Search for molecules involved in the formation of the nerve-muscle synapse

INAUGURALDISSERTATION

Zur
Erlangung der Würde eines Doktors der Philosophie
Vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
Der Universität Basel

Von

Soizic Carnejac
aus Französisch

Basel, 2005
Pharmazentrum der Universität Basel

CONTENTS

TABLE OF ABBREVIATIONS

aa	amino acid
ACh	acetylcholine
nAChR	nicotinic acetylcholine receptor
ARIA	acetylcholine receptor-inducing activity
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor (bFGF)
CAM	cell adhesion molecule
CASK	calcium/calmodulin-dependent serine kinase
CG	chick ciliary ganglion
CGRP	calcitonin-gene-related peptide
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CT-1	cardiotrophin-1
DG	dystroglycan
DGC	dystrophin-glycoprotein complex
Dvl	dishevelled
EC	embryonal carcinoma cells
ECM	extracellular matrix
EGF	epidermal growth factor
ErbB	epidermal growth factor-related receptor tyrosine kinase
FasII	fasciclin II
FBS	foetal calf serum
GABP	GA-binding protein
GDNF	glial cell line-derived neurotrophic factor
Ig	immunoglobulin
IGF	insulin-like growth factors
IgSF	immunoglobulin superfamily
LIF	leukemia inhibitory factor
MASC	muscle-associated specific component
MuSK	muscle-specific tyrosine kinase

NCAM	neural cell adhesion
NgCAM	neuron-glia cell adhesion molecule
NMJ	neuromuscular junction
NRG	neuregulin
Nup50	nucleoprotein 50
PAK	p21-activated kinase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PKC	protein kinase C
PNS	peripheral nervous system
poly-A	polyadenylated
RDD	RNA differential display
RT-PCR	reverse-transcription polymerase chain reaction
SAGE	serial analysis of gene expression
SV2B	synaptic vesicle glycoprotein 2B
syt	synaptotagmin
SynCAM	synaptic cell adhesion molecule
TM-agrin	transmembrane-agrin-expressing cells

SUMMARY

Efficient synaptic transmission requires a high local specialization of pre- and postsynaptic cells: the presynaptic nerve terminal must be competent to secrete neurotransmitter substance in response to an invading action potential, and the postsynaptic cell must express in the synaptic portion of its cell membrane a high density of receptor molecules for neurotransmitter which for signal transduction into the postsynaptic cell. The reciprocal signalling mechanisms that regulate and coordinate pre- and postsynaptic differentiation during synapse formation are only poorly understood. At one synapse in the peripheral nervous system, the neuromuscular junction (NMJ), some of the signalling cascades involved are known. Specifcally, Agrin, a heparansulfate proteoglycan that is secreted by motor neurons and interacts with a muscle specific receptor tyrosine kinase (MuSK) has been shown to trigger the differentiation of a postsynaptic membrane in the muscle fiber in the absence of a nerve.

However, experiments in our laboratory have shown that the secretion of Agrin by motor neurons and the expression of its receptor MuSK in the fiber surface are not sufficient to induce motor neurons to make synapses on muscle fibers. Furthermore, it is know from classical experiments that muscle fibers must be denervated, i.e. electrically inactive, to be susceptible to motor innervation. Therefore, we hypothesized that denervated muscle fibers secrete factors or express molecules on their surface that promote neuromuscular synapse formation.

In attempt to identify such molecules, we performed differential display of mRNAs expressed in innervated and in denervated muscle fibers. In addition to many different genes whose mRNAs were up- or down-regulated by denervation and which are known to be involved in protein turnover, cytoskeletal rearrangements and energy metabolism, we also observed that the mRNA for a putative surface molecule of the immunoglobulin superfamily (IgSF), Embigin, was highly upregulated in denervated muscle. Quantitative Real-Time PCR and Northern blot analysis showed that the level of Embigin mRNA was increased between 50 and 150 fold in denervated rat and mouse muscles. Based on the homology of its extracellular domain with SynCAM an IgSF member recently shown to drive synaptic assembly in the central nervous system, we hypothesized that Embigin is involved in denervation-induced neuromuscular synapse formation.

The rat and mouse orthologs of Embigin were cloned, and the developmental expression pattern of Embigin mRNA in muscle was analyzed by Northern blot analysis and by quantitative RT-PCR. Importantly, Embigin mRNA increased upon denervation 1-2 days prior to ectopic endplate formation, a time course consistent with a role in neuromuscular synapse development. Furthermore, exogenous stimulation of denervated muscle repressed Embigin mRNA, again consistent with such a role. Embigin mRNA expression is also induced during differentiation of the mouse myogenic cell line C2C12 which are known to form synapses. Cell biological experiments using HEK293 cells transfected with an Embigin full length clone suggested, however, that unlike other IgSF members such as NgCAM, Embigin does not mediate adhesion via homophilic interactions. Finally, we tested whether Embigin expressed in surface of COS-7 cells induced the differentiation of presynaptic terminals by co-cultured chicken ciliary ganglion neurons. However, unlike with cells expressing a transmembrane isoform of Agrin, no effect could be seen. It is hypothesized that Embigin could make the motor neuron growth cone approaching the muscle fiber adhere transiently to the muscle fiber which would allow Agrin to be deposited locally and in sufficient quantities to activate MuSK, thus initiating the formation of a neuromuscular synapse.

Chapter I

1. Development of the neuromuscular junction: a general introduction

1.1. The synapse

The function of the nervous system is to receive signals from the outside world and from the body via sense organs and afferent nerves, to design appropriate responses and to exert them via the efferent nerves that drive the activity of the peripheral target organs such as glands and muscles. This function depends on the development of an appropriate wiring pattern between its building blocks, the neurons, and on transmission of signals between them. To fulfill their function, neurons have a characteristic shape with neuritic processes, axon and dendrite which are extended from the cell body. The axon is responsible for sending out signals to other neurons whereas the dendrite is involved in receiving signals from other neurons. During nervous system development, the neurons respond to various extracellular signals to extend their neurites and innervate their specific targets. At the point where two neurons communicate, there is a specialized structure called synapse. Most commonly, synapses are chemical. In chemical synaptic transmission, an action potential invading the presynaptic nerve terminal causes the opening of voltage-gated Ca^{2+} channels resulting in a localized fast Ca^{2+} influx into the terminal. This Ca^{2+} ions influx in turn initiates the fusion with the plasma membrane of presynaptic vesicles that contain the neurotransmitter. By this process the neurotransmitter is released into the synaptic cleft and diffuses across the synaptic cleft to the postsynaptic cell where it binds to specific receptor molecules expressed at high density in the subsynaptic cell membrane and associated with a variety of extracellular, transmembrane and cytoplasmic proteins that have adhesive, structural and signalling roles. Neurotransmitter binding to subsynaptic receptors elicits a response in the postsynaptic membrane. Although the principles and mechanisms of synapse formation are likely to be similar in the peripheral nervous system (PNS) and the central nervous system (CNS) much of our understanding about the mechanisms of synapse formation arises from studies of the vertebrate neuromuscular synapse: the neuromuscular junction (NMJ), i.e. the synapse between spinal motor neurons and skeletal muscle fibers. Its size, its simple structure, its easy experimental accessibility and its large numbers in periphery make the NMJ a powerful system for the analysis of synaptic development (Kandel et al., 2000).

The NMJ is a highly specialized structure which ensures an accurate and rapid transmission of electrical impulses from motoneurons to muscle fibers (Sanes and Lichtman, 1999). The neurotransmitter mediating the impulse transmission of the NMJ is acetylcholine (ACh) which binds to so-called nicotinic acetylcholine receptors (nAChR) in the postsynaptic

membrane of the muscle fiber. The nAChR is an ionotropic receptor forming an ion channel permeable mainly to Na^+, K^+ and Ca^{2+}. As a result of positive charge influx the muscle membrane is locally depolarized. This depolarization termed endplate potential elicits an action potential in the muscle fiber which is propagated along its entire length in both directions from the endplate resulting in a contraction of the fiber.

1.2. Cells types present at the NMJ

Three types of cells interact at the NMJ: motor neuron, muscle fiber and Schwann cell (Couteaux, 1973). All three cells originate from different precursor cells. During development all these three cells travel long distances to meet at the synapse. Motoneurons arise from somata in the neural tube and Schwann cells from the neural crest. The skeletal muscle goes through different stages of development (Brand-Saberi et al., 1996): commitment of mesodermal cells to myogenic cells, division of myogenic cells and differentiation into myoblasts. When myoblasts reach sites where muscles will form, they fuse to form myotubes, centrally multinucleated cells. As they fuse, they are approached by targeting motor axons followed by Schwann cells. Initially growth cones from several motor axons innervate single myotubes. Once a motor axon's growth cone contacts a newly formed myotube, differentiation of the NMJ begins with the formation and assembly of a highly differentiated presynaptic nerve terminal and a highly specialized postsynaptic apparatus which leads to a high efficient synaptic transmission between nerve and muscle (Dennis, 1981). By birth, the NMJ is fully functional (Fig. 1.1). After a period of polyneural innervation, the muscle fiber will be innervated by a single motor axon and the terminal bouton will be capped by a Schwann cell.

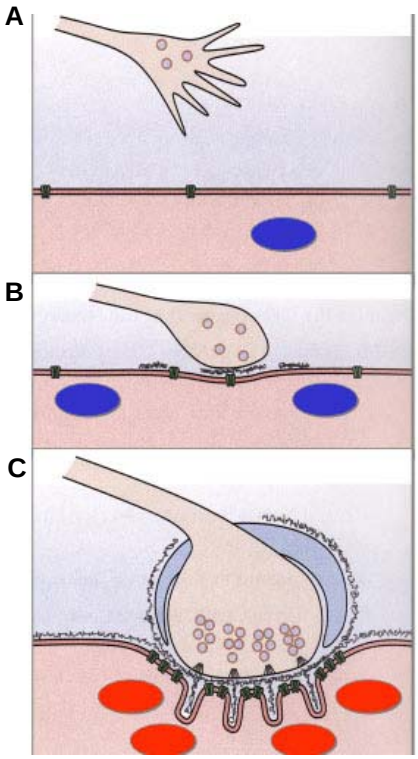


Fig. 1.1. Different stages of neuromuscular junction development.
A, The growth cone of a motor axon is approaching a newly formed myotube. B, Later in embryogenesis, vesicles accumulate in the nerve terminal and quantal content increases but transmission remains prone to failure and the action potential is broad. C, In the adult, at the area of contact, the axon differentiates into a motor nerve terminal that is specialized for transmitter release, it is capped by Schwann cells, and the muscle forms a complex postsynaptic apparatus that ensures a high safety factor of impulse transmission. Adapted from Sanes and Lichtman, 1999.

