Activity-Dependent Regulation of Gene Expression in Muscle and Neuronal Cells

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Abstract

In both the central and the peripheral nervous systems, impulse activity regulates the expression of a vast number of genes that code for synaptic proteins, including neuropeptides, enzymes involved in neurotransmitter biosynthesis and degradation, and membrane receptors. In recent years, the mechanisms involved in these regulations became amenable to investigation by the methods of recombinant DNA technology. The first part of this review focuses on the activity-dependent control of nicotinic acetylcholine receptor biosynthesis in vertebrate muscle, a model case for the regulation of synaptic protein biosynthesis at the postsynaptic level. The second part summarizes some examples of neuronal proteins whose biosynthesis is under the control of transsynaptic impulse activity.

The first, second, and third intracellular messengers involved in membrane-to-gene signaling are discussed, as are possible posttranscriptional control mechanisms. Finally, models are proposed for a role of neuronal activity in the genesis and stabilization of the synapse.

Index Entries: Nicotinic acetylcholine receptor; synapse; neuronal activity; synaptic protein expression; membrane to gene signaling; transsynaptic control; synapse stabilization; neuromuscular junction.

Introduction

The nervous system, like any organ of the body, consists of an organized assembly of cells. Yet, it differs from nearly all the other tissues by its much higher complexity. Each individual neuron, instead of being bound to a few partners by the facets of its cell surface, is specifically linked to hundreds or thousands of them via synaptic connections: moreover, within a given category, each individual cell can be distinguished from its neighbor by its precise connectivity and chemical composition. In other words, each neuron possesses its own individuality or "singularity" (Changeux, 1983, 1986). Such sophisticated architecture progressively develops during embryogenesis and postnatal development as a result of the differential and sequential expression of genes. Early on, functional synaptic contacts may form in neuronal networks, and electrical activity arises, first spontaneously, and subsequently as a consequence of the interaction with the outside world (Preyer, 1885: Hamburger, 1970). An important question is to what extent such propagated activity regulates gene expression in the course of neurogenesis. In particular, how important is the part played by activity in generating and stabilizing the ultimate communication channel, the synapse, and what are the molecular mechanisms engaged in this regulation?

The aim of this review is not to cover all the experimental and theoretical work done on this issue (*see*, for example, Changeux and Danchin, 1976: Purves and Lichtman, 1980: Black et al., 1987). We will deliberately limit its scope to: 1) systems where tools from recombinant DNA technology are available to study gene expression: 2) proteins that are characteristic components of the synapse, such as the postsynaptic receptors for neurotransmitters, and the enzymes responsible for the biosynthesis of neurotransmitters or of neuropeptides that co-exist (Hökfelt et al., 1986) with them.

The first part of this paper focuses on the expression of some postsynaptic proteins of the vertebrate motor endplate, examplified by the nicotinic acetylcholine receptor. The second part deals with the regulation of the expression of neuronal proteins that play a role in synaptic function.

Regulation of Synaptic Protein Expression at the Developing Neuromuscular Junction

Molecular Architecture of the Motor Endplate

The junction between motor neuron and skeletal muscle, or motor endplate, is a particularly convenient system to analyze synapse formation at the molecular level for several reasons.

1. Its anatomy has been studied in detail (Couteaux, 1978);
2. It is of easy access for electrophysiological recordings (Katz, 1967);
3. Its pharmacology and the biochemistry of its major components are rather well understood (Changeux, 1981); and
4. The cDNAs and chromosomic genes of several endplate proteins have been cloned and sequenced (review Numa et al., 1983; Stroud and Finer-Moore, 1985; Cunningham et al., 1987; Frail et al., 1987; Soreq and Gnatt, 1987; Changeux et al., 1984, 1987a).

The motor endplate in vertebrates develops from the juxtaposition of two basic structures (Fig. 1): the ending of the motor nerve and a subneural domain, separated by a 50–100 nm cleft. The nerve terminal contains 30–60 nm clear vesicles (filled with the neurotransmitter acetylcholine), which form linear arrays on top of membrane specializations referred to as "active zones." On the opposite side of the cleft, the muscle membrane makes repeated foldings with, at the top of the folds, thickenings located in front of the active zones and composed of closely packed acetylcholine receptor (AChR) molecules (about 10,000 mol/μm^2). A few microme-

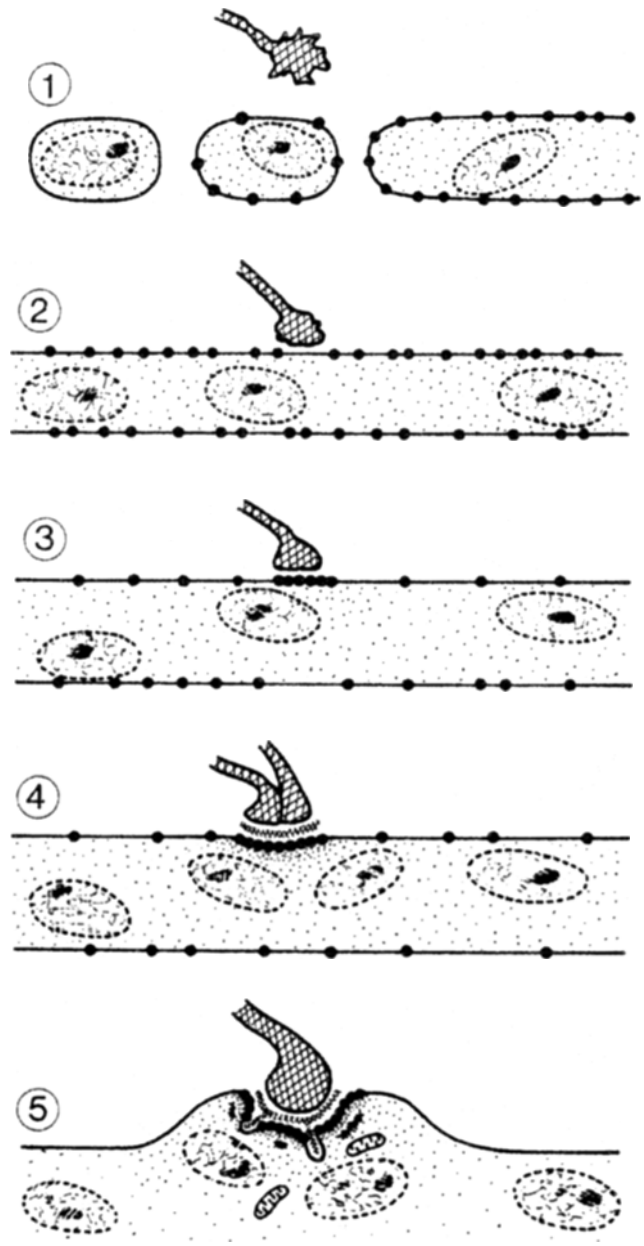

Fig. 1. Expression of the acetylcholine receptor (AChR) during formation of the neuromuscular junction (black dots: AChR). 1) Fusion of myoblasts into myotubes: AChR biosynthesis is enhanced: 2) The exploratory motor axon approaches: 3) The growth cone contacts the myotube, a subneural cluster of AChR forms: 4) Several motor nerve endings converge on the subneural cluster of AChR: 5) One motor nerve ending becomes stabilized: subneural folds develop: interactions with the cytoskeleton become apparent (modified from Changeux et al., 1987a).

ters away from the endplate, the density of AChR drops to less than 10 mol/μm^2. The postsynaptic membrane is covered, on the cleft side, by an electron dense layer, the basal lamina, which contains collagen, heparan sulfate proteoglycan, laminin, several endplate specific antigens (Burden, 1987), and the tailed forms of acetylcholinesterase (Massoulié and Bon, 1982). On the cytoplasmic side, a peripheral protein of mol wt 43,000 dalton underlies the AChR molecules to which it tightly binds with a 1-to-1 stoichiometry (*references* in Kordeli et al., 1986; Frail et al., 1987). The whole subsynaptic domain is anchored to a complex network of filaments that includes actin, α-actinin, vinculin, and filamin (*review* in Bloch and Hall, 1983). The cytoplasm of the muscle fiber is slightly raised at the level of the terminal arborization of the motor nerve where mitochondria and 4–8 muscle nuclei, named "fundamental nuclei" by Ranvier (1875), are accumulated.

The highly organized postsynaptic domain of the motor endplate thus results from a local differentiation of the muscle surface. Denervation experiments indicate that the adult motor endplate is a rather stable structure (*review* Bourgeois et al., 1978a; Salpeter and Loring, 1985) and this raises the question of how such a sophisticated supramolecular architecture arises.

Expression of Nicotinic Acetylcholine Receptors During Endplate Formation

The nicotinic AChR of the vertebrate neuromuscular junction is a major component of the endplate postsynaptic membrane. Composed of four homologous transmembrane subunits (α, β, γ, and δ) with the stoichiometry $\alpha_2 \beta \gamma \delta$, it contains the ACh binding sites, the ion channel, and all the structural elements required for the regulation of its opening by ACh (Popot and Changeux, 1984; Stroud and Finer-Moore, 1985; Hucho, 1986; Changeux et al., 1987a). Cloning of the cDNAs and genes coding for AChR subunits from different species revealed that each subunit is encoded by a single copy gene (*reviewed* by Stroud and Finer-Moore, 1985; Changeux et al., 1987a). The availability of molecular probes for AChR genes opened the way to an in-depth analysis of the mechanisms by which the biosynthesis of a synaptic protein can be regulated transsynaptically.

During the development of the neuromuscular junction, as well as in adult muscle, motor innervation exerts a profound influence on the number, the distribution, and the functional properties of the AChR (*reviewed* by Fambrough, 1979; Changeux, 1981; Merlie, 1984; Salpeter and Loring, 1985; Changeux et al., 1987a,b; Klarsfeld, 1987; Schuetze and Role, 1987).

Before the arrival of the exploratory motor axons, an important increase in the number of AChRs occurs. Following the fusion of myoblasts into myotubes, AChR molecules are diffusely distributed over the entire surface of the muscle membrane. Such embryonic AChR molecules are metabolically unstable (half-life 17–22 h), undergo significant lateral motion, and exhibit a mean channel open time of 3–10 ms. With the onset of motor innervation, AChRs start to cluster beneath the nerve endings and progressively become immobile and metabolically stable (half-life 10 d or more). At the same time, extrajunctional AChRs are eliminated, such that at the adult motor endplate, AChRs are present at levels up to 10,000-fold higher than in extrasynaptic regions of the sarcolemma. Adult AChRs also differ functionally from embryonic receptors: They possess a larger conductance and a shorter channel open time (*review* Salpeter and Loring, 1985; Schuetze and Role, 1987).

Schematically, the evolution of AChR content during muscle development can be described as a succession of four main phases (Fig. 1).

1. An initial increase in AChR numbers;
2. The local concentration of AChRs under the motor nerve ending;
3. The disappearance of extrajunctional receptors; and
4. The "maturation" of the motor endplate, involving changes in AChR metabolic stability

and channel properties, as well as the continued, preferential insertion of AChRs into the subsynaptic sarcolemma.

Various changes in the distribution and properties of AChR thus occur at different stages of endplate development, and are probably mediated by distinct regulatory mechanisms. These regulations involve the combined action of extracellular "anterograde" factors released from the motor nerve endings, and the neurally evoked electrical activity of the muscle fiber. Some of these alterations, such as the clustering of AChRs under the nerve endings, and possibly their metabolic stabilization, may involve either posttranslational modifications of *preexisting* receptor molecules, or of changes in the interactions between AChRs and other subsynaptic proteins. On the other hand, some of the developmental changes affecting AChR number and distribution clearly result from activity-dependent control of the biosynthetic pathways that lead to the appearance of functional AChRs in the muscle membrane. The subsequent chapters will focus mainly on some possible molecular mechanisms for the activity-dependent regulation of AChR biosynthesis. For the purpose of clarity, such a discussion is bound to rely on a much simplified and schematized view of motor endplate development. For additional accounts of AChR biosynthesis and developmental regulation, the reader is referred to the recent reviews by Salpeter and Loring (1985), Merlie and Smith (1986), and Schuetze and Role (1987).

AChR Biosynthesis During Early Muscle Differentiation

The dramatic increase of AChR number during the early stages of embryonic muscle development (Betz et al., 1977, 1980; Bourgeois et al., 1978b; Burden, 1977) occurs independently of neural activity, since it is not affected by chronic in ovo administration of neuromuscular blocking agents (Giacobini et al., 1973; Burden, 1977; Bourgeois et al., 1978b; Betz et al., 1980). This process starts in the myotomes at early stages of myoblast differentiation (Baldwin et al., 1988; Fontaine and Changeux, 1989). It can also be observed in primary cultures of embryonic muscle cells (Patrick et al., 1972; Merlie, 1984), where it largely coincides with the fusion of myoblasts into myotubes. The developmental increase of AChR levels involves *de novo* synthesis of receptor molecules, as demonstrated by incorporation of radiolabeled or heavy isotope-labeled amino acids (Merlie et al., 1975, 1978; Devreotes and Fambrough, 1975; Devreotes et al., 1977). The degradation rate of AChR does not significantly change throughout this process (Merlie et al., 1976).

The burst of AChR synthesis that occurs during myogenesis is accompanied by an increase in mRNA levels for the α-, β-, γ-, and δ- subunits (mouse and rat: Buonanno and Merlie, 1986; Evans et al., 1987; chick: Shieh et al., 1988; *Xenopus laevis:* Baldwin et al., 1988). It is, at least in part, due to enhanced transcription of the genes coding for AChR subunits, as demonstrated for the α- and δ-subunits by nuclear run-on assays (Buonanno and Merlie, 1986). Further evidence for the developmental regulation of AChR gene transcription comes from the finding that muscle differentiation is accompanied by the appearance of DNase I-hypersensitive sites in the DNA sequences flanking the δ- and α-subunit genes (Crowder and Merlie, 1986, 1988).

Experiments are underway to identify the putative *cis*-acting regulatory sequences that control the expression of AChR genes. As a first step in this direction, the 5'-flanking region of the α-subunit gene of chick muscle AChR was mapped and sequenced (Klarsfeld et al., 1987). It was found to contain a TATA and a CAAT box, as well as a potential Spl binding site. When inserted in front of the bacterial chloramphenicol acetyltransferase (CAT) gene, this promoter, including 850 bp of upstream sequence, was able to direct CAT expression in transfected myotubes, but not in myoblasts or nonmyogenic cells, indicating that it contains an element that confers tissue-specificity and developmen-

tal regulation to the α-subunit gene (Klarsfeld et al., 1987). Similar studies subsequently identified two sequences of 1.1 kb and 148 basepairs at the 5' ends of the mouse muscle AChR γ-subunit (Gardner et al., 1987) and δ-subunit (Baldwin and Burden, 1988) genes, respectively, that control cell-type specific and differentiation-dependent expression. A domain of significant homology (11 out of 13 basepairs) with the α-subunit gene was found at position –290 in the γ-subunit gene (Gardner et al., 1987) and at position –289 in the α-subunit gene, and this motif is also present in the chick cardiac actin gene at position + 32 (*see* Klarsfeld et al., 1987). Moreover, the first intron of the γ-subunit gene contains sequence elements strikingly homologous to the core sequence of the SV40 enhancer (Weiher et al., 1983) and of the Spl recognition site (Dynan and Tjian, 1985) but in an inverted orientation. Similar sequences are also contained in the chick α-subunit promoter (Klarsfeld et al., 1987).

