of immune system

In order to prevent infections or to fight them when they occurs, human beings developed a powerful and specialized system of cells and mediators that can recognize potential pathogens, induce cell-to-cell communication, promote activation and migration of specific cells: the immune system.

There are two distinct classes of immune response: innate immunity, which is the first to be involved in protecting organisms from pathogens and tissue damage and provide an immediate albeit aspecific level of defence, and adaptive immunity, which is antigen-specific and therefore able to successfully fight the different infections that can occur.

These two distinct yet related phases of immune response are mediated by several different cells that originate from common progenitors: all the cellular elements of the blood, in fact, including the cells of the immune system, arise from pluripotent hematopoietic stem cells in the bone marrow. These pluripotent cells divide to produce two types of stem cells: a common lymphoid progenitor that gives rise to natural killer (NK) cells, T- and B-lymphocytes, and a common myeloid progenitor that gives rise to different type of leukocytes (granulocytes, macrophages and mast-cells), to dendritic cells, erythrocytes and megakaryocytes (platelets progenitors).

T- and B-cells are distinguished by their site of differentiation, the thymus and the bone marrow respectively, and by the possession of antigen receptors, whereas NK cells lack antigen specificity; mature T- and B-lymphocytes circulate between blood and peripheral lymphoid tissues so that they can recognize infected or damaged tissues.

Granulocytes include neutrophils, eosinophils and basophils (with respect to the specific reactivity of their cytoplasmatic granules); they circulate in the blood and enter the tissues only at sites of inflammation. More precisely, neutrophils are recruited to phagocytose bacteria, eosinophils and basophils are involved in defense processes against parasites.

Immature dendritic cells travel through the blood to enter peripheral tissues, where they can mature after interaction with potential pathogens, and then migrate to lymphoid tissues in order to activate antigen-specific T-lymphocytes. Monocytes also enter tissues, where they differentiate into phagocytic macrophages; mast-cells also complete their maturation in inflamed tissues and are important mainly in allergic responses.

As previously mentioned, there are two levels of immune response: innate and adaptive immunity.

Leukocytes and dendritic cells, as well as physical barriers like skin and mucoses, contribute to the first level of protection, whose task is to prevent infections or to limit them while activating further more specific responses.

When pathogens enter the body, in fact, they are first engulfed by macrophages and neutrofiles which try to destroy them; immature dendritic cells are also phagocitic, but after engulfing a pathogen they migrate to lymphoid tissues and present specific antigens to lymphocytes in order to activate them and start an-antigen specific immune response: the adaptive immunity. Macrophages can also present antigens to T-cells and activate them.

Once activated, T-cells give rise to two different lineages of effector cells: CD8+ cytotoxic and CD4+ helper T-lymphocytes. The former directly kills infected cells, the latter produces factors important to enable phagocytic cells to kill engulfed pathogens or further activates B-cells to antibody-producing plasma cells.

In summary, different types of immune cells travel through the blood or migrate to tissues in order to provide defense against infections; some of these cells are important in peripheral tissues to give rise to a first level barrier to pathogens whose task is to limit infections as well as to activate specific response.

The other components are fundamental for antigen specific immune response, which is several times much more powerful than innate, aspecific immune response.

Because of its great potency, adaptive immunity needs to be finely tuned in order to give immediate and maximal response very quickly as well as to be rapidly silenced after pathogens removal, otherwise it can be seriously dangerous for healthy tissues and cells.

In such a context, T-lymphocytes play a pivotal role since they can differentiate in effector cells with a great variety of functions.

4.2 T-Cell receptor (TCR) and antigen recognition: one of the adaptive immunity starting point

The main event in T-lymphocytes activation is the interaction between T-Cell receptor and specific protein antigens derived from pathogens; the specific antigen recognition, together with all the co-stimulatory signals which contributes to such a complex interaction, determines the activation of T-lymphocytes to mature effector cells.

TCR structural and functional properties are therefore crucial for this process.

TCR RECEPTOR STRUCTURAL FEATURES

As assessed by using clonotypic antibodies, each T-cell has been shown to bear about 30000 identical antigen receptor molecules on its surface: those molecules, named the T-cell receptor (TCR), are responsible for the clonal activation of T-lymphocytes during inflammation.

The TCR is composed of six different polypeptide chains: two main chains, each made of a constant and a variant portion and termed TCRα and TCRβ, that are linked by disulfite bond; and four CD3 chains (Fig. 4.1). The specificity of antigen binding is dictated by the clonotypic TCRα and TCRβ chains, which arise from a process of genetic rearrangement that results in millions of receptor variants. The several possible combinations at genomic level allow T-cell population to produce a wide range of different antigen specificities, thus enabling the organism to face several different pathogens.

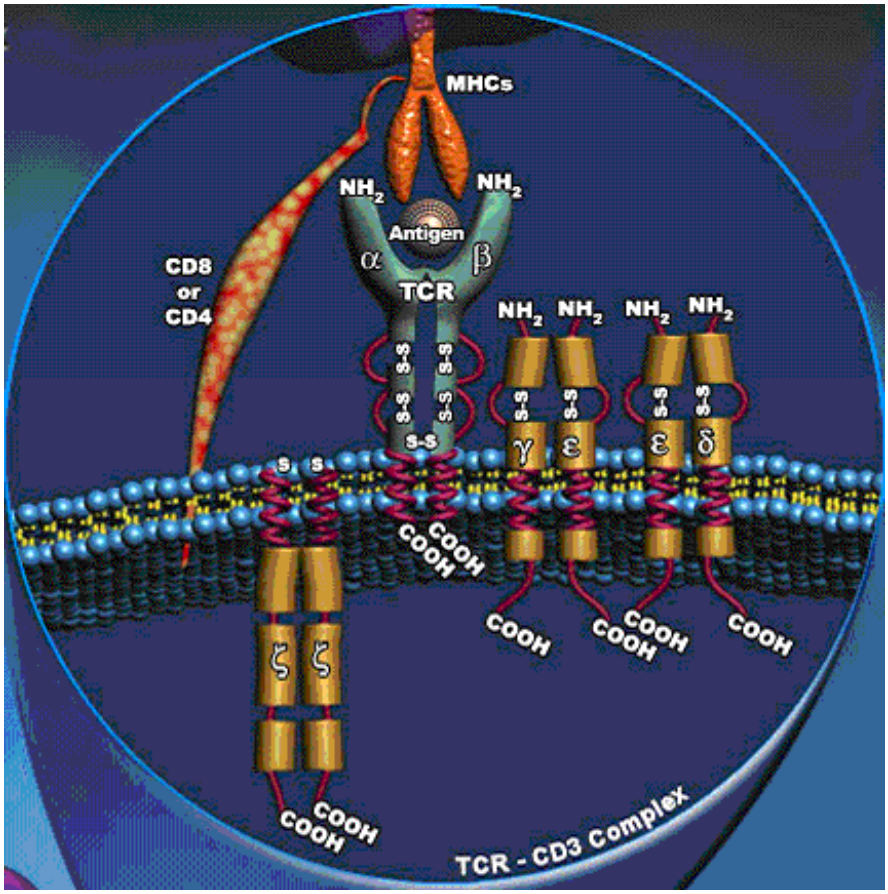


Fig. 4.1 *Schematic representation of molecular structures involved in antigen recognition: TCRα and TCRβ chains, CD3 molecules and co-receptors.*

TCR interacts with short contiguous amino acid sequences, thus needing a processed peptide rather than a whole protein, as an antigen; therefore, specialized antigen presenting cells, such as

dendritic cells and mocrophages, hydrolyse pathogen proteins into small peptides to be exposed on their cell surface in the context of major histocompatibility complex (MHC) (Fig. 4.1).

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) AND ANTIGEN RECOGNITION

TCRα and TCRβ chains form a heterodimer that binds directly to an antigen-bearing major histocompatibility complex (MHC) exposed on antigen presenting cells' surface (Fig. 4.1).

There are two classes of MHC molecule which differ in their structure and expression pattern on tissues of the body: MHC-classI and MHC-classII.

Their distinct distribution among cells reflects the different effector functions of the T-cells that recognize them. MHC-I presents peptides from intracellular pathogens, such as viruses, to cytotoxic T-lymphocytes; since viruses can infect any nucleated cell, almost all such nucleated cells express MHC-I on their surface, albeit at different levels.

In contrast, the main function of T-cells that recognize MHC-II is to activate other effector cells of the immune system; thus MHC-II molecules are normally found on specialized immune cells such as B-lymphocytes, dendritic cells and macrophages.

ROLE OF TCR-ASSOCIATED CD3 CHAINS

Communication of TCRαβ engagement by peptide-MHC to the intracellular signalling machinery occurs via the TCR-associated CD3 chains, which are arranged into three dimers: γε, δε, and ζζ (Alarcon et al., 2003) (Fig. 4.1).

Each CD3 chain consists of one extracellular immunoglobulin-like domain and a short cytoplasmatic tail; they are fundamental for TCR mediated cell signalling, as TCRα and TCRβ chains can themselves recognize antigens but they are not directly coupled with any signal transducer.

Each CD3 chain contains, in fact, immunoreceptor tyrosine-based activation motifs (ITAMs) which can be phosphorylated and can function as docking sites for signal trasnduction molecules.

TCR CO-RECEPTOR MOLECULES AN CO-STIMULI

There are two different classes of T-lymphocytes that can be distinguished by the different co-receptor molecule expressed on their cell surface: CD4 and CD8. Those molecules are defined as co-receptors because their interaction with the TCR/MHC complex is required for the T-cell to make an effective response.

CD4+ and CD8+ T-lymphocytes differ also for their function after TCR engagement by antigen and for the specific MHC molecule they interact with: CD8+ T-cells are in fact cytotoxic T-lymphocytes that kill the infected cell after interacting with the antigen expressed on its surface in the context of MHC-I; CD4+ T-cells, on the contrary, activate specialized immune effector cells

following interaction with antigen presented by the MHC-II molecules expressed on the membrane of these latter cells.

As CD4 interacts strongly with a cytoplasmic tyrosine kinase called Lck, by interacting with the TCR/MHC-II complex it delivers this kinase into close proximity to the ITAMs of the TCR complex; furthermore, CD4 contributes to stabilize TCR/MHC-II interaction as it binds strongly to such a complex.

CD8 is also associated to Lck, so that it can bring the kinase in close proximity to the TCR/MHC-I complex, whose stabilization it also contributes to; therefore CD8 function is almost identical to the one mediated by CD4 co-receptor molecule.

4.3 TCR signalling pathway: the complex molecular network for T-lymphocytes activation

In order to fully respond to infections, T-cells need to be engaged by different but simultaneous stimuli: TCR recognizes a specific antigen in the context of MHC molecules and co-receptors significantly contribute in stabilizing this interaction and in bringing signal transducers in close proximity to TCR/antigen/MHC complex (Fig. 4.2).

Other transmembrane proteins, such as CD28, present on T-cell surface interact with co-stimulatory molecules exposed on antigen-presenting cells, thus providing further stimuli that are important for the induction of a full immune response (Fig. 4.2).

This series of events initiates a complex network of signal transduction whose aim is to promptly activate T-cell by inducing gene expression and modifying cytoskeleton.

Given that T-cell activation determines very powerful responses, it is very important that TCR signalling cascade is silenced following infection removal, in order to prevent undesired tissue damages and improper immune responses; similarly, the maintenance of TCR at a resting state is also crucial. Therefore, TCR signalling comprises also TCR silencing pathways.

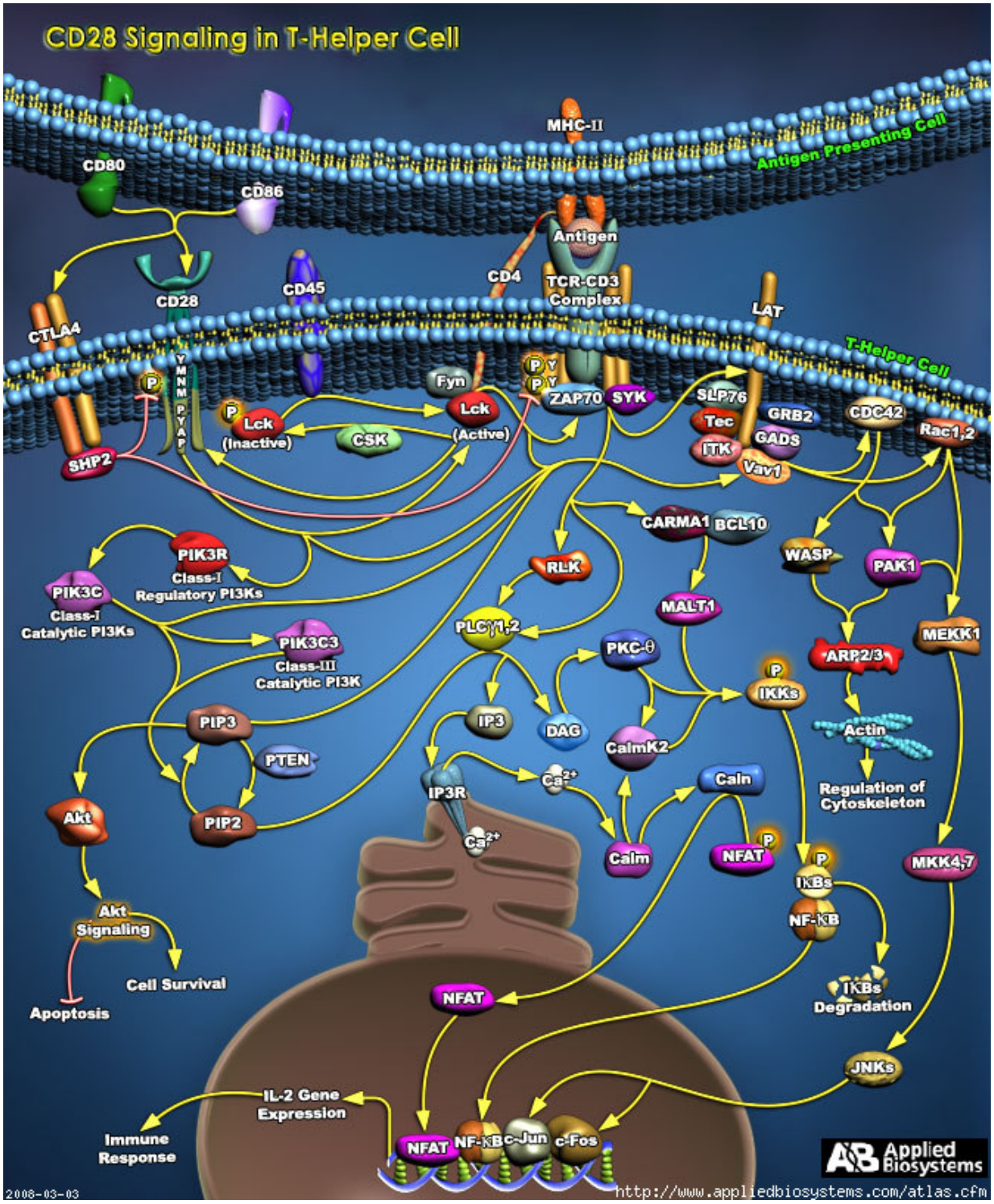


Figure 4.2 *Schematic representation of TCR and MHC/antigen interaction and the following signal transduction pathways, the formation of immunological synapse and the signalling induced by co-stimuli such as CD28*

TCR ENGAGEMENT: TYROSINE KINASE CASCADE, PHOSPHORILATION OF LINKER PROTEINS AND ASSEMBLY OF SIGNALOSOME

Given the primacy of ITAM phosphorylation by Lck in TCR signalling, an especially important question to answer is how is ITAM tyrosine phosphorylation maintained below the signalling threshold prior to TCR engagement.

Current data suggest that multiple mechanisms act in concert to block spontaneous TCR signalling. At the first level, there is a physical sequestration of Lck away from the TCR, in resting T-cells, by virtue of differential partitioning of Lck and the TCR into lipid rafts (Cherukuri et al., 2001); lipid rafts are heterogeneous lipid microdomains relatively enriched in sphingomyelin, glycosphingolipids, and cholesterol that spontaneously form in cell membranes as a consequence of the biophysical properties of its different lipids. Lck constitutively partitions to the lipid rafts, whereas the unstimulated TCR is largely excluded from this fraction.

The CD3 ITAMs are also maintained in a sub-critical state of tyrosine phosphorylation by tyrosine phosphatases: these enzymes, in fact, have ready access to the TCR prior to TCR stimulation, but more limited access following TCR stimulation (Stefanova et al., 2003). In addition, prior to TCR engagement, Lck is maintained in an inactive state by the combined actions of Csk (Fig. 4.2) and PEP, a tyrosine kinase that phosphorylates the negative regulatory C-terminal tyrosine residue of Lck and a phosphatase that dephosphorylates the activation loop tyrosine of Lck, respectively.

Following TCR stimulation, there is increased distribution of TCR to the lipid rafts and sequestration of negative regulatory tyrosine phosphatases away from the TCR (Cherukuri et al., 2001). Concurrently, Lck becomes activated via dephosphorylation of its regulatory C-terminal tyrosine in response to increased exposure to CD45 (Fig. 4.2), a transmembrane phosphatase that dephosphorylates the negative regulatory site, and decreased exposure to Csk (Hermiston et al., 2002). In addition, because a portion of Lck is constitutively associated with the CD4 co-receptor, the antigen-MHC-induced co-localization of TCR with CD4 results in an increased local concentration of Lck around the TCR (Fig. 4.2). Therefore, the ITAM sequences of the CD3 chains subsequently become fully tyrosine-phosphorylated, thus serving as binding sites for other signal transducers such as ZAP-70 (Fig. 4.2).

ZAP-70 binds to phosphorylated ITAMs via its tandem SH2 domains (Wange and Samelson, 1996); then it is activated via Lck-mediated tyrosine phosphorylation of its activation loop tyrosine (Tyr-493).

Activated ZAP-70 autophosphorylates at tyrosines 292, 315, and 319, serving these sites to recruit various positive and negative signalling effectors to the TCR complex (Wange, 2004). In addition to serving as a scaffold via self-phosphorylation, ZAP-70 also phosphorylates a restricted set of substrates following TCR stimulation, including, Vav-1, VHR, Shc, Gab2, LAT, and SLP-76 (Fig. 4.2) (Wange, 2004).

These latter two substrates in particular have been recognized to play a pivotal role in TCR signalling: when phosphorylated, in fact, both LAT and SLP-76 act as linker/adapter proteins, which serve as nucleation points for the construction of higher-ordered multi-molecular signalling complexes, often referred to as the signalosome (Lindquist et al., 2003) (Fig. 4.3).

Acting in concert, these linker/adapter proteins regulate the activation of PLCγ1, thus inducing several down-stream signalling cascades, and of another TCR-activated protein-tyrosine kinase, Itk. Activated PLCγ, in fact, cleaves membrane-bound phosphatidylinositol bisphosphate (PIP2) into inositol trisphosphate (IP3) and diacylglycerol (DAG) (Fig.3).

IP3 molecules interact with their receptors in the endoplasmic reticulum, thus releasing Ca^{2+} from its intracellular storage site into the cytoplasm and therefore increasing immediately intracellular levels of Ca^{2+} (Fig. 4.2). Depletion of the endoplasmic reticulum calcium stores triggers the opening of calcium channels on cell membrane, thus determining a further increase in intracellular calcium levels (Fig. 4.2). Free calcium ions interact with calmodulin and then binds to and activates the protein phosphatase calcineurin, which in turn dephosphorilates and activates the transcription factor NFAT.

DAG, on the other hand, activates Protein Kinase C (PKC), which in turn activate the transcription factor NF-kB (Fig. 4.3).

Itk has been implicated in PLCγ1 activation, regulation of TCR-stimulated actin cytoskeleton reorganization via its involvement in regulating the Vav-1/Cdc42/WASP pathway, and in so-called “inside-out” signalling (Miller and Berg, 2002) (Fig. 4.2).

The LAT and SLP-76 signalling complexes also support the activation of multiple other signalling proteins, including mitogen-activated protein kinases (p38, Jnk, and Erk) and small molecular weight G proteins (Ras, Rac, Rho, and Cdc42) (Fig. 4.3).

A key feature of LAT is that it is a transmembrane adapter protein that is constitutively targeted to the lipid rafts (Lindquist et al., 2003). It has no protein interaction domains other than the multiple tyrosine residues that are phosphorylated by ZAP-70 following TCR engagement.