1.3. Development of the neuromuscular junction

1.3.1. Prepatterning

The patterning of skeletal muscle is thought to depend upon signals provided by motor neurons as they contact developing muscle. Acetylcholine receptors (AChRs) constitute the best-studied class of proteins that become localized to this small patch of the muscle fiber membrane, and their restriction to synaptic sites (Fambrough and Hartzell, 1972) during development is a hallmark of the inductive events of synapse formation. The spatial patterning of AChRs on skeletal muscle cells has classically been thought to depend on focal signals provided by motor axon terminals (Burden, 1998; Mc Mahan, 1990; Sanes and Lichtman, 1999). However it has been shown that *AChR* gene expression and AChR clusters are concentrated in the central region of embryonic skeletal muscle even in the absence of

innervation (Braithwaite and Harris, 1979; Fischbach and Cohen, 1973; Yang et al., 2001; Yang et al., 2000). Thus a AChR prepattern exists before the arrival of the nerve.

1.3.2. Structure of AChR and their developmental regulation

To ensure a high and efficient transmission of the information between nerve and muscle, a high concentration and mature AChRs are needed in the postsynaptic membrane in order to bind the neurotransmitter, the ACh, released at high concentration from the overlying neuron (Salpeter and Loring, 1985). The AChR of muscle is a large integral transmembrane glycoprotein composed of the subunits α, β, δ, γ or ε, each encoded by a different gene (Mishina et al., 1986). These subunits may combine in two different stochiometries $\alpha_2\beta\gamma\delta$ and $\alpha_2\beta\varepsilon\delta$ to form two different subtypes of functional AChR channels, the fetal and adult AChR subtypes, respectively (Mishina et al., 1986; Takai et al., 1985; Witzemann et al., 1989b). The subunits are arranged in a pentameric ring-like structure whose core forms the channel (Raftery et al., 1980; Unwin, 1993; Unwin, 2000). During synaptogenesis, the number, the distribution and subtypes of AChRs expressed in the muscle fiber vary in a characteristic manner. Mononucleated myoblasts have only few fetal types AChR on their surface. During continued myogenic differentiation α–, β–, δ– and γ –subunit genes are activated along their entire length (Anderson and Cohen, 1977; Braithwaite and Harris, 1979; Frank and Fischbach, 1979; Kues et al., 1995a; Kues et al., 1995b) as part of the myogenic program. During NMJ development, following innervation of the myotubes, the mRNAs of the α–, β–, δ– and γ –subunit genes accumulate in synaptic and begin to decrease in extrasynaptic fiber regions (Anderson and Cohen, 1977; Bevan and Steinbach, 1977; Braithwaite and Harris, 1979; Frank and Fischbach, 1979). Soon thereafter, ε-subunit mRNA becomes expressed selectively in subsynaptic nuclei (Brenner et al., 1990; Kues et al., 1995a) while γ-subunit mRNA begins to disappear in response to the onset of electrical muscle activity (Mishina et al., 1986). During postnatal development α–, β– and δ–subunit transcript levels are further reduced predominantly in extrasynaptic fiber segments, whereas the γ-subunit mRNA disappears completely within the first postnatal week (Kues et al., 1995a; Kues et al., 1995b). At the mature NMJ only α–, β–, δ– and ε –subunit mRNAs are expressed, and they are largely concentrated at the synapse (Mishina et al., 1986). This

suggests that α–, β–, δ– and ε-subunit genes expression at the synapse are primarly under control of a neurotrophic factor (Brenner et al., 1990).

The nerve plays an important role in the control of junctional and extrajunctional AChR density (Fig. 1.2). The precise apposition of postsynaptic specializations to the motor nerve terminal arises in one of two ways: the motor nerve might induce AChR clusters or clusters might form aneurally and then be recognized by the ingrowing axon. Spontaneous "hot spots" forming on aneurally myotubes support the first idea. Neurites contact myotubes at random then new clusters form at sites of contact (Anderson and Cohen, 1977; Frank and Fischbach, 1979) indicating that motor axons can localize and increase postsynaptic components generated by myotubes on their own to form a postsynaptic apparatus at the site of nerve contact. Muscle activity and neural trophic factors released from nerve regulate AChR number and distribution through three different processes: clustering of diffusely distributed AChR in the postsynaptic membrane, transcriptional activation of AChR subunit genes in subsynaptic nuclei and transcriptional repression of AChR subunit genes in non synaptic myonuclei.

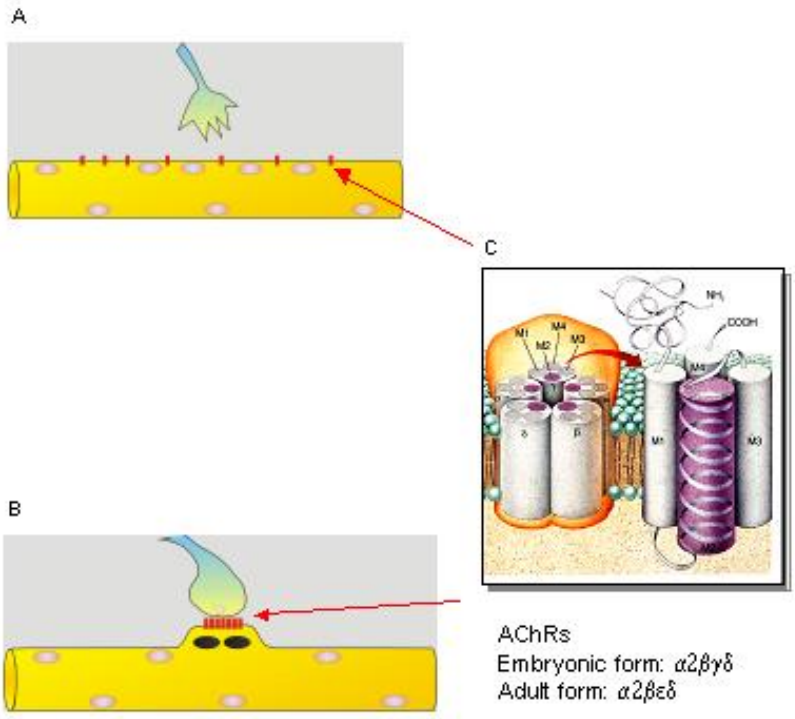


Fig. 1.2. Accumulation of AChRs in the post-synaptic membrane.
A, AChRs are initially expressed constitutively as part of the myotube's developmental program. B, The nerve sends then three signals that affect AChRs distribution: Agrin, Neuregulin and Acetylcholine. Nuclei accumulate under the differentiated preterminal nerve. AChRs are highly concentrated in the post-synaptic membrane and virtually absent extrasynaptically. This clustering involves both redistribution of AChR proteins and localized synaptic synthesis of AChRs. C, The AChR is a pentameric transmembrane protein composed of four subunits (α, β, δ, γ or ε) each encoded by a different gene. Adapted from Bezakova and Ruegg, 2003; Changeux, 1993.

1.3.3. Clustering of AChR

On the basis of the idea that motor neurons use chemical messengers to organize the postsynaptic membrane, many groups sought molecules that can cluster AChR. Elements associated with the extracellular matrix have been implicated. A basal lamina ensheaths each muscle fiber, occupies the synaptic cleft and reaches into the junctional folds. The major components of muscle basal lamina are molecules like Collagen isoforms, Laminin, Fibronectin and Heparan sulphate proteoglycans (Sanes and Lichtman, 1999). The first evidence for a function of synaptic basal lamina in NMJ development and maintenance came from experiments in which axons of motor neurons and myotubes were destroyed and new fibers were allowed to regenerate in the absence of the nerve inside old basal lamina sheaths from satellite cells. When such fibers regenerated, synaptic structures including AChR clusters formed at the sites of the old synapses (Burden et al., 1979; Sanes et al., 1978). In further experiments in which axons of motor neuron, muscle fiber and Schwann cell are destroyed, the basal lamina was identified as the inductive source of re-accumulation of AChRs in regenerating myofiber at the original synaptic site (McMahan and Slater, 1984). Furthermore, the synaptic portion of the myofiber's basal lamina induces synapse-specific expression of AChR genes (Brenner et al., 1992). Thus factors that induce the synapse-specific expression of AChR genes are stably bound to synaptic basal lamina.

Several groups used cultured myotubes to seek clustering molecules. Several active agents were identified including Neuregulin, Transferrin, Collagen, Laminin, Ascorbic acid, Calcitonin-gene-related peptide (CGRP), Midkine, Fibroblast growth factors, Pleiotrophin and Agrin (Falls et al., 1993a; Fontaine et al., 1987; Knaack et al., 1986; Mc Mahan, 1990; Oh and Markelonis, 1982; Peng et al., 1991; Vogel et al., 1983; Zhou et al., 1997). However, only for Agrin there is strong evidence for a direct role in synaptogenesis *in vivo*. Another neural signalling molecule identified, Neuregulin, is thought to mediate the neural regulation of differentiation of the postsynaptic apparatus by stimulating the synapse-specific AChR gene expression (Jessell et al., 1979).