Wang et al. (1988) further restricted the domain responsible for tissue- and stage-specific expression of the chick α-subunit gene to the –116 to –81 region. In the mouse C2C12 muscle cell line, this 36 bp fragment activates transcription in a distance—and orientation—independent manner and thus fulfills the criteria of an enhancer.

Piette et al. (1989) recently confirmed that the –110 to –45 segment controls the expression of the chick α-subunit gene during myotube differentiation in primary cultures. In agreement with these functional assays, Piette et al. (1989) found by DNase I footprinting and gel retardation assays, that within the 800 basepair upstream sequence of the α-subunit gene, only the most proximal 140 nucleotides display significant interactions with nuclear proteins prepared from cultured myotubes or embryonic muscle. Three domains of interactions referred to as ARI, II, and III were identified in this region, adjacent to the transcription start point. The levels of several of the factors interacting with these DNA elements were found to change during fusion of myoblasts into myotubes (ARIIb and III) and, also, as a consequence of denervation (ARIIb and III). The identification of these DNA-binding factors is in progress. In summary, a rather short segment of DNA appears to be involved in the regulation of AChR gene transcription. Yet, it is still not known whether this sequence is sufficient to control the localization and stabilization of the AChR at the adult endplate.

Clustering of AChR at Early Stages of Motor Endplate Formation

The first sign of endplate construction, which follows the contact of the growth cone with the embryonic muscle fiber is, within hours, the formation of a high density subneural cluster of AChR (Anderson et al., 1977; Anderson and Cohen, 1977; Frank and Fischbach, 1979; *for review, see* Salpeter and Loring, 1985; Peng and Poo, 1986; Englander and Rubin, 1987). AChR clusters can develop spontaneously in muscle cells in culture but such aneural cluster formation has not been reported to occur during normal muscle development in higher vertebrates. A variety of cholinergic neurons in culture establish functional neuromuscular contacts; however, only endings from motor neurons and not from sensory or sympathetic neurons cause subneural clustering of AChR (Cohen and Weldon, 1980).

In *Xenopus*, the local increase of AChR density at synaptic sites unambiguously forms out of a preexisting surface pool of AChR by lateral redistribution in the plasma membrane (Anderson and Cohen, 1977; Kuromi and Kidokoro, 1984). Moreover, in *Xenopus* neuromuscular cocultures, dispersion of spontaneous aneural clusters precedes the formation of the junctional clusters (Kuromi and Kidokoro, 1984). On the other hand, at chick nerve–muscle contacts, newly formed AChR molecules become locally inserted into the subsynaptic domain (Role et al., 1985; Ishikawa et al., 1988).

AChR clustering in cocultures takes place in the presence of curare (Cohen, 1972) and flaxedil (Bourgeois et al., 1978) and is, therefore, not elicited by ACh binding and channel activation of the AChR. Yet, it can be triggered by electric fields along the cathode facing surface of muscle cells (Orida and Poo, 1978), raising the possibility of a regulation of AChR distribution by the early electrical activity of the developing fiber.

The signals provided by the developing motor axons for the initiation of AChR clustering have not been identified as yet. In *Xenopus* muscle, silk threads (Jones and Vrbova, 1974) and latex beads coated with a basic polypeptide can cause a local aggregation of AChR at the contact with the muscle fiber surface (Peng et al., 1981; Peng and Cheng, 1981). Proteins extracted from the basement membranes of the synapse rich electric organ of *Torpedo californica*, such as agrin, cause the formation of AChR clusters on chick myotubes (Nitkin et al., 1983, 1987; Godfrey et al., 1984; Fallon et al., 1985; Reist et al., 1987). Agrin-like immunoreactivity has been detected in the basal lamina at the adult neuromuscular junction in vivo (Fallon et al., 1985; Reist et al., 1987). It is present in embryonic muscles before the formation of AChR clusters (Fallon, 1987) and cell bodies of motor neurons contain agrin-like molecules (Magill et al., 1987). Whether this protein plays a physiological role at the initial stage of endplate formation remains to be established (*review* Burden, 1987).

Early ultrastructural observations disclosed a close association of the subsynaptic membrane domain with the cytoskeleton (Couteaux, 1978) and several cytoskeletal proteins such as actin, α-actinin, vinculin and filamin have been detected at this level (Bloch and Hall, 1983). A regulation of the interaction AChR-cytoskeleton has, therefore, been suggested to play a role in AChR cluster formation. A network of thin actin filaments forms at the level of latex beads–muscle contacts one hour prior to the aggregation of AChR (Peng and Phelan, 1984). Furthermore, the cytoskeletal 43 kDυ_1 protein (Sobel et al., 1977) was found associated with AChR clusters formed beneath latex beads (Peng and Froehner, 1985) or nerve endings (Burden, 1985). Release of the 43 kD protein by brief pH 11 treatment from *Torpedo* postsynaptic membranes (Neubig et al., 1979) or from AChR clusters in rat myotubes (Bloch and Froehner, 1987) enhances the susceptibility of the AChR molecule to heat denaturation (Saitoh et al., 1979) or proteolytic degradation (Klymkosky et al., 1980) and increases its rate of rotation (Rousselet et al., 1979, 1980, 1982; Lo et al., 1980) and lateral diffusion (Barrantes et al., 1980; Bloch and Froehner, 1987). However, in *Torpedo marmorata*, at an early stage (45 nm embryo) of electrocyte differentiation, a large AChR cluster develops at one pole of the cell without any detectable association of the 43 kD protein (Kordeli et al., 1988). The 43 kD protein seems, therefore, not to be necessary for the initial aggregation of the AChR, but may consolidate the cluster once formed, for instance, by promoting its interaction with the cytoskeleton (Nghiêm et al., 1983; Cartaud et al., 1983; Kordeli et al.,1986, 1988).

Activity-Dependent Repression of AChR Biosynthesis

The elimination of extrajunctional AChR occurs in the embryo soon after neuromuscular contacts and AChR subneural clusters are established. In chick embryos, it can be prevented by chronic paralysis of the muscle (Giacobini-Robecchi et al., 1975; Burden, 1977; Bourgeois et al., 1978b; Betz et al., 1980). In adult muscle, extrajunctional AChR reappears over the entire myofiber surface when the motor nerve is cut. This effect, which is responsible for the enhanced sensitivity of denervated muscle to acetylcholine ("denervation hypersensitivity") (*see* Axelsson and Thesleff, 1959; Miledi, 1960) can be reversed by direct electrical stimulation of the muscle (Lømo and Rosenthal, 1972; Lømo and Westgaard, 1975; Bevan and Steinbach, 1977; *for review, see* Fambrough, 1979). These and

other results (*for review, see* Fambrough, 1979; Salpeter and Loring, 1985) clearly show that the neurally evoked electrical activity of the developing muscle fiber contributes to the disappearance of the AChR in extrajunctional areas.

The elimination of extrajunctional AChR is not accompanied by an alteration of its metabolic stability (Betz et al., 1977, 1980; Burden, 1977) and thus results from the repression of AChR biosynthesis. The mechanisms responsible for activity-dependent regulation of AChR biosynthesis can be studied in cultured muscle cells, which exhibit spontaneous (nonneurogenic) electrical activity and contractions (Cohen and Fischbach, 1973; Shainberg and Burstein, 1976; Betz and Changeux, 1979). Blocking the spontaneous activity of cultured chick myotubes with tetrodotoxin, an inhibitor of voltage-sensitive sodium channels, results in an increase of AChR levels. Conversely, when the spontaneous activity is enhanced, either by the sodium channel agonist veratridine, or by direct electrical stimulation, AChR levels fall below control values (Shainberg and Burstein, 1976; Betz and Changeux, 1979). Since firing of action potentials is accompanied by mechanical contraction of the muscle fiber, biochemical events linked to muscle contraction may regulate AChR biosynthesis. This possibility is ruled out by the analysis of the mutation *muscular dysgenesis* in the mouse. In this mutant, muscle cells fire action potentials but do not contract. Yet, AChR biosynthesis is repressed by electrical activity even in dysgenic myotubes, demonstrating that it is the electrical activity of the myotubes, and *not* their mechanical contraction, that regulates AChR biosynthesis (Powell and Friedman, 1977).

Potentially, there exist multiple levels in the pathway leading to the synthesis, assembly, and surface expression of a functional AChR at which regulation by electrical activity may occur. These include transcription into mRNA, mRNA processing and stability, translation into polypeptide chains, posttranslational modifications, transport and assembly of the subunit (α, β, γ, and δ) into the mature oligomer ($\alpha_2\beta\gamma\delta$), and insertion into the sarcolemma (Merlie and Smith, 1986). Recent studies from several laboratories indicate that activity may regulate AChR biosynthesis by both transcriptional and posttranslational mechanisms.

Transcriptional Regulation

As mentioned above, transcriptional activation of the genes coding for AChR subunits appears to be responsible for the burst of AChR expression that occurs in culture upon fusion of myoblasts into multinucleated myotubes (Buonanno and Merlie, 1986), a process that does not, however, directly depend on junctional innervation or electrical activity. Transcriptional regulation has also been implicated in the activity-dependent control of AChR expression.

Blocking the spontaneous electrical activity of cultured chick myotubes with tetrodotoxin was found to cause an up to 13-fold increase in the levels of mRNA coding for the α-subunit of the AChR (Klarsfeld and Changeux, 1985). Likewise, denervation of adult chicken, rat, and mouse muscles led to important rises in α-subunit mRNA content (Merlie et al., 1984; Klarsfeld and Changeux, 1985; Goldman et al., 1985) as well as in the levels of mRNAs coding for the other receptor subunits (mouse and rat: Evans et al., 1987; chick: Moss et al., 1987; Shieh et al., 1987,1988). These effects could result either from an enhanced transcription rate of the α-subunit gene, or from alterations in mRNA processing or degradation. Experiments with actinomycin D, an inhibitor of RNA synthesis, suggested that activity-dependent regulation occurs, at least in part, at the level of gene transcription (Fambrough, 1970; Shainberg et al., 1976). A similar conclusion was reached by Shieh et al. (1987) on the basis of their findings that the levels of α-subunit mRNA precursors, as well as the ratio of precursor/mature mRNA, increased upon denervation of chick skeletal muscle. Furthermore, Fontaine and Changeux (1989) have shown by *in situ* hybridization stud-

ies with intronic probes that unspliced α-subunit precursor mRNA accumulates in cultured myotubes after blocking spontaneous firing by tetrodotoxin, a finding recently confirmed by nuclease protection experiments (Klarsfeld et al., 1989). Final proof for control of AChR gene transcription by electrical activity must, however, await the direct determination of transcription rates using nuclear run-on assays.

Since the AChR is composed of four subunits, the expression of the four genes coding for these polypeptides has to be coordinated during development (Merlie and Smith, 1986). However, since several subunits are required in order to form a functional receptor (Mishina et al., 1984), the repression of a single subunit gene may be sufficient to account for the decrease of AChR that occurs upon innervation. The question thus arises as to whether or not a *common* regulatory sequence may exist, which would control in *cis* the expression of all subunit genes clustered at a common chromosomal locus. The linkage between the genes coding for the γ and δ subunits (Nef et al., 1984) indeed pointed to such a possibility. However, the recent chromosomal localization of the four genes encoding the α, β, γ, and δ subunits in the mouse revealed that while the γ and δ subunit genes cosegregate and are located on chromosome 1, the α- and β-subunit genes are found on chromosomes 2 and 11, respectively (Heidmann et al., 1986; Siracusa et al., in preparation).* The partial dispersion of these genes argues against the existence of a common *cis*-acting regulatory sequence. Consistent with this view, denervation of adult rat muscle elicited a relatively higher increase of the steady-state level of α, γ, and δ than β transcripts (Evans et al., 1987). In the chick, the levels of α transcripts increased more than those of the other subunits upon denervation (Shieh et al., 1988); moreover, the γ subunit messenger was not detected in innervated adult muscle (Moss et al., 1987; *see, however,* Shieh et al., 1988), but was present after denervation and in the embryo. In cultured chick myotubes, various agents that cause an increase in α-subunit mRNA levels, such as tetrodotoxin, 8-bromo-cAMP, forskolin, and ARIA were reported not to change the levels of γ- and δ-subunit transcripts (Harris et al., 1988; Shieh et al., 1988). Finally, a novel subunit referred to as ε probably replaces the γ-subunit at late stages of synapse maturation in mammals (Takai et al., 1985; Mishina et al., 1986). Distinct regulations may thus individually affect the genes coding for each of the AChR subunits.

Posttranscriptional Regulation

Much of our current knowledge concerning the posttranscriptional effects that activity exerts upon AChR biosynthesis comes from the studies of Merlie and colleagues (*reviewed by* Merlie, 1984; Merlie and Smith, 1986). Using pulse-chase labeling followed by immunoprecipitation with monoclonal antibodies specific for either native or immature receptor subunits, as well as velocity sedimentation analysis of newly synthesized α-subunits, these authors demonstrated that tetrodotoxin treatment of cultured rat myotubes caused a fourfold increase in the rate with which the newly synthesized α-subunit becomes assembled with the other receptor chains. Since the rate of α-subunit synthesis rose only twofold under the same conditions, it appears that in addition to its effect on mRNA availability, activity may control the processing of AChR subunits in this culture system (Carlin et al., 1986). This idea was corroborated by the finding that spontaneously active cultures contained a 5S precursor pool of the α-subunit that was absent in inactive cultures, where it was rapidly converted into as-

*The initial allocation of the α-subunit gene to chromosome 17 (Heimann et al., 1986) was based on its linkage with the α-cardiac actin gene, incorrectly assigned to chromosome 17 by Czosnek et al. (1983). Recent unpublished work by Siracusa et al. (in preparation) demonstrated linkage of the AChR α-subunit gene with the Abl gene, located on mouse chromosome 2.

sembled 9S receptor. Two mechanisms could account for the observed results (Carlin et al., 1986). First, activity may directly regulate a specific posttranslational event in the maturation of the α-subunit. Since tetrodotoxin treatment did not affect the acquisition by newly synthesized α-subunit of the ability to bind the AChR antagonist α-bungarotoxin, regulation must occur after this stage of maturation and immediately prior to assembly (Carlin et al., 1986). The relevant posttranslational modifications should occur in the endoplasmic reticulum, since that is where formation of the α-bungarotoxin binding site, as well as assembly of the receptor subunits take place (Smith et al., 1987). As pointed out by Smith et al. (1987), covalent posttranslational modifications of AChR subunits that may occur in the endoplasmic reticulum include disulfide bond formation and trimming of the oligosaccharide tree normally associated with AChR subunits. Fatty acid acylation has also been implicated in the regulation of AChR assembly (Olson et al., 1984a). Finally, in cultured chick muscle cells, the δ-subunit was found to be more highly phosphorylated in its unassembled vs assembled form, raising the possibility that a phosphorylation–dephosphorylation reaction plays a role in the regulation of AChR subunit assembly (Ross et al., 1987). It should be noted that an alternative hypothesis to account for activity-dependent modulation of the association between the α-subunit and other receptor chains is that this process is limited by the availability of the β, γ, or δ subunits, which may be regulated by distinct pre- or posttranslational mechanisms (Evans et al., 1987; Shieh et al., 1988).