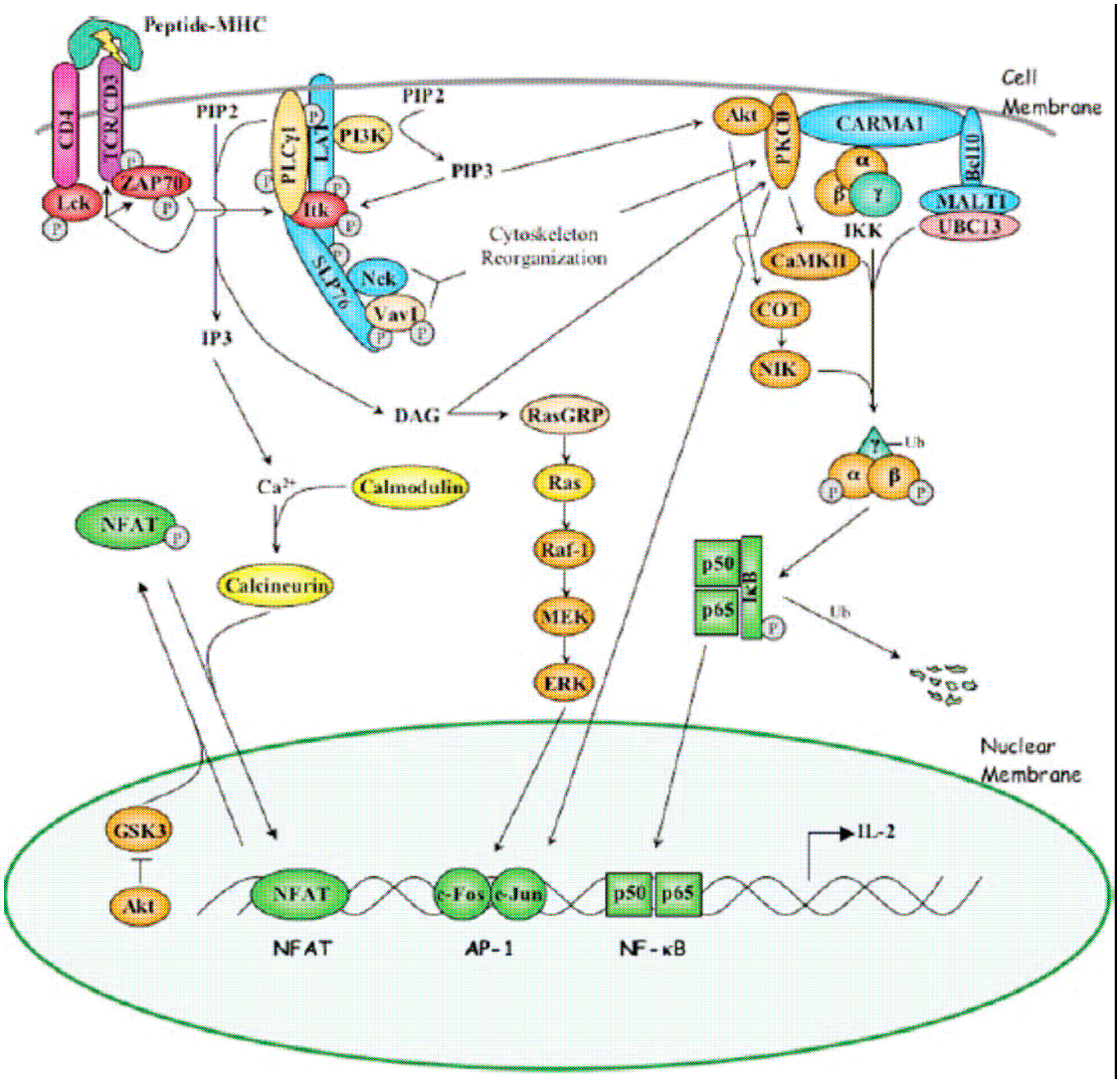


Fig. 4.3 *T cell receptor signalling events leading to activation of transcription factors. This figure presents an overview of some of the key signalling events linking the binding of peptide-MHC to the T-cell antigen receptor (TCR/CD3) and the CD4 co-stimulatory receptor.*
The existence of key phosphorylation events is indicated on some of the signalling proteins by small grey circles labelled P; Ub designates ubiquitination. The nature of the different interactions is described in the accompanying text. For greater graphical clarity some important signalling events are not depicted; most notably the small molecular weight linkers Gads and Grb2 are excluded from the LAT/SLP-76 signalosome complex. Also not shown are the signals contributed from the CD28 co-stimulatory receptor toward the activation of NF-kB and the Vav-1-PI3K positive feedback loop.

When phosphorylated, these tyrosines serve as binding sites for specific SH2 domain-containing proteins. There are a total of eight tyrosine residues in LAT that are conserved between humans, mice, rats, and bovines (Wange 2000). The C-terminal five tyrosine residues play critical roles in the ability of LAT to bind to PLCγ1 and to the linker proteins GADS and Grb2, which can in turn recruit SOS and then Ras, thus activating mytogen-activated protein kinases (MAPKs) cascade (Fig. 4.2 and Fig. 4.3). This latter signal transduction pathway consists of a series of subsequent protein phosphorylations (Ras-Raf-MEK1/2-Erk1/2) and leads to the activation of

several transcription factors, including Fos (Fig. 4.3); this latter transcription factor is very important during T-cell activation as it is part of the transcription factor AP-1 (dimer Fos-Jun) (Fig. 4.3).

The MAPK pathway that activates Jun, and therefore allows the formation and induction of AP-1, is induced in T-Cells by the co-stimulatory molecule CD28, which recognises specific surface molecules induced in antigen presenting cells (Fig. 4.2); this process underlines that distinct but simultaneous and converging signals are fundamental for a full response by T-lymphocytes (Fig. 4.2).

SLP-76 associates with LAT via GADS and can also directly bind to PLCγ1, indicating the existence of higher-ordered interactions between these and possibly other signalling proteins in the signalosome.

In addition to the proteins already mentioned, phosphorylated LAT also binds to PI3K, Grap, 3BP2, Shb , c-Cbl, Vav-1, and Itk, localizing these molecules in close proximity and a defined orientation to one another within the lipid raft domain of the plasma membrane (Fig 2 and Fig. 4.3).

Vav-1 can activate another small G protein, Rac, which in turn can induce MAPK cascade as well as Ras, thus providing activated TCR of another connection point to MAPKs cascade.

Vav and Rac, moreover, can also contribute to changes in actin cytoskeleton, thus linking the signalling on cell surface to cell shape and motility (Fig. 4.2).

Unlike LAT, SLP-76 is a cytosolic protein (Clements, 2003) which bears an acidic region and three sites of tyrosine phosphorylation (Tyr-113, Tyr-128, and Tyr-145); when phosphorylated, these sites can bind the SH2 domains of Vav-1, Nck, and Itk, whereas an extended proline-rich area close to the acidic region binds the SH3 domains of GADS, Itk, and PLCγ1, thus providing another integration point for the high-ordered TCR signalling complex (Fig. 4.2). Furthermore, the C-terminal portion of SLP-76 is comprised of a single SH2 domain that binds primarily to ADAP, a key protein in inside-out signalling to integrins (Griffiths and Penninger, 2002). It is supposed, in fact, to increase integrin affinity.

As it appears from what has been reported above, ITAMs phosphorylation by co-receptor associated Lck following TCR engagement determines the subsequent recruitment and activation of ZAP-70, which in turn recruits and activates LAT and SPL-76 and the subsequent formation of an high-ordered signalling complex which induces transcription factors activation, trough at least three different cascades, and cytoskeleton remodelling.

TCR SIGNALLING PATHWAY: CYTOSKELETAL REORGANIZATION AND THE FORMATION OF IMMUNOLOGICAL SYNAPSE

As well as changing the pattern of transcription within the cells, signals from cell surface receptors change cells' behaviour also in other ways: adaptor proteins LAT and SLP-76 interact, in fact, with cytoskeleton and influence cell shape and motility (Fig. 4.2).

Mainly three different of these pathways have been described, thus confirming the high complexity of TCR cell signalling. Furthermore, a new layer of complexity in this process has come to light in recent years in the form of the immunological synapse (IS), which has also been referred to as the supramolecular activation complex (SMAC). This is a dynamic yet highly ordered structure that forms at the site of T-cell contact with an antigen presenting cell (Huppa and Davis, 2003). The mature IS is characterized by a central region (c-SMAC) that is enriched in clustered TCR and PLCγ, surrounded by a peripheral ring (pSMAC) of adhesion factors such as LFA-1, and a distal ring (dSMAC) containing proteins such as the tyrosine phosphatases CD148 and CD45. The IS, although not required for initiating TCR signalling, is required for sustained signalling, IL-2 production, and cell proliferation (Huppa and Davis, 2003). IS has also the ability to act as a positive or negative servo, either amplifying weak TCR signals or attenuating strong signals (Lee et al, 2003).

Clustering of lipid rafts at the contact site and formation of the IS is an active process that requires several upstream signalling events to occur. Most important of these are the signals that lead to reorganization of the actin cytoskeleton, whose proximal catalyst is the recruitment and activation of the Arp2/3 complex (Fig. 4.2). In fact, it catalyses the formation of new nucleation sites for actin polymerisation. A key upstream regulator of Arp2/3 is WASP, which is rapidly recruited to lipid rafts following TCR/CD28 co-stimulation (Fig. 4.2) (Cannon and Burkhardt, 2002). WASP is constitutively present at high stechiometry in a complex with WIP and CrkL. WIP-bound WASP is refractory to activation. Upon TCR stimulation, WIP/WASP complex is recruited to the TCR (and consequently to the lipid rafts and the antigen-presenting cell contact site) via the binding of the CrkL SH2 domain to tyrosine-phosphorylated ZAP-70 (Sasahara et al., 2002). Co-localization of this complex with activated PLCγ at the lipid rafts results in the phosphorylation of WIP and disruption of the WIP-WASP association, thereby facilitating activation of WASP by Cdc42 (Sasahara et al., 2002). WASP can also be recruited to the lipid rafts via SH3 domain-mediated binding of Nck to a proline-rich region of WASP (Zeng et al., 2003). In this case, the raft recruitment and activation of WASP are coordinated by SLP-76,

which functions as a targeting scaffold, bringing WASP into close proximity with Cdc42 that has been activated by SLP-76-bound Vav-1. Cdc42 in this way modulates T-cell polarization.

As previously reported, Itk plays a critical role in this process too, apparently at the level of supporting Vav-1 recruitment to the plasma membrane (Grasis et al., 2003) and the subsequent activation of Rac, which in turn is involved in the creation of novel actin fibers at the edge of moving cells (Fig. 4.2). On the contrary, Rho, another small G protein recruited to the signalosome, contributes to the reorganization of existing cytoskeleton at the trailing edge.

STOPPING THE TCR SIGNALLING PATHWAY: INHIBITION OF T-CELL ACTIVATION

After TCR engagement several cellular responses are induced in T-cells in order to strongly and quickly activate them; when the cause of T-cell activation is removed, stopping TCR signalling is a crucial step to prevent unwanted damages.

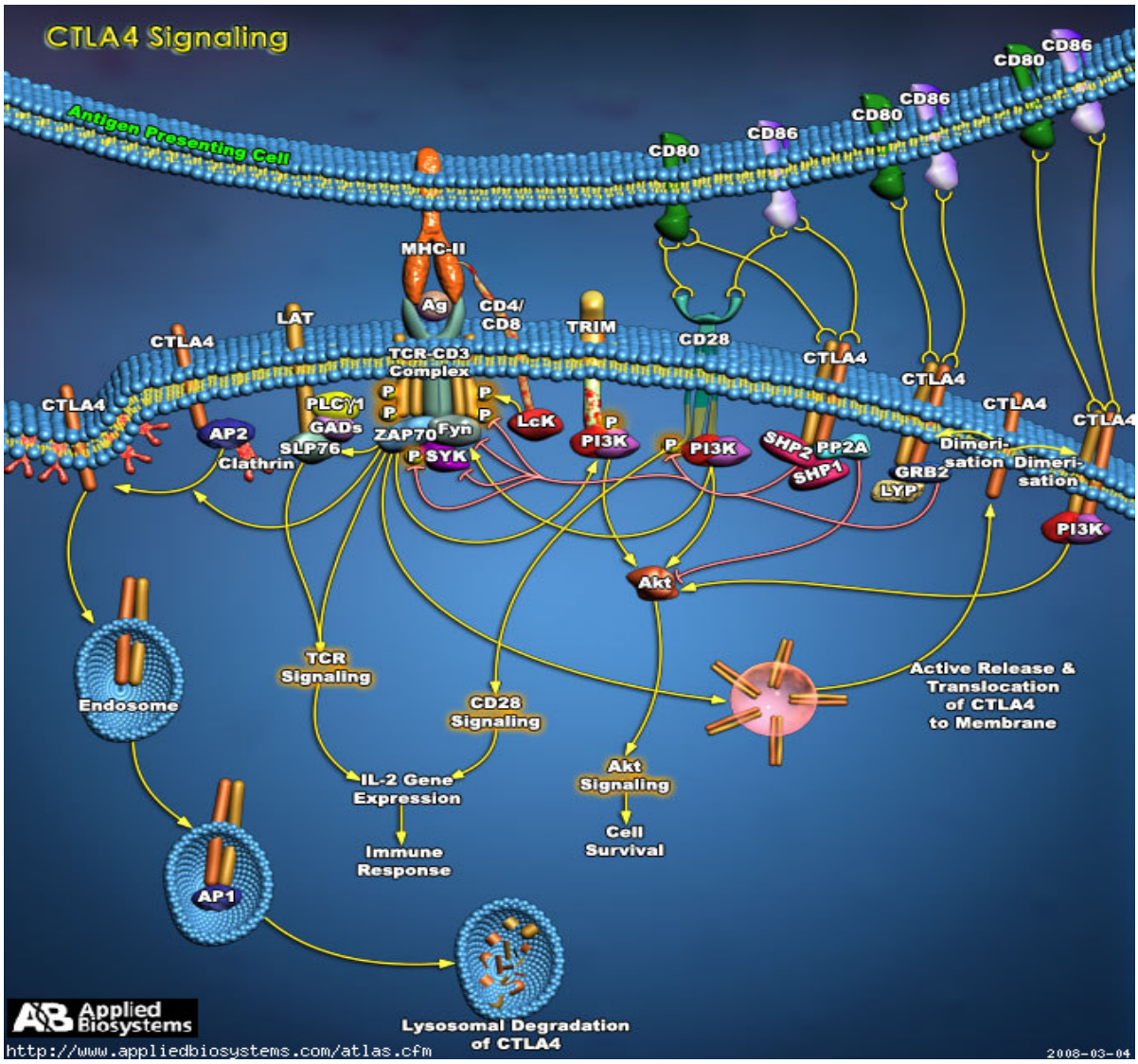


Fig. 4.4 *TCR engagement by antigen/MHC complex and co-stimuli activates also inhibitory signals: the same activator pathways trigger TCR signalling silencing, thus providing a negative feed-back loop which is important to rapidly stop T-cell activation.*

Inhibitory signals that can counteract and modify activating ones are delivered through receptors that bear distinct motifs called immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails. Phosphorylated ITIMs recruit protein phosphatases SHP-1 or SHIP, which remove the phosphate groups added by tyrosine kinases or the 5'phosphate from IP3, respectively.

In T-lymphocytes the transmembrane protein CTLA-4 is induced following TCR activation and then plays a crucial role in regulating T-cell signalling (Fig. 4.4); in fact, it binds to the same co-stimulatory molecules as TCR, such as CD28, where it recruits phosphatases, thus inhibiting TCR signal transduction pathways and stopping the T-cell effector functions (Fig. 4.4).

These regulatory processes provide a negative feed-back loop that contribute to silence TCR signalling following its activation; in this way the same group of stimuli that promotes TCR engagement, and the subsequent T-cell quick and potent activation, controls also the end of such signalling pathways (Fig. 4.4).

4.4 Transcription factors induced by TCR activation

Changes in gene expression represent the culmination of the TCR signalling pathway and are required for the T cell to gain full proliferative competence and the ability to produce effector cytokines.

Three transcription factors have been mainly found to play a key role in TCR-stimulated changes in gene expression; these are NF-kB, NFAT, and AP-1.

NF-KB ACTIVATION AND FUNCTIONS

NF-kB transcription factors are ubiquitously expressed in mammalian cells. These proteins are highly conserved across species, and in mammals the NF-kB family (also known as the Rel family) consists of five members. Activation of NF-kB is primarily controlled via the nuclear versus cytoplasmic partitioning of NF-kB (Fig. 4.3). In the absence of an activating signal, NF-kB is retained in the cytoplasm by tight binding to an inhibitory I-kB protein (Fig. 4.3). Numerous stimuli including TNFα, IL-1, and TCR/CD28 co-stimulation activate an I-kB kinase (IKK) complex containing two kinase subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (also known as NEMO) (Fig. 4.3). The IKK complex phosphorylates I-kB and targets it for ubiquitination and proteolysis via the 26 S proteasome complex. The degradation of I-kB unmasks the nuclear localization sequence of NF-kB, allowing its translocation to the nucleus, where NF-kB regulates the activity of its target genes (Fig. 4.3) (Ghosh et al., 1998).

Remarkably, TCR stimulation uses a completely different pathway for activating IKK than other stimuli (e.g. TNFα and IL-1), and many of the early signalling proteins that have been described above are required, including Lck, ZAP-70, SLP-76, PLCγ1, and Vav-1 (Schmitz et al., 2003) (Fig. 4.2).

A key step in NF-kB activation is the induction of PKCθ and its translocation to lipid rafts (Fig. 4.5). PKCθ is a member of the "novel" class (DAG-responsive, Ca^{2+}-independent) of PKCs, it is selectively expressed primarily in T cells, and is the only PKC isozyme that is known to translocate to the immunological synapse (Altman and Villalba, 2003).

PKCθ does not directly phosphorylate IKK and may act through calmodulin-dependent kinase II (Altman and Villalba, 2003).

A critical upstream element in raft recruitment and activation of PKCθ is Vav-1-mediated activation of G proteins belonging to the Rho family and the subsequent reorganization of the actin cytoskeleton (Fig. 4.5) (Altman and Villalba, 2003). Raft localization of PKCθ also requires Lck, PI3K, PDK1, SLP-76, PLCγ1, and CARMA-1 (Fig. 4.5) (Schmitz et al., 2003).

In this process, PLCγ1 is likely acting as a scaffolding protein in this pathway, possibly in a multi-molecular complex with SLP-76 and Vav-1. PKCα but not PKCβ, both "conventional" PKC isotypes (responsive to DAG and Ca^{2+}), also plays an important but as yet undefined role upstream of PKCθ in TCR/CD28-costimulated, but not TNFα-stimulated, NF-kB activation(Trushin et al., 2003).

More recently, Vav-1 has been implicated as an upstream regulator of PI3K, so Vav-1 may function in a positive feedback loop for PI3K activation.

There are also additional kinases that clearly play a role in NF-kB activation. These include calmodulin-dependent kinase II, MEKK1, MEKK2, MLK3, COT/Tpl-2, and NIK (Schmitz et al., 2003). The described diversity of NF-kB inducers highlights an intriguing aspect of its regulation, namely the ability of many different signal transduction pathways emanating from a wide variety of induction mechanisms to converge on a single target (Fig. 4.2).

It has been well documented that activation of NF-kB plays a pivotal role in many cellular processes, including inflammation, cell proliferation and apoptosis.

NF-kB activation leads to the up-regulation of several genes involved in inflammation and immune response such as cytokines and surface receptors; in this way a sustained stimulation of T-cell is provided, thus completing the full immune response required following TCR engagement by antigen/MHC complex.

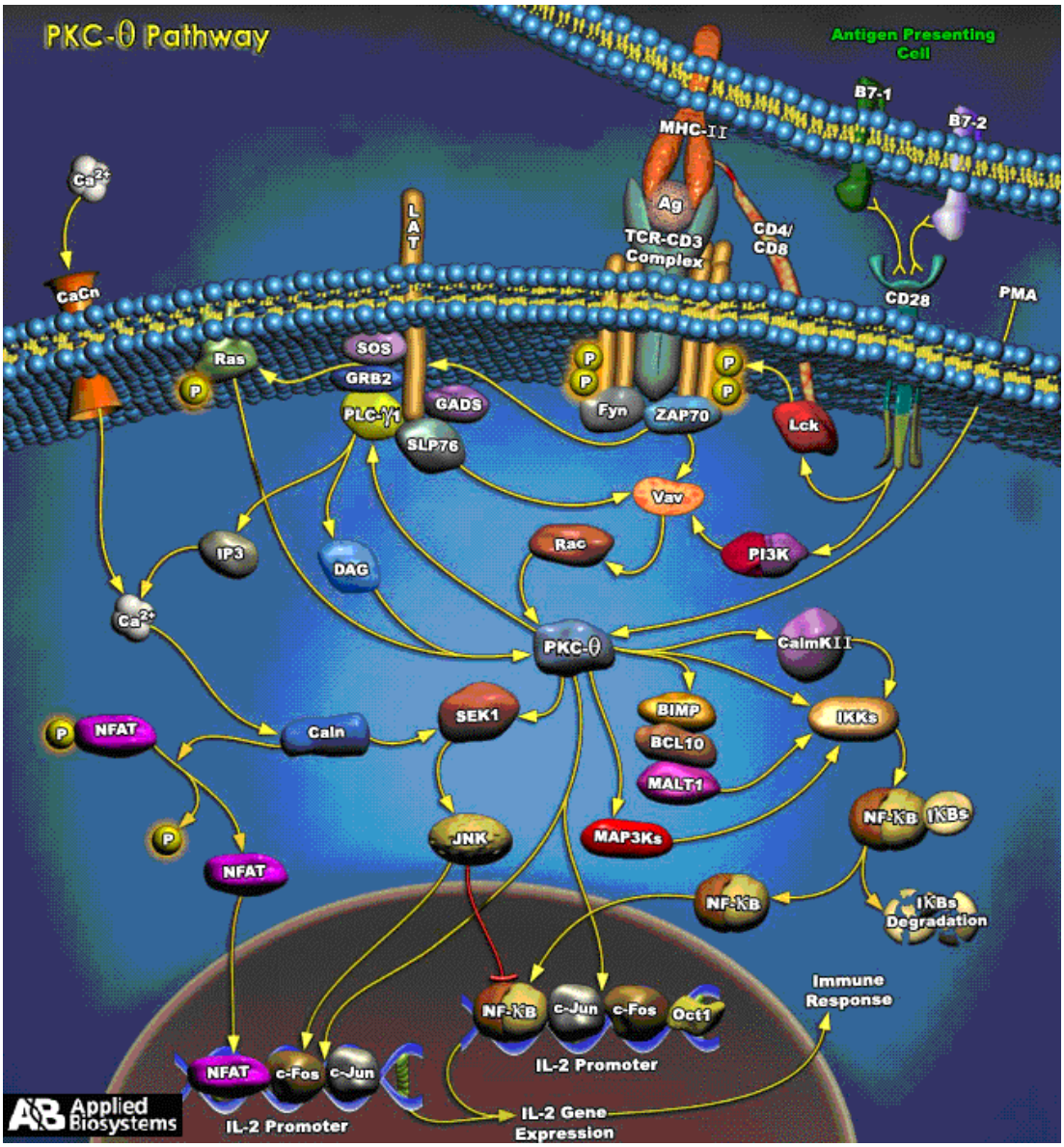


Figure 4.5 *PKCθ is a key regulator of NF-kB activation induced by TCR engagement and it is an important connection point among several different signalling pathways that leads to the induction of transcription factors.*

NFAT ACTIVATION, FUNCTION AND INTERACTION WITH OTHER TRANSCRIPTION FACTORS

The NFAT family consists of five members: four of these proteins are regulated by calcium signalling whereas all the isoforms have a highly conserved DNA-binding domain that is structurally related to the DNA-binding domain of the Rel-family transcription factors.