1.3.4. Clustering of AChR: Agrin signalling

McMahan and colleagues isolated the protein Agrin from the basal lamina of the electric organ of the marine ray *Torpedo californica* (Godfrey et al., 1984; Nitkin et al., 1987). The Agrin gene was cloned from chick and mammals (Ruegg et al., 1992; Rupp et al., 1991). Agrin is a heparan sulphate proteoglycan with a protein core of a predicted molecular weight of 225 kDa (Tsen et al., 1995). It is highly glycosylated to reach 400-600kDa (Denzer et al., 1995). It is synthesized by motor neurons, transported down motor neurons and released from the nerve terminals where it stably associates with the basal lamina of the synaptic cleft (Mc Mahan, 1990). Alternative splice variants generate Agrin isoforms with different activities in receptor clustering (Ferns et al., 1992; Hoch et al., 1993; Ruegg et al., 1992). At the N-terminal part, alternative splicing results in secreted isoforms that have a strong affinity to Laminin conferring attachment of Agrin to basal lamina (Denzer et al., 1995; Denzer et al., 1997) and in a transmembrane type-II isoform attached to the nerve cell membrane whose function is not known (Burgess et al., 2000; Neumann et al., 2001). The C-terminal region which includes EGF-like repeats and Laminin G-like domains contains the AChR clustering activity (Ruegg et al., 1992). This carboxy-terminal part is subjected to alternative splicing at both sites A and B in chick and y and z in rodents and mammals. Of particular importance, Agrin expressed by motor neurons contains an eight amino acid insert at the B/z site which is the most active isoform in receptor clustering assays. The B+/z+ isoforms of Agrin which is a thousand times more potent than B-/z- *in vitro*, is only expressed in neurons (Gesemann et al., 1995; Ruegg et al., 1992). The B-/z- isoform of Agrin is synthesized by muscle (Fallon and Gelfman, 1989). No AChR clusters were observed with muscle-specific isoforms of Agrin lacking B/z insert at the C-terminal splice site *in vitro* (Gesemann et al., 1995) and *in vivo* (Meier et al., 1997). Furthermore, Agrin -/- muscles transplanted to wild-type muscles were reinnervated by Agrin expressing axons and showed normal synapses. When the B+/z+ exon is deleted, the chimeric synapses seem to be normal, in contrast to other neural isoforms lacking C-terminal and all muscle-specific isoforms (Burgess et al., 1999). Thus nerve-derived B+/z+ Agrin is essential for postsynaptic differentiation.

In cultured myotubes, neural Agrin induces AChR aggregation but clusters also other postsynaptic components (Wallace, 1989). This led to the “Agrin hypothesis” first proposed by McMahan that Agrin is a critical nerve-derived organizer of postsynaptic differentiation at the NMJ (Mc Mahan, 1990). Several gain and loss of functions support this idea. Neural

Agrin knock-out mice fail to form normal neuromuscular synapses (Gautam et al., 1996). Ectopic overexpression of Agrin in the absence of the nerve in muscle fibers (Cohen et al., 1997; Jones et al., 1997; Meier et al., 1997) induces the formation of ectopic AChR clusters. Thus Agrin is necessary as well as sufficient for postsynaptic differentiation.

In the Agrin hypothesis, McMahan describes one major component of this hypothesis. Agrin induces clustering of AChR and other postsynaptic components by interacting with a muscle fiber receptor (Mc Mahan, 1990). Several molecules have been shown to interact with Agrin. These include MuSK, Dystroglycan, Glycoconjugates, Integrins, Pleiotropin, Heparan sulfate proteoglycans, N-CAM and Laminins (Bowe et al., 1994; Campanelli et al., 1994; Daggett et al., 1996; Denzer et al., 1997; Gee et al., 1994; Gesemann et al., 1996; Hopf and Hoch, 1996; Martin and Sanes, 1997; Martin and Sanes, 1995; Mook-Jung and Gordon, 1995; O'Toole et al., 1996; Storms et al., 1996; Sugiyama et al., 1994; Valenzuela et al., 1995). Of these only MuSK seemed to be indispensable for the formation of a postsynaptic apparatus (Valenzuela et al., 1995), whereas others did not prevent but impaired postsynaptic differentiation to varying degrees. MuSK is a transmembrane receptor tyrosine kinase homologous to an orphan tyrosine kinase previously cloned in the electric organ of the marine ray *Torpedo californica* (Jennings et al., 1993). MuSK is selectively expressed by skeletal muscle where it is colocalized with AChR in the postsynaptic membrane (Meier et al., 1997; Valenzuela et al., 1995) and becomes rapidly phosphorylated upon addition of neural Agrin to cultured myotubes (Glass et al., 1996). Furthermore, MuSK -/- knock-out mice showed no postsynaptic differentiation in muscles and thus have similar, if not more severe, neuromuscular defects than Agrin knock-out mice (DeChiara et al., 1996). In these mutant mice, muscle fibers lack all known features of postsynaptic differentiation at all stages of development. This led to the hypothesis that MuSK is the receptor of Agrin. However it has been impossible to demonstrate direct binding of purified Agrin to purified MuSK or to MuSK expressed in non muscle cells, leading to the hypothesis that MuSK is part a multisubunit receptor (Glass et al., 1996). In support of this idea, constitutively active MuSK isoform overexpressed *in vivo* in the absence of Agrin induces AChR clusters in the absence of Agrin. Thus MuSK acts downstream of Agrin (Jones et al., 1999). MuSK signaling requires a muscle-associated specific component MASC not yet identified, as in fibroblasts expressing MuSK, Agrin does not phosphorylate MuSK (Glass et al., 1996). Presynaptic differentiation is also aberrant in MuSK mutant mice (DeChiara et al., 1996) as well as in

Agrin mutant mice (Gautam et al., 1996), as motor axons fail to stop or differentiate and instead are present throughout the muscle.

Agrin stimulation of MuSK leads not only to the clustering of AChR but also of critical muscle-derived proteins such as MuSK and Laminin β2, and to activation of synapse-specific gene expression (Jones et al., 1997; Meier et al., 1997; Moore et al., 2001). Laminin β2 has been proposed to be a retrograde signal for presynaptic differentiation. Activation of MuSK in injected muscle fibers induced localized deposits of Laminin-β2 which was not accompanied by the accumulation of endogenous muscle Agrin (Jones et al., 1999; Kummer et al., 2004). Laminin-β2 has been shown to be a synaptic component of the extracellular matrix important in the differentiation of the presynaptic nerve terminal (Patton et al., 1997; Porter et al., 1995).

1.3.5. Molecules involved in Agrin/MuSK-induced AChR clustering

1.3.5.1. Rapsyn

A crucial effector of the postsynaptic differentiation downstream of MuSK is Rapsyn. Rapsyn is a 43 kDa membrane-associated cytoplasmic protein. It is present at the NMJ as soon as AChR clusters appear and co-localizes with AChR in adult NMJ. Rapsyn which is associated with synaptic AChR β-subunit in 1:1 stoichiometry (LaRochelle and Froehner, 1986; Noakes et al., 1993) is a required intermediate on the pathway that couples MuSK activation to AChR clustering. In Rapsyn-/- knock-out mice, no AChR clusters develop on the surface of the muscle (Gautam et al., 1995). However, other aspects of synaptic differentiation are relatively normal in Rapsyn mutant mice. MuSK is localized to Rapsyn mutant synaptic sites. AChR gene expression is enriched in the synaptic region of Rapsyn mutant muscles (Gautam et al., 1995). Thus Rapsyn is essential for clustering of AChR but not of MuSK suggesting that MuSK is a primary scaffold to which Rapsyn then recruits additional components.

1.3.5.2. Dishevelled-PAK1

Because MuSK is absolutely essential for the pre- and postsynaptic differentiation at the NMJ, extensive efforts have been invested to understand of how MuSK transduces the signal. Using the intracellular region of MuSK as bait in yeast-two hybrid screen, Dishevelled (Dvl) was identified as a MuSK binding protein (Luo et al., 2002). Dvl was originally discovered in *Drosophilia* for its role in the development of coherent arrays of polarized cells (Perrimon and Mahowald, 1987). *Drosophila* are composed of set of repetitive units each of which is the composite of two different lineages, one for the anterior structures of segments and one for the posterior structures of segments. The first repetitive units that can be detected in the embryo are not segmental but parasegmental. Each parasegment is the composite of an anterior and a posterior compartment belonging to the different segmental units. Soon after their formation, the parasegmental units disappear and segmental boundaries are formed. Searches for genes involved early in establishing the segmental pattern have led to the identification of three classes of embryonic lethal *loci*: the gap, pair rule and segment polarity genes. The segment polarity phenotype displays a repeated pattern defect in which part of each segment is deleted and a mirror-image duplication of the remaining pattern element forms. Dvl is a late zygotic lethal mutation which produces a segment polarity phenotype (Perrimon and Mahowald, 1987). Dvl is enriched at the NMJ (Luo et al., 2002) and inhibition of Dvl function or expression attenuates Agrin-induced AChR clusters and the formation of the neuromuscular synapse in culture (Luo et al., 2002). Interestingly, MuSK shares a conserved extracellular cysteine-rich domain with the Wnt receptor Frizzled which signals through Dvl leading to the activation of Rho GTPases in the cell polarity events (Dann et al., 2001; Habas et al., 2001; Masiakowski and Yancopoulos, 1998; Xu and Nusse, 1998). Thus the role of the Wnt signalling pathways was investigated in Agrin-induced AChR clusters. Indeed, it has been shown that GTPases of the Rho Family and cytoskeletal proteins play important roles in AChR clustering (Dai et al., 2000; Weston et al., 2000). Candidate of small GTPase effectors that regulate cytoskeleton includes p21-activated kinase (PAK). Indeed, it has been shown that PAK is activated by Agrin and is required for AChR clustering (Luo et al., 2002). Importantly, Agrin-induced PAK activation is attenuated in muscle cell expressing mutant Dvl, suggesting the involvement of Dvl in this event.

1.3.5.3. Abl

In the late 1990's, it has been suggested that signalling downstream from MuSK requires a kinase to mediate tyrosine phosphorylation and clustering of AChRs (Fuhrer et al., 1997). Indeed Finn and colleagues provided some evidence that Abll and/or Abl2, two structurally related non-receptor tyrosine kinases are involved in these events (Finn et al., 2003). Abl kinases transduce signals downstream of growth factor stimulation (Plattner et al., 1999) and shape the cytoskeleton through phosphorylation of regulatory proteins (Lanier and Gertler, 2000; Pendergast, 2002). Abl kinases are localized at the developing NMJ and inhibition of their kinase activity inhibits Agrin-stimulated clustering of AChRs (Finn et al., 2003). Abl kinase activity induces also the increase of MuSK tyrosine phosphorylation. Moreover, because Abl family kinase activity is required for agrin-induced AChR clustering and enhances MuSK tyrosine phosphorylation, the interaction between Abl kinases and MuSK was investigated. Indeed, MuSK and Abl1 form a complex, when overexpressed in non muscle cells after Agrin stimulation and effected reciprocal tyrosine phosphorylation. Abl kinases contain binding domains for F-actin and G-actin (Pendergast, 2002). Furthermore it was shown that Agrin stimulates Cdc42 and Rac, both of which are required for AChR clusters (Weston et al., 2003; Weston et al., 2000). Thus Abl kinases are involved in synapse formation with the kinase activity required for signal amplification and the intrinsic cytoskeletal regulatory capacity for assembly and remodelling.