Several other findings are consistent with the idea that AChR expression may be regulated at some step posterior to mRNA synthesis. Thus, following treatment of cultured myotubes with pharmacological agents that mimic the effects of electrical activity, AChR synthesis declined with a faster rate than that expected from inhibition of mRNA synthesis (Pezzementi and Schmidt, 1981). Furthermore, tetrodotoxin treatment of cultured chick myotubes led to a 13-fold increase in α-subunit mRNA content, which contrasted with an only twofold increase in the number of surface AChRs (Klarsfeld and Changeux, 1985). Similarly, during differentiation of the BC3H-1 muscle cell line (Olson et al., 1983,1984b) and development of the mouse muscle cell line C2 (Evans et al., 1987) discrepancies were observed between variations in the levels of translatable α-subunit mRNA and the actual changes in sarcolemmal AChR content. Finally, the finding that treatment of cultured chick myotubes with the tumor promoter 12-0-tetradecanoyl phorbol-13-acetate (TPA) reduced the number of surface AChR receptors without affecting α-subunit mRNA levels (Fontaine et al., 1987) also points to the existence of a posttranscriptional control mechanism.

Intracellular Signaling Mechanisms

Even though intensive efforts have been directed at the identification of the intracellular messengers that mediate the transsynaptic control of AChR biosynthesis, the mechanisms involved in this regulation are not securely identified to date. The reasons for the difficulty of these studies are severalfold. The first stems from the nonselectivity of most pharmacological tools used in such experiments. Furthermore, since pharmacological treatments may differentially affect any one of the steps involved in AChR biosynthesis, it may not be sufficient to analyze the changes in surface AChR alone, as has mostly been done so far. (These studies were only recently extended to the mRNA and mRNA precursor level; *see* Shieh et al., 1988; Klarsfeld et al., 1989.) Finally, the complex interactions between different cellular signaling systems (Nishizuka, 1986; Rasmussen et al., 1986) severely complicate the interpretation of the results. With these reservations in mind, the studies summarized below can be viewed as

a first step toward the identification of the chain of events linking neural activity to the characteristic changes in AChR biosynthesis.

Intracellular Ca^{2+}. The activity-dependent repression of AChR biosynthesis has been proposed to be mediated by Ca^{2+} ions, whose cytoplasmic concentration is known to rise transiently following depolarization of a muscle fiber. This idea is supported by the following line of evidence. AChR synthesis in skeletal muscle cells is reduced by pharmacological treatments that cause an increase in cytoplasmic Ca^{2+} concentration, including:

1. Electrical stimulation (Shainberg and Burstein, 1976) and veratridine, an agonist of voltage-sensitive Na^+ channels (Betz and Changeux, 1979; Shieh et al., 1988); and
2. The calcium ionophore A23187, which causes Ca^{2+} influx and depletes intracellular Ca^{2+} stores (Forrest et al., 1981).

Moreover, the tetrodotoxin-induced rise of AChR (Cohen and Fischbach, 1973; Shainberg and Burstein, 1976; Betz and Changeux, 1979; Klarsfeld and Changeux, 1985), which presumably results from a reduction of cytoplasmic Ca^{2+} concentration, is prevented by A23187 (McManaman et al., 1982; Rubin, 1985; Klarsfeld et al., 1989).

Conversely, AChR synthesis is increased by agents that are expected to cause a reduction of cytoplasmic Ca^{2+} concentration, including the calcium channel blockers D-600 (Shainberg et al., 1976; Shieh et al., 1983, 1988) and verapamil (Klarsfeld et al., 1989), and dantrolene sodium, a drug that inhibits the release of Ca^{2+} from the sarcoplasmic reticulum (Birnbaum et al., 1980). AChR appearance rate on the surface of cultures chick myotubes is also enhanced by trifluoperazine and chlorpromazine, two known inhibitors of calmodulin function (Schneider et al., 1984). However, based on drug combination experiments, these authors concluded that the effect of the phenothiazines was due to their ability to block voltage-dependent calcium channels, rather than to inhibition of calmodulin action. It might be added that these compounds have also been shown to inhibit protein kinase C activity (Schatzman et al., 1981). This example illustrates one of the above-mentioned difficulties of interpretation inherent in this kind of experiment.

The notion that a rise in cytoplasmic Ca^{2+} level shuts down receptor synthesis is seemingly contradicted by the finding that AChR levels in cultured chick myotubes rose in response to elevated extracellular Ca^{2+} concentrations (4–10 m*M*) and that low extracellular Ca^{2+} (20–50 μM) prevented the effect of tetrodotoxin in this system (Birnbaum et al., 1980). Moreover, in cultured rat (but not chick) myotubes, AChR levels decreased following incubation in calcium-deficient medium (McManaman et al., 1981; Birnbaum et al., 1980). These results were interpreted in the sense that an activity-dependent *decrease* of Ca^{2+} concentration in the sarcoplasmic reticulum or other cellular compartments, such as the nucleus, may trigger a reduction of AChR synthesis (Birnbaum, et al., 1981; McManaman et al., 1981). Interestingly, it has recently been shown that a depletion of intracellular Ca^{2+} stores, rather than a change in cytoplasmic Ca^{2+} concentration, is responsible for the induction of a group of genes by Ca^{2+} ionophores in hamster fibroblasts (Drummond et al., 1987). A regulation of this kind may involve posttranslational control mechanisms, since the endoplasmic reticulum, which is the main cellular Ca^{2+} store, is also one of the places where processing of newly synthetized poly-peptide chains takes place. It should be pointed out, however, that the effect described by Birnbaum et al. (1980) was not specific for Ca^{2+} ions, since a similar increase in AChR levels was obtained by elevating the extracellular Mg^{2+} concentration. Furthermore, it is not known to what extent external Ca^{2+} modulates the concentration and distribution of Ca^{2+} in cultured skeletal muscle cells. In analogy with other excitable tissues (smooth muscle: Williams et al., 1985; neurosecretory nerve endings: Brethes et al., 1987), myotube

Ca^{2+} levels may in fact remain relatively constant over a wide range of extracellular Ca^{2+} concentrations. The possibility exists, therefore, that nonphysiological variations of extracellular Ca^{2+} or Mg^{2+} indirectly affect AChR synthesis, for instance, by interfering with voltage-dependent channel gating (Hille, 1984). In any case, the evidence summarized above clearly indicates that AChR biosynthesis can be modulated by variations of the intracellular Ca^{2+} concentration. The relative importance of cytoplasmic Ca^{2+} vs that stored in intracellular organelles remains to be clarified, for instance, by using fluorescent dyes to follow the Ca^{2+} movements in cellular compartments (Williams et al., 1985) in parallel to the measurement of AChR appearance rate.

Protein Kinase C

Another signaling mechanism that appears to be involved in the regulation of AChR biosynthesis by muscle activity is the activation of the Ca^{2+}—and phospholipid—dependent protein kinase C (Nishizuka, 1986). Treatment of spontaneously active cultured chick myotubes with nanomolar concentrations of the protein kinase C activator TPA markedly decreased both the total content of cellular AChRs and the number of AChR on the surface of the myotubes (Fontaine et al., 1987). TPA reduced the rate of appearance of newly synthesized AChRs on the myotube surface but was without effect on AChR degradation. The phorbol ester appears to modulate AChR synthesis at more than one step. On the one hand, AChR α-subunit mRNA levels were not reduced by TPA in spontaneously active muscle cultures, indicating that the observed decrease of AChR expression took place at the posttranscriptional level. On the other hand, TPA did prevent the rise of α-sub-unit mRNA normally elicited by tetrodotoxin, suggesting that the phorbol ester can reduce AChR transcript levels, under conditions where they are not already maximally repressed by spontaneous discharges of the myotube (Fontaine et al., 1987).

Recent experiments provide further support for a role of protein kinase C in the repression of AChR biosynthesis. Indeed, it was shown that AChR precursor and mature mRNA levels, as well as AChR protein content of cultured myotubes, rose in response to the protein kinase C inhibitor staurosporine, and following prolonged exposure of the cells to TPA, which presumably results in downregulation of the kinase (Klarsfeld et al., 1989).

By affecting both mRNA levels and subsequent steps of AChR biosynthesis, TPA appears to mimic the effects of muscle activity. This finding is consistent with recent reports showing that ACh treatment, as well as electrical stimulation of skeletal muscle cells, stimulate the phospholipase C-mediated hydrolysis of polyphosphoinositides (Adamo et al., 1985; Vergara et al., 1985; Asotra and Vergara, 1986). One of the products of this reaction is diacylglycerol, an endogenous activator of protein kinase C (Nishizuka, 1986). Moreover, electrical stimulation of the sciatic nerve was shown to lead to the association of protein kinase C with particulate fractions of rat skeletal muscle, a process associated with activation of the kinase (Richter et al., 1987). The regulation of AChR biosynthesis may therefore involve the following signaling sequence: muscle activity —> stimulation of phosphoinositide-specific phospholipase C —> production of diacylglycerol —> activation of protein kinase C —> phosphorylation of regulatory proteins that, directly or indirectly, modulate the rate of AChR expression.

The precise mechanisms by which Ca^{2+} ions and protein kinase C regulate AChR biosynthesis are under current investigation. Four main possibilities can be considered:

1. The most conservative one is that the kinase is activated by the concerted action of Ca^{2+} ions, entering the cell via voltage-gated channels and/or released from the sarcoplasmic reticulum,

and diacylglycerol, which is produced upon stimulation of phospholipase C by muscle depolarization (Vergara et al., 1985).

2. The influx of Ca^{2+} ions may by itself suffice to activate protein kinase C, as proposed in other systems (Brocklehurst et al., 1985; Ho et al., 1988).
3. Activation of protein kinase C may enhance the entry of Ca^{2+} ions into the muscle cells (Navarro, 1987), possibly via phosphorylation of voltage-dependent calcium channels (O'Callahan et al., 1988).
4. Ca^{2+} ions may use additional pathways, perhaps independent of protein kinase C, e.g., involving calmodulin (Schneider et al., 1984).

Cyclic GMP. Cyclic nucleotides have also been implicated in the regulation of AChR biosynthesis. In many systems, a rise in cytoplasmic Ca^{2+} concentration is associated with an increase in cGMP concentration (Rasmussen et al., 1986). This was found to be also the case in cross-striated muscle of the giant barnacle, where cGMP levels rose in response to KCl depolarization or nerve stimulation (Beam et al., 1977). Similarly, in vertebrate skeletal muscle, activation of nicotinic AChR or direct electrical stimulation caused increases in cellular cGMP content (Nestler et al., 1978). These results pointed to the possibility that the nucleotide may play a role in the repression of AChR biosynthesis by muscle activity (Betz and Changeux, 1979). Support for this notion came from the finding that membrane-permeant analogs of cGMP reduced the number of AChRs on the surface of cultured chick myotubes without affecting their metabolic stability (Betz and Changeux, 1979). However, dissimilar results were reported by other authors (Blosser and Appel, 1980; Forrest et al, 1981; McManaman et al., 1982) possibly reflecting the fact that, under different experimental conditions, "basal" AChR biosynthesis may be repressed to different extents by spontaneous electrical activity. Thus, the question as to whether or not cGMP is involved in the control of AChR biosynthesis remains unanswered for the time being (*see also* Betz, 1980, 1983).

The Role of Neural Factors in the Persistence of AChR Biosynthesis at the Motor Endplate

The local increase of AChR density in the subsynaptic sarcolemma, which occurs soon after innervation of embryonic muscle fibers, forms out of a preexisting surface pool of AChR, in some species, but may also result in part from the preferential insertion of newly synthetized AChRs into the junctional membrane (Bursztajn and Fischbach, 1984; Role et al., 1985; *for review, see* Salpeter and Loring, 1985; Schuetze and Role, 1987). The simplest explanation for this phenomenon is that the motor nerve controls the local rate of AChR synthesis and/or membrane insertion in the immediate vicinity of the nerve-muscle contact. This idea is corroborated by the findings that intracellular AChRs (Pestronk, 1985), as well as α- and δ-subunit mRNAs (Merlie and Sanes, 1985), are enriched in the endplate regions of innervated rodent muscles. Local synthesis of mRNAs coding for the subunits of junctional AChR may occur in those nuclei that were found to be closely associated with AChR clusters (Bruner and Bursztajn, 1986; Englander and Rubin, 1987). Recent *in situ* hybridization studies demonstrated a highly preferential localization of AChR α-subunit mRNA at neuromuscular junctions, and, most importantly, on or around subsynaptic nuclei of 15-d-old chick skeletal muscles. As expected, denervation increased the level of α-subunit mRNA, and resulted in its distribution over the entire length of the muscle fiber, where it was localized around about 10% of the myonuclei (Fontaine et al., 1988) (*see* Fig. 2). Conversely, AChR α-subunit mRNA is distributed throughout chick muscle fibers at early stages of development and becomes progressively restricted to the endplate regions at later stages (Fontaine and Changeux, 1989). Similar results were obtained with strictly intronic probes. Moreover, the distribution of the label over muscle nuclei was found to vary

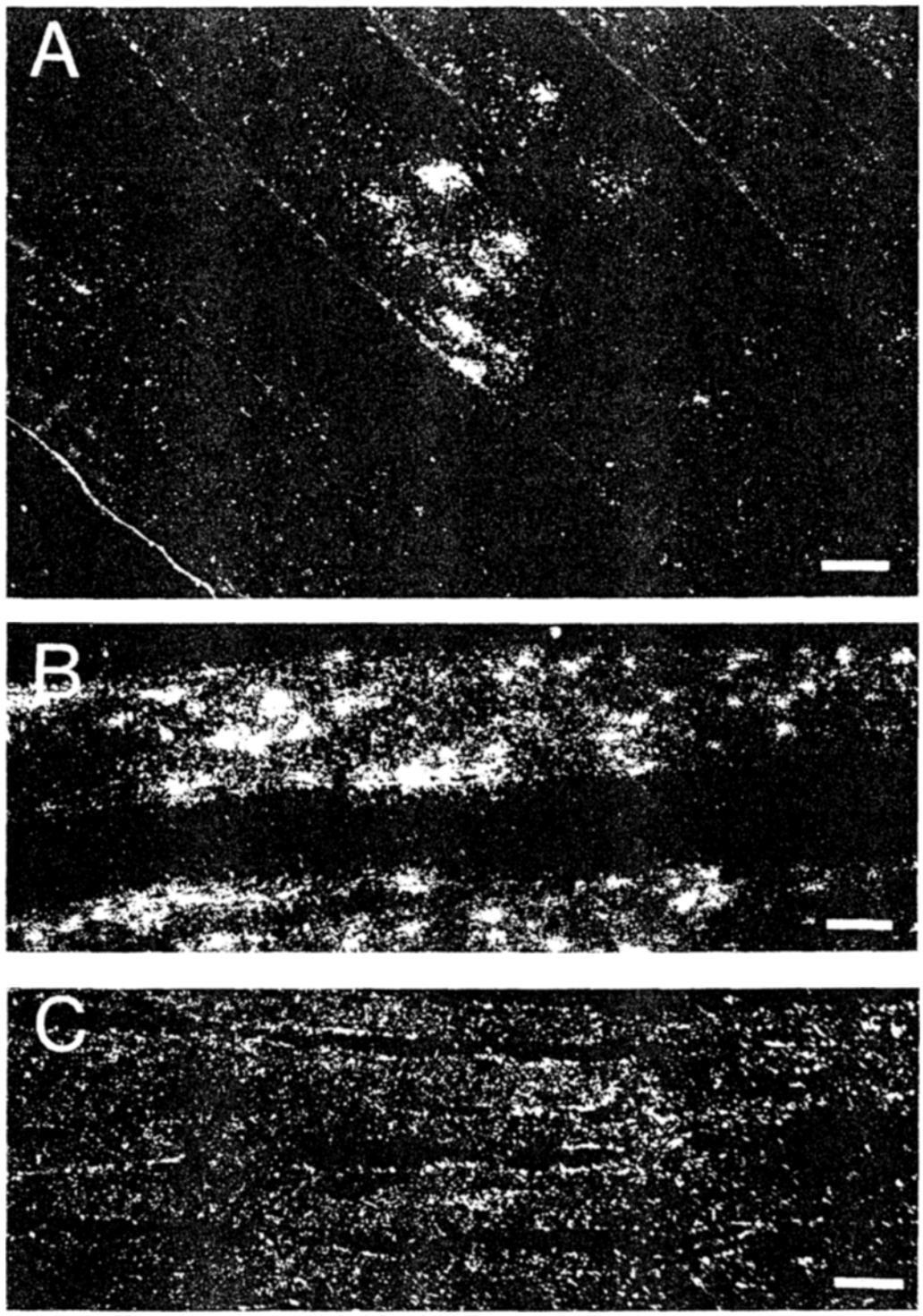

Fig. 2. Detection by *in situ* hybridization of AChR α-subunit mRNA in *posterior Latissimus dorsi* muscle of 15-d-old chick (from Fontaine et al., 1988). A. Innervated muscle. B. Denervated muscle. C. Innervated muscle hybridized with an actin probe.

in a discrete all-or-none fashion: some of the nuclei fully expressing the mRNA coexisted, in the same fiber, with almost completely silent ones.