The conserved core region of NFAT proteins consists of two tandem domains: a regulatory domain, which is also known as the NFAT-homology region (NHR); and the Rel-homology region RHR, which binds DNA. The NHR contains many serine residues that are phosphorylated in resting T-cells. It also includes the docking sites for calcineurin and the NFAT kinases, which regulate the activation of NFAT proteins by determining the

phosphorylation status of the serines. The RHR domain shares structural homology with REL proteins and confers the DNA-binding specificity that characterizes NFAT family members (Chen et al., 1998).

In comparison with NF-kB, the pathway leading to activation of NFAT is much simpler (Hogan et al., 2003).

The rate-limiting step in NFAT activation is the removal of key phosphate groups from the N-terminus of the NFAT protein (Fig. 4.3). Phosphorylation of these residues masks the nuclear localization sequence on NFAT, and when the phosphates are removed NFAT can translocate to the nucleus and regulate the expression of various genes (Fig. 4.3).

The dephosphorylation of NFAT is specifically carried out by the Ca^{2+}-calmodulin-regulated phosphatase, calcineurin (Fig. 4.3). Consequently, TCR-stimulated activation of PLCγ1, with the subsequent production of IP3 and increase in intracellular Ca^{2+}, is a critical component of NFAT activation. The activity of calcineurin is controlled not only by calcium and calmodulin but also by several calcineurin inhibitors, which have been identified during the past few years (Fig. 4.6).

These include calcineurin-binding protein 1 (CABIN1; also known as CAIN), the A-kinase anchor protein AKAP79 (also known as AKAP5) and members of the Down's syndrome critical region (DSCR)/modulatory calcineurin-interacting protein (MCIP) family of calcineurin inhibitors, which are known as calcipressins (Fig. 4.6) (Rothrmel, 2000). Vav-1 is also recognized as playing a key role in NFAT activation (Altman and Villalba, 2003). During T-cell activation, it is the engagement of the T-cell receptor (TCR) that activates the calcium/calcineurin/NFAT pathway. Interestingly, NFAT proteins then cooperate with other transcriptional partners, which are activated in response to TCR and co-stimulatory receptor engagement, and thereby induce the expression of cytokines and many other T-cell-activation-induced proteins (Macian, 2005).

Because NFAT proteins can interact with different transcription-factor partners in the nucleus, they are important integrators of calcium signalling with many other signalling pathways in T cells (Fig. 4.6). The structures of monomeric NFAT-DNA complexes emphasize the high flexibility of the linker region that is located between the N-terminal domain of the RHR, which contains the DNA-binding loop and confers base-specific recognition, and the C-terminal domain, which makes contact only with the phosphate backbone of DNA (Stroud and Chen, 2003).

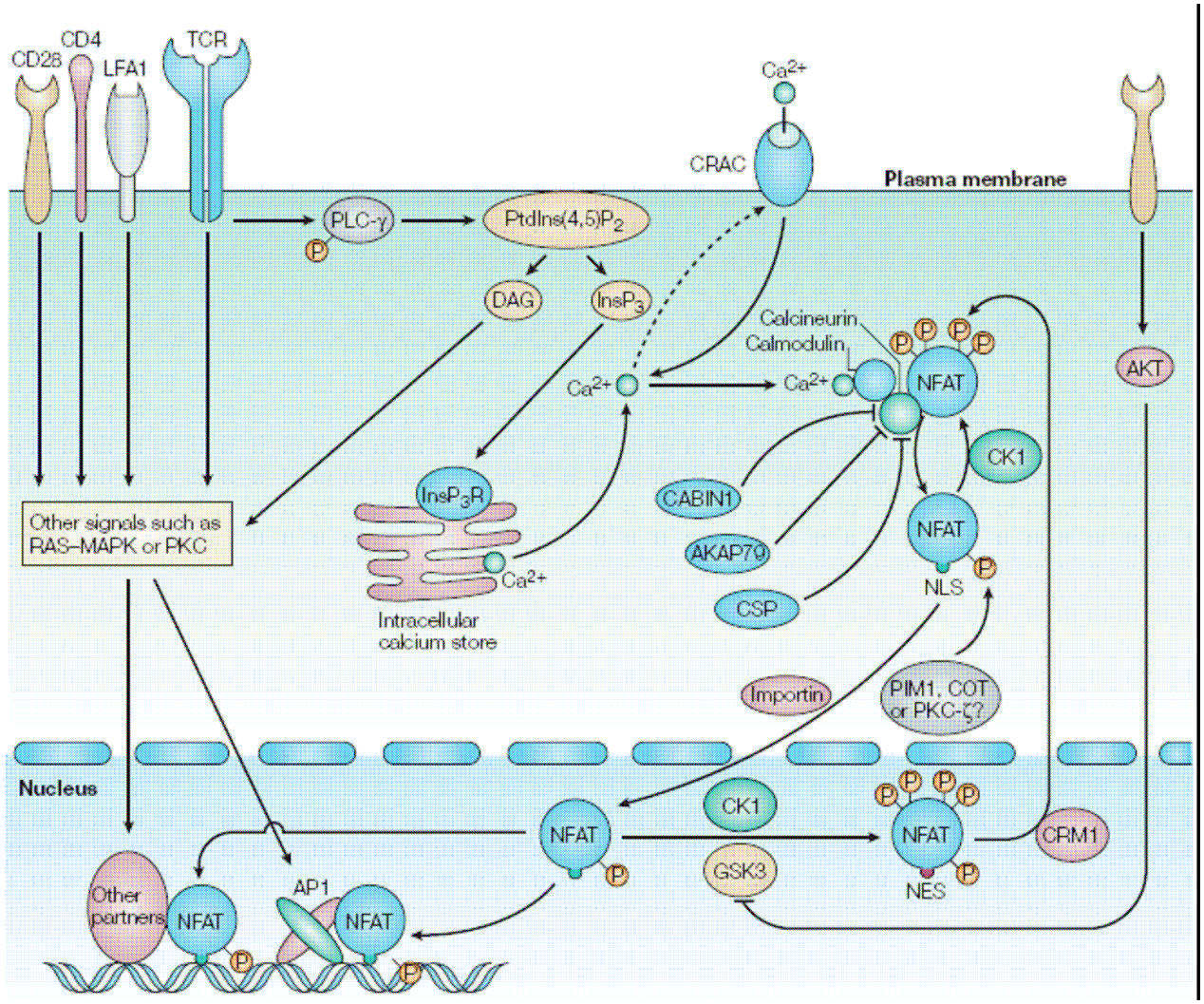


Figure 4.6 *Regulation of NFAT activation. TCR engagement triggers the activation of receptor-associated tyrosine kinases that lead to the activation of phospholipase C-γ (PLC-γ). Activated PLC-γ causes the hydrolysis of PIP2, which generates IP3 and DAG. IP3 binds to its receptor and induces an increase in intracellular calcium levels that is caused by the depletion of intracellular stores. This increase triggers, through poorly characterized mechanisms, the opening of calcium-release activated calcium (CRAC) channels in the plasma membrane, which leads to a sustained increase in intracellular calcium levels.*
Calcium binds calmodulin and activates calcineurin. Activated calcineurin dephosphorylates NFAT proteins,
which exposes their nuclear-localization signal (NLS) and induces their nuclear translocation. The activity of calcineurin is also negatively regulated by calcineurin-binding protein 1 (CABIN1), calcipressins (CSPs) and A-kinase anchor protein 79 (AKAP79). After it has entered the nucleus, NFAT interacts with AP1 and other transcriptional partners to promote gene transcription.
The activity of NFAT is also regulated by kinases, such as casein kinase 1 (CK1) and glycogen-synthase kinase 3 (GSK3), which help to maintain NFAT in a phosphorylated state in the cytosol (maintenance kinases) or induce the rephosphorylation of nuclear NFAT to expose a nuclear-export signal (NES) and translocate NFAT back to the cytosol (export kinases). GSK3 is negatively regulated by the kinase AKT, the activation of which is coupled to CD28 engagement.

This peculiar features of NFAT allow interaction with several transcriptional partners. In fact, although cooperation between NFAT and AP1 proteins is of crucial importance during T-cell activation, Fos and Jun proteins are not the only NFAT transcriptional partners. There are also many reports of functional synergy and protein-protein interactions between NFAT and proteins belonging to several other families of transcription factors, including MAF and GATA-binding proteins. Binding to different transcription factors allows NFAT proteins to cooperate with them and integrate different signalling pathways to activate specific programmes of gene expression in response to various stimuli.

As could be inferred from the fact that the DNA-binding domain of NFAT is structurally homologous to the REL domain, NFAT proteins can also function as dimeric transcription factors at quasi-palindromic sites that resemble NF-kB binding elements (Macian, 2005).

By virtue of its different binding partners or its homodimerization, therefore, NFAT can modulate different and sometimes opposite responses following TCR engagement, from full immune response to anergy; this peculiar properties of NFAT are fundamental in T-cell development, T-helper cell differentiation into specific sub-types, T-cell activation and T-cell tolerance.

NFAT, therefore, provides very different effects and represents also the connection point of distinct signal transduction pathways, namely calcium signalling and MAPKs cascade.

The NFAT activation/deactivation cycle is completed by the rephosphorylation of NFAT, which causes NFAT to repartition into the cytosol. This reaction can be carried out by several different serine/threonine kinases including CK1, CK2, Jnk, Erk, and p38; however, it is the GSK3 serine/threonine kinase that appears to play the dominant role in inactivating NFAT in T cells (Fig. 4.6) (Hogan et al., 2003). The prominent role of GSK3 in NFAT deactivation also provides an additional pathway by which PI3K can lead to increased or sustained NFAT activation, thus generating another interaction point between the different signalling pathways induced following TCR engagement (Fig. 4.6). GSK3, in fact, is basally highly activated, so that it keeps NFAT inactive, but can be inactivated when phosphorylated upon an N-terminal serine residue by kinases like Akt, which are particularly effective at phosphorylating and inactivating GSK3 (Fig. 4.6). Thus, PI3K activation induces Akt activation, phosphorylation, and inactivation of GSK3: as a consequence, NFAT is inactivated at a slower rate.

As observed for NF-kB, also NFAT activation and inactivation are tightly regulated in T-cells in order to modulate promptly and precisely all the required cellular responses and to shut off them when they are no more necessary.

AP-1 ACTIVATION AND FUNCTION

Like NF-kB activation, the activation of AP-1 requires PKCθ induction (Fig. 4.5), as PKCθ null mice fail to activate AP-1 in response to TCR stimulation (Altman and Villalba, 2003). The AP-1 transcription factor is composed of dimers of c-Jun and c-Fos family proteins and can be activated both by phosphorylation of c-Jun by Jnk and by up-regulation of c-Fos and c-Jun expression (Foletta et al., 1998). AP-1 is also activated by PKCθ-independent pathways including the Ras/Raf/Mek/Erk pathway, which signals for increased expression of c-Fos. An important aspect of AP-1 function is its ability to form complexes with the NFAT and NF-kB transcription factors. It is particularly notable that the proximal NFAT binding sites of the IL-2 promoter cooperatively bind both NFAT and AP-1. Likewise the CD28RE site of the IL-2 promoter is a cooperative binding site for NF-kB and AP-1 (Altman and Villalba, 2003).

Actually, AP1 proteins are the main transcriptional partners of NFAT during T-cell activation (Fig. 4.6) (Macian et al., 2001): dimers of Fos and Jun form quaternary complexes with NFAT and DNA on NFAT/AP1 composite sites, which contain two adjacent binding motifs for both transcription factors and are present in many genes that are induced during T-cell activation (Macian et al., 2001). These complexes have an extensive network of protein/protein contacts, which explains their stability and cooperative nature (Chen et al., 1998). NFAT/AP1 cooperation during T-cell activation is responsible for a specific pattern of gene expression, which induces the functional changes that characterize an activated T cell. In the absence of AP1, different sets of genes are activated by NFAT proteins, which might result in a completely different functional outcome (Macian et al., 2000).

T-CELL ACTIVATION AND THE FUNCTIONAL INTERPLAY BETWEEN NF-KB, NFAT AND AP-1

The full activation of T cells requires the engagement not only of the TCR but also of various co-stimulatory receptors, including the co-receptors CD4 and CD8, the integrin lymphocyte function-associated antigen 1 (LFA1), and co-stimulatory molecules such as CD28 and inducible T-cell co-stimulator (ICOS). Some of these couple mainly to calcium influx and NFAT activation, whereas others couple to AP1 and NF-kB activation, although most probably influence both pathways to varying extents. The CD4 and CD8 co-receptors bind LCK, which initiates a cascade of tyrosine phosphorylation that leads to PLC-γ activation and calcium signalling (Palacios and Weiss, 2004). LFA1 increases calcium signalling (Wulfing et al, 1998) but also couples to AP1 activation (Perez et al., 2003).

CD28 has several effects, which are consistent with its role as a co-stimulatory receptor. It induces calcium signalling through phosphatidylinositol 3-kinase (PI3K) activation of TEC-family tyrosine

kinases, which are required for the optimal phosphorylation and activation of PLC-γ in T cells (Fig. 4.2) (Diehn, 2002). CD28 also activates Akt through the PI3K pathway, one of the downstream targets of which is GSK3 (Fig. 4.2). Normally, the activation of GSK3 results in NFAT nuclear export; however, under conditions of AKT activation, GSK3 undergoes an inhibitory phosphorylation on a serine residue close to its N-terminus, which diminishes NFAT nuclear export and prolongs the overall time of NFAT residence in the nucleus (Diehn, 2002). CD28 is also coupled to NF-kB and AP1 activation (Fig. 4.2) (Wang et al., 2004).

4.5 TCR-activation and the different cellular responses induced in T-lymphocytes

The adaptive phase of the immune response begins with engagement on CD4+ helper T cells of the TCR by its ligand (Alarcon et al., 2003), a small foreign peptide bound to a cell surface protein of the class II major histocompatibility complex (peptide-MHC) expressed on an antigen-presenting cell. This engagement initiates a series of biochemical events that can differentially signal the naive T-cell to: either enter into a pathway leading to generation of effector T cells with the onset of rapid proliferation and production of effector cytokines; or enter into a state of antigenic non-responsiveness known as anergy (Fig. 4.8) (Huang and Wange, 2004).

The type of response elicited depends on multiple factors including the affinity of the interaction, the duration of the interaction, and the presence or absence of various co-stimulatory signalling inputs such as those provided by the CD4 co-receptor and the CD28 co-stimulatory receptor (Huang and Wange, 2004).

NFAT IS A KEY REGULATOR OF T-CELL DEVELOPMENT AND FUNCTION

All the transcription factors induced by TCR engagement contribute to such responses, especially NFAT that plays crucial roles in the development and function of the immune system.

In T-cells, in fact, NFAT proteins not only regulate activation but also are involved in the control of thymocyte development, T-cell differentiation and self-tolerance. The functional versatility of NFAT proteins can be explained by their complex mechanism of regulation and their ability to integrate calcium signalling with other signalling pathways (Macian, 2005), as it has been extensively discussed in the previous paragraphs.

In the thymus, immature precursors that are generated in the bone marrow differentiate into mature T cells, which can develop into effector cells on antigen encounter. Double negative (DN) T-cell precursors, that do not express CD4 or CD8, mature and first rearrange the β-chain of the TCR, which together with the invariant pre-TCR α-chain forms the pre-TCR, and then rearrange the α-chain of the TCR.

DN thymocytes then become double positive (DP) cells that express both CD4 and CD8. CD4+CD8+ DP thymocytes undergo positive and negative selection to successfully generate CD8+ or CD4+ single positive (SP) T cells with an antigen receptor that can interact with self-MHC molecules (positively selected) but is unable to recognize self-antigens (negatively selected). Mature T cells that express either CD4 or CD8 are then released into the periphery (Starr et al., 2003).

Calcium and calcineurin signals are involved in the regulation of thymocyte proliferation and the development of immature DN thymocytes into mature SP cells.

In addition, pre-TCR signalling induces an increase in intracellular calcium levels that results in the activation of both NFAT and NF-kB (Aifantis et al., 2001), although the exact targets of these transcription factors in immature thymocytes remain to be identified.

As for many transcription factors, NFAT proteins can regulate gene expression at two different levels.

In a locus that is open and available for immediate transcription, such as the IL-2 locus, NFAT proteins and other transcription factors bind and promote (or repress) transcription. By contrast, in a precursor cell type that is poised to differentiate along one or another pathway of cell-lineage specification, the loci that control genes of a distinct lineage are closed and require remodelling such that transcription factors can gain access to the promoter to drive gene expression. As such, inducible transcription factors, such as NFAT proteins, might be required to remodel the locus itself and also to induce the transcription of lineage-specific transcription factors that commit the cells to one or the other lineage.

A good example of this is the choice that is made by naive T-helper (TH) cells in the periphery to proceed along either the TH1 or TH2 pathway (Fig. 4.7). This is a process of antigen-driven differentiation that transforms these cells into two distinct populations with characteristic patterns of cytokine expression and, therefore, specific immune functions. TH1 cells are characterized by the expression of interferon-γ (IFN-γ) and participate in the clearance of intracellular pathogens.

They also contribute pathologically to inflammation and autoimmune disease. By contrast, TH2 cells express IL-4, IL-5 and IL-13, constitute a defence against extracellular pathogens, and are important in atopy and asthma.

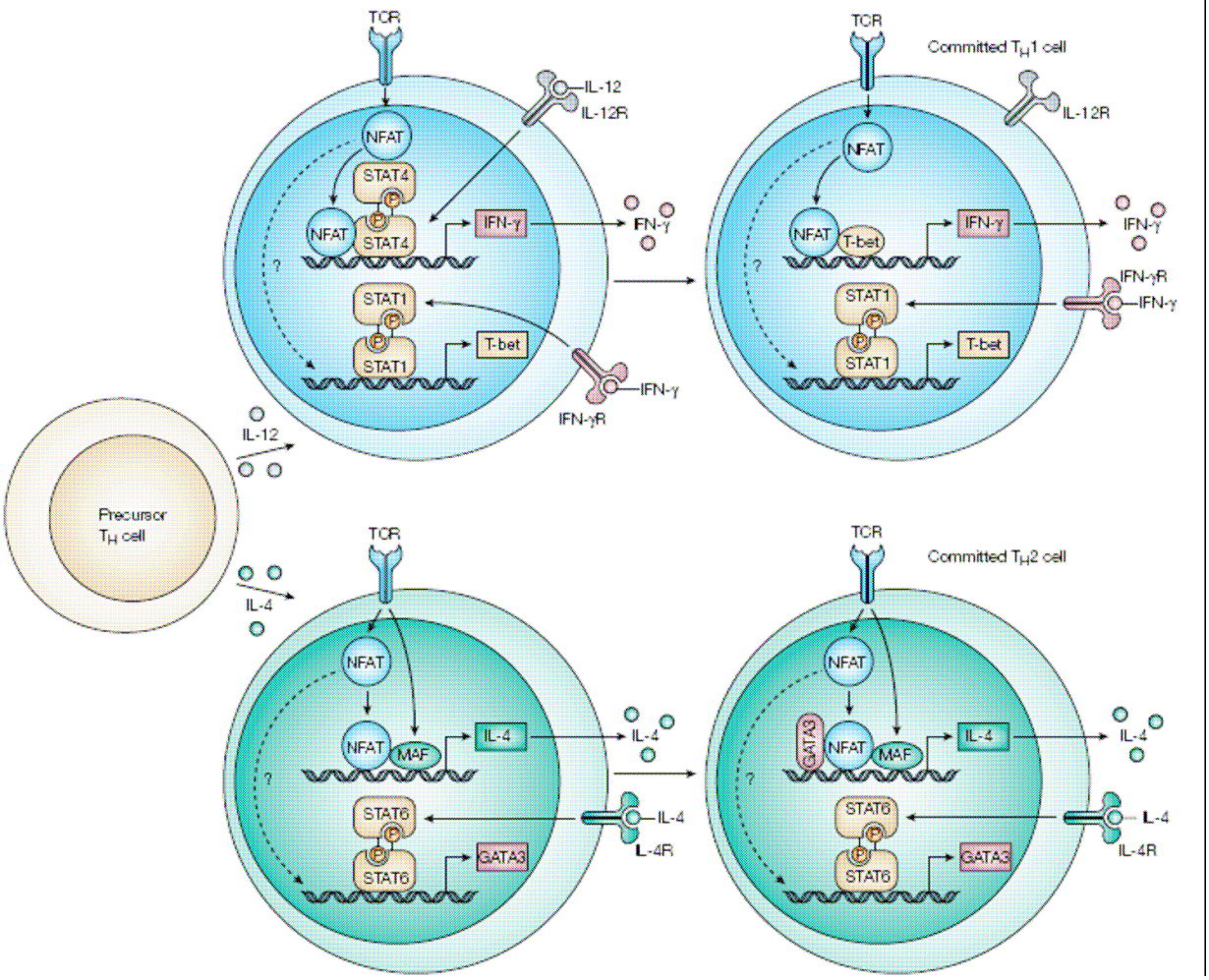


Figure 4.7 *NFAT and T-helper-cell differentiation. On encountering antigen-presenting cells presenting cognate peptide–MHC complexes that engage the TCR, naive T-helper (TH) cells differentiate into effector TH1- or TH2-cell populations. The outcome of this process depends on the nature of the stimulus (type of antigen, intensity of stimulation and type of cell that presents the antigen) and the signals that are received from specific cytokines. This process can be envisioned as occurring in two steps. In the first step, transcription factors that are activated by TCR engagement cooperate with signal transducer and activator of transcription (STAT) proteins to induce the expression of cytokine genes and lineage-specific transcription factors.*

In TH1 cells, NFAT proteins cooperate with STAT4, which is activated in response to interleukin-12 receptor (IL-12R) engagement, to induce the expression of interferon-γ (IFN-γ). Signalling through the IFN-γ receptor induces STAT1 activation and translocation to the nucleus, where it induces the expression of T-bet.