1.3.5.4. β-dystroglycan

Dystroglycan (DG) is transcribed from a single gene and is post-translationally modified to yield two glycoproteins, namely α- and β-dystroglycan. The transmembrane β-DG links α-DG by a tight but non-covalent link to the Actin cytoskeleton via Dystrophin (Ervasti and Campbell, 1991) or Utrophin (Matsumura et al., 1992). DG is part of the Dystrophin-glycoprotein complex (DGC). The accumulation of molecules of the DGC at the postsynaptic membrane of the NMJ and their co-distribution with AChR clusters *in vitro* suggested a role of the DGC in synaptogenesis (Yang et al., 1993). Furthermore, Rapsyn and β-DG have been shown to interact in the postsynaptic membrane (Apel et al., 1995; Cartaud et al., 1998). However myotubes generated from DG-/- embryonic stem cells formed normally and retained

the ability to form numerous AChR clusters following treatment with Agrin (Grady et al., 2000). Thus DG is dispensable for myogenesis and Agrin signalling. However DG may have an extracellular role. The specialized basal lamina that is associated with AChR clusters in control myotubes does not form in DG-/- myotubes. Thus DG may play a role in synapse maturation of the postsynaptic apparatus that involves the DG-dependent assembly of a synaptic basal lamina (Grady et al., 2000).

1.4. Selective gene transcription at the synapse

The synaptic accumulation of AChRs in electrically active fibers results not only from the clustering of AChRs but also from the selective transcription of genes encoding AChR subunits by subsynaptic myonuclei, thus ensuring activity-resistant AChR expression at the synapses.

In vitro, application of brain and spinal cord extracts leads to increased synthesis of AChRs on skeletal myotubes in culture (Jessell et al., 1979). These results suggest that the nerve provides a localized signal that stimulates AChR gene expression in subsynaptic nuclei.

ARIA (AChR-inducing activity), an isoform of the secreted growth factor Neuregulin 1 (NRG 1) was isolated from chicken brain in a search for neural factors that stimulate AChR accumulation in cultured myotubes (Usdin and Fischbach, 1986). NRG 1 is a product of *neuregulin 1* gene which by alternative RNA splicing codes for a number of growth and differentiation factors with a multitude of function in neural development (Buonanno and Fischbach, 2001). Subsequently, ARIA has been shown to be a member of the Neu ligand family (differentiation factor) (Falls et al., 1993b) and homologous to Glial growth factor (Marchionni et al., 1993). Thus, these factors are alternatively spliced variants of *nrg* 1 gene. Like Agrin, Neuregulin is transported down the motor axon, incorporated into the synaptic basal lamina (Goodearl et al., 1995) through binding to Heparin sulphate proteoglycans (Loeb and Fischbach, 1995). Like MuSK, Neuregulin receptors are transmembrane tyrosine kinases, Epidermal growth factor-related receptor tyrosine kinase ErbB 2, 3, and 4 which are concentrated in the postsynaptic membrane at the NMJ (Moscoso et al., 1995; Zhu et al., 1995).

ARIA has no effect on AChR clustering but increases AChR subunit mRNA levels in cultured myotubes suggesting a transcriptional effect (Martinou et al., 1991). Moreover, a Neuregulin

response element is contained in the same cis-regulatory region of AChR genes that confers synapse-specific expression in mice (Gundersen et al., 1993). An important element called a N-box that conforms to a consensus-binding site for Ets transcription factors is required for the synaptic expression of AChR δ and ε subunit genes (Duclert et al., 1996; Koike et al., 1995). Members of the Ets transcription factor family GA-binding protein (GABP) α and GABP β were found to bind AChR genes (Fromm and Burden, 1998; Sapru et al., 1998; Schaeffer et al., 1998). They have been shown to be involved in the expression of AChR ε gene *in vivo* (Briguet and Ruegg, 2000). In culture myotubes, ErbB kinases signal through a generic cascade of kinases including Ras, Raf, Erk and Phosphatidylinositol-3-kinase (Altiok et al., 1995; Si et al., 1996; Tansey et al., 1996).

However it has been difficult to demonstrate the role of NRG mediating the neural control of synapse-specific transcription *in vivo*. NRGs act via activation of ErbB2-4 receptors tyrosine kinases. Because mice lacking Neuregulin, ErbB2 and ErbB4 die due to defects in cardiac development during embryogenesis before neuromuscular synapse formation (Lee et al., 1995; Meyer and Birchmeier, 1995) it has been difficult to determine whether Neuregulin-mediated signalling is required for synapse specific gene expression even when genetic tricks (transgene rescue targeted to the heart, deletion of a neuron-specific isoform, or conditional mutagenesis) were used to circumvent embryonic lethality for ErbB3 and ErbB4. Nevertheless, adult mice that are heterozygous for the immunoglobulin allele of Neuregulin (Neuregulin $^{Ig+/-}$) have a mild deficiency in synaptic transmission and 50% less AChR at their neuromuscular synapse (Sandrock et al., 1997). However, the interpretation of these phenotypes is complicated because Neuregulin-ErbB signalling is essential for the proliferation, migration and survival of Schwann cells (Garratt et al., 2000) and muscles as well as motoneurons synthesize Neuregulin (Moscoso et al., 1995). Moreover, in the absence of nerves, Agrin binds to a basal lamina substrate to induce AChR gene expression in cultured myotubes (Jones et al., 1996). Extrasynaptic regions of electrically active muscle fibers injected *in vivo* with Agrin expression plasmids develop postsynaptic specializations identical to normal synapses. These ectopic specializations include the accumulation of ErbB2 and ErbB3, AChR ε subunit (Jones et al., 1997; Meier et al., 1997) as well as Neuregulin (Meier et al., 1998b) in the absence of motoneurons. Indeed, it has been recently confirmed with help of genetic mouse models that AChR transcription and clustering occur in the absence of

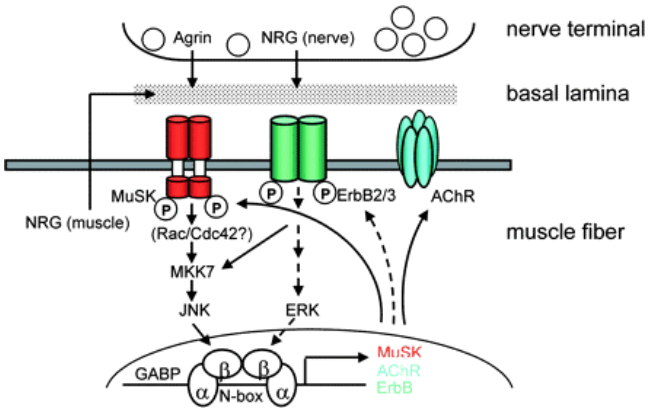


Fig. 1.3. Model for stabilization of synaptic gene expression through stabilization of *musk* expression by Agrin from motor nerve terminal.
Agrin secreted from nerve terminal activates preexisting MuSK to induce expression of *musk* via its N-box (i) by organizing an NRG/ErbB pathway, involving MuSK-induced recruitment of ErbB receptors and of muscle-derived NRG and (ii) by MuSK-induced activation of JNK (via Rac/Cdc42). With *musk* expression stabilized, the same pathways are used for *AChR* and *erbB* expression. Expression may be strengthened by NRG-1 secreted from nerve terminal. Lacazette et al, 2003.

neurally supplied NRG, indicating that neural NRG is not required to induce synapse specific transcription (Yang et al., 2001). Nevertheless muscle-derived Neuregulins could be involved in this regulation (Meier et al., 1998b).

Nerve-associated transcriptional specialization are also absent from MuSK -/- muscles (DeChiara et al., 1996) but present in Rapsyn -/- muscles (Gautam et al., 1995). Thus Agrin-MuSK signalling activates two pathways: a Rapsyn-dependent pathway for clustering AChR and a Rapsyn-independent pathway for localized transcription. Muscle-derived NRG could act in an autocrine fashion essentially as second messengers to nerve-derived Agrin to potentiate AChR transcription.

During development, the expression mRNA for MuSK is regulated similarly as AChR genes i.e. it also accumulates at the synapse with synapse-specific activation of its gene by the nerve (Valenzuela et al., 1995) and extrasynaptic down-regulation by muscle activity (Bowen et al., 1998; Valenzuela et al., 1995). This raises the question, how synaptic MuSK expression is stabilized. Agrin/MuSK and NRG-1 pathways could be involved in the nerve-induced expression of MuSK mRNA at the synapse (Lacazette et al., 2003). Indeed *Musk* is activated through an N-box present in the *musk* promoter by neuronal Agrin via the activation of MuSK. The Agrin-induced *musk* expression is controlled by a secondary NRG/ErbB pathway which is organized by Agrin/MuSK and by a novel path independent of NRG/ErbB in which MuSK signals to the muscle nuclei through Rac and c-Jun NH_2-terminal kinase (JNK) (Fig. 1.3). AChR ε subunit expression has been shown to be regulated via Rac and Cdc42 (Weston

et al., 2000). Thus the nerve uses identical pathways to regulate key genes in neuromuscular synapse formation.

While these studies begin to piece together messengers that lead to AChR clustering and synapse-specific transcription of AChR subunit genes and key genes in neuromuscular synapse many elements involved in the formation of neuromuscular synapse are still unknown.

1.5. Transcriptional repression in extrasynaptic regions

Once myoblasts fuse to form myotubes, AChRs start to cluster at the synapse whereas in extrasynaptic regions, the density of α, β and δ-subunit transcript levels of AChRs decrease. This density increases again after denervation of muscle fiber leading to an increase of transcription of AChR genes by extrasynaptic nuclei (Tsay and Schmidt, 1989). Two explanations are possible: electrical activity of the muscle regulates the expression of AChR mRNAs and/or the nerve supplies a repressive factor to the muscle.

Electrical activity reduces the density of extrasynaptic AChRs in denervated muscle fibers (Lomo and Rosenthal, 1972). This is due to the down-regulation of AChR subunit mRNAs in muscle fibers *in vivo* in non synaptic regions (Goldman et al., 1988) suggesting that the down-regulation of AChR in non-synaptic regions of embryonic myotubes is due to the onset of impulse activity upon NMJ formation.