Concerning the mechanisms by which motoneurons might stimulate the accumulation of subsynaptic AChR, experiments with cocultures of spinal cord explants and myogenic cells indicated that diffusible factors released from the motor nerve endings and different from ACh may be responsible for increased receptor synthesis (Cohen and Fischbach, 1977; Podleski et al., 1978). Several soluble factors of neural origin have been reported to increase the number of AChRs on the surface of cultured muscle cells without affecting their metabolic stability (Jessel et al., 1979; Neugebauer et al., 1985; Knaack et al., 1986; Fontaine et al., 1986; New and Mudge, 1986; and *for review, see* Salpeter and Loring, 1986; Schuetze and Role, 1987). For instance, ascorbic acid has been identified as the substance responsible for the AChR increase on rat L_5 myogenic cells, elicited by fetal calf brain extracts (Knaack and Podleski, 1985; Knaack et al., 1986). Ascorbic acid treatment also caused an increase in AChR α-subunit mRNA levels in L_5 cells (Knaack et al., 1987). Other brain-derived substances that can stimulate AChR synthesis in muscle cells include a low molecular weight, trypsin-sensitive peptide (Buc-Caron et al., 1983), as well as ARIA ("AChR-inducing activity"), a polypeptide of 42 kD purified from chicken brain (Usdin and Fischbach, 1986). Treatment of cultured chick myotubes with ARIA was shown to result in a specific increase in the levels of AChR α- (but not γ- or δ-) subunit mRNA, as well as of a putative nuclear precursor of the α-subunit mRNA, suggesting that the polypeptide stimulates transcription of the α-subunit gene (Harris et al., 1988). To date, it is not known whether any of the factors mentioned above is contained in, and/or can be released from, spinal cord motoneurons upon depolarization. Their possible involvement in motor endplate formation thus remains to be established.

One neuronal messenger has been identified, which is present in spinal cord motoneurons, and whose application to chick myotube cultures increases AChR synthesis (Fontaine et al., 1986; New and Mudge, 1986). Calcitonin gene-related peptide (CGRP), a neuropeptide of 37 amino acids, coexists (Hökfelt et al., 1986) with acetylcholine in several vertebrate motor systems (Rosenfeld et al., 1983; Gibson et al., 1984; Fontaine et al., 1986; New and Mudge, 1986), including nerve terminals of rodent neuromuscular junctions (Takami et al., 1985a,b). Treatment of cultured chick myotubes with CGRP (10^{-8}–$10^{-6}M$) led to a 30–50% increase in both sarcolemmal and total AChR levels, without affecting receptor degradation (Fontaine et al., 1986; New and Mudge, 1986). Moreover, CGRP elicited a threefold elevation of α-subunit mRNA levels (Fontaine et al., 1987). Interestingly, these responses occurred independently of the rise in AChR numbers elicited by tetrodotoxin treatment of the myotubes, indicating that different intracellular signaling mechanisms are involved in the regulation of AChR biosynthesis by CGRP and electrical activity, respectively (Fontaine et al., 1986, 1987). As in the case of other putative "anterograde" factors, an in vivo role for CGRP in the development of the neuromuscular junction has not been proven as yet.

There is increasing evidence suggesting that cAMP may serve as one of the second messengers that mediate the increase in subsynaptic AChR numbers elicited by motoneuronal "anterograde" factor(s). Indeed, AChR synthesis is increased by a variety of agents that are known to elevate cAMP levels in skeletal muscle cells, including membrane permeant analogs of cAMP (Betz and Changeux, 1979; Blosser and Appel, 1980), prostaglandin E1 (Betz and Changeux, 1979), β-adrenergic agonists (Blosser, 1983), and cholera toxin (Blosser and Appel, 1980). Treatment of cultured chick myotubes with cholera toxin, forskolin, or CGRP led to similar increases in AChR α-subunit mRNA content (Fontaine et al., 1987; Harris et al., 1988). Moreover, CGRP

was recently found to activate sarcolemmal adenylate cyclase, and to elevate muscular cAMP content (Takami et al., 1986; Kobayashi et al., 1987; Laufer and Changeux, 1987). The time— and concentration—dependence of these effects suggested that cAMP was responsible for the CGRP-induced stimulation of AChR biosynthesis (Laufer and Changeux, 1987).

Since the increase in AChR synthesis elicited by CGRP and other cAMP-mobilizing agents occurs even in the presence of tetrodotoxin or TPA, it appears that AChR synthesis can be regulated in parallel by cAMP-dependent and by Ca^{2+}-dependent mechanisms (Fontaine et al., 1986, 1987). These results, therefore, support the idea of a dual regulation of AChR biosynthesis, namely Ca^{2+}-dependent repression by muscle activity and Ca^{2+}-independent stimulation by "anterograde" factors released from the motoneuron. It should be emphasized that the action of other nerve-derived factors, such as ARIA, may not be mediated by cAMP (Harris et al., 1988), and that other, as yet unidentified second messengers, could be involved in the stimulation of AChR biosynthesis. It is, for instance, conceivable that some anterograde factors simply reverse the activity-dependent repression of AChR expression by antagonizing one of the signaling steps activated by membrane depolarization.

It was recently found that CGRP and other cAMP-mobilizing agents stimulate, in a Ca^{2+}-dependent fashion, the turnover of inositol phospholipids in cultured chick myotubes (Laufer and changeux, 1989). This effect is not likely to account for the increase in AChR biosynthesis elicited by CGRP, since stimulation of the phosphoinositide signaling system is expected to lead to activation of protein kinase C and thereby to diminish receptor levels. However, the possibility exists that in addition to the cAMP-dependent, Ca^{2+}-independent mechanism by which neural factor(s) proposedly stimulate the local expression of AChR genes under the nerve ending (Laufer and Changeux, 1987), cAMP may also play a role in the potentiation of Ca^{2+}-dependent signaling events triggered by muscle depolarization.

Regulation of AChR Channel Properties

In rat and several other species (but not in chicken), AChRs undergo a change in their channel properties from a slow opening embryonic type (mean open time 5 ms) to a fast opening adult type (mean open time about 1 ms) that also possesses a higher channel conductance (Katz and Miledi, 1972; Dreyer et al., 1976a,b; Neher and Sakmann, 1976; Fischbach and Schuetze, 1980). At birth, both channel types coexist, but during the first 3 wk of postnatal life, embryonic channels are gradually eliminated. Similarly to the elimination of extrajunctional AChRs, the appearance of fast AChR channels in rat muscle appears to depend on muscle activity, but not on the continuous presence of motoneurons (*for review, see* Salpeter and Loring, 1986; Schuetze and Role, 1987).

Three hypotheses have been advanced to account for the developmental change of AChR channel properties:

1. The existence of a single channel type, whose functional properties depend on its membrane environment (Michler and Sakmann, 1980);
2. The conversion of an embryonic-type channel into an adult-type channel by posttranslational modifications; and
3. The existence of two distinct gene products.

As discussed in detail by Schuetze and Role (1987), there is evidence in support of each one of these mechanisms, but the latter appears to make the most significant contribution in mammals. Indeed, recent experiments indicate that the different forms of AChR expressed during muscle development may arise from the replacement of the AChR γ-subunit by another subunit, termed ε, which was discovered in calf muscle, and shows higher homology to the δ-subunit than to any other AChR subunit (Takai

et al., 1985; Mishina et al., 1986). In *Xenopus*, oocytes injected with a combination of mRNAs coding for calf muscle α, β, γ, and δ subunits, AChRs possessing the functional properties of embryonic receptors were expressed. Conversely, adult-type receptors were expressed when the oocytes were injected with a mixture of α, β, ε, and δ mRNAs (Mishina et al., 1986; Witzemann et al., 1987). In addition, RNA blot hybridization experiments showed that the developmental expression of the γ- and ε-subunits corresponded with the relative abundances of fast and slow opening AChR channels. Thus, the content of mRNA coding for the γ-subunit decreased during fetal development and was not detectable after birth, whereas the opposite was true for the ε-subunit mRNA (Mishina et al., 1986; Witzemann et al., 1987).

Expression of Other Proteins at the Motor Endplate

During the development of the motor endplate, several muscular proteins become specifically enriched in subsynaptic regions of the muscle fiber, suggesting that their expression may be coregulated with that of the AChR (Changeux et al., 1987a,b; Klarsfeld, 1987). These "synaptic" proteins include (i) the heavy (16 S in mammals) asymmetric form of acetylcholinesterase (Massoulié and Bon, 1982; Rotundo, 1987); (ii) the voltage- and tetrodotoxin-sensitive sodium channel (Beam et al., 1985; Angelides, 1986; Dreyfus et al., 1986); (iii) the neural cell adhesion molecule (N-CAM; Cunningham et al., 1987), an integral membrane protein that is thought to mediate nerve–muscle interactions (Covault and Sanes, 1985); (iv) some basal lamina components (Sanes and Lawrence, 1983); and (v) the 43 kD protein (Sobel et al., 1977; Neubig et al., 1979; Frail et al., 1987).

As in the case of the AChR, motor innervation was shown to control the "production" of most of these proteins. Thus, muscle denervation leads to an increase in the biosynthesis of N-CAM, which shows striking parallels with that observed for the AChR. In particular, denervation causes reexpression of N-CAM in extrajunctional areas of the muscle fiber (Covault and Sanes, 1985; Covault et al., 1986). The density of tetrodotoxin-sensitive sodium channels increases following denervation of rat muscle between postnatal d 5 and 11 (Sherman and Catterall, 1982), and decreases upon denervation of adult rat and chick muscle (Colquhoun et al., 1984; Barchi and Weigele, 1979; Schmid et al., 1984). Furthermore, denervation of rat muscle induces the appearance of a type of voltage-sensitive Na^+ channel that possesses low affinity for tetrodotoxin (Redfern and Thesleff, 1971; Pappone, 1980). Enhanced expression of this channel may account for the 2–3-fold increase in the levels of total (tetrodotoxin-sensitive and insensitive) Na^+ channel mRNAs observed upon denervation of adult rat muscle (Cooperman et al., 1987). In the case of the heavy asymmetric form of acetylcholinesterase, denervation results in diminished protein levels (Massoulié and Bon, 1982; Rotundo, 1987). After denervation or chronic paralysis, the enzyme disappears (Giacobini et al., 1973; Betz et al., 1980; Rubin et al., 1980; Vigny et al., 1976); it reappears at the endplate after electrical stimulation (Lomo and Slater, 1980). It is not known, at present, whether the expression of the 43 kD υ_1 protein is subject to transsynaptic control (Frail et al., 1987).

The regulatory pathways involved in these biosynthetic control mechanisms appear to share some common features with those involved in AChR expression. Ca^{2+}-dependent signaling mechanisms have been implicated in the regulation of acetylcholinesterase and voltage-sensitive Na^+ channels by muscle activity (De La Porte et al., 1984; Sherman and Catterall, 1984; Rubin, 1985; Powell et al., 1986). Moreover, cAMP was shown to stimulate the biosynthesis of voltage-sensitive sodium channels in a depolarization-independent manner (Sherman et al., 1985) and to cause an increase in acetylcholinesterase levels of skeletal muscle (Lentz, 1972).

It is interesting to mention in this context that muscle activity and intracellular cAMP also modulate the expression of several other, non-synaptic proteins, such as ion transport systems that are thought to be involved in excitation-contraction-coupling, proteins of the contractile apparatus, and enzymes of muscular energy metabolism (*see, for instance,* Schmid et al., 1984, 1985; Schmid-Antomarchi et al., 1985; Matsuda et al., 1984; Lawrence and Salsgiver, 1984; Lebherz, 1984; Leberer et al., 1986; Wolitzky and Fambrough, 1986; Williams et al., 1987; Weydert, 1988). Moreover, cAMP reportedly promotes the formation of synapses between cocultured neuronal and skeletal muscle cells (Nirenberg et al., 1984), as well as the maintenance of sarcolemmal postsynaptic specializations (Lentz, 1972), suggesting that the nucleotide plays an important role in the development of the motor endplate.

Models for the Regulation of Synaptic Protein Expression During the Development of the Neuromuscular Junction

In summary:

1. In the course of skeletal muscle development, the biosynthesis of the AChR is submitted to several distinct regulations: i) an early increase of AChR biosynthesis related to myoblast differentiation, ii) an activity-dependent repression of AChR biosynthesis in extrajunctional areas, and iii) a persistence of AChR biosynthesis at the level of endplate "fundamental" nuclei.
2. There is evidence for transcriptional control of AChR gene expression in the case of regulations i and ii mentioned above.
3. Electrical activity may, to some extent, affect posttranscriptional processing of AChR subunits, and redistribution of AChR molecules on the surface of the myotubes.
4. Distinct second messengers systems are, most likely, involved in the subneural *positive* regulation of AChR biosynthesis and in its activity-dependent *negative* regulation outside the endplate.
5. Discoordinate expression of the AchR subunit genes and of other synaptic proteins may take place during endplate formation and maturation.

Thus, a complex network of regulatory interactions controls the expression of synaptic protein genes in the course of the terminal differentiation of skeletal muscle and the formation of the motor endplate, but only some of them depend upon functional innervation. On this basis, the proteins involved can be grouped into a minimum of three main families (Changeux et al., 1987a,b).

A vast ensemble of proteins does not significantly change after denervation before the onset of muscle atrophy. This *Family I* includes the "housekeeping proteins" and most (but not all) contractile proteins.

A second group of proteins referred to as *Family II* evolves in a manner similar if not identical to the AChR and their "production" increases upon denervation. It includes voltage-sensitive Ca^{2+} channels (Schmid et al., 1984), calcium-sensitive K^+ channels (Schmid-Antomarchi et al., 1985), the cell adhesion molecule N-CAM, some *basal lamina* components, the neurite outgrowth promoting factor active on chick spinal neurons (MNGF) (Henderson et al., 1983), and some contractile proteins and enzymes of energy metabolism. The cytoskeletal 43 kD protein, which selectively interacts with the AChR on its cytoplasmic face is also a potential candidate for such a regulation. It remains to be determined whether, as in the case of the AChR, these postdenervation increases reflect the relief from electrical activity-dependent gene repression (*see, however,* Covault et al., 1986). Such "negative" regulation by electrical activity should, of course, be counteracted by "anterograde" factor(s) (distinct from acetylcholine) at the level of the endplate, where these *Family II* proteins persist in the adult.