In TH2 cells, MAF and NFAT proteins induce the expression of IL-4. IL-4R signalling causes the activation of STAT6, which promotes GATA-binding protein 3 (GATA3) expression. It is possible that NFAT proteins might also cooperate with STAT factors to induce the expression of T-bet and GATA3. In the second step, NFAT proteins cooperate with T-bet in TH1 cells and GATA3 in TH2 cells to maintain and commit to TH-cell differentiation through the induction of IFN-γ or IL-4. Auto-regulatory positive-feedback loops ensure the expression of T-bet and GATA3.

The choice of differentiation into TH1 or TH2 cells is determined by the nature and intensity of the antigenic stimulus, the type of antigen presenting cell that delivers it and the signals that are received from specific cytokines (IL-12 for TH1 cells and IL-4 for TH2 cells; Fig. 4.7) (Murphy and Reiner, 2002).

Epigenetic changes that are induced by TCR signalling and cytokine-mediated signalling are responsible for the establishment of specific patterns of cytokine expression. The evidence supports a model in which NFAT proteins act together with signal transducer and activator of transcription (STAT) factors to determine the TH1/TH2 lineage choice: STAT1 and STAT4 downstream of IL-12 and IFN-γ, respectively, for TH1 cells; and STAT6 downstream of IL-4 for TH2 cells (Fig. 4.7).

The simultaneous engagement of the TCR and co-stimulatory molecules (such as CD28) at the surface of T cells initiates a genetic programme of expression that leads to full T-cell activation (Diehn, 2002). The importance of NFAT proteins in T-cell activation is underscored by genetic data. In two human families, the inability to activate NFAT proteins because of a defect in store-operated calcium entry was associated with severe immunodeficiency (Feske et al., 2000), whereas in mice deficiency in both NFAT1 and NFAT2 in T-cells is associated with grossly impaired production of many cytokines, including IL-2, IL-4, IL-10, IFN-γ, granulocyte/macrophage colony-stimulating factor and TNF. Another important aspect of T-cell function that NFAT might regulate is cell-cycle control.

For many years, NFAT has been considered to be a key regulator of T-cell activation through its interaction with proteins of the AP1 family of transcription factors.

The discovery that NFAT proteins can also form transcriptional complexes with other partners, and can even be transcriptionally active by themselves, has introduced the possibility of defining new roles for NFAT proteins in T-cells (Fig. 4.8). In the classical two-signal model, the stimulation of T-cells by engagement of their TCR (signal 1) and co-stimulatory molecules (such as CD28; signal 2) results in full productive T-cell activation, whereas signalling through the TCR in the absence of co-stimulation leads to anergy (Macian et al., 2004).

The absence of CD28 co-stimulus leads to the induction of calcium-activated signalling pathways in the absence of the full induction of other pathways (Fig. 4.8): for example, pathways that are regulated by the RAS–MAPK pathway, protein kinase C (PKC) or IKKs.

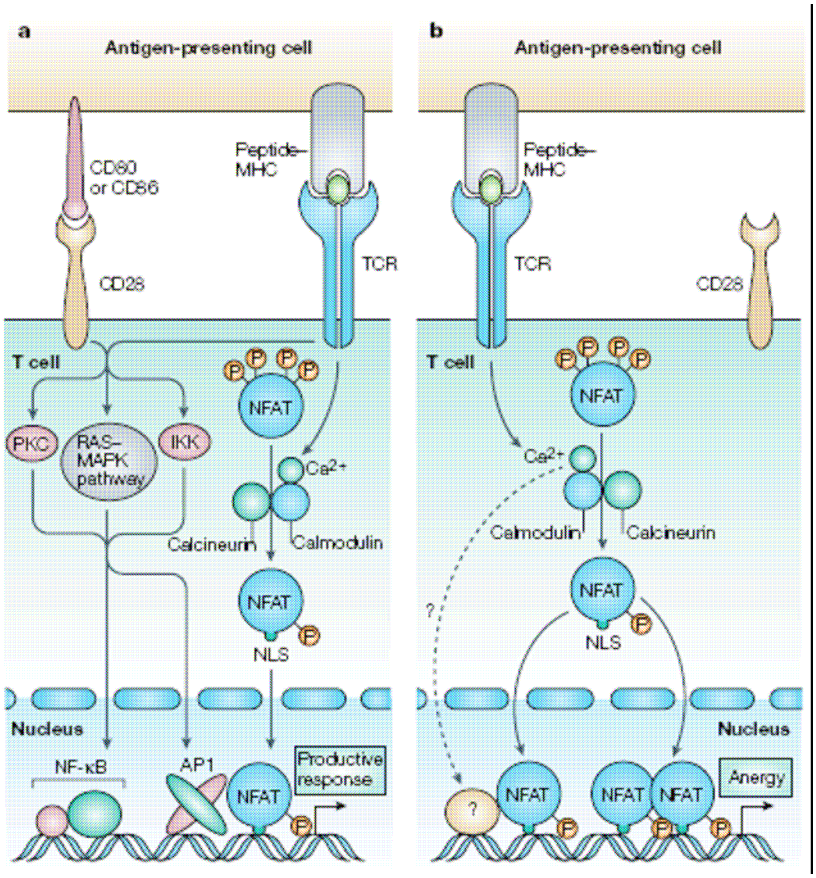


Figure 4.8 *NFAT-activated programmes of gene expression: T-cell activation versus T-cell anergy.*
a) Signals that are delivered by the engagement of the T-cell receptor (TCR; signal 1) and co-stimulatory molecules (such as CD28; signal 2) induce different signalling pathways that result in the activation of several transcription factors. In the nucleus, nuclear factor of activated T cells (NFAT) proteins cooperate with activator protein 1 AP1 and other transcription factors to induce a programme of gene expression that is characteristic of a productive immune response.
b) When TCR engagement (signal 1) occurs in the absence of co-stimulation, calcium-mediated signals induce the activation of NFAT proteins without concomitant AP1 activation. In the absence of cooperative binding to Fos and Jun, NFAT proteins, which might form dimer complexes or cooperate with other calcium-induced transcription factors, elicit the expression of a distinct set of anergy-inducing genes. The products of these genes inhibit T-cell function at different levels and induce a status of T-cell unresponsiveness.

This unbalanced activation ultimately results in the presence of different sets of transcription factors in the nucleus: under these circumstances, in fact, NFAT, in the absence of AP1 proteins and possibly other transcriptional partners, directs the transcription of a specific programme of gene expression that might be responsible for the block in TCR signalling that characterizes anergic T-cells (Fig. 4.8) (Macian et al, 2002).

TCR ENGAGEMENT, CO-STIMULI AND MISSING SIGNALS: T-CELL FULL ACTIVATION VERSUS ANERGY

Tolerance in vivo and its in vitro counterpart, anergy, are defined as the state in which helper T- lymphocytes are alive but incapable of producing IL-2 and expanding in response to optimal antigenic stimulation (Appleman et al., 2000). Anergy is induced when the TCR is engaged by

antigen in the absence of co-stimulation or IL-2. This leads to unique intracellular signalling events that stand in contrast to those triggered by co-ligation of the TCR and co-stimulatory receptors. Specifically, anergy is characterized by lack of activation of Lck, ZAP 70, Ras, Erk, Jnk, AP-1, and NFAT. In contrast, anergizing stimuli appear to activate the protein tyrosine kinase fyn, increase intracellular calcium levels, and activate Rap1. In human T-cells it is also observed that during the induction of anergy CD3 is not phosphorylated whereas TCR is only partially phosphorylated and is associated with activated fyn but not Lck or ZAP-70. In contrast, the induction of productive immunity results in phosphorylation of CD3 and hyperphosphorylation of TCR, both of which are associated with Lck and ZAP-70 (Boussiotis et al., 1996). These results suggest that fyn, which is constitutively associated with TCR, is the only protein tyrosine kinase (PTK) that becomes activated by an anergizing signal and the only one that has an active role in the induction and maintenance of the anergic state. Further studies have shown that the anergy-inducing signalling events result in activation of Rap1, a small GTP-binding protein of the Ras family which functions as an inhibitor of IL-2 gene transcription (Boussiotis et al, 1997). The induction of helper T-cell clonal anergy, therefore, requires active biochemical events initiated following TCR ligation by antigen, which result in distinct pattern of tyrosine phosphorylation of the TCR, distinct pattern of intracellular protein tyrosine phosphorylation, and active inhibition of IL-2 transcription. The mechanism by which IL-2 gene transcription is blocked in T-cell anergy has been the topic of extensive studies. Early work suggested that the defect of the anergic cell in IL-2 production exists at the translational level because stimulation of the anergic cells resulted in normal activation of IL-2 transcription factors and induction of IL-2 mRNA but no IL-2 production (Go and Miller, 1992). However, availability of new assays led to the understanding that anergic cells cannot up-regulate protein binding and transactivation of NFAT and AP-1, two critical IL-2 enhancer elements (Mondino et al., 1996). This appears to be the consequence of TCR-proximal biochemical signalling defects, specifically blockade of Ras but also blockade of CD28 signalling and subsequent defective activation of JNK.

4.6 Immune system and nervous system: an important cross-talk in health and illness

NEUROTROPHINS AND IMMUNE SYSTEM

Neurotrophic cross-talk between the immune and nervous systems might have far-reaching consequences for physiological as well as pathological conditions. Inflammation, a universal reaction to tissue destruction, is crucial for defense and repair (Noseworty et al., 1999). Immune cells are known to infiltrate the CNS after injury due to trauma, ischemia, and degeneration

(Singer and Clark, 1999). On the one hand, there is clear evidence that the neutralization of toxic inflammatory mediators may improve the outcome in experimental models of CNS damage (McGeer and McGeer, 1997). On the other, some studies claimed that suppression of inflammation led to a reduction of lesion size and improvement of the clinical outcome (Riepe et al., 1996).

In the healthy mature nervous system, neurotrophins regulate cellular changes underlying neuronal plasticity. They trigger adaptive changes in adult neuronal morphology (McAllister, 2000), modulate functional properties of synapses by both presynaptic and postsynaptic mechanisms and even initiate fast synaptic responses (Kafitz et al., 1999).

In view of their crucial functions in the nervous system, it initially was surprising to discover that some neurotrophins and their receptors are produced and act in the immune system, so that it is conceivable that immune cells also can be the target of autocrine or paracrine neurotrophin actions, as they express some of the neurotrophin receptors. It therefore appears likely that neurotrophins can mediate bidirectional cross-talk between the nervous and immune systems (Kerschensteiner et al., 2003).

The idea that inflammatory reactions may not always be harmful but under certain conditions even confer tissue protection and repair could have important consequences for the design of immunomodulatory therapies for multiple sclerosis and other diseases (Kramer et al., 1995). We now know that immune cells can supply several neuroprotective mediators including the NGF, the GDNF, and the neuropoietic cytokine families. It is equally evident, however, that many neurotoxic and proinflammatory mediators are produced and released by immune cells.

In fact, immune cell infiltration of the CNS occurs not only after traumatic injury, but also in primarily neurodegenerative diseases such as Parkinson's disease, motor neuron disease, and Alzheimer's disease (Perry et al., 1993). It is currently unclear whether the detrimental or beneficial side of inflammation prevails in these conditions.

In some circumstances there is evidence that the neutralization of proinflammatory signals can be beneficial in experimental models of neurodegeneration, such as organotypic or transgenic models of motor neuron disease (Jones et al., 2002). However, several observations argue that inflammation has a protective effect in neurodegenerative diseases.

Thus, detrimental as well as beneficial components of inflammation seems to have a role in the CNS after injuries and in neurodegenerative diseases; on the other hand, also neurotrophins modulate immune cells, and not only neuronal cells. At this level, therefore, an important cross-talk between nervous system and immune system occurs.

CROSS-TALK BETWEEN CHEMOKINES AND NEURONAL RECEPTORS

Chemokine receptors, a family of Gi protein-coupled receptors responsible for cell migration, are widely expressed by cells of immune and nervous systems. Activation of receptors on the surface of leukocytes, such as opioid, vasoactive intestinal peptide, or adenosine receptors, often has inhibitory effects on chemokine receptors by a mechanism termed heterologous desensitisation, resulting in suppression of immune responses. Conversely, activation of chemokine receptors also induces heterologous desensitisation of μ-opioid receptors (MOR), a class of key analgesic receptors on neurons. Furthermore, prior exposure of neuronal cells to chemokine treatment enhances the sensitivity of transient receptor potential vanilloid 1 (TRPV1), a heat- and ligand-gated calcium channel, which is critical for sensing of pain. Consequently, during inflammation, activation of chemokine receptors on neurons contributes to hyperalgesia by inhibiting MOR and concomitantly sensitising TRPV1 via Gi protein-mediated signalling pathways. These observations suggest that the cross-talk between chemokine receptors and neuropeptide membrane receptors serves as a bridge between the immune and nervous systems (Fig. 4.9) (Zhang and Oppenheim, 2005).

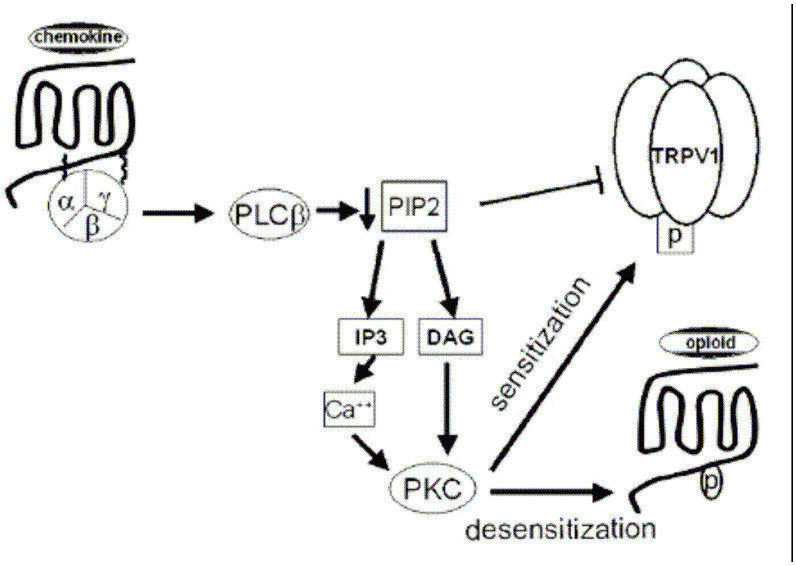


Figure 4.9 *Cross-talk among chemokines, opioid, and TRPV1 receptors. In sensory neurons, treatment with proinflammatory chemokines down-regulates opioid receptor function through PKC, resulting in hyperalgesia. CCL3 also enhances the sensitivity of TRPV1, a "pain" receptor, through a signal transduction cascade involving Gi protein, PLC b and PKC.*

5. Aim of the research

As it emerges from introductive chapters, OPRM1 transcription and, therefore, MOPr expression, are modulated by several endogenous as well exogenous compounds both in neuronal and in immune cells; neuronal differentiating agents such as RA and PMA up-regulate MOPr expression in neuronal cells, thus arising questions about any possible role for MOP receptors in mature neurons. Recently studies proposed a neuroprotective action for opioid receptors in differentiated neuronal cells: in SH-SY5Y human neuroblastoma cells as well as in cortical neurons, opioid agonists like DAMGO and morphine prevented apoptosis induced by serum deprivation (Iglesias et al., 2003). Furthermore, other authors observed that in SH-SY5Y cells and primary hippocampal neurons DAMGO determined neuroprotection trough processes involving Cycline dependent kinase-5 (CDK5) (Wang et al., 2006).

The existence of a neuroprotective action mediated by MOPr and its ligands, as it is emerging from literature data, points to a possible interconnection between opioids and other neurotrophic factors like IGF-I.

IGF-I and its receptors are widely distributed throughout the nervous system during development, and their involvement in neurogenesis and neuronal differentiation has been extensively investigated (Arsenijevic *et al.* 1998; van Golen and Feldman 2000); long-term exposure to IGF-I has been also shown to decrease REST transcript levels, after an initial up-regulation of this transcription factor (Di Toro et al., 2005). Given the important differentiative action of IGF-I in neurons, therefore, it could be interesting to investigate whether IGF-I influences MOPr expression and the role of the transcriptional repressor REST in such process.

MOPr expression levels are also important from a pharmacological point of view: opioid drugs are commonly used in pain therapy, but determine dramatic side effects, such as tolerance and addiction, which can significantly alter the responsiveness to opioid administration.

Understanding how transcriptional activator can be induced in order to up-regulate OPRM1 transcription and, ultimately, MOPr number on neuronal cell surface, therefore, could be of great interest to improve efficacy of opioid therapy and counteract or, at least, delay the onset of the mentioned side effects.

Inflammatory mediators like IL-4, IL-6 and TNF-α can induce OPRM1 transcription in neurons (Kraus et al., 2001; Börner et al., 2004; Kraus et al., 2003) as well as neurodifferentiating agents; these findings suggest a possible induction of the endogenous analgesic system by the same pro-inflammatory cytokines that are responsible for nociception during inflammation.

Interestingly, IL-4, IL-6 and TNF-α can induce OPRM1 transcription in immune cells as well, thus underlining the tight connection between immune system and nervous system and suggesting a potential role for MOP receptors in such a context.

Moreover, MOPr agonists like morphine have been demonstrated to exert immuno-modulator effects: therefore, it is intriguing to hypothesize that MOPr plays an important role in the bi-directional cross-talk between immune cells and neurons, process in which it can contribute to the balance between pro-inflammatory and nociceptive stimuli and protective and analgesic effects.

Considering the complex scenario above described, aim of this thesis has been to functionally characterize the transcription factors that can activate or inhibit OPRM1 transcription and, therefore, MOPr expression, in neuronal cells as well as in immune cells.

Concerning OPRM1 transcriptional regulation in neurons, in the present study we investigated whether IGF-I may act as an activator of OPRM1 gene transcription: more precisely, we focused our attention on the transcriptional repressor REST, which plays a key role in neuronal differentiation, as extensively explained in chapter 1, and on the Signal Transductor and Activator of Transcription 3 (STAT3), which has been related to IGF-IR signaling (Yadav et al., 2005) and has been demonstrated to bind the STAT1/STAT3 responsive element in OPRM1 promoter (Börner et al., 2004).

As regards to OPRM1 transcriptional modulation in immune cells, in the present study we investigated whether T-cell receptor activation in itself can up-regulate MOPr; such a question arises from the observation that in a cytokine-activated environment OPRM1 has been shown to be induced in several immune cell types. We pointed to the activation of T-cell, and in particular of CD4+ T-helper lymphocytes, because their engagement by antigen presenting cells is a key step in the development of a full adaptive immune response and also because opioid agonists determine many of their immuno-modulator effects on this class of immune effectors.

The present thesis aims to contribute to a more accurate comprehension of the processes responsible for OPRM1 transcriptional modulation, which is important for a more detailed knowledge of MOPr expression regulation that may find interesting pharmacological application considering the important role of opioid in pain management.

Furthermore, a better understanding of the transcription factors involved in the modulation of OPRM1 expression may shed light to some novel aspects of opioid function in both neurons and immune cells: neuroprotection on one side, and the cross-talk between nervous system and immune system on the other.

6. Materials and Methods

6.1 Cell culture and reagents

Human neuroblastoma SH-SY5Y cells (European Collection of Cell Culture, Salisbury, UK) were grown as monolayers in MEM and Ham's F12 (1:1) medium supplemented with 10% (v/v) fetal calf serum, L-glutamine (2 mM), pyruvic acid (1 mM), 1x non-essential amino acids and 1x antibiotic-antimycotic solution. Rat pheochromocytoma PC12 cells (European Collection of Cell Culture) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated (55°C, 30 min) horse serum, 5% fetal calf serum and 1x antibiotic-antimycotic solution. Cells were cultured at 37°C in a humidified atmosphere of 5% CO2. Culture media and sera were obtained from Invitrogen (Carlsbad, CA, USA) or Cambrex (Verviers, Belgium). Wild type Jurkat cells were cultivated in RPMI-1640 medium (Cambrex Bio Science S.p.r.l., Verviers, Belgium) supplemented with 10% fetal calf serum (PAN-BIOTECH GmbH, Aidenbach, Germany) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Cambrex Bio Science). Wild type Jurkat cells were kindly provided by Camilla Merten and Burkhart Schraven (Institute of Immunology, University of Magdeburg).

Recombinant human IGF-I was purchased from Peprotech Inc. (Rocky Hill, NJ, USA). All-trans-retinoic-acid (RA) was obtained from Sigma (Steinheim, Germany). The monoclonal antibody 12C11-IBII was raised against the N-terminal region of REST and was a kind gift from Dr. D.J.Anderson (Caltech, Pasadena, CA, USA); monoclonal β actin antibody was from Sigma. Polyclonal anti-histone H1 antibody was purchased from ProSci (Poway, CA, USA). Rabbit polyclonal anti STAT3 and mouse monoclonal anti phospho-STAT3 were from Upstate (Lake Placid, NY, USA). Mouse monoclonal antibody anti STAT1 and rabbit polyclonal antibody anti phospho-STAT1 were purchased respectively from AbNova (Taipei, Taiwan) and Upstate (Lake Placid, NY, USA). Oligonucleotides were obtained from Invitrogen (Carlsbad, CA, USA). Restriction enzymes were obtained from New England Biolabs Ltd. (Hertfordshire, UK) or Roche (Mannheim, Germany). [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) was purchased from Bachem (Weil-am-Rhein, Germany) and [^{3}H]-DAMGO from GE Healthcare (Milan, Italy). All other reagents were of analytical grade, or of the highest purity available, purchased from Sigma or Roche.