The signalling pathway for extrasynaptic repression follows the scheme of synaptic transmission which causes muscle contraction: release of ACh activates AChR leading to a depolarizing synaptic potential which reaches threshold and triggers an action potential. The action potential propagates the repressive signal of depolarization along the length of the muscle fiber which allows calcium to enter into the muscle fiber through voltage-gated Ca channels. The calcium influx activates muscle contraction and represses AChR gene transcription (Huang et al., 1994). The calcium influx activates a serine/threonine kinase PKC which mediates the effect of electrical activity on AChR gene transcription (Huang et al., 1992). The targets of PKC are myogenic factors of the basic helix loop helix family of transcription factors which are required for muscle specific expression of AChR subunit genes (Berberich et al., 1993; Gilmour et al., 1991; Numberger et al., 1991; Prody and Merlie, 1991; Prody and Merlie, 1992; Simon and Burden, 1993). In the non phosphorylated state, these

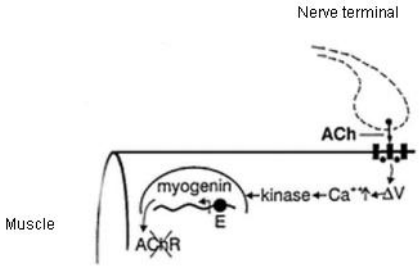


Fig. 1.4. Transcriptional repression of AChR in extrasynaptic regions.
Acetylcholine, which activates AChR generates a voltage- and calcium-dependent signal that represses AChR subunit gene expression in extrasynaptic nuclei. This effect is mediated in part by binding of MyoD-family transcription factors to E-box sequences in AChR gene promoters. Adapted from Sanes and Lichtman, 1999.

myogenic factors can bind to a short consensus site called E-box, which confers activity-dependence on reporters. E-boxes are present in the promoter regions of AChR subunit genes as well as the genes encoding the factors leading to the activation of their transcription (Fig. 1.4). Their phosphorylation by the activated PKC inhibits their binding to E-boxes of AChR genes leading to the inhibition of the transcription of AChR genes (Mendelzon et al., 1994). In addition electrical activity represses myogenin transciption (Eftimie et al., 1991) by reducing myogenin activation of its own promoter which contains also E-boxes (Buonanno et al., 1993). Thus electrical activity represses AChR genes synthesis in non synaptic regions by inhibiting the synthesis of myogenic factors (Eftimie et al., 1991) as well as by phosphorylating them (Mendelzon et al., 1994).

1.6. Cellular interactions required for synapse formation

Complex interactions between motor neurons, muscle and Schwann cells culminate in the formation of a highly differentiated presynaptic terminal and a highly specialized postsynaptic membrane. The precise organization of molecules in presynaptic and postsynaptic membranes suggests that reciprocal interactions requiring spatially restricted signalling between presynaptic and postsynaptic cells are required to assemble the synapse and to coordinate presynaptic and postsynaptic differentiation. An important observation is, that only electrically inactive muscle fibers are susceptible to innervation (Lomo and Slater, 1978).
Three types of interactions that could be involved are described below.

1.6.1. Neurite outgrowth/attachment

Muscle cells may have a role in directing motor axons toward the appropriate muscle once the axons are within striking distance of their target (Burden, 1998). Denervated muscles may secrete factors at limited distances (Slack and Pockett, 1981) that stimulate motor neurons to make synapses or Schwann cells to induce motor neurons to make synapses. Indeed both processes have been observed. Partial muscle denervation induces the spread of Schwann cells and their processes away from the synaptic zone implying that these cells do not target axons directly to the endplate (Reynolds and Woolf, 1992). Adjacent motor axons navigate along the Schwann cells processes to re-innervate the neighbouring denervated endplates (Son and Thompson, 1995a; Son and Thompson, 1995b; Tam et al., 2001). Extracts of denervated muscles can support neuronal survival and neurite outgrowth far better than equivalent material from innervated adult muscles (Henderson et al., 1983; Rassendren et al., 1992).

Cardiotrophin-1 (CT-1) and the Insulin-like growth factors (IGFs) are candidate target-derived motoneuron survival factors as both are expressed in muscle during naturally occurring motoneuron death and, applied systemically, support the survival of developing motoneurons. It has been shown recently that they are retrogradely transported from muscle to nerve cell body *in vivo* (Rind and von Bartheld, 2002). However, these factors have not been described in neurite outgrowth. Glial cell line-derived neurotrophic factor (GDNF) is the most potent motoneuron survival factor known to date (Henderson et al., 1994). Exogenous GDNF injected into muscles is retrogradely transported to motoneuron cell bodies (Steljes et al., 1999; Yan et al., 1995). It has been generally assumed that motoneurons respond to GDNF delivered by retrograde axonal transport from their target muscles (Springer et al., 1995; Suzuki et al., 1998). Recently it has been found that GDNF supports motoneuron survival in a retrograde, target-derived fashion, as opposed to a local paracrine route or an indirect route via sensory afferents (Chen et al., 2003). Although GDNF was initially characterized for its ability to prevent cell death of subsets of neurons during development, evidence accumulated that it can also regulate proliferation and differentiation (Taraviras et al., 1999). Indeed in the absence of GDNF signalling, motor neurons that innervate specific muscles are abnormally positioned in the spinal cord and muscle invasion by their axons is dramatically reduced (Haase et al., 2002).

Several observations suggest that secretion of growth factors cannot alone account for the different susceptibilities of innervated and denervated muscle to synapse formation. For

example, only nerve terminals adjacent to denervated muscle fibers form sprouts after partial denervation (Brown et al., 1981; Slack and Pockett, 1981). This indicates at least one signal provided by denervated fibers has a short effective range and may not be freely diffusible. Thus surface molecules might act along with soluble factors to signal the denervated fiber's susceptibility to innervation. Molecular candidates for this role are cell adhesion molecules.

There are three main families of adhesion molecules concentrated at neuron-neuron synapses. Little is known about their role at the NMJ. Cadherins, concentrated in the synaptic cleft of neuron-neuron synapses is involved in adhesion at the synapse mainly by mediating homophilic interactions (Tepass et al., 2000). They are not yet described at the NMJ. Integrins are present at the NMJ where they presumably interact with basal lamina (Cohen et al., 2000). They are clearly involved in growth and function of the *Drosophilia* NMJ (Beumer et al., 2002; Suzuki et al., 2002).

Several immunoglobulin superfamily (IgSF) members have been shown to be involved in axonal growth and guidance (Mueller, 1999; Walsh and Doherty, 1997). Recent data presume a role of such molecules at synapses. N-CAM has three fibronectin type III domains and five Ig-domains. It is involved both in early synaptogenesis and subsequent synaptic maturation by mediating homophilic and heterophilic interactions. In N-CAM knock-out (ko) mice, many aspects of transmission are normal and thus many presynaptic and postsynaptic molecules have assembled properly in the absence of N-CAM (Rafuse et al., 2000). On the other hand, in *Drosophilia*, the N-CAM ortholog fasciclin II (FasII) is a key regulator of growth and guidance of motor axons (Grenningloh et al., 1990; Grenningloh et al., 1991). At the NMJ, overexpression of Fas II postnatally has been shown to stabilize ectopic connections in postembryonic life. In the CNS, overexpression of Fas II pre- and postsynaptically causes a reduction in synaptic input to the motor neurons concerned (Baines et al., 2002). Another class of IgSF proteins that have been shown to be implicated in synapse formation are the Nectins. Nectins are composed of three Ig-domains, a transmembrane domain and a short intracellular domain (Takai and Nakanishi, 2003). The Actin-binding and PDZ-domain containing protein Afadin which is an Actin-binding protein in the C-terminal part of Nectins provide a direct link between the transmembrane Nectin proteins and the cytoskeleton. This Nectin/Afadin complex is implicated in synapse formation (Mizoguchi et al., 2002). Further experiments *in vivo* in particular have to be performed to corroborate these *in vitro* results. While searching for vertebrate proteins that share structural homologies with the FasII IgSF protein, SynCAM was identified (Biederer et al., 2002). It composes of three Ig-domains. It is

a relatively small, transmembrane N-glycosylated protein. SynCAM, localized on both sides of certain synapses in the brain, has been shown to be involved in the alignment between pre- and post-synaptic specializations by making homophilic interactions (Biederer et al., 2002). Furthermore SynCAM has been shown to drive synaptic assembly in *in vitro* assay (Biederer et al., 2002).

Axons regenerate to reinnervate denervated skeletal muscle fibers precisely at original synaptic sites (Kasthuri and Lichtman, 2003; Letinsky et al., 1976). Thus there must be some factors at or near the original postsynaptic surface that axons recognize. The synaptic basal lamina has been shown to contain clues that guide selective reinnervation of synaptic sites and differentiation of growth cones into motor nerve terminals at those sites (Sanes et al., 1978; Walsh and Lichtman, 2003).

Tenascin and Fibronectin which are synthesized by fibroblasts in peripheral synaptic areas (Gatchalian et al., 1989) may promote or direct growth of regenerating axons as they approach their targets (Caroni and Schneider, 1994; Sanes et al., 1986). Synaptic Laminins are also involved in promoting outgrowth and its ability to promote neurite outgrowth is higher than Fibronectin (Gundersen, 1987; Porter et al., 1995). Indeed, synaptic Laminins appear to organize the apposition of nerve terminal and muscle endplate *in vivo* (Noakes et al., 1995). Synaptic β2-laminins regulate nerve terminal differentiation *in vitro* and *in vivo* (Patton et al., 1997; Porter et al., 1995; Son et al., 1999).

1.6.2. Synapse inducers

Once a motor axon's growth cone contacts a newly formed myotube, differentiation of the NMJ begins with the differentiation of the presynaptic nerve terminal and the specialization of the postsynaptic apparatus which leads to a high efficient synaptic transmission between nerve and muscle (Dennis, 1981). Adult muscles, on denervation, develop characteristics very like those of embryonic muscle fibers. Both have AChR and MuSK distributed all over their surface (Salpeter et al., 1988). The density of AChR increases in the extrasynaptic region of a denervated muscle compared to innervated muscle. Nerve terminals differentiate where they contact muscle fibers (Sanes et al., 1978). The synapses formed are functional (Marshall et al., 1977). Release of motor neuron-derived signals, including Agrin, induces synapse

formation by interacting with receptor tyrosine kinases which in turn initiates concentration of ACh receptors and other proteins at the developing synapse (see 1.3.4. Clustering of AChR: Agrin signaling).