A third group of proteins, named *Family III*, is typified by the heavy 16–19.5 S form of acetylcholinesterase, whose levels decrease after denervation and whose accumulation at the endplate requires muscle activity. Tentatively, one may include in this family the AChR ε-subunit, whose expression is positively regulated by the activity of the muscle fiber, albeit at a much later stage than acetylcholinesterase.

Several mechanisms may be involved in controlling the expression of genes coding for these proteins, i) at the level of transcription, and ii) at various steps following transcription.

Regulation at the Level of Transcription

The model we will first discuss (Changeux et al., 1987a,b) deals exclusively with the *transcriptional regulation* of genes coding for proteins from Family II and III that have already been *determined*, or "committed," i.e., where the chromatin is in a "ready to be transcribed" state. Such regulation involves a *minimum* of five distinct components:

1. Extracellular first messengers;
2. Intracellular second messengers;
3. *Trans*-acting regulatory proteins binding to specific DNA regulatory sequences;
4. *Cis*-acting DNA regulatory sequences; and
5. Different categories of sarcoplasmic nuclei, according to their topological distribution in subneural or in nonjunctional areas.

The essential proposal of the model is that, within the same sarcoplasm, nuclei may exist under several discrete states of differentiation identified by the pattern of genes actually transcribed (and those switched off) that represent *"selections"* among the set of "open"or determined genes that characterize the committed myoblast cell lineage (*see* Changeux, 1986, for a discussion). These states may be classified, in a highly simplified manner, as follows:

In the *committed myoblast precursor* nuclei, the "housekeeping"and muscle-specific proteins from Family I are synthesized, but the transcription rates of the determined genes coding for Families II and III are negligible. In the *differentiated myoblast and myotube nuclei*, the genes coding for Families I and II are actively transcribed. A set of *trans*-acting proteins binding to *cis*-acting DNA sequences referred to as M (or muscle specific), should play a critical role at these stages. Such regulatory proteins might be encoded by genes such as the recently identified MyoD (Davis et al., 1987) or myd (Pinney et al., 1988) loci. The first and second messengers involved are not identified with certainty.

In the *adult extrajunctional nuclei*, the genes of Family I are switched on, whereas those of Family II and III are switched off. As a consequence, the muscle fiber becomes refractory to innervation. The first messenger is the electrical activity of the muscle fiber, and the putative second messenger is Ca^{2+}, and/or diacylglycerol. The *trans*-acting proteins involved are assumed to bind to putative *cis*-acting elements referred to as A (or activity-responsive) (*see* Fig. 3).

In the *junctional nuclei*, the genes of Family II (and possibly also some of Family I and III) are switched on. In other words, junctional nuclei express the genes coding for proteins that accumulate at the endplate region of the muscle fiber. "Synapse-specific" regulation might be conferred by *cis*-acting DNA sequences that respond to regulatory proteins encoded by hypothetical "synapse differentiation genes." These can be envisaged as being analogous to the muscle differentiation genes (such as MyoD) referred to above, but their expression would coincide with the "ultraterminal" differentiation of the muscle fiber, namely, the formation of the subsynaptic domain.

Once the "synapse-specific" genes have been turned on, their continuous expression in junctional nuclei depends on the release of various factors (such as, ARIA, ascorbate, or CGRP) from the motor nerve endings. The second messengers that mediate the regulation by neural factors are not identified with certainty, but cyc-

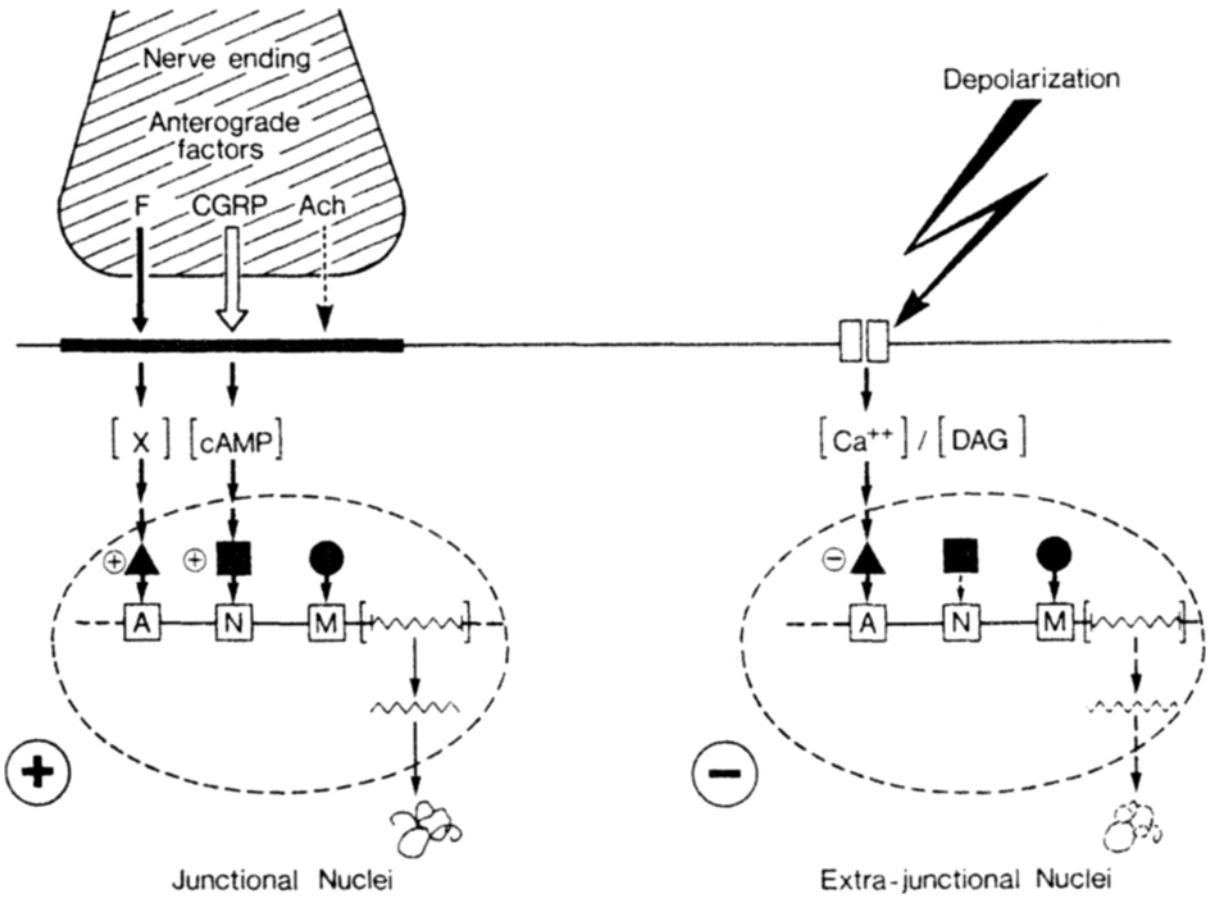

Fig. 3. Model for the regulation of AChR α-subunit gene expression in subneural and extrajunctional areas of the developing neuromuscular junction (modified from Changeux et al., 1987a). A, N, M represent regulatory DNA sequences that respond to muscle activity, nerve-derived factor, and muscle differentiation factors, respectively.

lic AMP appears as one plausible candidate (among others) (Laufer and Changeux, 1987). Specific regulatory DNA sequences (labeled N, or nerve-factor-responsive) are hypothesized to control the expression of the genes coding for synaptic proteins. In addition, anterograde factors released from the motoneurons may reverse the activity-dependent repression of AChR gene expression at the level of the subsynaptic nuclei by interfering with one of the signaling events triggered by the depolarization of the muscle fiber (*see* Fig. 3). This could, for instance, be achieved by inhibition of Ca^{2+} entry, or downregulation of protein kinase C (Niedel and Blackshear, 1986) in subneural regions.

The model presented above supposes the existence of *trans*-acting factors, which regulate the transcription of genes coding for synaptic proteins, and respond to the second messengers produced by muscle depolarization and neuronal messengers. These proteins may *not* be specific of the synapse. On the other hand, they

may interact with DNA regulatory elements in combination with the products of synapse differentiation genes, thus creating a "critical stage" or "sensitive period," where the expression of a given gene becomes sensitive to electrical activity and/or neural factors.

An important feature of the development of the postsynaptic domain is the existence of a time-order in gene expression, exemplified by the (not) —> γ—> ε transition of the AChR subunit genes in calf and rat. The mechanisms involved in such timing relationships are not known. One possibility is that the product of a given differentiation gene regulates "en cascade" the expression of other regulatory genes (*see* Blau, 1988).

Another original aspect is the discrete expression of the AChR α-subunits gene in some, but not all, nuclei of the same muscle fiber (Fontaine et al., 1988; Fontaine and Changeux, 1989). Such all-or-none switches might originate from closed feedback loops where, for instance, the product of a given regulatory gene activates its own transcription (*see* Monod and Jacob, 1961).

These schemes obviously correspond to oversimplified and schematized representations of the regulatory mechanisms actually involved in motor endplate genesis and maturation. The gene families, states of the nuclei, and DNA-binding proteins involved might be more numerous than postulated and might evolve in both sequential and parallel manner during development. For instance, if the change of mean channel open time, which occurs postnatally in amphibian and mammalian endplates, results from a switch in the expression of the gene coding for the γ-subunit to that coding for the ε-subunit (Mishina et al., 1986), then substates of the endplate nuclei have to be postulated between the newborn and the adult. One may then distinguish between "juvenile" and "adult" nuclei, which would express, respectively, the γ- and ε-subunit genes.

These simple-minded schemes, nevertheless, point to important issues: the identification of eventual "synapse differentiation" genes, of "second messenger regulation" genes and of the *cis*-acting sequences involved.

An important aspect of the model is the postulate that in the *same* cytoplasm, nuclei may coexist that are in *different* states of gene activation. This situation imposes constraints on the production, diffusion, and degradation of the first and second messengers from and into the subneural region of the muscle fiber. In particular, if one postulates that the anterograde signals liberated by the nerve ending positively regulate AChR gene expression in subneural areas, then a *change* of *sign* of the regulation must occur in the immediate vicinity of the endplate. This may simply result from the fact that different first messengers and independent intracellular pathways are involved in subneural vs extrajunctional regulation. Alternatively, or in addition, the *same* first messenger (for instance, CGRP or electrical activity) may switch on (or off) *different* second messengers in junctional vs extrajunctional areas as a consequence of a differential distribution of proteins involved in the genesis of these second messengers (such as Ca^{++} channels, G proteins, or protein kinases).

Another question concerns the *reversibility* of the state of differentiation of the subneural nuclei, for instance after denervation (*see* Loring and Salpeter, 1980; Salpeter and Loring, 1985) or in a more general manner, the *stability* of the whole synapse. Family II includes components of the postsynaptic domain but also proteins involved in cell surface adhesion (N-CAM, basal lamina antigens) and in transsynaptic "retrograde" signaling such as the MNGF(s) (Henderson, 1987). The maintenance of MNGF production by the endplate nuclei may create a *positive* feedback loop upon the motor nerve ending which, in combination with an anterograde *positive* factor, like CGRP, will constitute a closed circuit. The whole synapse may then be in a far-from-equilibrium but stable steady-state, which becomes resistant to protein turnover (*for a discussion, see* Changeux and Heidmann, 1987; Changeux et

al., 1987b). Such a mechanism may be utilized for the selection of synapses in the course of development (Gouzé et al., 1983).

Several aspects of the model may be experimentally tested, for instance, by the identification of the first and second messengers involved in the regulation of the patterns of mRNA expressed in subneural vs extrajunctional nuclei, by the analysis of the regional distribution of mRNA primary transcripts (e.g., by *in situ* hybridization with intronic probes), by the identification of the *cis*-acting DNA sequences, *trans*-acting proteins, and putative "synapse differentiation " genes.

Posttranscriptional Regulation

Regulation at the transcriptional level is only one among several regulatory processes that may contribute to the development and maturation of the postsynaptic domain. There are several steps that could be subject to posttranscriptional control.

1. Regulation of mRNA stability and transport have been described in several systems, but so far not in the case of synaptic proteins;
2. The processing of the translated subunits, their conformational maturation (Merlie and Smith, 1986) and their assembly into a functional oligomer; phosphorylation-dephosphorylation of the subunits (*review* Changeux et al., 1987b; Greengard, 1987; Huganir and Greengard, 1987) may, for instance, control their assembly (Ross et al. 1987) and could be the target of an activity-dependent regulation via the second messengers mentioned above;
3. The targeting of the assembled oligomer from the Golgi apparatus toward the postsynaptic membrane may also be regulated by muscle activity; and
4. The number of surface AChR molecules is, as in the case of other membrane proteins, regulated by an internalization process that may lead to degradation of the protein. A possible mechanism for the metabolic stabilization of the AChR, which takes place during the late development of the endplate, might be the setting out of a device that protects against such internalization. The development of the subneural foldings and their consolidation by a specialized cytoskeleton may play a role in this process. There, again, phosphorylation-dephosphorylation reactions might be involved.

Regulation of Neuronal Protein Biosynthesis

In both the central and the peripheral nervous system, stimulation or inhibition of neuronal activity are often associated with characteristic long-term changes in the "sensitivity" of postsynaptic neurons to neurotransmitters and pharmacological agents (*see, for example,* Schuetze and Role (1987) and Nathanson (1987) for reviews on the regulation by innervation of acetylcholine sensitivity in nicotinic and muscarinic neurons, respectively). The efficiency of synaptic transmission may be modulated by innervation-dependent changes in the levels of neurotransmitter receptors (Schwartz et al., 1983), of enzymes involved in neurotransmitter biosynthesis (Zigmond and Bowers, 1981; Mallet et al., 1983; Thoenen and Acheson, 1987) and degradation (Koelle and Ruch, 1983), of neuropeptide precursors (Black et al., 1987), or of neuronal growth factors (Henderson, 1987). These changes can be brought about by posttranslational modifications (e.g., phosphorylation) of preexisting receptor or enzyme molecules (examplified by the "acute" regulation of tyrosine hydroxylase activity by depolarization or neuropeptides; *review* Zigmond, 1985; Rittenhouse et al., 1988), or by activity-dependent regulation of one of the steps involved in the biosynthesis of a particular neuronal protein. We will be concerned here only with the latter process, which in recent years became amenable to investigation by the methods of recombinant DNA technology.

The following chapters focus on the most extensively studied cases of activity-dependent control of gene expression in the nervous sys-

tem, namely, the transsynaptic regulation of enzymes involved in catecholamine biosynthesis and of neuropeptide precursors. This section also deals with control mechanisms involved in the biosynthesis of neurotransmitters and hormones in the adrenal medulla as well as in the anterior and intermediate lobes of the pituitary gland. Indeed, since these organs resemble central nervous tissues in many aspects, but are better characterized and more accessible from an experimental point of view, they have been widely used as model systems for activity-dependent regulation of gene expression in the central nervous system.

Tyrosine Hydroxylase

Tyrosine hydroxylase (TH) catalyzes the rate-limiting reaction in the biosynthesis of catecholamines and, therefore, plays a key role in adrenergic transmission. Reflex stimulation of the sympathetic nervous system by environmental stress or administration of reserpine leads to a 2–3-fold increase in TH activity in sympathetic ganglia and adrenal medulla. TH levels maximally rise within 48 h of a brief (30 min) stimulation, and remain elevated for several days. The rise in TH activity is mediated by transsynaptic stimulation of adrenergic cells, since it can be prevented by cutting the preganglionic cholinergic fibers, and induced by directly stimulating the afferent nerves (for recent reviews, *see* Zigmond, 1985; Thoenen and Acheson, 1987; Black et al., 1987).