6.2 Plasmid constructs

All reporter plasmids are based on the pGL3Basic vector system (Promega, Madison, WI, USA) containing the luciferase reporter gene. First, a fragment of the human OPRM1 promoter, ranging from -1672 to +64 (designated +1 at translation start codon, GenBank™ accession number AF153500), was amplified by PCR using SH-SY5Y cell genomic DNA as the template. A forward oligonucleotide (5'-GTAGGTCGACGTGTCTTGTCTTCCACAT-3'), and a reverse oligonucleotide (5'-GTACTCATGAAACTTGAGTACGCCAAGG-3'), bearing the *BspHI* site at its 3' terminus, were used. This PCR product, named -1672/+64, was cloned into the pCR-Blunt-II-TOPO plasmid (Invitrogen), sequenced (BMR Genomics, Padua, Italy), digested with *SacI/BspHI* and cloned into the pGL3Basic vector. By subsequent digestion of pCR-Blunt-II-TOPO+(-1672/+64) with *PstI*, a shorter OPRM1 promoter fragment from -1672 to -254 was generated and cloned into pGL3Basic between the *SacI/XhoI* sites. Another OPRM1 promoter fragment, from -1672 to +4, was obtained by enzymatic restriction of pGL3+(-1672/+64) with *SacI* and *NcoI* and cloned into the pGL3Basic vector. The pGL3+(-1672/+4) was digested with *NdeI* and *EagI* and the excised fragment was inserted into pCR-Blunt-II-TOPO+(-1672/-254) between the *NdeI* and *NotI* restriction sites; this way another OPRM1 promoter fragment ranging from -1672 to -10 was generated. The latter fragment was cloned into pGL3Basic between the *SacI* and *XhoI* restriction sites. Finally, an OPRM1 promoter fragment, ranging from -1556 to -40, was obtained by PCR amplification of pGL3+(-1672/-10) with the following primers: 5'-TTTGTTAGTCTCTAGGAAATCTCTG-3' and 5'-AGGAGCA CCGAGACTTTTC-3'. The PCR product was cloned into the pCRII-Blunt-TOPO plasmid, sequenced and cloned into pGL3Basic vector between the *SacI* and *XhoI* restriction sites. In summary, five OPRM1 promoter/luciferase reporter gene constructs were built.

EGFP-MOPr encoding plasmid was a kind gift by Herman Hammer (Institute of Veterinary Medicine, University of Munich).

6.3 Cell transfection and reporter gene assays

SH-SY5Y were plated in 24-well dishes and at 50-60% confluence were transiently transfected with each OPRM1 promoter/luciferase reporter plasmid (1.5 μg/ well) and pSV-ßGal (0.5 μg/well; Promega) using the EXGEN 500 Transfection Reagent (Fermentas, Hanover, MD, USA). Cells were cultured in serum-free medium for 16-18 h then exposed to IGF-I or left untreated for 24 h. Finally, cells were lysed in Reporter Lysis Buffer (Promega) and samples were collected.

PC12 cells were plated in collagen IV (Sigma)-coated 24-well dishes, transfected and treated as above. In another set of experiments PC12 cells were transiently transfected with 1.4 μg/well of pGL3+(-1672/+64), 0.7 μg/well of the plasmid pCMV6XL4+REST (Origene, Rockville, MD, USA) containing the cDNA coding sequence of human REST, and 0.4 μg/well of pSV-ßGal; alternatively, the mock plasmid pcDNA 3.1 (Invitrogen) was used as a control instead of the REST- expressing plasmid. Luciferase and beta-galactosidase activities of cell lysates were measured with the Bright-Glo™ Luciferase Assay System or Beta-Glo™ Assay System (Promega), according to the manufacturer's instructions.

In some experiments, SH-SY5Y cells were differentiated by RA (10 μM; 5 d), transfected with 5 μg/dish of either pCMV6XL4+REST or pcDNA3.1 empty vector, exposed to IGF-I (10 nM; 24 h) or left untreated. hMOPr mRNA levels were evaluated by real-time PCR as described below.

Transfection of Jurkat cells has been performed by electroporation with 15 μg of DNA per 5 x 10^6 cells according to a preset protocol on a Gene Pulser Xcell (Bio-Rad Laboratories, Munich, Germany).

6.4 Total RNA preparation and real-time reverse transcription (RT)-PCR analysis

For real time RT-PCR experiments, SH-SY5Y cells were collected from tissue culture flasks, centrifuged (500 g for 5 min) and rinsed with phosphate-buffered saline. Total cellular RNA was extracted with Trizol® reagent (Invitrogen) and digested with RNAse-free DNAse (Invitrogen) for 15 min at 25°C according to the manufacturer's instructions. A 2-μg sample was reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Milan, Italy) according to the manufacturer's instructions. Real-time PCR was employed for relative quantification of MOPr transcripts using the Light Cycler Instrument (Roche Diagnostics) and the SYBR Premix Ex Taq (Takara Bio Europe S.A., Gennevilliers, France). This 'hot start' reaction mix contains *Takara Ex TaqHS* DNA polymerase, dNTP mix and the fluorescent dye SYBR Green I for real-time detection of double-stranded DNA. Reactions were set up in 20 μL including 10 ng of target DNA. To amplify the hMOPr cDNA, a sense primer (5'-CTGGGTCAACTTGTCCCACT-3') and an antisense primer (5'-TGGAGTAGAGGGCCATGATC-3') were used at 2.5 nM final concentration for amplifying a 146-bp fragment (327 to 472 bp) from GenBank™ Accession no. NM_000914. As a control, a 169-bp fragment of the human L19 ribosomal protein gene was amplified with a sense primer (5'-CTAGTGTCCTCCGCTGTGG-3') and an antisense primer (5'-AAGGTGTTTTTCCGGCATC3') at 5 nM final concentration, producing a fragment (62 to

230 bp) from GenBank™ Accession no. BC062709. Amplification was as follows: 95°C for 10 s followed by 40 cycles of 95°C for 5 s, 62°C for 20 s and 72°C for 6 s. After that, the temperature was lowered to 40°C for 30 s and the specificity of the reaction was verified by analysis of the melting curve once the appropriate double-stranded DNA melting temperature had been reached.

Relative expression of RT-PCR products was determined using the $\Delta\Delta C_t$ method (Winer *et al.* 1999): fold induction = $2^{-\Delta\Delta Ct}$; where C_t is the threshold cycle, i.e. the cycle number at which the sample's relative fluorescence rises above the background fluorescence and $\Delta\Delta C_t$ = [C_t gene of interest (unknown sample) – C_t L19 (unknown sample)] – [C_t gene of interest (calibrator sample) – C_t L19 (calibrator sample)]. One of the control samples was chosen as the calibrator sample and used in each PCR. Each sample was run in triplicate and the mean C_t was used in the $\Delta\Delta C_t$ equation.

Total RNA from T cells was extracted using the Nucleospin RNA II kit from Macherey-Nagel (Düren, Germany). One microgram of total RNA was used for cDNA synthesis with Moloney murine leukemia virus reverse transcriptase, RNase H minus (Promega, Mannheim, Germany), and diluted to 50 µl. Two microliters of cDNA was used for RT-PCR reactions. Quantitative real-time RT-PCR was performed in a total volume of 20 µl on a LightCycler instrument using the LightCycler-Fast Start DNA Master SYBR Green I kit (both from Roche). Conditions were as follows: β-actin, 5'-GGTCCACACCCGCCACCAG-3' and 5'-CAGGTCCAGACGCAGGATGG-3' primers; preincubation, 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 60°C, and 22 s at 72°C. The sequences of

mu-opioid receptor primers were 5'-GATCATGGCCCTCTACTCCA-3' (located at position 216 in exon 1) and 5'-GCATTTCGGGGAGTACGGAA-3' (located at position 557 in exon 2, to avoid amplification of genomic DNA). Data collected from real time PCR carried out in immune cells were analysed by the $\Delta\Delta C_t$ method, using β-actin as calibrator.

6.5 CD3/CD28 stimulation of T-cells and decoy oligonucleotide approach.

Wild type Jurkat cells were seeded at $1x10^6$ cells/sample and exposed to CD3/CD28 antigen stimulation for 30 min at 37°C. After that, antigen mixture was washed away and treated cells were plated again in normal growth medium for 24 h before extracting mRNA.

The transcription factor decoy oligonucleotide approach, its efficiency, and specificity were described in detail in previous publications (Kraus et al., 2003; Börner et al., 2004). In general, in the decoy oligonucleotide approach, short double-stranded oligonucleotides with specific binding

sequences for transcription factors are introduced into living cells. In the cells, transcription factors interact with the excess of decoy oligonucleotides rather than bind to the natural regulatory motifs of genes. Thus, the decoys selectively disrupt the function of a desired transcription factor. Because the decoys act within living cells, they are highly specific. The sequences of the decoy oligonucleotides used in this study were as follows were as follows:

AP-1, 5'-CGATTGACTCAGTACTGAGTCAATCG-3';

NF-kB,5'-AAAGTTGAGGGGACTTTCCCAGGCCT-3';

NFAT, 5'-CGAGTTGAGGAAAACTGCCCGAGAC-3';

STAT5, 5'-GATCGCATTTCGGAGAAGACG-3'

Cells were incubated with decoy oligonucleotides (160 nM) for 16 h before and 24 h after stimulation

6.6 Western blotting analysis

Cells were scraped in cold phosphate-buffered saline, pelleted, and resuspended in 100 μL of CER I buffer (NE-PER™ Extraction Reagent; Pierce, Rockford, IL, USA). After 10 min on ice, 5.5 μL of CER II buffer was added and cells were resuspended by vortexing, then incubated on ice for 1 min and resuspended again. The cytoplasmic fraction was separated by centrifugation at 16000 g for 5 min. To obtain the nuclear extract, the cell pellet was resuspended in 50 μL of NER buffer and incubated on ice for 40 min. Soluble proteins were separated by centrifugation at 16000 g for 10 min at 4°C. The protein concentration was quantified by BCA protein assay (Pierce). Nuclear or cytoplasmic extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cruz marker (sc-2035; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was also loaded on the gel as molecular weight standard. Proteins were then transferred to Hybond™ ECL™ nitrocellulose membranes (Amersham Biotec, Milan, Italy), which were blocked in a 5% non-fat milk - TBS (10 mm Tris-HCl, pH 8, containing 150 mm NaCl) - 0.1% Tween 20 solution for 1.5 h at room temperature (25°C). Blots were then probed with the same non-fat milk solution and anti-REST monoclonal antibody for 3 h at room temperature. Antibodies were diluted as follows: REST antibody, 1:50; STAT3 antibody, 1:1000; phospho-STAT3 antibody, 1:200; STAT1 antibody, 1:200; phospho-STAT1 antibody, 1:1000.

Membranes were incubated with peroxidase-conjugated anti-mouse secondary antibodies (Santa Cruz Biotechnology) at room temperature for 1.5 h and the blots were developed with SuperSignal West Pico chemiluminescent substrate according to the manufacturer's protocol

(Pierce). Blot images were digitally acquired by an LAS3000 Imager (Fujifilm Corporation, Stamford, CT, USA). Protein expression was analyzed semiquantitatively using AIDA (Raytest Isotopenmessgeräte GmbH, Mannheim, Germany).

6.7 Electrophoretic mobility shift assay (EMSA)

Experiments were carried out as previously described (Di Toro *et al.* 2005). Briefly, nuclear extracts were prepared using the NE-PER™ extraction kit (Pierce). The protein concentration of nuclear fractions was determined by BCA protein assay (Pierce), and all extracts were stored at -70°C until assay. EMSAs were done using 15 μg of nuclear protein extract from SH-SY5Y cells. The probe was a 60-bp biotin end-labeled, duplex oligonucleotide containing the 21-bp RE1 sequence of the human OPRM1 gene (sense sequence: 5'CTTGGTTGCGGTGGGGCCGGGTCAGTACCATGG ACAGCAGCCGGCGGCTGGCGGGGAGA-3'; the RE1 sequence is underlined) or the partial RE1 sequence generated in pGL3+(-1672/+4) fused with the luciferase cDNA sequence (sense sequence:
5'CTTGGTTGCGGTGGGGCCGGGTCAGTACCATGGAAGACGCCCGGCGGCTG GCGGGGAGA-3'; the part corresponding to an RE1 consensus sequence is underlined). DNA binding reactions were set up employing the LightShift™ chemiluminescent electrophoretic mobility shift assay kit (Pierce), the nuclear extract and 62.5 fmol of biotin-labeled oligonucleotide duplex. In some experiments, 15 μg of nuclear protein extract were pre-incubated at room temperature with or without 10 μg of an anti-REST monoclonal antibody (Upstate) for 30 min before adding the probe. The reaction mixture was loaded onto 4% non-denaturing PAGE with 0.5% TBE buffer and electrophoresed for 60 min at 100 V. The gel was transferred to a nylon membrane (Nytran™; Schleicher & Schuell Italia, Legnano, Italy) and detection was by a chemiluminescent procedure, as indicated by the manufacturer.

6.8 Antisense oligonucleotide experiments

The antisense phosphorothioate oligodeoxynucleotide (AS-ODN) used in this study was complementary to nucleotides 390–411 of human REST mRNA: (5'-TGTTGCCACTGCTGGTAAACAG-3', GenBank™ Accession no. NM_005612). A scrambled ODN (Scr-ODN) was also designed for use as negative control: (5'-GGTTACCTCTCCTAGCCATCGG-3'). A GenBank™ search indicated that these ODNs were not complementary to mRNA sequences in any other human gene entered in the database so far.

ODNs were reconstituted and stored as previously described by our group (Di Toro *et al.* 2005). ODNs (10 μM; 48 h) were diluted to the desired concentration and added to SH-SY5Y cells as a complex with oligofectamine (Invitrogen), according to the manufacturer's protocol. Cultures were maintained for 48 h in the presence of ODNs. IGF-I (10 nM; 24 h) was added 24 h after ODNs.

6.9 Small interfering RNA (siRNA)

A validated duplex siRNA for human STAT3 (STAT3 Stealth™ RNAi; 5'-GCAGUUUCUUCAGAGCAGGUAUCUU-3') was purchased from Invitrogen. A Stealth™ RNAi negative control duplex with a GC content similar to that of siRNA and a scrambled siRNA duplex (5'-GCACAUGGUACAUUGGAGAUACGUU-3') were used as negative controls. Duplex siRNAs were transfected into SH-SY5Y cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. The transfection efficiency of each duplex siRNA was confirmed using the Block-IT™ fluorescent oligo (Invitrogen). About 80-90% of cells were transfected, as also confirmed by flow cytometry (data not shown).

SH-SY5Y cells were transfected with either control siRNAs or specific STAT3 siRNA (50 nM; 48 h) and with pGL3+(-1672/-10) as previously described; they were then either exposed to IGF-I (10 nM; 24 h) or left untreated. Luciferase and β-galactosidase activities were measured. Alternatively, SH-SY5Y cells were differentiated with RA (5 μM; 5 d), transfected with either control siRNAs or specific STAT3 siRNA (50 nM; 48 h), and exposed to IGF-I (10 nM; 24 h) or left untreated. hMOPr mRNA levels were measured by real-time RT-PCR.

6.10 hMOPr binding assay

SH-SY5Y cell membranes were prepared by homogenizing cells in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol and 1 mM benzamidine, with a Polytron homogenizer. After centrifugation (1000 x g for 10 min at 4°C), supernatants were centrifuged (18000 x g for 30 min at 4°C) and the pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$. Protein concentration was determined by BCA assay (Pierce).

For saturation binding experiments, cell membranes (100 μg/assay tube) were incubated in 100 mM Tris-HCl (pH 7.4) containing 0.3% BSA with increasing concentrations of [^{3}H]-DAMGO (0.1-5 nM). Non-specific binding was determined in the presence of DAMGO (10 μM). After 90 min incubation at 25°C, bound ligand was isolated by rapid filtration on Whatman GF/B filters

(Schleicher & Schuell). Filters were washed with 20 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and left in scintillation fluid for 8 h before counting.

Data were fitted by non-linear least-square regression and the LIGAND program (Munson and Rodbard 1980) was used to calculate receptor density (B_{max}), Hill slopes and ligand affinity (K_d). Data are expressed as fmol of [^{3}H]-DAMGO bound and normalized to cell protein content.

6.11 Confocal microscopy

48 h after transfection, wild type Jurkat cells electroporated with the EGFP-MOPr encoding plasmid were exposed to 10 μM of either DAMGO or morphine, or left untreated; at different time points they were analyzed by TCS-NT laser scanning confocal microscope (Leica, Heidelberg, Germany).

6.12 Statistical analysis

In reporter gene experiments, each sample was assayed in triplicate, and each RT-PCR sample was amplified in duplicate. All data are presented as mean ±SEM for the number of experiments indicated. Statistical significance was determined by Newman-Keuls test after ANOVA using GraphPad Prism, version 3.0 (GraphPad Software, Inc., San Diego, CA, USA). P values <0.05 were considered significant.

For confocal microscope analysis at least ten identical cell per sample were considered for the interpretation of the result; pictures of representative cells were collected.

Results

Part I: regulation of MOPr expression in neuronal cells exposed to IGF-I

7.1 IGF-I induces transcription of the OPRM1 gene in neuronal cells

In the first set of experiments I utilized the human neuroblastoma cell line SH-SY5Y, which constitutively expresses hMOPr (Yu and Sadee, 1988) and is a common model for neuronal cells (Pahlman *et al.* 1990). These cells also express IGF-I receptors (van Golen and Feldman 2000), contain functional signaling pathways for this growth factor [e.g. p42/44 mitogen-activated protein kinase (Di Toro *et al.* 2005) and STAT3 (Zong *et al.* 2000)], and express REST in the nuclear compartment (Di Toro *et al.* 2005). The constructs containing various lengths of the OPRM1 promoter fused to the luciferase gene were cloned into a pGL3-basic plasmid (a promoter-less luciferase reporter vector), and the transcriptional activity of these plasmids was examined in transient transfection assays.

As shown in Fig. 7.1A, IGF-I (10 nM) did not increase OPRM1 transcription in SH-SY5Y cells transfected with pGL3+(-1672/+64) which includes the entire RE1 DNA binding element (-9 /+12 bp), or in cells transfected with the pGL3+(-1672/+4) plasmid, bearing only a partial segment of the RE1 element (-9/+4 bp) fused with the luciferase cDNA sequence. However, this latter construct maintains a substantial similarity with the reference RE1 consensus sequence (Schoenherr *et al.* 1996); in fact, the first 8 bp of the luciferase cDNA sequence resemble the lacking RE1 sequence (from +5 to +12 bp) as confirmed in the EMSA experiments. More extensive *in situ* mutagenesis disrupted the REST interaction with the RE1 element on mouse oprm1 promoter, whose sequence is almost identical to the RE1 on the OPRM1 promoter (Kim *et al.* 2004). Following a consecutive 3'-end deletion of the OPRM1 promoter, IGF-I significantly enhanced OPRM1 transcription in cells transfected with pGL3+(-1672/-10) or pGL3+(-1672/-254), both lacking the RE1 sequence (Fig. 7.1A). To define any positive contribution of the promoter region, including the STAT1/3 binding site, to IGF-I-induced OPRM1 transcription, I synthesized the pGL3+(-1556/-40) construct lacking 116 bp of the 5'-flanking region of the OPRM1 promoter, as well as the RE1 binding element. After transient transfection of SH-SY5Y cells with this construct, IGF-I induced no real changes in luciferase activity (Fig. 7.1A), suggesting that the deleted 5'-region of the promoter contains a positive regulatory element.

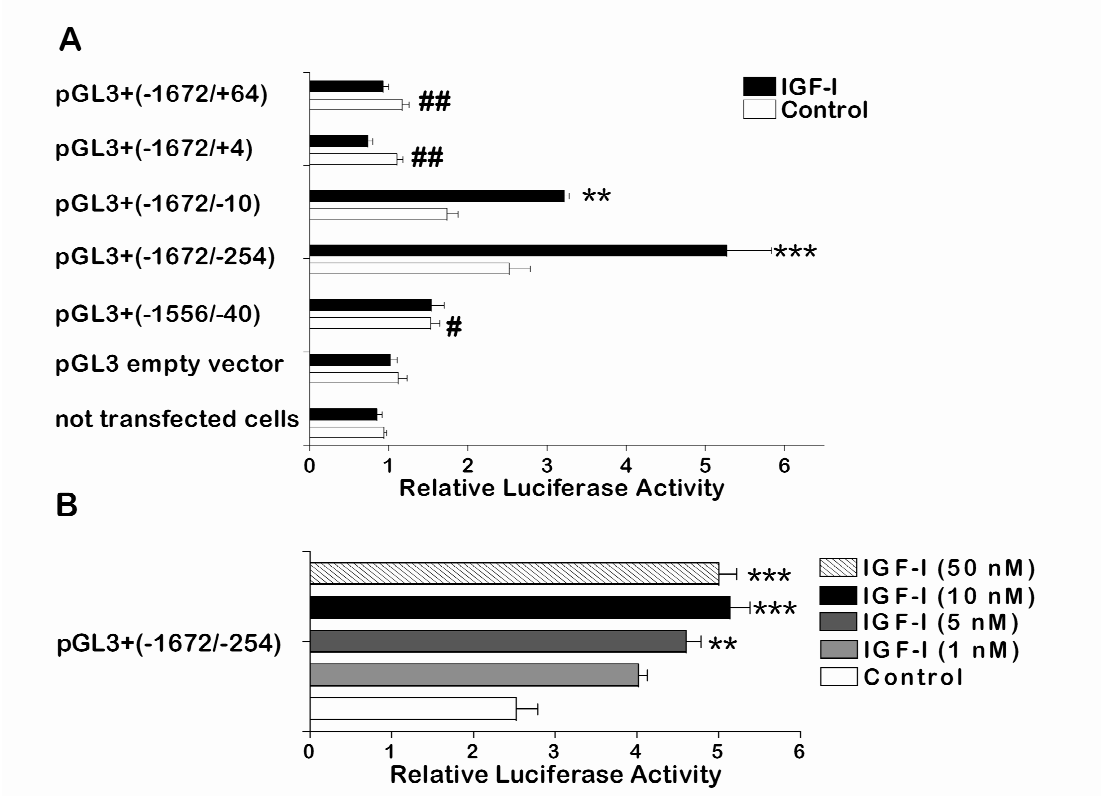


Fig. 7.1 *Effect of IGF-I on the transcriptional activity of different fragments of the OPRM1 promoter in SH-SY5Y cells. Cells were transiently transfected with OPRM1 reporter gene constructs inserted into the pGL3-basic, promoter-less luciferase plasmid vector. Alternatively, cells were transfected with the empty pGL3 plasmid or not transfected. After 24 h, cells were left untreated (control) or exposed to IGF-I for 24 h; thereafter, luciferase activity was measured in cell lysates. The transcriptional activity of each construct is expressed as luciferase activity relative to the activity of not transfected cells, which had assigned a value of 1.0. Transfection efficiencies are normalized to β-galactosidase activity by co-transfection of the internal control plasmid, pSV-βGal. The data are presented as mean ±SEM of six independent experiments carried out in triplicate, with at least three different plasmid preparations. A. Effect of 10 nM IGF-I on the transcription of different OPRM1 promoter fragments. B. Concentration-dependent effect of IGF-I (1 - 50 nM; 24 h) on transcriptional activity of the construct pGL3+(-1672/-254). ** p<0.01; *** p<0.001 compared to the respective control; ## p<0.01 compared to cells transfected with pGL3+(-1672/-254) or with pGL3+(-1672/-10) and not treated with IGF-I (control); # p<0.05 compared to cells transfected with pGL3+(-1672/-254) and not treated with IGF-I (control).*

As a consequence of the suppressive action of endogenous REST on OPRM1 transcription, the relative luciferase activity in control cells transfected with constructs bearing the RE1 binding elements pGL3+(-1672/+64) and pGL3+(-1672/+4) was significantly lower than in the constructs deficient in the RE1 binding element, namely pGL3+(-1672/-10) and pGL3+(-1672/-254) (Fig. 7.1A). Luciferase activity was also significantly reduced in control cells transfected with the plasmid deficient in a regulatory element residing in the promoter region from -1672 to -1556 bp, pGL3+(-1556/-40), in comparison to cells transfected with pGL3+(-1672/-254), which contain this element (Fig. 7.1A).