1.6.3. Synapse elimination by electrical muscle activity

Functional NMJ form immediately and in abundance such that fibers come to be innervated by axons from several motoneurons (polyneuronal innervation). Few days later, synapse elimination results in a reduction of polyneuronal innervation and thus to the formation of mature NMJ. Polyneuronal innervation can persist for months if muscles are transiently paralyzed during the time when synapse elimination would occur shortly after birth (Brown et al., 1982) or several weeks following nerve crush in reinnervated muscles of adult rats (Barry and Ribchester, 1995; Costanzo et al., 1999). Thus blockade of electrical activity delays synapse elimination. The excess inputs are eliminated by competition between axons resulting from the interaction of receptors expressed in axons that compete for a limited supply of neurotrophic factors produced by muscle fibers in proportion to activity (Bennett and Robinson, 1989; Thompson, 1985). Several trophic factors have been shown to delay synapse elimination, support motor axon branching, or cause motor unit enlargement when applied *in vivo* including IGF-1, Basic fibroblast growth factor (bFGF), Ciliary neurotrophic factor (CNTF), GDNF, Leukemia inhibitory factor (LIF) (Caroni and Becker, 1992; English and Schwartz, 1995; Jordan, 1996; Jordan et al., 1995; Keller-Peck et al., 2001; Kwon et al., 1995; Kwon and Gurney, 1996; Nguyen et al., 1998). A striking hyperinnervation of NMJ has been shown in transgenic mice in which GDNF is overexpressed in skeletal muscle (Nguyen et al., 1998; Zwick et al., 2001). In these GDNF overexpressing transgenic mice each endplate receives more synaptic inputs than the age-matched controls. Subcutaneous injection of GDNF also results in hyperinnervation (Keller-Peck et al., 2001). These neurotrophic factors have been shown to be also involved in neurite survival and thus could be involved in both processes. Despite these intriguing results the specific involvement of neurotrophic factors in naturally occuring synapse elimination has not been demonstrated.

1.7. Aim of the thesis

Electrical muscle activity is not only involved in synapse elimination but it also appears to regulate the susceptibility of muscle fibers to become innervated (Lomo and Slater, 1978). Following denervation, ectopic NMJs form between transplanted axons and extrajunctional regions of muscle fibers are made receptive to innervation. Specifically, when a foreign nerve is transplanted onto a non synaptic muscle region, it will not make synapses. However, when the muscle's own nerve is cut or paralyzed, it will form new synapses in extrasynaptic fiber regions. NMJ formation in this model recapitulates essential aspects of normal NMJ formation in the embryo. Thus denervated skeletal muscle fibers may express secreted soluble and surface membrane bound molecules that induce motor neurons to contact the muscle fiber and allow the formation of functional ectopic synapses (Lomo and Slater, 1978).

In an attempt to identify muscle-derived molecules that may promote synapse formation in a manner-dependent on electrical muscle activity, we used mRNA Differential Display approach (RDD) in normally innervated and denervated muscles. This method allowed us to analyze the differential expression of transcripts in four-day-denervated rat soleus muscles compared to innervated muscles. We were particularly interested in mRNAs that were up-regulated upon denervation as AChR, the hallmark of inductive events of synapse formation, are up-regulated following denervation. RDD permitted us to isolate low to high abundant transcripts present in the muscle samples. We isolated one gene, Embigin, which encodes a transmembrane protein highly up-regulated upon denervation. These experiments are described in Chapter II.

In chapter III, I investigated whether Embigin plays a role in the formation of the neuromuscular synapse. The results showed that Embigin mRNA is regulated by muscle activity, is transcribed during differentiation of C2C12 cells and its expression precedes ectopic synapse formation, i.e. as predicted for a surface molecule involved in synapse formation. However, an involvement of Embigin in NMJ formation could not be demonstrated.

Chapter II

2. mRNA differential display and validation by quantitative RT-PCR

2.1. Introduction

The aim of this research was to identify novel factors present at the surface of the muscle or secreted by the muscle involved in neurite outgrowth/attraction and attachment between the presynaptic nerve terminal and the postsynaptic membrane. Denervation is one of the best-defined systems to identify "sprouting signals" released from muscle that can diffuse limited distances within the muscle or present in the muscle surface and induce new growth of established axons (Brown, 1984). We chose mRNA Differential Display (RDD) to isolate such genes as this technique is designed to search for low to medium abundance of novel genes with high reproducibility (Liang and Pardee, 1992). Detectable differences between samples analysed by RDD is comprised between 1.1 fold and more (Liang and Pardee, 1992; Stein and Liang, 2002). The principle of the technique is described in Fig. 2.1. The general strategy is to amplify partial cDNA sequences from subsets of mRNAs by reverse transcription and the polymerase chain reaction (PCR). The key element of this method is to use a set of oligonucleotide primers, one being anchored to the polyadenylate tail of a subset of mRNAs, the other being short and arbitrary sequence so that it anneals at different positions relative to the first primer.

One drawback of the RDD is that analysis of large populations of mRNA (i.e. functional transcriptomics) would require about 240 different primer combinations (Stein and Liang, 2002) per sample to achieve 95% coverage of the transcriptome. If extensive gene coverage is desired other methods should be used for global analysis of gene expression such as microarrays and serial analysis of gene expression (SAGE). Quantitatively, the RDD method requires a very low amount of poly-A RNA.

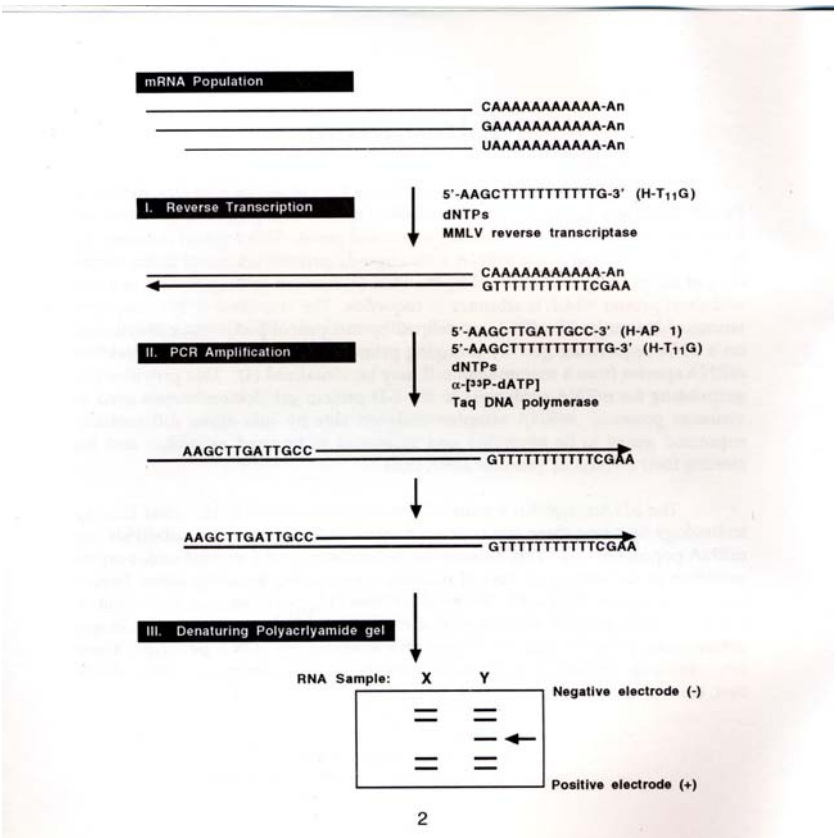


Fig. 2.1. Principle of the mRNA Differential Display.
Total RNA are prepared from samples used for RDD. An RT-PCR was then performed on these total RNA using oligo dT primers. The RT-PCR is followed by a PCR amplification using H-AP primers that represent consensus sequences. The weakly radioactive PCR products are then loaded on a PAGE.

2.2. Material and Methods

2.2.1. Sample preparation

Soleus muscles of 7-week old Wistar rats were denervated by cutting the sciatic nerve at a distance of about 2 cm of the muscle. 4 days after denervation, rats were sacrified and soleus muscles isolated. For control samples, soleus muscles were isolated from age-matched rats (innervated samples). The soleus weight from rat muscles was 162,3 ± 3,2 mg and from 4-day denervated soleus rat muscles was 164,7 ± 11 mg. Soleus muscles were transferred into green-capped FastPrep™ tubes containing lysing matrix D (Bio101, Qbiogene) and filled with 1 ml of TRIReagent® (Molecular Research Products), then snap-frozen in liquid nitrogen and kept at -80° C until homogenization. Muscles were homogenized by four 20 sec

shaking cycles on the FastPrep™ FP120 apparatus (Bio101, Qbiogene). RNA extraction was performed according to the manufacturer instructions (Molecular Research Products).

2.2.2. mRNA differential display

Total RNA from each three innervated and denervated rat soleus muscles was treated with DNAse I (MessageClean®, GenHunter® Corporation) before proceeding to mRNA differential display. mRNA differential display was performed using RNAimage® kits (GenHunter® Corporation) containing H-AP primers 1 to 16 according to manufacturer instructions (GenHunter® Corporation). However the following modifications were made. PCR amplifications were performed with α-[^{33}P]dATP (NEN) and AmpliTaq polymerase (Roche) in a final volume of 10 μL. Reamplified PCR fragments were gel-purified with QIAEXII (Qiagen) and subcloned into pGEM®-T and pGEM®-T Easy vector systems (Promega). Sequencing was performed on both strands on an ABI377 DNA Sequencer (Applied Biosystems). The obtained sequences were blasted against the non-redundant public databases using the NCBI website.

2.2.3. Quantitative RT-PCR

Total RNA was treated with DNAse I (Ambion) and reverse-transcribed with an oligo-dT primer using 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche). For quantitative PCR, cDNA was amplified with qPCR™Mastermix Plus for SYBR® Green I (Eurogentec). Primer sequences used in PCR reactions were chosen based on the sequences available in GenBank. Primers (Microsynth AG) were designed to generate a PCR amplification product of 160-220 bp. Only primer pairs yielding unique amplification products without primer dimer formation were subsequently used for Real Time PCR assays. The mRNA expression of all genes reported is normalized to ribosomal protein L8 expression.