Early experiments had shown that the increase in TH activity could be prevented by inhibitors of RNA and protein synthesis (Mueller et al., 1969) suggesting that it was due to specific stimulation of TH biosynthesis. This was later confirmed by pulse-labeling and immunotitration experiments showing that transsynaptic stimulation of adrenergic cells results in enhanced production of TH molecules (Joh et al., 1973; Reis et al., 1975; Zigmond et al., 1978). Finally, it was established that transsynaptic stimulation causes a specific increase of TH mRNA levels in rat adrenal (Mallet et al., 1983; Tank et al., 1985; Stachowiak et al., 1985) and superior cervical ganglion (Black et al., 1985), which precedes the rise in enzyme activity (Faucon-Biguet et al., 1986).

Activity-dependent stimulation of TH synthesis is not restricted to the periphery, but also occurs in the central nervous system. Reserpine treatment or transneuronal stimulation increased TH levels in catecholaminergic cells of rat locus coeruleus (Reis et al., 1974; Zigmond et al., 1974), olfactory bulb (Baker et al., 1983), cerebellum and frontal cortex (Black, 1975). In the rat locus coeruleus, reserpine treatment caused a fourfold increase in TH mRNA levels after 2 d, which preceded a twofold rise in enzyme activity. This effect is remarkably long-lasting, as TH mRNA levels in the locus coeruleus of reserpine-treated animals remained significantly elevated for up to 18 d after drug administration (Faucon-Biguet et al., 1986). A similar increase of TH expression was observed in a recent study in which TH mRNA was localized in catecholaminergic neurons of the rat locus coeruleus by the *in situ* hybridization technique (Han et al., 1987). Thus, TH mRNA levels can be regulated by impulse activity in both the central and peripheral nervous systems. At present, however, it is not known whether the increased mRNA levels reflect a net increase in the rate of mRNA synthesis or a specific stabilization of TH mRNA (the former has, however, been shown to occur in response to an elevation in intracellular cAMP; *see below*).

Since the proposal by Axelrod (1971) of a gene regulation hypothesis to account for the transsynaptic stimulation of TH activity, there have been efforts to identify the putative second messengers mediating the membrane to gene signaling mechanism. The initial event in transsynaptic regulation of TH production in the sympathetic nervous system has been shown to be the activation of nicotinic cholinergic receptors by ACh released from the preganglionic

nerves (Boenisch et al., 1980; Chalazonitis et al., 1980). Cyclic AMP has been put forward as a second messenger that links nicotinic action to enhanced TH synthesis in adrenal medulla (Guidotti and Costa, 1977). This hypothesis was inferred from the observations that transsynaptic stimulation led to an increase in adrenal cAMP levels, and that treatment of cultured chromaffin cells with 8-Br-cAMP resulted in increased TH synthesis. Moreover, since 8-Br-cAMP as well as transsynaptic stimulation in vivo also enhanced cAMP-dependent protein kinase activity and the phosphorylation of nuclear proteins, it was proposed that these events are involved in the regulation of TH synthesis (Guidotti and Costa, 1977; Kumakura et al., 1979). Although the physiological significance of these findings has been disputed (Thoenen and Acheson, 1987), it seems that at least in adrenal medulla, cAMP can mimic the effect of transsynaptic stimulation. Treatment with 8-Br-cAMP was shown to increase TH mRNA levels in rat pheochromocytoma cells (Lewis et al., 1983) by stimulating the rate of transcription of the TH gene (Lewis et al., 1987). Furthermore, a 5' flanking sequence of the TH gene (nucleotides –272 to +27) was found to confer cAMP responsiveness when placed upstream of the bacterial CAT gene and transfected into rat pheochromocytoma and GH4 pituitary cell lines (Lewis et al., 1987). This DNA fragment comprises a highly conserved sequence (at –44 to –37), which is present in the regulatory regions of other cAMP-responsive genes (*see below*).

In sympathetic ganglia, different mechanisms appear to be involved in TH regulation, since dibutyryl-cAMP and the adenylate cyclase activator cholera toxin failed to increase TH levels in cultured sympathetic neurons (Hefti et al., 1982). In this system, TH activity (Hefti et al., 1982) and mRNA levels (Raynaud et al., 1987) rose in response to depolarization with high K^+ medium. The depolarization-evoked increase of TH levels could be prevented by calcium channel blockers and calmodulin inhibitors, suggesting that Ca^{2+} ions play a role in this process (Hefti et al., 1982). However, depolarization *per se* does not increase TH levels in sympathetic ganglia in vivo or in organ culture (Otten and Thoenen, 1976; Thoenen and Acheson, 1987). The situation may be complicated by the possible existence of distinct regulatory mechanisms operating at different developmental stages (Thoenen and Acheson, 1987).

Proenkephalin

Pharmacological or surgical blockade of synaptic transmission to the rat adrenal gland leads to increases in adrenal enkephalin content (Fleminger et al., 1984; La Gamma et al., 1984), with an accompanying rise in the levels of pro-enkephalin mRNA coding for the precursor protein of enkephalin peptides (Kilpatrick et al., 1984). This effect could be reproduced in vitro by explanting rat adrenal medullae into culture, thereby relieving them from the influence of nerve activity. After 2–3 d, in explant culture, enkephalin levels rose 50-fold, and this increase could be inhibited by depolarizing agents, which presumably mimic the effects of neuronal impulse activity (La Gamma et al., 1985). Nuclear run-on assays have recently shown that the rate of transcription of the proenkephalin gene is specifically and strongly inhibited by culturing explanted adrenal medullae in depolarizing, high K^+ medium (Black et al., 1987). It thus appears that regulation occurs, at least in part, at the level of gene transcription. No information is available so far as to the intracellular signaling mechanisms involved in this regulation.

Enkephalin biosynthesis can be modulated by various pharmacological treatments in cultured bovine adrenal chromaffin cells (Kley, 1988). In this system, however, nicotinic receptor stimulation elevates rather than reduces, proenkephalin mRNA and Met-enkephalin levels (Eiden et al., 1984a). The effect of nicotinic receptor stimulation appears to be mediated by Ca^{2+} ions entering the cell via voltage-gated

channels activated by membrane depolarization (Eiden et al., 1984a; Kley et al., 1986; Kley et al., 1987). Proenkephalin mRNA levels can also be increased by cAMP (Eiden et al., 1984a,b; Quach et al., 1984) and by activators of protein kinase C (Kley, 1988). Consistent with these findings, the 5'-flanking sequence of the human proenkephalin gene was found to contain two cAMP-and phorbol ester-responsive regulatory elements, designated ENKCRE-1 and ENKCRE-2, which bind distinct *trans*-acting factors. The latter element contains the CGTCA sequence common to cAMP response elements in a variety of genes (Comb et al., 1986, 1988). Kley et al. (1987) suggested that the nucleotide acts by stimulating Na^+ and/or Ca^{2+} influx into the chromaffin cells. However, based on pharmacological evidence, these authors concluded that although a rise in intracellular cAMP may contribute, besides the increase in Ca^{2+} entry, to the effect of nicotinic receptor stimulation, the nucleotide probably plays no major role in the regulation of proenkephalin gene expression by impulse activity. It is, however, conceivable that cAMP will be found to mediate some hitherto undiscovered modulating effects of coexisting neuronal messengers.

Impulse activity regulates enkephalin biosynthesis in the central nervous system as well. After 10 d of daily administration of electroconvulsive shocks to rats, proenkephalin mRNA levels in the hypothalamus were found to be 75% higher than in untreated controls (Yoshikawa et al., 1985). Lesion of the dentate gyrus hilus of the rat, which causes recurrent hippocampal seizures, elicited a 14–15-fold rise in proenkephalin mRNA levels in dentate gyrus granule cells after 24 h (White et al., 1987). A qualitatively similar effect was observed by *in situ* hybridization, following electrical stimulation of the rat dentate gyrus in vivo (Morris et al., 1988). Chronic administration of a dopamine receptor antagonist (Sabol et al., 1983; Tang et al., 1983) or pharmacological lesion of mesencephalic dopamine neurons (Young et al., 1986) led to increased proenkephalin mRNA levels in the rat caudate putamen, which receives dopaminergic innervation. It has been hypothesized (Yoshikawa et al., 1987) that a rise in proenkephalin mRNA levels may be the result of a feedback regulation triggered by stimulation of transmitter releases from enkephalinergic neurons. It should be noted, however, that in the case of other hormones and neurotransmitters, such as somatostatin and growth hormone, regulation of precursor biosynthesis can occur independently of transmitter release (Barinaga et al., 1985; Montminy et al., 1986).

Prolactin

The biosynthesis of prolactin in the pituitary gland is regulated by a variety of hormones and neurotransmitters, including epidermal growth factor (EGF), glucocorticoids, dopamine, vasoactive intestinal polypeptide, and thyrotropin releasing hormone (TRH)(Tashjian, 1979). Measurements of nuclear prolactin mRNA precursors and of mRNA synthesis by isolated nuclei demonstrated that the dopamine agonist ergocryptine decreased, whereas TRH increased the rate of transcription of the prolactin gene in pituitary cells (Maurer, 1981; Potter et al., 1981; Murdoch et al., 1983). Further evidence for transcriptional regulation of prolactin gene expression by TRH comes from gene transfer studies. Chimeric genes constructed by fusing 5'-flanking sequences of the bovine prolactin gene to the bacterial CAT gene were transfected into the GH_3 rat pituitary cell line, where their expression could be stimulated by TRH. Deletion experiments showed that the DNA sequence necessary to confer hormonal responsiveness to the fusion gene lies within 250 base pairs of the prolactin gene promoter. Interestingly, this short DNA sequence also contains the regulatory regions that respond to EGF and glucocorticoid treatment (Camper et al., 1985).

The signaling mechanisms involved in the regulation of prolactin gene expression seem to

call into play the actions of several interacting second messengers. Transcription of the prolactin gene could be induced by elevating intracellular cAMP levels (Maurer, 1981; Murdoch et al., 1982). Stimulation or inhibition of pituitary adenylate cyclase by vasoactive intestinal polypeptide and dopamine, respectively, may thus underlie the opposed effects of these agents on prolactin gene expression (Maurer, 1981; Gourdji et al., 1979). Increases in intracellular cAMP and prolactin gene transcription were associated with the phosphorylation of two chromatin-associated proteins, designated BRP and CBP (Murdoch et al., 1982; Waterman et al., 1985). However, no evidence exists so far as to the possible involvement of these nuclear phosphoproteins in the regulation of prolactin gene transcription. Interestingly, cAMP mobilization also led to increased transcription of the growth hormone gene in GH4 cells, but this effect seems to be regulated by a distinct molecular mechanism (Waterman et al., 1985). Thus, cAMP-induced transcription of the prolactin gene, as well as phosphorylation of BRP, one of the nuclear proteins referred to above, could be inhibited by Co^{2+}, suggesting that these responses involve a Ca^{2+}-dependent reaction. In contrast, Co^{2+} potentiated cAMP-induced transcription of the growth hormone gene and phosphorylation of CBP. It appears, therefore, that cAMP can simultaneously regulate the expression of different genes by activating two distinct signaling pathways, one of which is Ca^{2+}-dependent (Waterman et al., 1985).

A Ca^{2+}-sensitive pathway seems to be activated by TRH as well. Stimulation of prolactin gene transcription by TRH required cytoplasmic Ca^{2+} (White and Bancroft 1983) and was inhibited by agents that antagonize various Ca^{2+}-dependent processes (Murdoch et al., 1985). The same is true for TRH-stimulated phosphorylation of the chromatin-associated protein BRP (Murdoch et al., 1983, 1985). Since TPA, an activator of Ca^{2+}-dependent protein kinase C mimicked the nuclear actions of TRH, it has been proposed that these involve stimulation of phosphoinositide breakdown, and ensuing activation of protein kinase C by diacylglycerol as primary signaling events (Murdoch et al., 1985).

Gene transfer experiments, in which a rat prolactin-growth hormone fusion gene was introduced into a human epidermal carcinoma cell line, demonstrated that regulatory sequences necessary for TPA- (and EGF)-evoked stimulation of transcription lie within –3.0 kb to + 0.8 kb of the rat prolactin gene (Supowit et al., 1984). Deletion analysis of this sequence localized the *cis*-active regulatory element responding to TPA and EGF to the –78 to +34 fragment (Elsholtz et al., 1986). The regulatory sequence was found to act as an inducible enhancer element (Maniatis et al., 1987) when placed in either orientation upstream or downstream of CAT fusion genes under the control of the rat growth hormone or herpes simplex virus thymidine kinase promoters. Even though further deletions of the –78 to +34 sequence greatly reduced responsiveness to TPA or EGF, a fragment as short as the –78 to –30 sequence was found to confer regulation to the CAT gene when placed in the inverted orientation in front of the thymidine kinase promoter. Interestingly, a similar effect was obtained with two tandem copies of the –79 to –30 fragment in the direct orientation (Elsholtz et al., 1986). It thus appears that even though its adjoining sequences are necessary for optimal regulation, the –79 to –30 fragment contains elements that respond to hormonal stimulation. This short sequence also interacted with nuclear proteins from GH4 cells, which may correspond to hormonally regulated transacting factors (Elsholtz et al., 1986).

Proopiomelanocortin

The production of proopiomelanocortin (POMC), the biosynthetic precursor of adrenocorticotrophic hormone, melanocyte-stimulating hormone and β-endorphin, is under the control of catecholamines and corticotropin re-

leasing factor (CRF) in the intermediate and anterior pituitary lobes, respectively (*review* Reisine and Affolter, 1987). Dopamine agonists reduced and dopamine antagonists increased POMC mRNA levels in rat intermediate lobe (Höllt et al., 1982; Chen et al., 1983; Cote et al., 1986). These effects are thought to be mediated by cAMP, since dopamine inhibits adenylate cyclase in pituitary cells, and intracellular cAMP increases POMC mRNA levels (Cote et al., 1986). Cyclic AMP seems to regulate POMC expression in the anterior lobe as well, where CRF, an activator of adenylate cyclase, increases POMC mRNA levels (Höllt and Haarmann, 1984; Affolter and Reisine 1985). The rise in POMC mRNA can be mimicked by Br-cAMP, and, interestingly enough, also by TPA(Affolter and Reisine, 1985), indicating that, as in the case of prolactin gene regulation, multiple second messenger systems may be involved in the control of POMC expression. Further support for this view comes from the experiments of Reisine et al. (1985), demonstrating that the introduction of an inhibitor of cAMP-dependent protein kinase into a pituitary cell line, using a liposome technique, specifically prevented the rise in POMC mRNA elicited by CRF and Br-cAMP, but not by TPA. POMC expression seems to be regulated at the transcriptional level, and a cAMP-responsive regulatory element has been identified at approximately 275 basepairs upstream of the site at which transcription of the POMC gene is initiated (Roberts 1986).

Other Proteins

The regulation of substance P biosynthesis in the rat superior cervical ganglion in vivo resembles that of enkephalin in the rat adrenal medulla. Substance P levels are increased by decentralization or blockade of nicotinc transmission, and reduced by stimulation of preganglionic nerves (Kessler and Black, 1982). Interestingly, TH levels in this system are regulated in the opposite way. Explantation of the rat superior cervical ganglion into culture led to an increase in substance P levels (Kessler et al., 1981), preceded by a rise in the levels of mRNA coding for the substance P precursor preprotachykinin. Both effects could be prevented by culturing the ganglia under depolarizing conditions (Roach et al., 1987). In the rat brain, preprotachykinin mRNA levels are controlled by the activity of dopaminergic neurons. Administration of a dopamine antagonist (Bannon et al., 1986, 1987) or lesion of dopaminergic neurons (Young et al., 1986) depressed preprotachykinin mRNA levels in basal ganglia. The molecular mechanisms involved in the regulation of tachykinin biosynthesis have not been elucidated as yet.