In SH-SY5Y cells transfected with the pGL3+(-1672/-254) plasmid and exposed to IGF-I (1-50 nM) for 24 h, transcription of OPRM1 increased at 5 nM, peaking at 10 nM (Fig. 7.1B). The effect of IGF-I (10 nM) appeared by 24 h, and was not observed in cells transfected with (-1672/-254) plasmid and exposed to 10 nM IGF-I for 12 or 16 h (data not shown).

To confirm that REST can bind the partial sequence of the RE1 element present in the pGL3+(-1672 /+4) plasmid, an EMSA was done. As shown in Fig. 7.2, nuclear proteins extracted from SH-SY5Y cells bound to a 60-bp duplex oligonucleotide containing the putative RE1 sequence present in the OPRM1 gene, to form a shifted band (indicated by an arrow, see lane 1). Similarly, they bound to a 60-bp duplex oligonucleotide containing the partial RE1 sequence (Fig. 7.2, lanes 2 and 3), which is present in the pGL3+(-1672/+4) plasmid vector. A competitive EMSA with a 200-fold excess of unlabeled double-strand oligonucleotide confirmed that the DNA-protein interaction was specific (Fig. 7.2, lane 4). Incubating nuclear extracts with a REST antibody substantially diminished the protein-oligonucleotide complex (Fig. 7.2, lane 5).

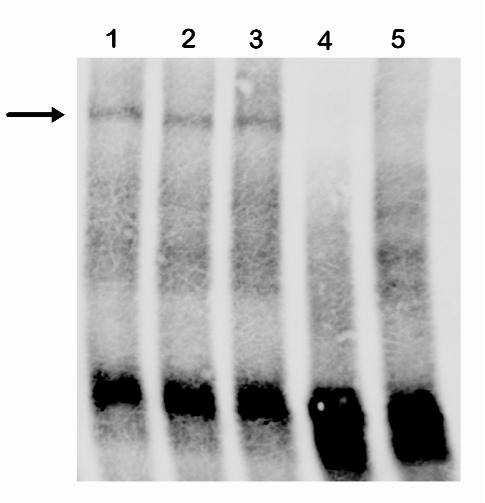


Fig. 7.2 *REST binds to the partial sequence of the RE1 element present in the plasmid pGL3+(-1672/+4). EMSAs were carried out using SH-SY5Y nuclear protein extracts. Nuclear cell extracts were incubated in the presence of a double-strand, biotin-labeled oligonucleotide containing a copy of the putative (lane 1) or of the partial RE1 sequence (lanes 2 and 3). A competitive EMSA with 200-fold molar excess of unlabeled double-strand oligonucleotide (12.5 pmol) containing the partial RE1 sequence prevented the formation of the retarded band (lane 4). Incubation with a REST antibody reduced the REST-oligonucleotide complex (lane 5). A representative experiment, repeated three times with similar results, is shown. The arrow indicates the REST-related specific complex. The biotin-labeled 60-bp duplex bearing the putative or the partial RE1 binding sequence incubated in the reaction mixture lacking of the nuclear protein extract did not produce any shifted band (data not shown).*

7.2 Identification of the OPRM1 gene IGF-I-inducible promoter element

As illustrated in Fig. 7.1A, IGF-I's action on OPRM1 transcription required a positive regulatory element in the promoter region which includes the STAT1/3 binding sequence. IGF-I can activate STAT3-regulated signaling in SH-SY5Y cells (Yadav *et al.* 2005), so I demonstrated that this transcription factor can also mediate the action of IGF-I on OPRM1 transcription. siRNA was employed to down-regulate endogenous STAT3 expression in SH-SY5Y cells. As shown in Fig. 7.3A and 3B, transfection of siRNA substantially lowered cytoplasmic STAT3 levels, whereas the transfection of a scrambled siRNA, or of a non-specific negative control siRNA, had no such effect, indicating high efficacy and specificity of the siRNA strategy.

To confirm that STAT3 siRNA, by lowering endogenous STAT3 levels, can prevent IGF-I-induced STAT3 activation, which requires the phosphorylation of this transcription factor (Reich and Liu 2006), SH-SY5Y cells were exposed to STAT3 siRNA for 48 h and then treated with IGF-I (10 nM) for 15 min. STAT3 was activated by phosphorylation, as this form increased in control cells treated with IGF-I, but it was only poorly detectable in cells exposed to STAT3 siRNA then treated with IGF-I, and in control cells (Fig. 7.3C and 3D). Thus, the siRNA strategy is effective for preventing IGF-I-mediated STAT3 signaling. In SH-SY5Y cells treated with siRNA, and transfected with pGL3+(-1672/-10), IGF-I did not raise MOPr mRNA levels, whereas it did in vehicle-treated cells and in cells treated with scrambled siRNA (Fig. 7.3E).

Finally, I ruled out the possibility that IGF-I acts by inducing phosphorylation of STAT1. As shown in Fig. 7.3F, STAT1 was detected in cytoplasmic lysates of SH-SY5Y cells, and was not affected by up to 30 min exposure to IGF-I (10 nM). Only a thin band of the phosphorylated form of STAT1 was detectable and was not influenced by IGF-I. Therefore, the observation that STAT1 is not phosphorylated by IGF-I indicates that this transcription factor is not involved in IGF-I signaling. Interestingly, STAT3 siRNA did not affect the cytoplasmic content of STAT1 (data not shown).

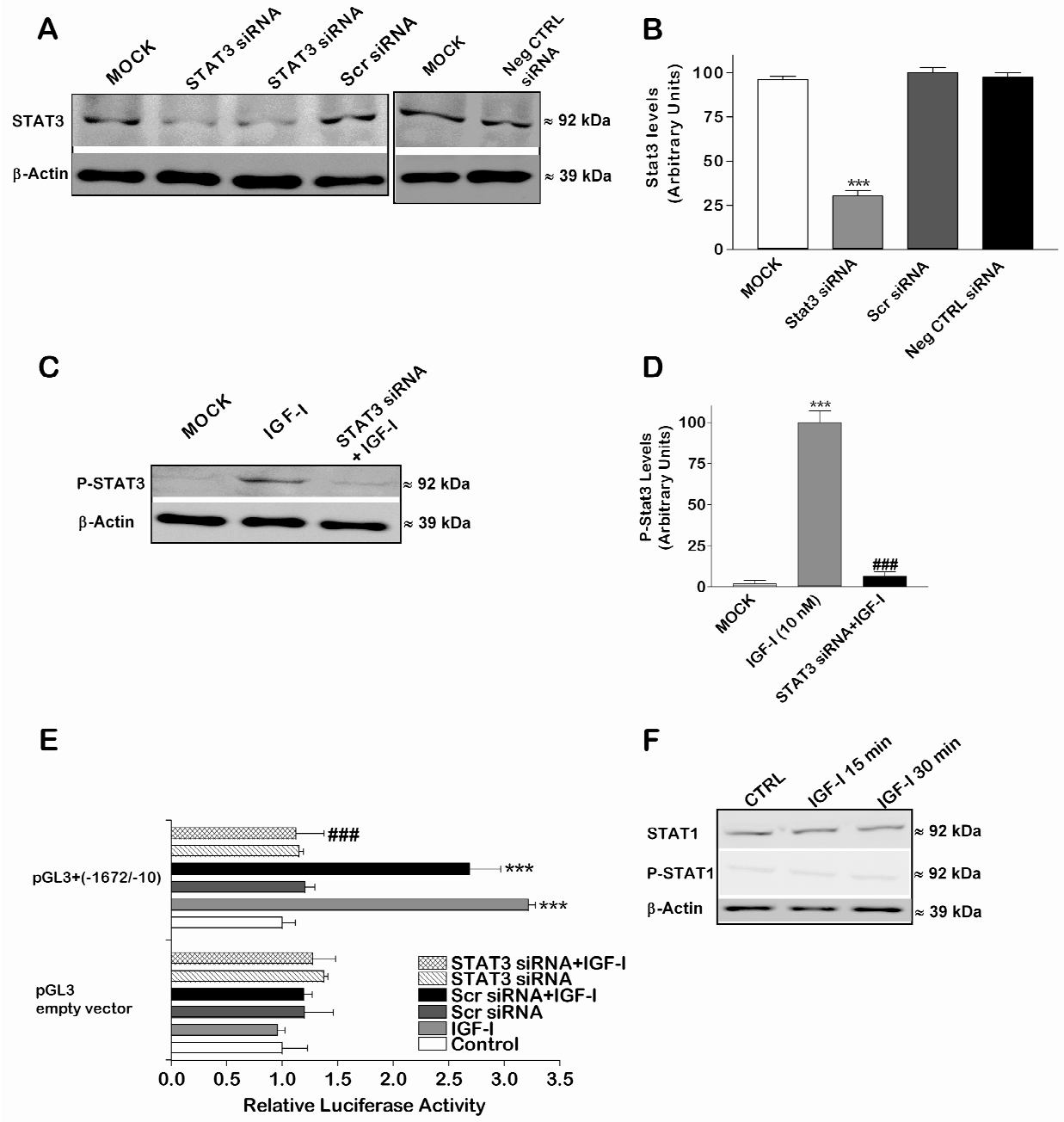


Fig. 7.3 *Knockdown of STAT3 protein by siRNA blocks IGF-I-induced increase of OPRM1 transcripts in SH-SY5Y cells transfected with pGL3+(-1672/-10). A. Specific reduction of STAT3 expression by STAT3 siRNA. A representative western blot experiment, carried out on cytoplasmatic cell extracts and repeated six times with similar results, is shown. Mock, transfection agent; STAT3 siRNA, specific STAT3 siRNA (50 nM); Scr siRNA, scramble control siRNA (50 nM); Neg CTRL siRNA, control siRNA (50 nM). The cells were treated for 48 h with mock or different siRNA constructs. β-actin was evaluated in the same samples and used for loading control. Approximate molecular mass of STAT3 and β-actin was determined by comparison with molecular mass standards. B. Densitometric analysis of the bands. OD refers to relative optic density of each band that was defined by normalization of the STAT3 band to the β-actin band (arbitrary units). Values are the mean ±SEM of at least six independent experiments carried out in duplicate. *** p<0.001 compared to mock, Scr siRNA or Neg CTRL siRNA. C. STAT3 siRNA prevents IGF-I-induced STAT3 phosphorylation (P-STAT3). SH-SY5Y cells were exposed to transfection reagent*

*(Mock), to IGF-I (10 nM) for 15 min (IGF-I) or transfected with STAT3 siRNA (50 nM; 48 h) and then treated with IGF-I (10 nM) for 15 min (STAT3 siRNA+IGF-I). A representative western blot experiment, carried out on cytoplasmatic cell extracts and repeated six times with similar results, is shown. D. Densitometric analysis of the bands. OD refers to relative optic density of each band that was defined by normalization of the P-STAT3 band to the β-actin band (arbitrary units). Values are the mean ±SEM of at least six independent experiments carried out in duplicate. *** p<0.001 compared to mock group; ### p<0.001 compared to IGF-I-treated cells. E. Transfection of SH-SY5Y cells with STAT3 siRNA resulted in a reduction of IGF-I-induced OPRM1 promoter transcription. Cells were transiently transfected with different siRNAs (50 nM; 48h) and either pGL3+(-1672/-10) or pGL3 empty plasmid. After 24 h of transfection, cells were left untreated (control) or exposed to IGF-I (10 nM) for 24 h; thereafter, luciferase activity was measured on cell lysates and is expressed as relative to the activity of the pGL3 empty vector, which had assigned a value of 1.0. The data are presented as mean ±SEM of six independent experiments carried out in triplicate, with at least three different plasmid preparations. *** p<0.001 compared to the respective control; ### p<0.001 compared to Scr siRNA+IGF-I or IGF-I alone. F. Exposure to IGF-I (10 nM) for 15 or 30 min did not change cytoplasmatic levels of STAT1 or its phosphorylated form (P-STAT1). A representative western blot experiment, carried out on cytoplasmatic cell extracts and repeated six times with similar results, is shown.*

7.3 REST regulates IGF-I-induced OPRM1 transcription through the RE1 binding motif of the promoter

Studies were carried out in PC12 cells, another *in vitro* model for investigating differentiation of neuronal cells, which do not express REST (Schoenherr *et al.* 1996) or MOPr (Ballas *et al.* 2001; Bedini *et al.* unpublished observations). In PC12 cells transiently transfected with different OPRM1 promoter constructs, IGF-I (10 nM, 24 h) induced a significant, superimposable increase of OPRM1 transcription regardless of the presence of the RE1 sequence in the construct (Fig. 7.4A). IGF-I (10 nM) did not influence OPRM1 transcription in PC12 cells transfected with the pGL3+(-1556/-40) construct lacking 116 bp of the 5'-flanking region of the OPRM1 promoter and the RE1 binding element (Fig. 7.4A). As a consequence of the fact that endogenous REST did not suppress OPRM1 transcription, the relative luciferase activity in control cells transfected with constructs bearing the RE1 binding elements, pGL3+(-1672/+64) and pGL3+(-1672/+4), was no lower than in the constructs deficient in the RE1 binding element, pGL3+(-1672/-254) (Fig. 7.4A).

To confirm the suppressive action of REST on OPRM1 transcription, I used the full-length human REST cDNA sub-cloned in the mammalian expression vector pCMV6XL4 for further functional analysis. When REST cDNA was co-transfected into PC12 cells with the full-length construct pGL3+(-1672/+64), REST protein was expressed (Fig. 7.4B and 4C) and was capable of repressing OPRM1 promoter activity in control cells or after exposure to IGF-I (Fig. 7.4D).

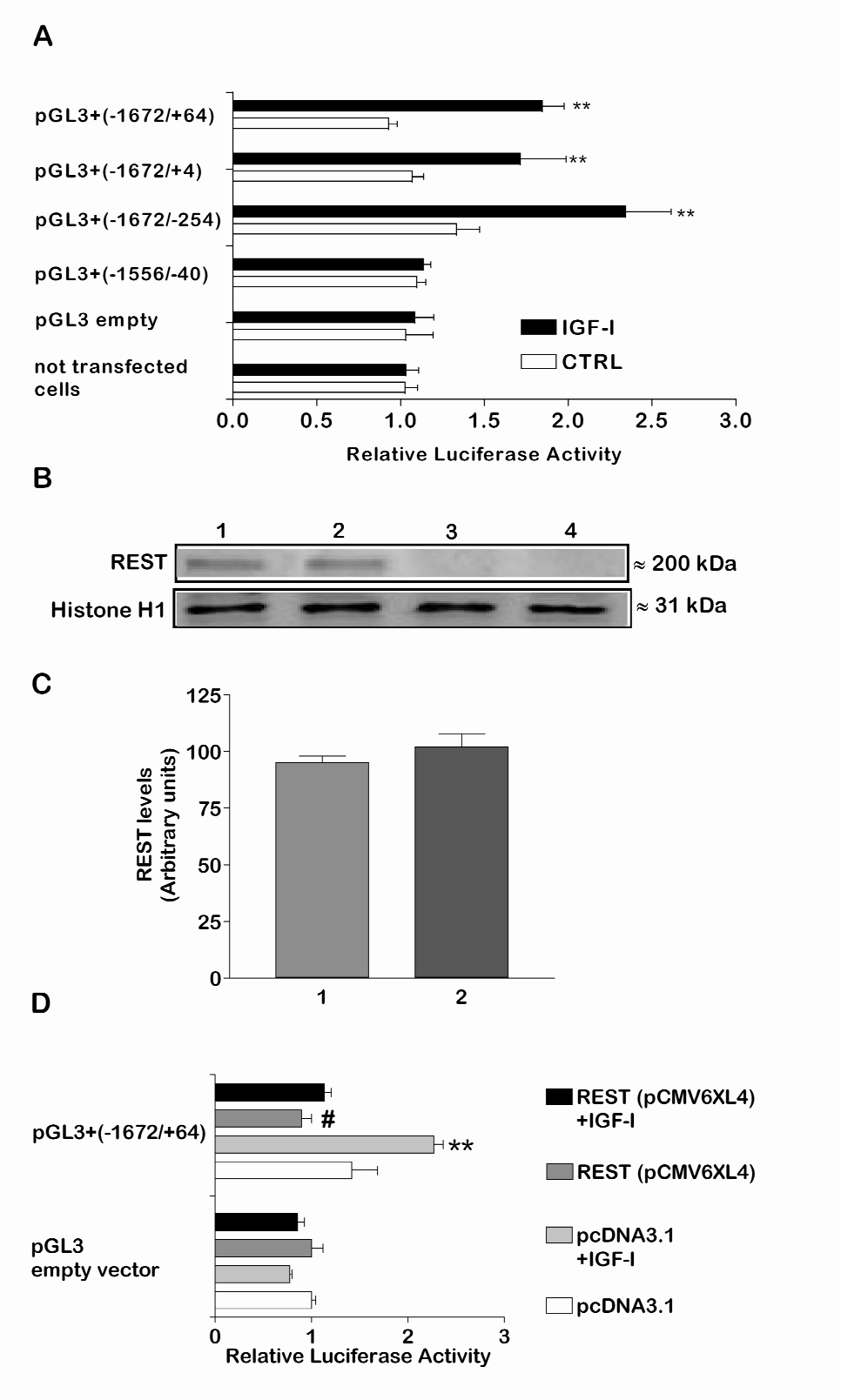


Fig. 7.4 *REST represses IGF-I-induced transcription of OPRM1 promoter fragments bearing the RE1 sequence in PC12 cells. A. Cells were transiently transfected with OPMR1 reporter gene constructs inserted into the pGL3-basic, promoter-less luciferase plasmid vector or with the empty pGL3 plasmid, or not transfected. After 24 h, cells were left untreated (control) or exposed to IGF-I (10 nM) for 24 h; thereafter, luciferase activity was measured in cells, presented as mean ±SEM of six independent experiments carried*

*out in triplicate, with at least three different plasmid preparations. ** p<0.01 compared to the respective control. B. Representative western blot analysis of REST nuclear protein levels in PC12 cells transiently co-transfected with the plasmid bearing human REST cDNA (pCMV6XL4) and pGL3+(-1672/+64) (lane 1) or pGL3 empty vector (lane 2). Control cells were co-transfected with pcDNA 3.1 plasmid and either pGL3 empty plasmid (lane 3) or pGL3+(-1672/+64) (lane 4). Histone H1 was evaluated in the same samples and used for loading control. Approximate molecular mass of REST (only a single band of ≈200-180 kDa was observed) and histone H1 was determined by comparison with molecular mass standards. A representative experiment, repeated six times with similar results, is shown. C. Densitometry of bands corresponding to lane 1 and 2. OD refers to relative optic density of each band that was defined by normalization of the REST band to the histone H1 band (arbitrary units). Densitometry of lanes 3, and 4 was omitted. Values are the mean ±SEM of at least six independent experiments carried out in duplicate. D. PC12 cells were transiently co-transfected with a REST-expressing plasmid pCMV6XL4 and the pGL3+(-1672/+64) or pGL3 empty plasmid. Alternatively, cells were transfected with pcDNA 3.1 plasmid and the pGL3+(-1672/+64) or pGL3 empty plasmid. After 24 h of transfection, cells were left untreated or exposed to IGF-I (10 nM) for 24 h; thereafter, western blot analysis was performed on nuclear extracts and luciferase activity was measured in cell lysates. Data are presented as mean ±SEM of six independent experiments carried out in triplicate, with at least three different plasmid preparations. ** p<0.01 compared to the respective control; # p<0.05 compared to pGL3+(-1672/+64) + pcDNA3.1.*

As expected, when the REST-expressing plasmid was co-transfected with a plasmid bearing the sequence of the OPRM1 promoter deficient in the RE1 DNA motif, pGL3+(-1672/-10) and pGL3+(-1672/-254), exposure to IGF-I resulted in a significant increase of OPRM1 promoter activity (data not shown).