2.3. Results

2.3.1. Transcript differences between innervated and denervated soleus

Analyzing RNAs from three innervated and denervated muscles showed differences in the expression level of 202 transcripts. To evaluate the significance of the observed mRNA changes, the results were compiled into functional categories (Table 1).

Table 1. Expression profile of genes identified by RDD.
Names and Genebank identifier (ID) of the transcripts detected in soleus rat muscles. The relative ratio (3 denervated versus 3 innervated muscles) are displayed. The P-Value has been also calculated.

Functional unit/Gene	ID	Induction Den/Inn	P-Value	Regulation
Nerve/muscle				
synaptotagmin-like protein	AK010527	0,800	0,251	down
Synapse elimination				
prothrombin	M81397	1,392	0,180	up
Vesicular transport				
synaptic vesicle glycoprotein 2B (Sv2b)	AF372834	0,435	0,060	down
Extracellular matrix				
alpha 8 integrin	gi47717126	1,479	0,164	down
annexin V (Anx5), Lipocortin V	gi 939850	0,316	0,178	down
extracellular matrix protein 2 (Ecm2), SPARC-like1, mast9, hevin	gi 6978788	0,581	0,003	down
procollagen, type VI alpha3 (type VI collagen alpha 3 subunit)	gi 13529550	0,202	0,038	down
lysyl oxidase (Lox)	gi 8393732	1,270	0,103	up
Cytoskeleton				
alpha-1 actin	V01218	0,647	0,160	down
alpha-actinin 2 (a-actinin skel. muscle isoform 2)	gi 12834316	0,422	0,043	down
dynactin 6 (Dctn6), WS-3 protein, dynactin subunit p27	gi 6756008	0,417	0,025	down
LIM and PDZ domain 1 (elfin) (Pdlim1)	U23769	0,394	0,036	down
myosin light chain 2 (MCL2), cardiac	gi 56682	0,164	0,028	down
myosin light chain 3 alkali cardiac ventricles (Myl3)	gi 56680	0,577	0,046	down
nonerythroid alpha-spectrin, alpha II spectrin, alpha-fodrin	gi 203013	0,107	0,066	down
palladin	XM_224797	0,300	0,024	down
smoothelin large isoform L2	BC002317	0,440	0,063	down
titin immunoglobin domain protein, myotilin (Ttid)	gi 16740675	0,347	0,056	down
titin, heart isoform N2-B	AK009965	0,623	0,082	down
titin, transcript variant N2-A	gi19747266	0,312	0,008	down

Table 1 to be continued

titin-cap protein (Tcap), telethonin	gi 6755725	0,626	0,259	down
troponin 1, slow skel. muscle troponin I	gi 20910382	0,397	0,093	down
troponin I, slow isoform, slow fiber troponin I	gi 8394462	0,411	0,035	down
troponin-T	M15202	0,466	0,115	down
2B myosin heavy chain	gi 288643	1,259	0,195	up
alpha-tropomyosin	M34134	8,675	0,004	up
dystonin isoform b, bullous pemphigoid antigen 1 (Bpag1)	gi 20845210	6,542	0,028	up
myopodin (synaptopodin2)	AI848603	1,333	0,041	up
myosin heavy chain 2B (MYHC-IIB)	gi 13177514	1,908	0,109	up
Actin monomer binding activity				
adenylyl cyclase-associated protein 2 (CAP2)	AK033071	0,848	0,080	down
Transmembrane proteins				
AMIGO 3	AK088051	0,484	0,004	down
chondroitin beta 1,4 N-acetyl galactosaminyltransferase	gi 20864357	1,015	0,341	down
L-type voltage-dependent Calcium channel alpha2delta	NM_012919	1,932	0,089	down
lymphocyte antigen 68 (Ly68), cell surface antigen AA4	gi 6754577	0,846	0,371	down
CD28	AK030812	1,045	0,052	up
Embigin	**gi16758533**	**9,475**	**0,01852**	**up**
Signal transduction				
guanine nucleotide binding protein beta polypeptide 2-like 1 (Gnb2l1)	gi 18543330	0,811	0,151	down
myo-inositol monophosphatase 1 (Impa1)	gi14091735	0,472	0,098	down
protein phosphatase 2A catalytic subunit	X14159	0,482	0,076	down
protein tyrosine phosphatase, non-receptor type 3 (Ptpn3)	gi 20828735	0,775	0,163	down
small G-protein RAP-2A-like protein	AL670230	0,721	0,221	down
sphingosine-1-phospate phosphatase 1	BC026814	0,580	0,124	down
acid sphingomyelinase-like phosphodiesterase 3b	BC009087	2,542	0,002	up
casein kinase II, alpha 2 polypeptide	AK004115	2,742	0,111	up
liver regeneration-related protein 2, TRAF6-binding protein (T6BP)	AF509819	1,576	0,088	up
protein phosphatase 2 (formerly 2A), catalytic subunit, α isoform	gi 8394017	1,516	0,076	up
sarcolipin	AK009809	1,927	0,061	up
calcyclin binding protein (Cacybp), homology with Cyp2A2	gi 19683987	2,604	0,090	up
Energy metabolism				
Fatty acid metabolism				
cytosolic malate dehydrogenase	gi 3747084	0,335	0,022	down
fatty acyl-CoA reductase	AK014486	0,695	0,055	down
heart fatty acid binding protein (H-FABP)	gi 204079	0,555	0,077	down
lipoprotein lipase (Lpl)	gi 6981167	0,249	0,019	down
methylmalonyl-CoA epimerase	gi 20832468	0,249	0,022	down
Citric acid cycle				
citrate synthase EC 4.1.3.7	gi20987214	0,425	0,049	down
isocitrate dehydrogenase 1	NM_031510	0,582	0,103	down
succinate dehydrogenase complex, subunit B	gi 17235761	0,494	0,003	down
Glycogen metabolism				

Table 1 to be continued

glycogen phosphorylase muscle isozyme	L10669	1,003	0,361	down
glycogenin	gi 13277959	0,817	0,322	down
muscle glycogen phosphorylase (MGP), CYP17	gi 310176	0,303	0,009	down
Oxidative phosphorylation				
adenine nucleotide translocator solute carrier family m25 (slc25a4), ANT1	D12770	0,132	0,006	down
ATP synthase subunit 6	X14848	0,511	0,027	down
ATP synthase subunit 9 (C) isoform 3 (Atp5g3)	AF315374	0,697	0,000	down
ATP synthase subunit gamma (ATP5L)		0,398	0,062	down
ATP synthase, H+ transporting, mitochondrial F1F0 complex, subunit e	gi 17978458	0,660	0,075	down
ATPase classII type 9A, ATPase 9A (Atp9a)	gi 13905301	0,804	0,032	down
cytochrome-c oxidase III	X14848	0,199	0,013	down
electron transfer flavoprotein beta subunit (BETA-ETF)	gi 2131203	0,584	0,012	down
F1-ATPase alpha subunit (EC 3.6.1.34)	gi 57028	0,531	0,031	down
NADH dehydrogenase (ubiquinone) 1 beta subcomplex 5 (Ndufb5)	gi19263528	0,504	0,006	down
NADH:ubiquinone oxidoreductase 17.2 kDa subunit	gi 12833603	0,502	0,067	down
ubiquinol-cytochrome c reductase iron-sulfur subunit, mitochondrial	gi 18044190	0,183	0,145	down
voltage-dependent anion channel VDAC1	AB039662	0,371	0,064	down
ATPase inhibitor protein (Atpi), mitochondrial IF1 protein	gi 286197	1,319	0,014	up
mitochondrial inner membrane translocase (Timm8b, DDP2)	AF196315	1,158	0,123	up
ubiquinol cytochrome c reductase complex 7.2 kDa protein	AK012489	2,754	0,063	up
Detoxification				
alcohol dehydrogenase class III (Adh-B2)	XM_215714	1,738	0,039	up
Amino acid metabolism				
4-hydoxyphenyl pyruvate dioxygenase	BC034099	0,343	0,008	down
fumarylacetoacetate hydrolase	XM_220229	0,287	0,067	down
Pentose phosphate pathway				
transaldolase 1 (Taldo1)	gi 13929151	1,370	0,160	up
Mitochondrial energy conversion beta-oxidation				
hydroxyacyl-CoA dH/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, α-subunit	gi 18677762	0,531	0,000	down
Portein turnover				
12S ribosomal RNA	S63167	0,204	0,005	down
BRIX	AK010171	0,566	0,008	down
mitochondrial 28S ribosomal protein S32 (MRP-S32)	gi 19353347	0,654	0,108	down
mitochondrial RNA-processing endoribonuclease (RMRP)	gi 20826446	0,151	0,092	down
proteasome 26S subunit ATPase 2 (Psmc2, MSS1)	D50694	0,287	0,018	down
ribosomal protein L10a (Rpl10a)	gi 135920008	0,686	0,033	down
ribosomal protein L21	gi 20339142	1,146	0,417	down
ribosomal protein L31 (Rpl 31)	X04809	0,477	0,067	down
ribosomal protein S8 (Rps8)	gi 13928985	0,617	0,077	down
TFIIH (Naip3)	AF242432	0,950	0,461	down
TFIIIA	gi 18448379	0,697	0,036	down
ubiquitin-like protein GDX (ubiquitin-like protein 4)	gi 20984330	0,723	0,048	down
Protein synthesis				