Other examples of neuronal proteins whose production is controlled by impulse activity include choline acetyltransferase (Ishida and Deguchi, 1983), acetylcholinesterase (Ishida and Deguchi, 1983; Koelle and Ruch, 1983), dopamine β-hydroxylase, which catalyzes the conversion of dopamine to norepinephrine (O'Malley et al., 1983; Thoenen and Acheson, 1987) and serotonin *N*-acetyltransferase, which is involved in the synthesis of melatonin from serotonin (Zigmond and Bowers, 1981). In the latter case, it has been shown that the circadian increase in enzyme activity that occurs in retina and pineal gland during darkness can be mimicked by elevating intracellular cAMP (Zigmond and Bowers, 1981; Iuvone and Besharse, 1986). In the pineal gland, the rise in cAMP is elicited by norepinephrine released from afferent sympathetic nerves at night (*review* Zigmond and Bowers, 1981). It should be noted that although transsynaptic stimulation has been shown to cause *de novo* synthesis of dopamine β-hydroxylase in rat adrenal medulla (Ciaranello et al., 1975) and brain (Sabban et al., 1987), this has not been demonstrated as yet for the other proteins mentioned above. In neither case have corresponding changes in mRNA levels been reported.

It should be stressed in this context that the expression of neuronal proteins can also be regulated by posttranscriptional mechanisms. For instance, the Ca^{2+}-dependent increase of mus-

carinic acetylcholine receptor numbers evoked by chronic membrane depolarization in cultured neuroblastoma cells (*review* Nathanson, 1987) is caused by an inhibition of receptor degradation, rather than by stimulation of its synthesis (Liles and Nathanson, 1987). An additional example of a posttranslational control mechanism is the cAMP-evoked increase in the proportion of functional acetylcholine receptors in the cell membrane of cultured neurons (Margiotta et al., 1987). However, the mechanisms involved in these processes are unknown for the time being.

An interesting case of activity-dependent regulation was described by Greenberg et al. (1986) who found that transcription of the c-*fos* protooncogene could be rapidly and transiently induced by nicotinic receptor stimulation in a nondividing, neuronally differentiated pheochromocytoma cell line. The induction of c-*fos* expression by cholinergic agonists appears to be mediated by the influx of Ca^{2+} ions via voltage-gated channels activated by membrane depolarization (Greenberg et al., 1986; Morgan and Curran, 1986). A Ca^{2+}-responsive regulatory sequence has been localized within the c-*fos* gene promoter (Sheng et al., 1988). In cultured rat cerebellar granule cells, the expression of c-*fos* is regulated by the neurotransmitter glutamate (Szekely et al., 1988). Since c-*fos* is thought to serve as a sort of switch for turning on other genes in response to various external stimuli, it has been proposed that its activity-dependent induction could play a role in tissue-specific gene expression or information storage in the nervous system (Greenberg et al., 1986; Morgan and Curran 1986).

cAMP-Regulated Genes

As illustrated above, neuronal gene expression can be regulated by depolarization- or receptor-mediated activation of transmembrane signaling systems. The second messenger whose effects on gene transcription have been most thoroughly studied is cAMP (Comb et al., 1987; Roesler et al., 1988). In addition to the examples presented above, cAMP stimulates the expression of the genes coding for the neuropeptides somatostatin and vasoactive intestinal polypeptide in neuronal cells (Hayakawa et al., 1984; Montminy et al., 1986a). These genes are flanked by short regulatory regions that confer cAMP responsiveness to heterologous genes (Montminy et al., 1986b; Tsukada et al., 1987) and possess properties similar to those of other inducible transcriptional enhancer elements (Yaniv, 1987; Maniatis et al., 1987). Cyclic AMP-responsive elements contain a highly conserved core motif that is identical or similar to the 8-basepair palindrome 5'-TGACGTCA-3', frequently flanked at its 5' end by a G + C-rich region, and at its 3' end by sequences containing the dinucleotide AG (Montminy et al., 1986b). Related or identical *trans*-acting factor(s) appear to be involved in the transcriptional regulation of these cAMP-responsive elements (Hyman et al., 1988; Deutsch et al., 1988). Cyclic AMP-responsive DNA binding proteins (designated CREB) have recently been purified (Montminy and Bilezikjian, 1987) and cloned (Hoeffler et al., 1988). These proteins possess the functional and structural properties of transcription factors (Yamamoto et al., 1988; Hoeffler et al., 1988).

Models of Activity-Dependent Regulation of Gene Expression

The Chemical Singularity of the Neuron

The foregoing examples show that, in the systems investigated, the state of activity of the neuron regulates the transcription of genes coding for neuropeptides and neurotransmitter-synthesizing enzymes. In other words, the biochemical phenotype of the neuron results to some extent from an "epigenetic" regulation of gene expression. The notion of "singularity" was formerly introduced to specify that within a given *category* of nerve cells the connectivity of an individual neuron exhibits a "fringe" of

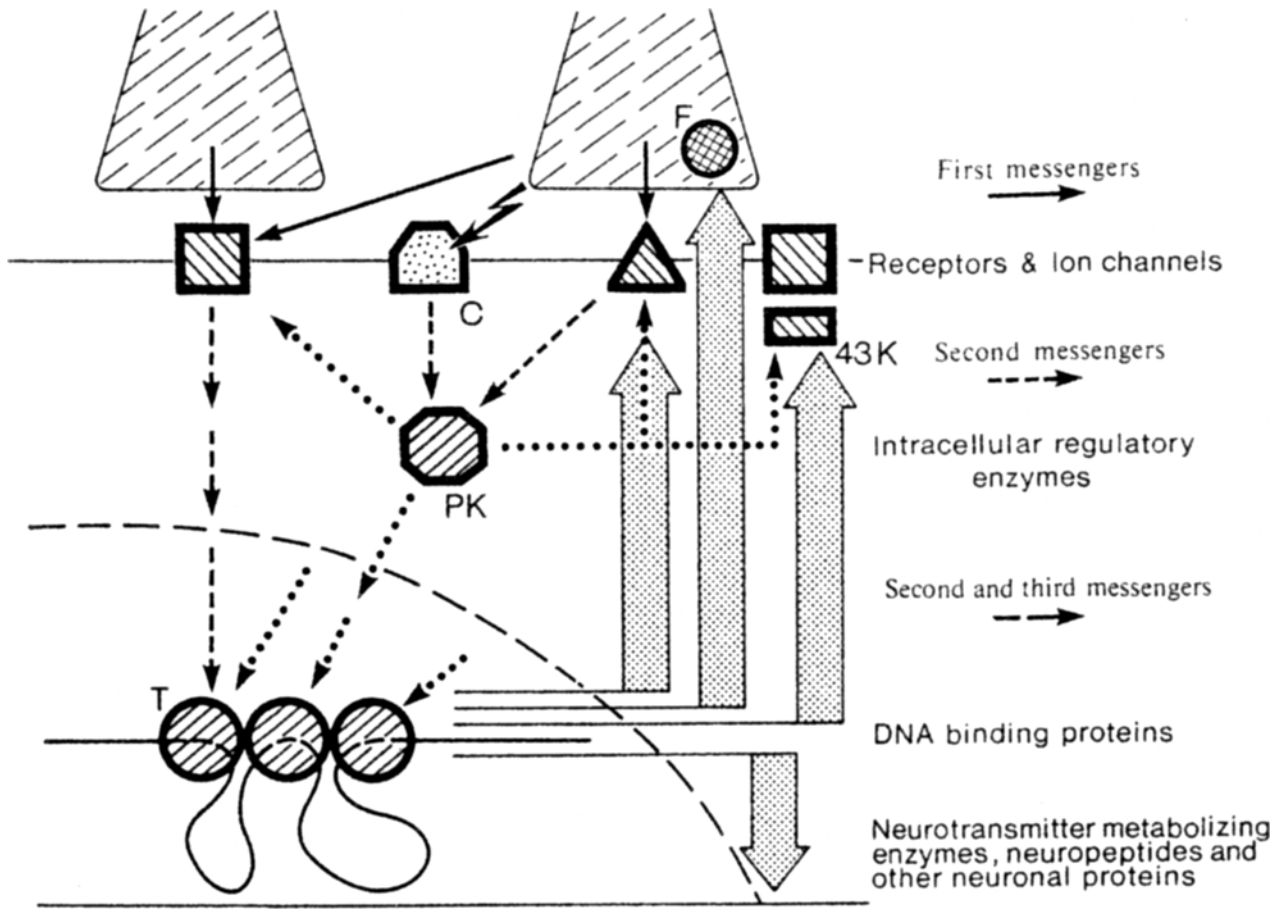

Fig. 4. Diagramatic representation of the basic elements involved in membrane-to-gene signaling. The two nerve endings represented on the top of the figure release first messengers, i.e., classical neurotransmitters and coexisting neuropeptides in an activity-dependent and/or spontaneous manner. These first messengers bind to membrane-bound allosteric receptors, which regulate the opening of ion channels and/or control the production of intracellular second messengers. In turn, these second messengers regulate the state of activity of intracellular allosteric proteins (in most instances, protein kinases (PK)). These enzymes may covalently modify allosteric *trans* acting proteins (T), which bind to DNA regulatory sequences acting in *cis* upon the transcription of the genes coding for neuronal proteins. They may also control, in a feedback manner, the state of activity (and/or stability) of the membrane-bound receptors and ion channels.

The DNA binding proteins may, in addition, be regulated, in a noncovalent manner, by second messengers acting as allosteric effectors. The cooperation between *several* of these proteins at the level of the DNA *cis* regulatory sequences may be required for transcriptional regulation to take place. The structural genes concerned code for neurotransmitter-metabolizing enzymes and neuropeptides, for their surface receptors and for associated proteins, such as the neural homologs of the 43 kD protein. They may also encode growth or stabilization factors (F), which may create positive feedback loops upon the afferent nerve ending. The second messengers and the regulatory enzymes under their control may affect, in addition to gene transcription, the several posttranscriptional steps that lead from the transcribed mRNA to the localized cell proteins.

variability (Changeux, 1983). Its definition as the precise set of connections the neuron establishes, can thus be legitimately extended to the particular repertoire of genes expressed by a given nerve cell (Changeux, 1986). This chemical individuality would then be determined, at least in part, by the spontaneous and/or evoked activity of the neuron and of the network to which it belongs, in concert with a variety of growth and differentiation factors acting in both anterograde and retrograde fashion (Changeux, 1986).

Such variability of the neuronal phenotype is bound within the limits of the genes that are in a *determined*, "ready to be transcribed" or "open" state. Tentatively, one may consider that the repertoire of *determined* genes specifies the neuronal category to which the nerve cell belongs. The acquisition of the terminal phenotype would then correspond to the *selection*, at a "sensitive" period of development, of a particular set among these open genes, and would manifest itself primarily (but not exclusively) by *different rates* of gene expression (*see* Changeux, 1986).

In contrast to the neuromuscular junction, where the target of the unique motor nerve ending is a multinucleated muscle fiber, synapses in the nervous system form between cells that possess a single nucleus, which determines the synthesis of all cellular proteins, synaptic or not. Therefore, in neuronal cells, the selection of the pattern of actively transcribed genes coding for synaptic proteins cannot possibly be determined, as it is in muscle cells, by a functional compartmentation of nuclei within the subsynaptic cytoplasm. Yet, the nerve cell displays a highly polarized organization from both a morphological and biochemical point of view. The dendritic, somatic, and axonal domains exhibit an anisotropic distribution of a vast amount of proteins, including in particular, neurotransmitters and their receptors as well as proteins involved in neurotransmitter biosynthesis, storage, and release. An anisotropic distribution of polyribosomes has been demonstrated at the level of particular dendritic spines (Steward, 1983). However, little evidence exists so far for a differential distribution of specific mRNAs in the axonal and dendritic domains (*see* Levinthal et al., 1987), except for the recently reported dendritic localization of mRNA for a microtubule-associated protein (Garner et al., 1988). Most of the biochemical anisotropy probably results from posttranslational phenomena such as sorting out at the level of the terminal Golgi apparatus, polarized transport, and targeting to the final location. An eventual activity-dependent regulation of these posttranslational steps remains to be demonstrated.

Intracellular Second Messengers Under the Control of Cell Surface Activity

An important issue concerning the activity-dependent regulation of gene expression is the nature of the intracellular pathway(s) involved in "membrane to gene" signaling (*see* Fig. 4). As described above, there are examples for the involvement of each one of the major intracellular second messengers, namely Ca^{2+}, diacylglycerol, and cyclic AMP (evidence for the implication of cyclic GMP is still lacking) in the regulation of neuronal gene expression. However, these molecules are rather ubiquitous and most often exert pleiotropic effects, either directly, by modulating the activity of a variety of regulatory proteins, or indirectly, by eliciting a cascade of enzymatic reactions within the cell. Moreover, the same second messenger may trigger divergent communication paths (as in the case of the regulation of prolactin and growth hormone gene expression) and "cross-talk" may occur between second messenger pathways in some systems (*see* Kley et al., 1987; Kley, 1988). At first sight, it looks as if a dramatic divergence and loss of specificity was occurring, in the transduction step from first to second messengers at the level of the cell membrane and in the subsequent effect of the second messenger within the cell.

The following observations and reasonings lead us to qualify this view:

1. As a counterpart of the extracellular network of synaptic connections established by any given neuron with its multiple partners, an *intracellular network* of second messengers exerting positive (stimulatory) and/or negative (inhibitory) effects develops.
2. Consequently, the *convergence* of a defined pattern or *combination* of *several* second messengers upon a given target may be required for a particular regulation to take place.
3. If such a target is an allosteric protein or a complex association of such proteins a *timing relationship* of the intracellular levels of these second messengers may be required to elicit their conformational transition.
4. The cell must be *competent* to carry out such intracellular regulations. In other words, it must be in a state of differentiation such that the regulatory protein(s), that respond to a particular pattern of second messengers, as well as the products of "differentiation genes" required for the timed expression of the gene considered, are present within the cell in sufficient amounts.

In other words, to the *extracellular network* of first messengers acting on the nerve cell surface corresponds a distinct intracellular network of second messengers and allosteric proteins. This is illustrated by the following examples. As mentioned, above, the stimulation by cAMP of proenkephalin gene expression in adrenal chromaffin cells requires the simultaneous opening of voltage-dependent Ca^{2+} channels, and can be enhanced by increasing the entry of Ca^{2+} ions into the cells (Kley et al., 1987). Similarly, in pituitary cells, cAMP enhances the expression of the prolactin gene in a Ca^{2+}-dependent fashion. In the latter cells, however, the nucleotide stimulates growth hormone gene transcription by a Ca^{2+}-independent mechanism (Waterman et al., 1985). The simultaneous release from presynaptic nerve terminals, of neurotransmitters that activate adenylate cyclase in pituitary cells, and of neurotransmitters that trigger membrane depolarization and/or Ca^{2+} influx into the cells can, therefore, be expected to switch on the transcription of both prolactin and growth hormone genes. In the absence of the latter stimulus, however, the growth hormone gene would be preferentially expressed. Similar considerations may apply to the cAMP- and Ca^{2+}-dependent regulation of proenkephalin gene expression (vs that of other, cAMP-inducible, but Ca^{2+}-insensitive genes) in adrenal chromaffin cells. In other words, some genes would be switched on, and other switched off, in such a *network,* depending on the particular *combination* of synaptic stimuli.