7.4 hMOPr regulation by IGF-I in RA-differentiated SH-SY5Y cells

To further explore the role of REST in regulating OPRM1 transcription by IGF-I, endogenous MOPr mRNA transcripts were evaluated by RT-PCR in SH-SY5Y cells differentiated by five days' exposure to RA. In these cells nuclear REST levels are substantially reduced and in SH-SY5Y cells exposed to RA followed by 24 h treatment with 10 nM IGF-I, this growth factor cannot up-regulate REST protein (Fig. 7.5A and 5B). Our group previously reported (Di Toro *et al.* 2005) that IGF-I raises REST nuclear levels in control, undifferentiated SH-SY5Y cells maintained for 16 h in serum-free medium then exposed to the growth factor for 24 h. Furthermore, exposure to IGF-I did not affect RA-induced cell differentiation, as ascertained by visual microscopic

analysis and western blot analysis of synapsin I and βIII tubulin, two markers of neuronal differentiation (Pahlman *et al.* 1990) (data not shown).

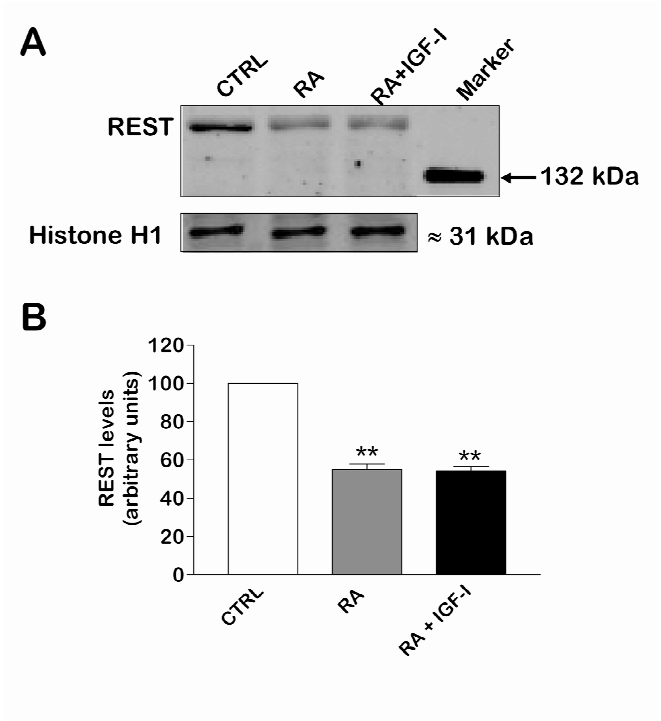


Fig. 7.5 *REST down-regulation induced by retinoic acid (RA) A. Representative western blot experiment showing a noteworthy reduction of REST in nuclear extracts of SH-SY5Y cells exposed to RA for 5 days or left untreated (CTRL). After 16 h of serum starvation, IGF-I (10 nM) was added for 24 h to cells exposed to RA for 5 days with no effect on REST levels. Histone H1 was evaluated in the same samples and used for loading control. B. Densitometric analysis of the bands. Values are the mean ±SEM of at least six independent experiments carried out in duplicate. ** $p<0.01$ compared to CTRL.*

In RA-differentiated SH-SY5Y cells hMOPr mRNA levels significantly increased and IGF-I, added for 24 h to five-day RA-treated cells, further raised them (Fig. 7.6A). This effect seems to be related to RA-induced nuclear REST down-regulation (see Fig. 7.5) as it was prevented in differentiated SH-SY5Y cells transfected with the REST-expressing plasmid pCMV6XL4, but not in cells transfected with a pcDNA3.1 empty vector. Moreover, in cells transfected with the REST-expressing plasmid, exposure to RA did not raise hMOPr mRNA (Fig. 7.6A). Western blot analysis confirmed the increase in REST nuclear levels in cells transfected with the pCMV6XL4 plasmid (Fig. 7.6B). In control, undifferentiated SH-SY5Y cells maintained for 16 h in serum-free medium then exposed for 24 h to IGF-I (10 nM), this growth factor did di did not cause any real increase in hMOPr mRNA levels (Fig. 7.6A).

Finally, transfection of RA-treated cells with STAT3 siRNA considerably reduced the IGF-I-induced hMOPr mRNA increase, whereas transfection of a scrambled siRNA had no such effect (Fig. 7.6A).

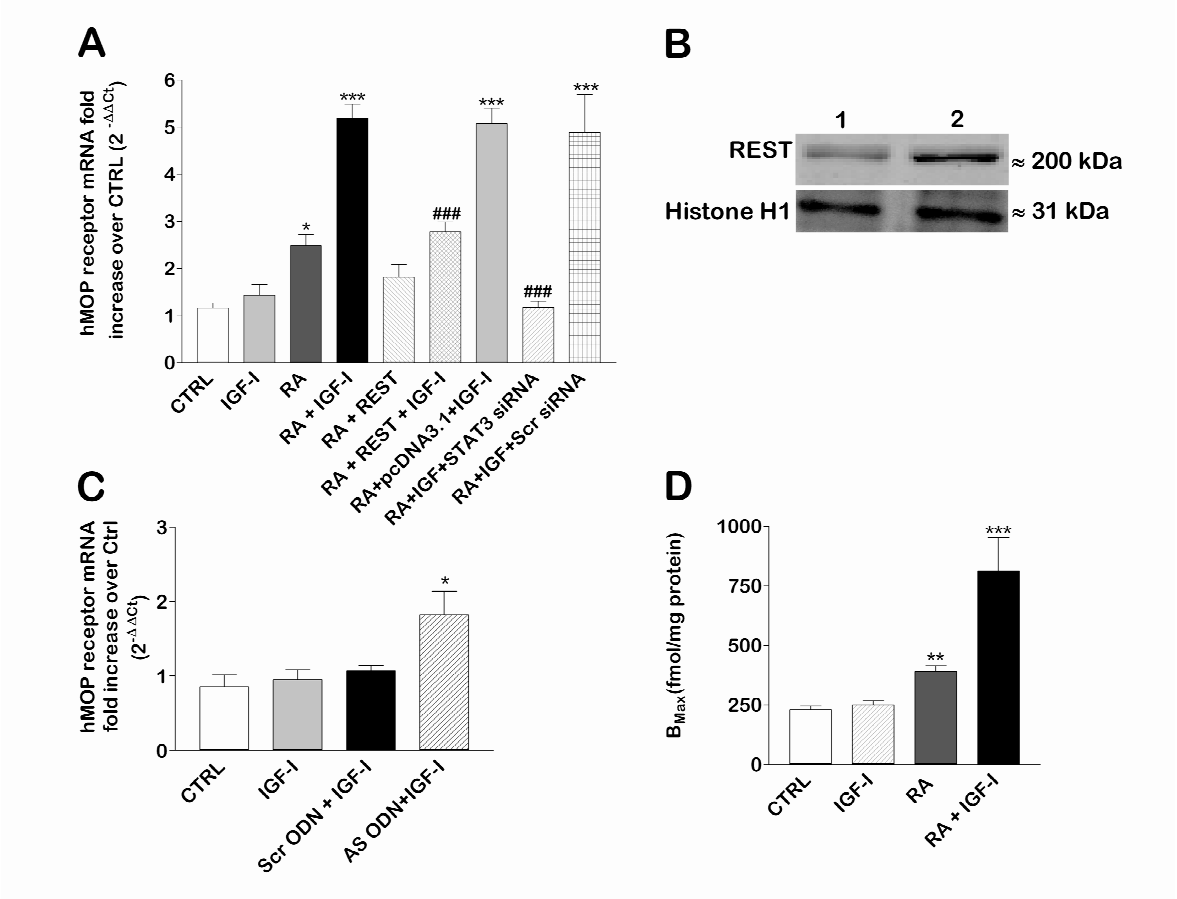


Fig. 7.6 *IGF-I exposure elevates hMOPr in RA-treated SH-SY5Y cells. A. Exposure to IGF-I (10 nM) for 24 h did not increase steady-state levels of hMOPr mRNA in treated cells compared to cells maintained in serum-free medium (CTRL). Cells were serum-starved overnight (16 h) before IGF-I treatment in serum-free medium for 24 h. Exposure to RA (10 μM) for 5 days elevates hMOPr mRNA, and IGF-I (10 nM), added for 24 h to 5-day RA-treated cells following a serum-starvation overnight (16 h), induces a further increase of hMOPr mRNA transcripts. This increase is prevented in cells transfected with the REST expressing plasmid (pCMV6XL4), whereas this effect is not observed in cells transfected with the pcDNA3.1 empty vector. In cells transfected with the REST expressing plasmid exposure to RA alone did not cause any significant increase in MOPr mRNA. Treatment of RA-exposed cells to STAT3 siRNA (50 nM; 48 h) resulted in a noteworthy reduction of IGF-I-induced increase of MOPr mRNA, whereas the transfection of a scrambled siRNA (Scr siRNA; 50 nM, 48 h) did not affect this action of IGF-I. Values are the mean ±SEM of at least six independent experiments carried out in triplicate. * p<0.05 compared to IGF-I and CTRL; *** p<0.001 compared to RA and RA+REST+IGF-I; ### p<0.001 compared to RA+IGF-I and RA+pcDNA3.1+IGF-I. B. Representative western blot analysis of REST protein in nuclear extracts from SH-SY5Y cells exposed to RA (10 μM; 5 d), transfected with pcDNA3.1 empty vector (lane 1) or with the REST expressing plasmid pCMV6XL4 (lane 2) and then both treated with IGF-I (10 nM; 24 h) after 16 h of serum starvation. Histone H1 was evaluated in the same samples and used as a loading control. Approximate molecular mass of REST (only a single band of ≈200-180 kDa was observed) and Histone H1 was determined by comparison with molecular mass standards. A representative experiment, repeated six times with similar results, is shown. C. Exposure to IGF-I (10 nM) for 24 h increases steady-state levels of hMOPr mRNA in SH-SY5Y treated with an antisense oligonucleotide complementary to REST mRNA (AS; 10 μM, 48 h), but not with a scrambled oligonucleotide (Scr-ODN; 10 μM, 48 h). IGF-I was added, in serum-free medium, 24 h after ODNs administration. Control cells were serum-starved overnight (16 h) before IGF-I treatment in serum-free medium for 24 h. Values are the mean ±SEM of at least six independent experiments carried out in triplicate. * p<0.05 compared to IGF-I. D. Effect of RA and IGF-I*

*on maximal density of hMOPr (B_{max}) measured in SH-SY5Y cell membranes. SH-SY5Y cells were serum-starved overnight (CTRL) and exposed to IGF-I for 48 h in serum-free medium. Alternatively, cells were exposed to RA for five days and then treated for 48 h with IGF-I after 16 h of serum-starvation. Saturation binding assays were conducted using [^{3}H]DAMGO (0.05-5 nM) on cell membranes. A single-site receptor binding model provided the best fit for data analysis. B_{max} values were estimated from nonlinear regression analysis. Data represent mean ±SEM of six experiments carried out in triplicate. ** $p < 0.01$ compared to CTRL; *** $p < 0.001$ compared to RA.*

In order to better investigate any role of REST in IGF-I's effects on OPRM1 gene transcription, I adopted an antisense (AS) strategy based on the use of ODNs to down-regulate its expression. In agreement with a previous study (Di Toro *et al.* 2005) western blot analysis indicated that exposure to AS-ODN (10 μM, 48 h) substantially lowered nuclear REST (76 ± 3% compared to control cells; mean ± SEM, n=3) whereas in cells cultured with a scrambled oligonucleotide (Scr-ODN; 10 μM), REST levels were similar to control cells maintained in serum-free medium for 16 h (data not shown). In SH-SY5Y cells treated with IGF-I (10 nM) for 24 h, previous exposure to AS-ODN (10 μM, 48 h), but not to Scr-ODN (10 μM, 48 h), significantly increased hMOPr mRNA transcripts whereas this did not occur in control cells exposed to IGF-I alone (Fig. 7.6C). Taken together, these data indicate that high nuclear levels of REST may counteract any positive effect of IGF-I on OPRM1 transcription. In agreement with previous studies (Yu and Sadee 1988; Jenab and Inturrisi 2002), prolonged exposure of SH-SY5Y cells to RA significantly increased the density of hMOPr (B_{max}) on cell membranes, as indicated by saturation binding assays. In cultures treated with RA for five days then exposed to IGF-I for 48 h, hMOPr density increased more than in cells exposed to RA alone for five days (Fig. 7.6D). In contrast, in SH-SY5Y cells maintained for 16 h in serum-free medium and exposed to IGF-I for 48 h, the density of hMOPr remained unchanged (Fig. 7.6D). Changes in hMOPr density had negligible effects on K_d (apparent affinity of the radioligand to hMOPr) (data not shown).

Part II: regulation of MOPr expression in resting and activated T-lymphocytes

7. 5 TCR engagement by CD3/CD28 antigens induces OPRM1 transcription in CD4+ T-cells

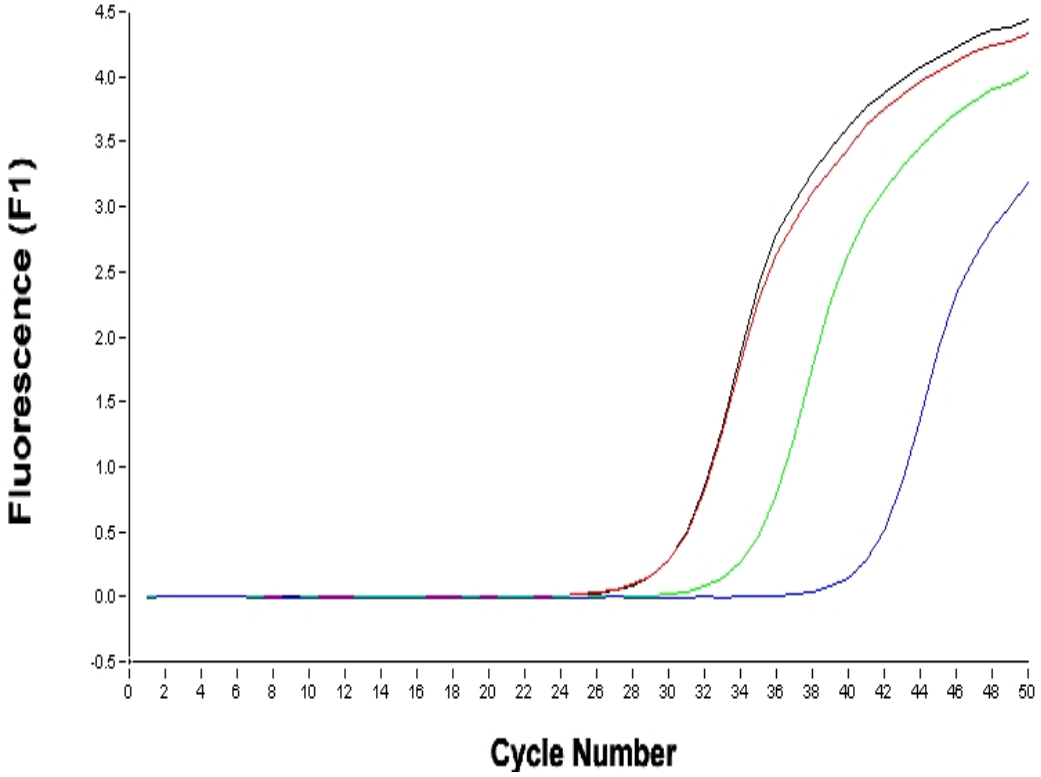
For this set of experiments I employed wild-type Jurkat T-cells, that expresses TCR; other sub-types of T-lymphocytic cell lines, in fact, like Jurkat E6.1 which are widely used, don't express it, therefore being useless to investigate TCR signalling

Wild type Jurkat cells where exposed to CD3 and CD28 (30 min at 37°C) and after that OPRM1 mRNA levels were evaluated by real time PCR (Fig. 7.7A). In untreated cells OPRM1 transcripts levels resulted very low or nearly undetectable: their amplification curves appeared between the 37^{th} and the 42^{nd} PCR cycle (Fig. 7.7A), having this latter cycle been calculated as the lowest detection limit in the experimental settings adopted (Kraus et al., unpublished observations).

CD3/CD28 stimulation of wild type Jurkat cells, on the contrary, resulted in a significant induction of OPRM1 transcripts, as confirmed by the respective amplification curves which resulted anticipated as compared to those of untreated control cells (Fig. 7.7A): amplification curves of treated samples were almost overlapping, with an exponential phase beginning at the 31^{st} PCR cycle.

As an internal control β-actin gene has been amplified by cDNA obtained from both treated and control cells; no significant changes in β-actin transcript levels have been observed.

Specificity of amplified products has been verified by melting curve analysis and by agarose gel electrophoresis; it is interesting to observe that, in the gel, bands relative to OPRM1 transcripts of untreated cells were either absent or very weak (Fig 7.7B), with a perfect correspondence to each respective amplification curve: very delayed curve for the undetectable sample, with exponential phase beginning around the 42^{nd} PCR cycle, for the undetectable control; amplification curve with exponential phase beginning around the 37^{th} PCR cycle for the slightly detectable band (Fig. 7.7A).



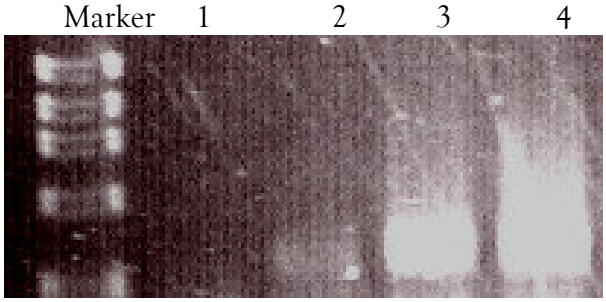


Fig. 7.7 *TCR engagement by CD3 and CD28 up-regulates OPRM1 transcription in CD4+ Jurkat cells.*
A) Amplification curves of OPRM1 transcripts in untreated Jurkat cells (blue and green lines) and CD3/CD28-stimulated Jurkat cells (red and black lines): the exponential amplification of OPRM1 transcripts in treated cells was significantly anticipated as compared to untreated ones.
B) Gel electrophoresis of amplified samples from both untreated (lanes 1 and 2) and CD3/CD28-treated (lanes 3 and 4) cells: bands relative to induced OPRM1 transcripts in treated cells are clearly visible as huge brilliant spots, whereas OPRM1 in control cells is slightly or even not detectable

7.6 OPRM1 transcription is induced in T-cells through the activation of transcription factors NF-kB, NFAT and AP-1

Wild type Jurkat cells were exposed either to vehicle (cell culture medium) or to 160 nM decoy oligodeossinucleotide (ODN) targeted to AP-1, NF-kB, NFAT (which are known to be activated following TCR engagement) or STAT5 (as a negative control) and then exposed to CD3/CD28 (30 min at 37°C); 24 hour later cDNA was prepared by each sample and OPRM1 transcript levels were evaluated by real time PCR.

AP-1, NF-kB and NFAT specific decoy ODNs completely abolished CD3/CD28-induced OPRM1 up-regulation (Fig. 7.8); OPRM1 transcript levels were, on the contrary, significantly increased by CD3/CD28 either alone or in the presence of STAT5 specific decoy ODN (Fig 7.8).

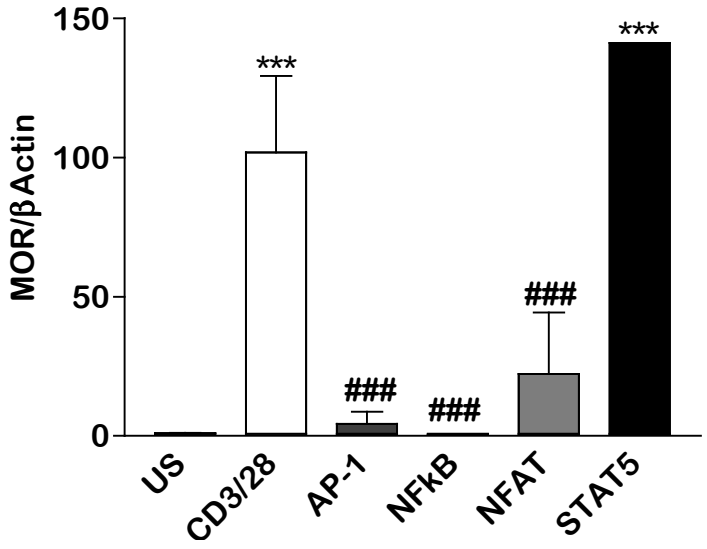


Fig. 7.8 *OPRM1 transcription is up-regulated following TCR engagement trough the activation of transcription factors AP-1, NF-kB, NFAT.*
*CD3/CD28 treated cells displayed a significant induction of OPRM1 transcripts; in Jurkat cells exposed to decoy oligodeossinucletides (ODN) specific for AP-1, NF-kB and NFAT and then treated with CD3/CD28 OPRM1 up-regulation was completely abolished, whereas decoy ODNs targeted to STAT5, a transcription factor which is not directly involved in TCR signaling, didn't affect the positive action of CD3/CD28 on OPRM1 transcription. ***p < 0.001 vs. US, AP-1, NF-kB and NFAT; ### p < 0.001 vs. CD3/28 and STAT5*

7.7 Over-expressed EGFP-MOPr in T-cells is internalised following exposure to DAMGO but not to morphine

In order to characterize MOPr turnover once it has been expressed in T-lymphocytes, Jurkat cells were electroporated with a plasmid bearing the coding sequence for MOPr fused with a portion of the Enhanced Green Fluorescent Protein (EGFP). 48h after transfection, cells were exposed to 10 μM DAMGO or morphine or left untreated and the cellular distribution of EGFP-MOPr was evaluated by confocal microscopy.

First of all EGFP-MOPr was effectively over-expressed in transfected cells and it was distributed mainly on cell surface, as it appears by the fluorescence that is distributed as a circle around the cells (Fig. 7.9); furthermore, DAMGO determined a significant and progressive internalisation of EGFP-MOPr, whereas morphine did not.

The maximal internalisation induced by DAMGO was observed after an exposure of 120 min, as indicated by the fluorescence of EGFP-MOPr that is all clustered in intracellular spots (Fig. 7.9); after that, the recombinant receptor started to be recycled on cell surface, this process being completed 120 min later (i.e.: after 240 min of exposure to DAMGO).