Allosteric Proteins Engaged in Intracellular Signaling

Among the vast population of regulatory proteins that may be under the control of intracellular second messengers, two major classes appear primarily concerned by the signaling pathways linking the cell membrane to the genome: the protein kinases and *trans*-acting DNA-binding proteins. At the genomic level, several distinct classes of regulatory sequences have been identified that act like inducible enhancer elements and respond to cAMP, Ca^{2+}, or phorbol esters, or to combinations of these agents (Angel et al., 1987; Comb et al., 1987, 1988; Imagawa et al., 1987; Mermod et al., 1988; Deutsch et al., 1988; Sheng et al., 1988; Roesler et al., 1988).

These regulatory elements specifically interact with DNA binding proteins such as the TPA- and cAMP-responsive transcription factors AP-1 (Angel et al., 1987; Deutsch et al., 1988) and AP-2 (Imagawa et al., 1987); the TPA-responsive transcription factors AP-3 (Chiu et al., 1987); and AP-4 (Mermod et al., 1988); the cAMP-responsive transcription factor(s) CREB (Yamamoto et al., 1988; Hoeffler et al., 1988); and the putative transcription factor ENKTF-1, whose interaction with the ENKCRE-1 DNA element appears necessary for TPA- and cAMP-responsiveness of the human proenkephalin gene (Comb et al., 1988). Second messengers may modulate the ability of certain *trans*-acting fac-

tors to bind to regulatory DNA elements, or the transcriptional stimulatory activity of DNA-binding proteins (Chiu et al., 1987). They may also control the interaction between sequence-specific transcription factors and other regulatory proteins, such as the c-*fos* gene product (Chiu et al., 1988).

Recent evidence indicates that the precise mechanisms by which second messengers regulate gene expression can be rather complex. This is best exemplified by the phorbol ester- and cAMP-mediated regulation of human proenkephalin gene transcription. First, the transcriptional activity of certain DNA-binding proteins (such as ENKTF-1) can be synergistically modulated by more than one second messenger (Comb et al., 1988). Second, a given regulatory DNA sequence may interact with multiple DNA-binding proteins. For instance, the ENKCRE-2 element of the human proenkephalin gene enhancer can bind AP-1, AP-4, and probably CREB (Hyman et al., 1988; Comb et al., 1988). Finally, several distinct DNA elements can act synergistically to confer second messenger-responsiveness to a given gene. For instance, the ENKCRE-1 and ENKCRE-2 elements act in concert with an AP-2 element to confer maximal response to cAMP and phorbol ester (Hyman et al, 1989). As a result of these complex interactions, the responsiveness of a gene to a particular combination of second messengers may depend not only on the nature of the second messenger-inducible enhancer elements, but also on the promoter, the cell type, and the physiologic state of the cell (Hyman et al., 1988; Deutsch et al., 1988). Obviously, this provides a means to generate a great variety of possible combinations of intracellular regulatory cues. As discussed in the preceding chapter, the resulting repertoire may be necessary to translate the information conveyed by a given combination of extracellular stimuli into a particular pattern of intracellular signals.

How is the information carried by the second messengers transmitted from the cytoplasm to the nucleus, where transcriptional regulation takes place? One possibility is that second messenger molecules bind to allosteric sites present on *trans*-acting factors, thereby modifying the interaction between the transcription factors and *cis*-acting regulatory DNA elements. Such a mechanism would be analogous to the regulation of prokaryotic gene expression by the cAMP receptor protein (de Combrugghe et al., 1984). Alternatively, the second messengers may activate a cascade of enzymatic reaction that result in the covalent modification (e.g., phosphorylation) of *trans*-acting transcription factors or proteins that interact with them. The fact that most known actions of cAMP, Ca^{2+}, and diacylglycerol are mediated via the stimulation of specific protein kinases (Greengard 1987) argues in favor of this possibility. As mentioned above, peptide hormone stimulation of gene expression was found to be associated with very rapid changes in the state of phosphorylation of certain nuclear proteins. In the case of the POMC and somatostatin genes, the transcriptional effects of cAMP were shown to require the activity of cAMP-dependent protein kinase (Reisine et al., 1985; Montminy et al., 1986b). Furthermore, the introduction into eukaryotic cells of a synthetic gene coding for a peptide inhibitor of cAMP-dependent protein kinase abolished the expression of a cotransfected fusion gene under the control of the cAMP-responsive element from the human proenkephalin gene (Grove et al., 1987).

Finally, microinjection of the catalytic subunit of cAMP-dependent kinase into cultured eukaryotic cells was recently found to stimulate the expression of genes containing cAMP-responsive enhancer elements (Riabowol et al., 1988). McLane et al. (1985) reported that nerve activity modulates the state of autophosphorylation of the regulatory (RII) subunit of muscular cAMP-dependent protein kinase in vivo. These authors proposed that phosphorylative modulations of RII may control the amount of free subunits of the kinase, each of which may play a role in transcriptional regulation. Indeed,

both the catalytic and the regulatory subunits of cAMP-dependent protein kinase translocate into the nucleus following a rise in intracellular cAMP (Kuettel et al., 1985, Sikorska et al., 1988), where they become associated with transcriptionally active chromatin (Sikorska et al., 1988). According to Schlichter et al. (1985), the nuclear effects of cAMP are mediated by the catalytic subunit of the kinase, which would stimulate the phosphorylation of proteins that directly or indirectly interact with *cis*-acting regulatory DNA sequences or other DNA-binding proteins. Alternatively, cAMP-stimulated gene transcription may be directly modulated by the regulatory (RII) subunit of cAMP-dependent protein kinase, which in its cAMP-bound and phosphorylated form was reported to possess intrinsic topoisomerase I activity (Constantinou et al., 1985; *see, however,* Sikorska et al., 1988). Since both Ca^{2+}-calmodulin-protein kinase (which is present in neuronal nuclei (Sahyoun et al., 1984)) and protein kinase C are able to phosphorylate and, in the latter case, to activate topoisomerase II in vitro, it was proposed that several second messengers may regulate gene transcription by modulating topoisomerase activities (Sahyoun et al., 1986).

In conclusion, protein phosphorylation most likely plays a key role in activity-dependent regulation of gene expression. Final proof for this idea must, however, await the characterization of nuclear proteins that bind to hormone- or depolarization-responsive regulatory DNA elements and whose state of phosphorylation is modulated by neuronal impulse activity in vivo. Phosphorylation of the CREB protein that selectively binds to the cAMP-responsive regulatory element of the somatostatin gene can be stimulated by elevating cAMP levels in PC12 cells (Montminy and Bilezikjian, 1987). Interestingly, this protein (which is distinct from the RII subunit of cAMP-dependent protein kinase, is a substrate for the catalytic subunit of the kinase in vitro, suggesting that its action may not necessitate a cascade of phosphorylative reactions. The mechanism by which the putative transcription factor modulates expression of the somatostatin gene remains unknown, since its phosphorylation was not found to be associated with enhanced binding to the regulatory DNA element (Montminy and Bilezikjian, 1987). Phosphorylation may, therefore, affect the transcriptional activity of the DNA-binding protein, once associated with an enhancer element (Yamamoto et al., 1988).

From Short-Term to Long-Term Regulation: The Stability of the Synapse

Under physiological conditions, synaptic activity and firing of action potentials take place in the ms to 0.1 s timescale, but repetitive activation of the cell may cause a progressive buildup of intracellular second messengers sufficient to modify transcription rates in a significant manner. Such effects should rapidly reverse after activity has ceased. However, as examplified by the transsynaptic control of tyrosine hydroxylase, brief stimuli can be converted into long-lasting changes in the rate of expression of a particular neuronal gene.

Speculative models about the transfer of short-term effects to long-term storage of information have been discussed in the case of classical conditioning paradigms with the simple nervous system of invertebrates [(*Aplysia* (Goelet et al., 1987) or *Hermissenda* (Alkon et al., 1987)] or, using appropriate experimental paradigms, with some vertebrate preparations [Hippocampus (Andersen, 1987) or cerebellum (Ito, 1987)]. Among vertebrate systems, the neuromuscular junction, adrenal medulla, pituitary, and autonomic neurons offer convenient experimental models to approach this issue since their biochemistry and molecular genetics are better understood than those of preparations from the central nervous system.

On the basis of the classical experiments of Flexner, Agranoff, and others with protein syn-

thesis inhibitors (recently revived by Kandel and collaborators (Goelet et al., 1987)), it is currently accepted that the storage of long term traces involves protein synthesis, whereas short-term effects do not. The paradigm underlying this idea is that new sets of proteins are synthesized in the course of the long-term storage step (Goelet et al., 1987). However, as illustrated in this review, a quantitative activity-dependent regulation of *already determined* genes may suffice for significantly changing the contribution of the neuron to intercellular communication processes. There may be no need for a qualitative modification of the pattern of genes expressed; long-term storage of information could be achieved by a *quantitative* regulation of the wide range of possibilities offered by the determined genes coding, for instance, for the multiple coexisting messengers.

If long-term storage was simply the consequence of a burst of protein biosynthesis elicited by a given transient pattern of activity, the timescale of the effect would be extended to the metabolic lifetime of the protein(s) involved and not longer than that. "Indefinite" persistence of the trace requires additional mechanisms which are *not* necessarily linked with, but, of course, may include the protein biosynthesis machinery. Such self-sustained mechanisms may develop at the level of circuits of neurons (*see* Hebb, 1949; Grillner et al., 1987, for a discussion) but also at the level of the single nerve cell and the synapse.

In a general manner, nerve cells and even synapses may be viewed as "open" thermodynamic systems that may exist under multiple *stable* steady-states (Prigogine, 1969). The logical structures that enable such systems to generate multiple discrete steady-states have been analyzed in detail. The simplest case is pure autocatalysis: a feedback loop with one positive element. This may be achieved with protein kinases (Lisman, 1985; Kennedy, 1987; *see also* Crick, 1984), allosteric receptors (Yeramian and Changeux, 1986), gene repressors and/or activators and *trans*-acting DNA binding proteins (Monod and Jacob, 1961), included in a metabolic chain with feedback loops. It can be shown that all these systems must possess either one positive feedback loop or negative ones, but in *even* number (Thomas, 1981). Under these conditions, stable steady-states may develop that resist to protein turnover (*see also* Changeux and Heidmann, 1987). Short-term patterns of nerve activity may be stored as a long-lasting trace by transitions from one steady-state to the other, a process that may *or not* require a reaction of protein biosynthesis.

Synapse Stabilization

Taking this reasoning further, one may argue that the establishment of closed regulatory circuits or loops, either transsynaptically, or within each of the pre- and postsynaptic neurons, provides an explanation for the maintenance of synapse stability. Feedback loops could control the expression of proteins directly or indirectly involved in synapse formation and stability, such as cell surface adhesion molecules and retrogradely acting growth factors (Henderson, 1987). Stable steady-state circuits are also expected to regulate the expression of gene repressors and/or activators and *trans*-acting DNA binding proteins (Monod and Jacob, 1961), thus leading to stable switches of gene patterns. Closed regulatory circuits, linking the cytoplasmic membrane to the gene transcription machinery, may thus transform discrete changes of cell surface activity into long-term stabilization of the synapse.

Neurotransmitter-Receptor Matching

One of the most intriguing biochemical questions concerning the interneuronal synapse relates to the complementarity between the type of neurotransmitter stored and released by a given nerve ending and the type of receptor densely accumulated on the opposite side of the synaptic cleft (even though receptor "mismatch"

sometimes occurs (Herkenham, 1987)). Such adequation requires, in addition to the ability of the two cell partners to express the genes coding for the relevant complementary proteins, and to the development of stabilization mechanisms, a more subtle regulation. Indeed, the presynaptic neuron is expected to express a large repertoire of neurotransmitters and coexisting peptides and the postsynaptic neuron a vast number of receptors on its cell surface. Yet, mostly the complementary receptor accumulates beneath the nerve ending of a given neurotransmitter type. The mechanisms involved in such "matching" are not known, but several possibilities may be considered (Changeux, 1986).

Anterograde Signals

As discussed above, in the case of the neuromuscular junction, the neurotransmitter does not seem directly involved in early subneural clustering of the AChR. Other neural factors released by the afferent neurons (such as agrin (Reist et al., 1987)) may play such a role. In addition, coexisting neuropeptides may regulate receptor biosynthesis in a positive manner and may contribute to the selection, at the transcriptional level, of the receptors expressed by the postsynaptic cell.

Yet, such receptors may become inserted in a random manner all over the surface of the postsynaptic cell. Some of the neural factors mentioned above may interact directly with the postsynaptic receptor. Accordingly, the genome of the presynaptic cell would code for a family of proteins, that one may refer to as "complementary images " of the neurotransmitter receptors, which would be expressed and released presynaptically and direct the subneural clustering of postsynaptic receptors. On the postsynaptic side, an analogous family of peripheral proteins consisting, for instance, of homologs of the 43 kD protein may exist. These proteins would serve to "anchor" a given receptor to a particular spot of the subneural membrane labeled by the release of a given anterograde factor. They may be specific of a given receptor and interact with a complementary sequence present on the cytoplasmic side of the receptor molecule. Alternatively, these peripheral proteins may be more ubiquitous and "shared" by different receptor species. "Consensus" sequences that are recognized by such proteins may exist on the cytoplasmic face of the diverse receptors and the "matching" conferred by additional regulatory mechanisms (such as phosphorylation-dephosphorylation reactions) triggered by the neurotransmitter and/or coexisting messengers.

Retrograde Signals

Conversely, once the functional contact has been established, the emission of "retrograde signals" by the postsynaptic cell (*see* Changeux and Danchin, 1976; Henderson, 1987) may vary with the type of postsynaptic receptor activated by a given neurotransmitter or coexisting peptide. Such retrograde signaling by the postsynaptic target may regulate the pattern of neurotransmitter and peptides synthesized and released by the presynaptic neuron. In other words, the matching might also result from the retrograde selection of the neurotransmitter or coexisting peptides synthesized by the presynaptic neuron (Changeux, 1986).

Timing Relationships Between Anterograde and Retrograde Signals

Finally, the biochemical complementarity between the two sides of the synapse may arise from a *reciprocal selection* of the genes and gene products expressed by the two cells in contact. This process requires an exchange of transsynaptic signals in *both* anterograde *and* retrograde directions that might depend on the impulse activity of the pre- *and* postsynaptic cells. Since the state of activity of each neuron is itself under control of that of the network to which it belongs, the reciprocal signaling will be sensitive to the neural *context* in which the partner cells are embedded. A fine matching between pre- and postsynaptic neurons may further be

achieved if, in addition, their respective firing coincides in time. It is noteworthy that such timing relationship is postulated in most current theories of learning as the basic algorithm for the change of synapse efficacy (Hebb, 1949; Ito et al., 1982; Heidmann and Changeux, 1982; Singer, 1987; Edelman and Finkel, 1987; Frégnac and Imbert, 1984; Bear et al., 1987), which can be, ultimately, related to intrinsic "integrative" properties, in time and space, of some allosteric protein (Changeux and Heidmann, 1987).

Conclusion

One of the most striking features of the functional organization of the synapse is that it unites two distinct categories of cells (sometimes even from different embryological origin), but nevertheless exhibits a strict biochemical complementarity between its pre- and postsynaptic sides. In this review, we have shown that the pattern of genes expressed may be qualitatively and quantitatively regulated by the state of activity of the cells in contact. The molecular mechanisms engaged in such activity-dependent regulations are under active investigation. Further progress in this field is expected to shed light on the contribution of neural activity to the development of the biochemical complementarity between the pre-and post-synaptic sides of the synapse.

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