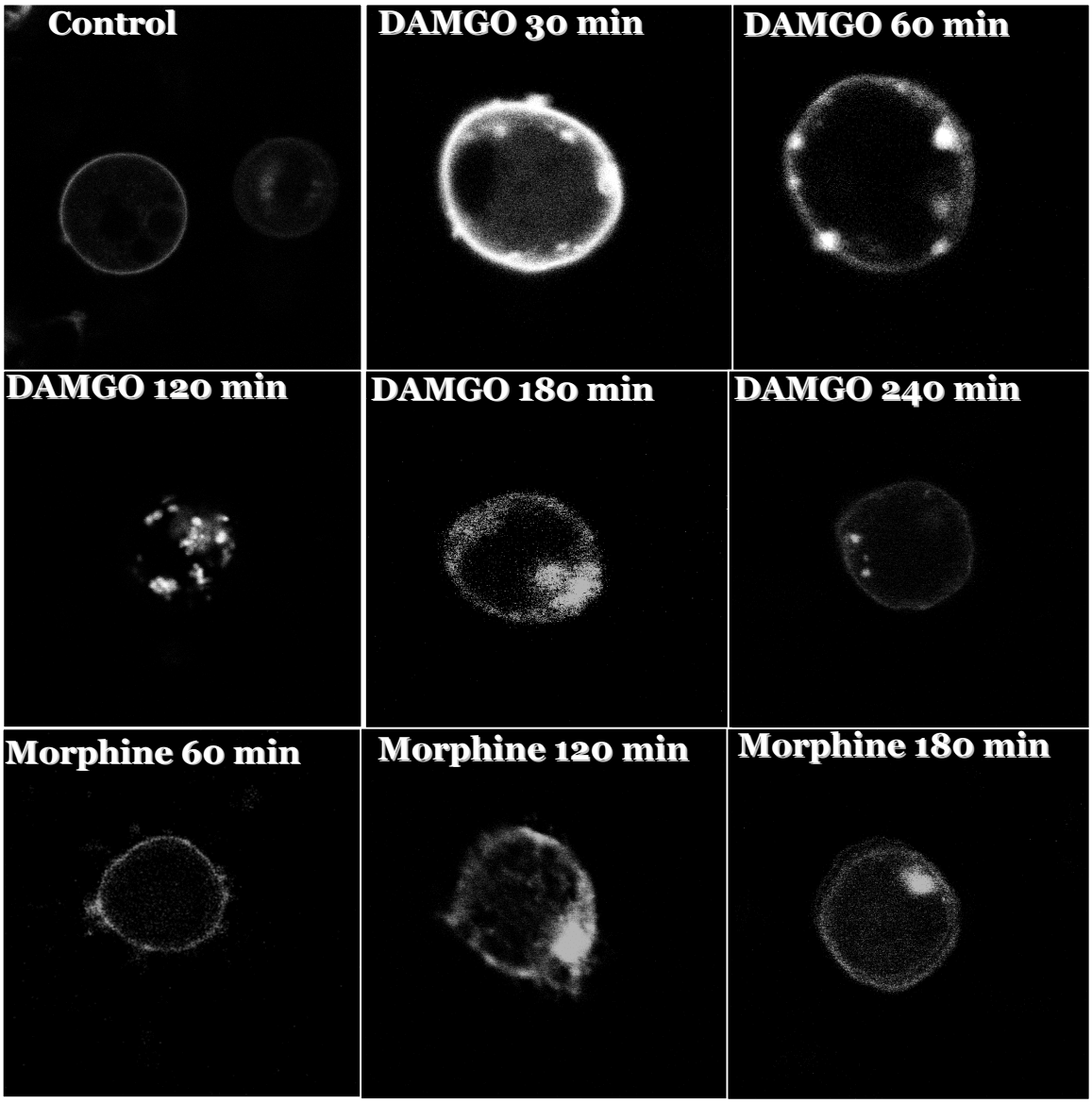


Fig. 7.9 *Recombinant EGFP-MOPr turnover in Jurkat T-cells exposed to different opioid agonists*
10 μM DAMGO induced a progressive and significant internalisation of EGFP-MOPr, with the maximum after 120 min of exposure; subsequently EGFP-MOPr is recycled on cell membrane and receptor recycling is complete at 240 min of exposure (i.e.: 120 min after the maximal internalisation observed).
10 μM morphine did not determine any significant internalisation of EGFP-MOPr in Jurkat T-cells

8. Discussion

OPRM1 TRANSCRIPTIONAL MODULATION IN NEURONAL CELLS

An accepted theory of neural gene transcription is based on the action of multiple factors that bind to different *cis*-acting DNA elements and either inhibit or activate transcription (Ma, 2006). In the first part of this thesis, I investigated the possibility that IGF-I may behave as an activator of OPRM1 gene transcription in neuronal cells when REST is absent, like in PC12 cells (Ballas *et al.* 2001; Bruce *et al.* 2006), or when it is down-regulated, like in RA-differentiated SH-SY5Y neuroblastoma cells. The data collected show that IGF-I can potentially contribute to increasing human MOPr expression in neuronal cells. Its action on OPRM1 transcription was further substantiated by work with a series of OPRM1-luciferase promoter/reporter constructs deficient in the RE1 binding element. The positive effect required the presence of a regulatory element residing in the promoter region from -1672 to -1556 bp. In this region there is a sequence of 9 bp (from -1583 to -1575) that can bind STAT1/3 proteins. These transcription factors form homodimeric or heterodimeric complexes that translocate to the nucleus and bind specific elements within the promoters of regulated genes. STAT proteins are acutely activated by tyrosine kinase signaling, which facilitates dimerization and consequent nuclear translocation, DNA binding, and activation of target gene expression (Reich and Liu, 2006). Börner *et al.* (2004) reported that the loss of STAT1/3 function by decoy oligonucleotides attenuates transcriptional induction of the OPRM1 gene in the presence of interleukin-6, indicating that this regulation is dependent on STAT1 and/or STAT3. However, these authors concluded that the question whether STAT1 or STAT3 homodimers or STAT1-STAT3 heterodimers bind to the OPRM1 promoter remains unanswered, because of the similarity in the molecular weight of these two STAT factors. In the present study, I demonstrated the substantial contribution of STAT3, but not STAT1, to IGF-I-mediated up-regulation of OPRM1 transcription using a siRNA strategy to suppress STAT3 expression in SH-SY5Y cells. A functional interaction between IGF-I/IGF-I receptor and STAT3 *in vitro* and *in vivo* has already been ascertained and the JAK/STAT3 pathway is involved in the survival of primary neural cells and in SH-SY5Y cells exposed to IGF-I (Yadav *et al.* 2005). Therefore, STAT3 appears to be essential for regulating IGF-I-modulated gene transcription in neuronal cells. The transcriptional repressor REST, interacting with the RE1 DNA element and possibly also recruiting co-repressors including mSin3A and CoREST (Battaglioli *et al.* 2002; Ballas *et al.* 2005), might block IGF-I's ability to activate the transcriptional machinery. Human MOPr expression, like that of other genes such as the

glutamate receptor gene (Okamoto *et al.* 1999) and the M4 muscarinic acetylcholine receptor gene (Wood *et al.* 1996), is detectable in the presence of REST and increases significantly in the absence of REST, and may require promoter/enhancer specific activators. In the present study, human MOPr expression inversely correlated with REST in SH-SY5Y cells exposed to RA and further elevated by exposure to IGF-I. It is becoming clear that during neuronal differentiation not only must certain genes be expressed when needed, but also the transcription of others must also be restricted at the right time (Hohl and Thiel 2005). RA plays a major role in regulating the transition of proliferating precursor cells to post-mitotic differentiated neurons (Giannini *et al.* 1997; Singh *et al.* 2003), an effect requiring 4-7 days, whereas short-term treatment (24-48 h) induces cell migration and invasion (Joshi *et al.* 2006). Jenab and Inturrisi adopted the SH-SY5Y human neuroblastoma cell line, a system widely used to investigate MOPr- and DOPr-mediated signal transduction mechanisms (Yu and Sadee 1988) and mechanisms that induce neurodifferentiation (Pahlman *et al.* 1990), to examine the effects of RA on human MOPr mRNA levels during neuronal differentiation. RA had a bimodal effect on human MOPr mRNA, with an initial decrease after 6-18 h of exposure followed by a doubling of mRNA by 168 h (Jenab and Inturrisi 2002), and of MOPr protein as measured by receptor binding (Yu and Sadee 1988). The latter finding is consistent with the increase in B_{max} of human MOPr I have reported here. Regarding REST expression, nuclear REST levels are largely reduced after neuronal differentiation of P19 cells exposed to RA for four days, whereas no such changes were reported in cells exposed for a shorter time (Bai *et al.* 2003). Earlier our group reported that nuclear REST increased in SH-SY5Y cells cultured in serum-free medium and exposed to IGF-I for two days, and declined in five-day treated cells concomitantly with progressive neurite extension (Di Toro *et al.* 2005). Therefore, in neuronal cells the expression pattern of REST appears to be time-, gene-, and cell type-dependent, which is consistent with the theory that during neuronal differentiation REST contributes to the repression of genes not yet required by the differentiation program, and declines later as its down-regulation may be needed for full elaboration of neuronal phenotypes (Ballas and Mandel 2005; Coulson 2005). REST continues to be expressed in many post-mitotic neurons, notably in the adult hippocampus, where both REST and its target genes are modulated in response to ischemic (Calderone *et al.* 2003) or epileptic insults (Spencer *et al.* 2006). REST also contributes to the down-regulation of MOPr expression in hippocampal neurons of rats subjected to transient global ischemia (Formisano *et al.* 2007). This implies that it acts as a potent repressor of target genes in multipotent cells and during neurogenesis, thereafter becoming a regulator in post-mitotic neurons. The finding that

IGF-I may up-regulate human MOPr expression in differentiating neuronal cells, and that this depends on REST down-regulation, is new and may help elucidate its role in neurogenesis and in post-mitotic neurons. Several studies have reported that OPRM1 gene expression is seen very early on in the embryonic rat brain with an increase during the critical period of neurogenesis, neuronal migration, and synaptogenesis, suggesting this opioid receptor is involved in brain development (Winzer-Serhan *et al.* 2003; Kivell *et al.* 2004; Howard 2005). MOPr regulates the survival of maturing neurons in adult hippocampal neurogenesis (Harburg *et al.* 2007), so it might be one of the target genes regulated by IGF-I in a time-dependent manner during neurogenesis. IGF-I, with its receptors and binding proteins, acts as a neurotrophic factor and plays an important role in the development of the nervous system, with effects on cell proliferation, differentiation, and survival of neuronal cells (van Golen and Feldman 2000). IGF-I has also emerged as a candidate for regulating neuronal production in adulthood and for providing constant trophic support to neuronal cells in the brain (Lichtenwalner *et al.* 2006). Clearly, further studies are needed to clarify whether IGF-I's positive effect on human MOPr expression, shown here, is also seen in other *in vitro* and *in vivo* models employed to investigate neurogenesis.

OPRM1 TRANSCRIPTIONAL MODULATION IN IMMUNE CELLS

Multiple immuno-modulator effects of opioids are transduced to immune effector cells mainly via three mechanisms: either indirectly via the central nervous system [e.g., by activation of the hypothalamus-pituitary-adrenal axis (Cabot et al., 2001; Roy et al., 2001)], directly via atypical opioid receptors [which were postulated because some opioid effects cannot be blocked by the classic opioid receptor antagonist naloxone (Roy et al., 1998b)], or directly via the classic opioid receptor subtypes μ, δ, and κ present on immune cells. It has been shown previously that classic MOP receptors are induced by IL-4 in various immune cells (Kraus et al., 2001) and by IL-1 in endothelial cells (Vidal et al., 1998). Furthermore, earlier studies demonstrated immunosuppressive and immuno-regulatory functions of opioid alkaloids like morphine and endogenous opioid peptides such as β-endorphin (Sacerdote et al., 2000).

The multiple effects of opioids on immune effector cells obtained from functional studies implicate that opioid receptors are expressed on these cells (Sacerdote et al., 2000). However, data obtained from different laboratories are rather contradictory: OPRM1 transcripts were detected in lymphocytes, monocytes, and macrophages by some researchers (Chuang et al., 1995), whereas other groups failed to detect MOPr expression in immune cells (Wick et al., 1996).

However, MOPr expression in immune cells is likely to be related to status of activation of the cells: in a cytokine-activated environment, in fact, OPRM1 transcription results significantly up-regulated.

Therefore, in the second part of this study I investigated the transcriptional induction of the OPRM1 gene in T lymphocytes in response to TCR engagement and characterized the role of AP-1, NF-kB and NFAT in such a process. Since TCR interaction with antigen/MHC complexes is the first step in T-cell activation, in fact, I wondered whether the signaling pathway induced can itself increase OPRM1 transcription.

I focused my attention on AP-1, NF-kB and NFAT because they are the transcription factors activated following TCR engagement and because OPRM1 promoter bears their responsive elements. The OPRM1 promoter, in fact, contains a distal AP-1 element located at nucleotide -2388 and a proximal site located at nucleotide -1434. The region containing the proximal element is conserved also in the rat mu-opioid receptor gene promoter, with a similar element at position -1367 (Kraus et al., 1995). The sequences corresponding to the distal element are not yet known for the rat gene.

AP-1-controlled transcriptional regulation can be of considerable importance for fine-tuning gene expression in response to various stimuli: the molecular mechanisms for the induction of OPRM1 mRNA by the cytokine IL-1β, for instance, could involve AP-1.

Two NFAT binding sites have been found on OPRM1 promoter by Xu and Carr at nucleotides from -1062 to -1056 and at nucleotides from -484 to -478, respectively (Xu and Carr, 2000).

Therefore it is conceivable that also NFAT can bind to OPRM1 and modulate its transcription.

Three NF-kB binding sites have been previously identified on OPRM1 promoter (Kraus et al., 2003); moreover, in the same study it was demonstrated that TNF-α could induce OPRM1 transcription through NF-kB activation. The presence of multiple NF-kB elements on the OPRM1 promoter most probably indicate the importance for regulation via this factor.

Regulation of MOPr by NF-kB may be important, for instance, within the complex of inflammation-induced analgesia. It is now established that immunocyte-derived endogenous opioids induce analgesia by activating peripheral opioid receptors at later stages during inflammation, which can be regarded as a physiological mechanism to counteract and reduce inflammatory pain (Stein et al., 1990; Cabot et al., 1997). Most probably, the relevant opioid receptors are of the μ-subtype, because these are up-regulated in dorsal root ganglia in inflammation (Ji et al., 1995).

In the second part of this thesis it has been shown that TCR engagement determines a significant increase in OPRM1 transcript levels and that this transcriptional effect is mediated by AP-1, NF-kB and NFAT, as hypothesised.

These findings are interesting considering the inflammation-induced analgesic process above described; in fact, it is turning out a scenario in which TCR activation by antigen presenting cells, as well as by cytokines produced after inflammation has occurred, not only sustains a specific and powerful immune response to the pathogen that caused the reaction, but also provides a significant increase in MOPr expression levels which can contribute to the attenuation of pain perception.

Therefore, MOPr seems to play a pivotal role in the cross-talk between immune system and nervous system, especially in modulating the balance between pro-inflammatory and pro-nociceptive stimuli and analgesia.

Considering the neuroprotective effect recently ascribed to opioids (Iglesias et al., 2003; Wang et al., 2006), it is intriguing to postulate that TCR engagement as well as cytokines induce OPRM1 up-regulation in both neurons and immune cells in order to protect neuronal cells from possible damages related to immune response and to provide a feed-back modulation of immune response.

In the second part of this thesis I also investigated MOPr turnover in T-cells exposed to DAMGO or morphine. Morphine is a poor inducer of MOPr internalisation, but a potent inducer of cellular tolerance, whereas DAMGO is known to internalise MOPr (Schultz et al., 2004). On the contrary, endomorphins were shown to induce rapid MOPr internalisation in SH-SY5Y human neuroblastoma cells (Horner and Zadina, 2004).

Currently, no data about MOPr turnover in immune cells are available; since I observed that TCR engagement significantly up-regulated MOPr, questions arose about the turnover of MOPr once its expression is induced in T-cells. To give an answer to such questions I transfected Jurkat T-cells with a plasmid bearing the coding sequence for a EGFP-MOPr fusion protein.

Confocal microscope analysis of transfected cells showed a prevalent expression of EGFP-MOPr on cell surface; furthermore, DAMGO, but not morphine, determined receptor internalisation and recycling, similarly to what has been observed in neuronal cells.

So far, these are the first, albeit preliminary, data on MOPr turnover in T-lymphocytes.

Further investigations will allow to better understand MOPr internalisation pattern in immune cells and its role in MOPr agonists-mediated immuno-modulator effects.

CONCLUDING REMARKS

In this thesis OPRM1 transcriptional modulation in neuronal and immune cells have been investigated and the transcription factors involved have been functionally characterized.

About regulation of MOPr expression in neurons we can conclude that when REST is expressed and active, as in undifferentiated SH-SY5Y cells, IGF-I does not up-regulate human MOPr expression, since it activates both REST and STAT3 (Fig. 8.1). On the contrary, when REST expression is reduced, as in RA-differentiated SH-SY5Y cells, or absent, as in PC-12 cells, IGF-I significantly increases OPRM1 transcription and MOPr expression on cell membrane (Fig 8.1). This effect is mediated by transcription factor STAT3, which is induced by IGF-IR and binds to OPRM1 promoter at the STAT1/3 binding site (Fig. 8.1).

About regulation of MOPr expression in T-lymphocytes we can conclude that when TCR is engaged by antigen/MHC complex and co-stimulator molecules a series of signal transduction processes begins and leads to the activation of AP-1, NF-kB and NFAT, which in turn up-regulate OPRM1 transcription (Fig. 8.1).

Once expressed on T-cell membrane, MOPr can be internalised and recycled by DAMGO but not by morphine.

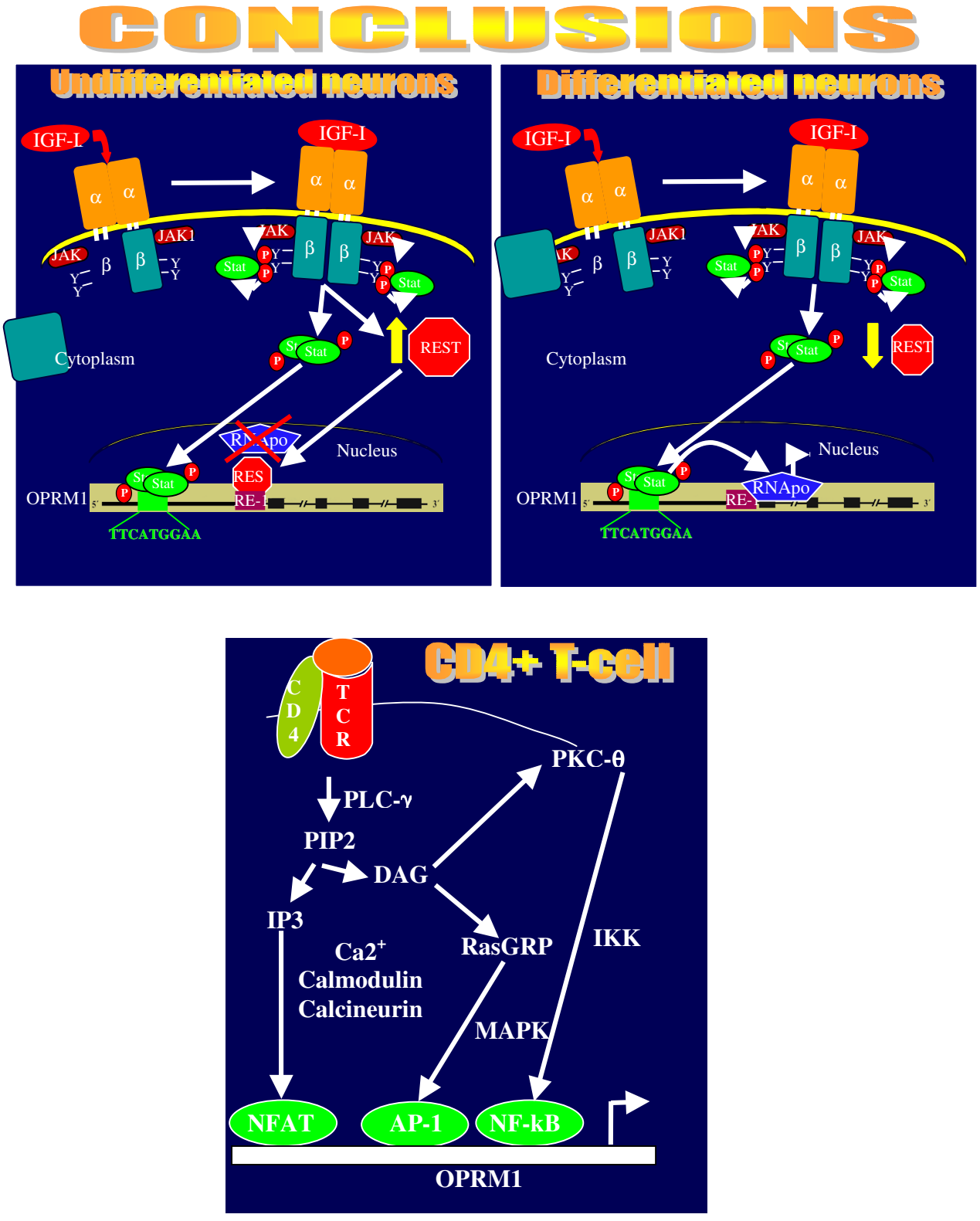


Fig. 8.1 *Transcriptional modulation of OPRM1 by IGF-I in neuronal cells.*
IGF-I activates STAT3, but it's unable to up-regulates OPRM1 transcription in undifferentiated neurons since REST is induced as well. REST expression levels significantly decrease along neuronal differentiation; therefore, in differentiated neurons, IGF-I-mediated activation of STAT3 can up-regulate OPRM1 transcription and subsequently MOPr expression.

TCR engagement by CD3/CD28 activates several transduction pathways that lead to the activation of AP-1, NF-kB and NFAT which in turn up-regulate OPRM1 transcription.

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