Table 1 to be continued

4-hydoxyphenyl pyruvate dioxygenase	BC034099	0,343	0,008	down
fumarylacetoacetate hydrolase	XM_220229	0,287	0,067	down
glutaminyl-tRNA synthetase	BC023023	0,185	0,046	down
ribosomal protein L14	XM_231617	0,350	0,011	down
elongation factor 1 alpha	X63561	1,461	0,012	up
endoplasmic reticulum protein 29 (Erp29)	gi13177514	1,081	0,269	up
eukaryotic translation initiation factor 3 subunit 4 subunit p42	gi 20884037	2,633	0,135	up
Protein degradation/folding/modifications				
prosome macropain 28 subunit alpha (Psme 1)	gi 8394087	1,202	0,029	up
cyclophilin A	gi 8394008	1,247	0,024	up
carbohydrate (chondroitin) synthase 1	XM_218758	3,545	0,084	up
Nedd4, WW domain-binding protein 5	gi 18042813	1,190	0,085	up
ubiquitin specific protease 28	gi 20888426	1,390	0,003	up
ubiquitin-specific protease 14 (Usp14)	gi 14919407	1,501	0,061	up
Transcription				
bromodomain-containing protein 7 (Brd7), BP75	gi 6755233	3,169	0,027	down
cell growth regulatory with Ring-Finger domain Cgr19	gi 16758771	0,384	0,031	down
constitutive androstane receptor (CAR, NR1I3)	AK028388	0,659	0,071	down
FK506 binding protein 3 (25 kD) (Fkbp3) (Fkbp 25)	gi 12805310	0,377	0,004	down
histone deacetylase 1 (HDAC1)	gi 12653070	0,337	0,026	down
Ring-finger protein 13	AK009868	0,746	0,097	down
signal transducer and activator of transcription 1 (STAT1)	gi 20846615	0,282	0,081	down
TFIIE	gi 26009942	0,552	0,171	down
calmodulin 1	gi 4010862	1,253	0,060	up
Swi/SNF related matrix associated actin dependent regulator of chromatin	gi 20809350	1,177	0,020	up
Zinc finger protein 265 (Znf 265) (Zfp 265)	gi 13928844	1,191	0,095	up
Zn-finger/Leu Zipper protein (AF10)	gi 20820758	1,857	0,150	up
Cell cycle				
Cell cycle growth/progression				
PTZ 17 (P311)	D45203	0,702	0,047	down
retinoblastoma-related gene (Rb2), p130, fused toes (Fts)	gi 13592040	0,381	0,002	down
rat growth and transformation-dependent mRNA	M17412	1,401	0,109	up
RGC 32 protein (response gene to complement 32) (Rpgc32)	gi 16758927	2,168	0,099	up
Apoptosis				
inhibitor of caspase-activated deoxyribonuclease	AK051011	0,338	0,027	down
castration induced prostatic apoptosis related protein-1 (CIPAR-1)	AJ010750	3,434	0,014	up
Intracellular signalling				
progesterone membrane binding protein, steroid receptor protein DG6	gi 20867173	1,688	0,112	up
transferrin receptor 1 (TFR1)	gi 21668434	0,356	0,007	down
Gametogenesis/meiosis/spermatogenesis				
spindlin 1	gi 20895182	0,682	0,001	down
protein kinase NYD-SP15	AK038926	0,907	0,054	down

Table 1 to be continued

Stress response				
Hsp90	S45392	4,071	0,109	up
Transport				
Endosome to Golgi trafficking				
sorting nexin 2 (Snx2)	gi 13385877	0,749	0,221	
Membrane trafficking				
FYVE and coiled-coil domain containing protein 1 (Fyco1), pMEM2	gi 18250725	0,577	0,147	down
Golgi-specific Brefeldin A-resistant guanine nucleotide exchange factor 1	gi 20889959	1,384	0,030	up
Nuclear protein export				
nucleoprotein 50 kDa (Nup 50)	gi 6981295	0,493	0,002	down
nuclear pore complex protein Nup107	gi 16758681	0,452	0,006	down
Nucleotide transport				
myeloid cell leukemia sequence 1 (Mcl1), EAT/MCL-1	gi 19774509	2,746	0,023	up
nucleoside diphosphate kinase (Nme 2)	S39901	0,972	0,363	down
Drug metabolism				
3'-phosphoadenosine 5'-phosphosulfate synthase 2 (Papss2)	gi 6754983	1,439	0,084	up
Thrombogenesis				
tissue factor pathway inhibitor	AK034752	0,703	0,058	down
prothrombin	M81397	1,392	0,180	up
Unknown				
hypothetical protein	XP_144930	0,518	0,155	down
hypothetical protein FLJ10702	AK050362	0,427	0,007	down
hypothetical protein FLJ12888	XP_225150	0,692	0,019	down
hypothetical T29315 protein (H.s. CGI-146 protein)	BC040687	0,679	0,201	down
mitochondrial genome D loop region	AY172581	0,630	0,171	down
secreted protein of unknown function	XM_213975	0,188	0,015	down
similar to KIAA0582 protein	BC027174	0,432	0,002	down
RIKEN D130062H10 (embry spinal ganglion, unique)	AK051657	3,124	0,008	up
hypothetical protein FLJ20984	BC021423	1,832	0,051	up
RIKEN 5730409K12 unclassifiable (embryo, brain)	gi 12856822	4,566	0,002	up
Glu-Pro dipeptide repeat lipid protein	gi 16758277	1,210	0,123	up
RIKEN 1110001E17 unclassifiable transcript	gi 20864635	2,732	0,057	up
unknown 5 (mouse chromosome 5)	AC011419	0,864	0,367	down
unknown 10 (mouse chromosome 2)	AL928963	0,438	0,091	down
unknown 25 (mouse chromosome 2)	AL714023	3,098	0,058	up
unknown 43 (mouse chromosome 2)	AC122202	1,471	0,015	up
unknown 95 (mouse chromosome 2)	AL732413	1,607	0,137	down
unknown 146 (mouse chromosome 4)	AL808103	1,170	0,115	up
unknown 89	AL606975	2,699	0,008	up
unknown 114	AC098709	0,527	0,014	down
unknown 143	U48809	0,623	0,186	down

Table 1 to be continued

unknown 149	BC024874	1,249	0,039	up
unknown 28		0,408	0,094	down
unknown 34		1,208	0,083	up
unknown 46		2,035	0,055	up
unknown 59		3,636	0,092	up
unknown 116		0,695	0,039	down
unknown 124		0,332	0,039	down
unknown 133		0,739	0,074	down
unknown 134		0,898	0,100	down
unknown 135		1,008	0,322	down
unknown 138		0,991	0,384	down
unknown 139		1,295	0,006	up
unknown 171		1,980	0,013	up
unknown 174		4,021	0,088	up

Brief general observations can be made before proceeding to the molecules potentially involved in NMJ formation. Three mechanisms of muscle atrophy are highlighted at the molecular level according to the number of genes found with altered expression in each category. We did not observe significant difference in muscle mass between innervated and denervated muscles 4 days after denervation. However the cellular processes underlying muscle atrophy probably have already started. A slow-twitch towards a fast-twitch partial transition has been widely demonstrated in the slow-twitch soleus muscle in atrophy (Diffee et al., 1991; McDonald et al., 1994) which is observed by a general down-regulation of genes encoding cytoskeletal components. The genes involved in energy metabolism were found to be down-regulated. This is in full agreement with the less aerobic energy supply of fast-twitch muscle fibers in atrophy (Thomason and Booth, 1990). Down-regulation of essential genes involved in protein synthesis, maturation and of transportation and the up-regulation of genes involved in protein degradation appear to be a basic component of the muscle atrophy process too (Goll et al., 1998).

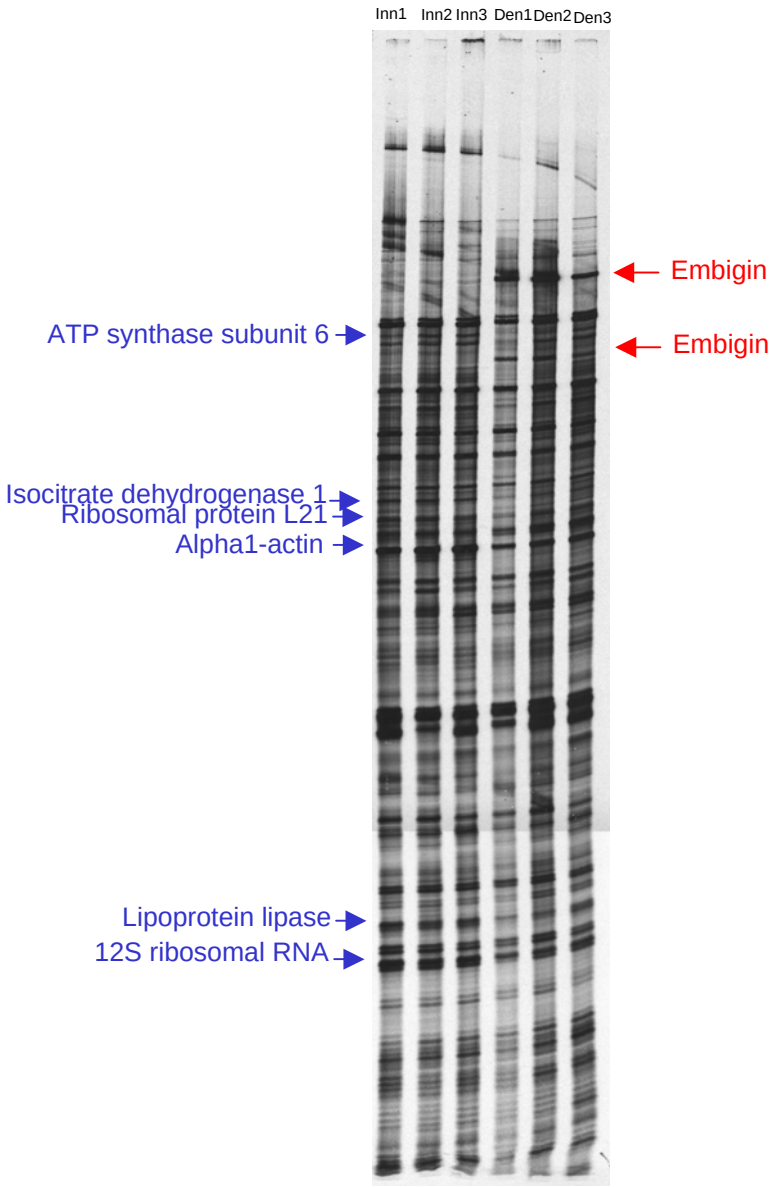


Fig. 2.2. Representation on a polyacrylamide gel of genes differentially regulated upon denervation following RDD analysis with one primer pair.
In red colour, Embigin which is up-regulated upon denervation. In blue colour, genes that are down-regulated after denervation.

The functional unit grouping genes encoding transmembrane proteins was of particular interest in our purpose. mRNAs coding for transmembrane proteins were in majority down-regulated upon denervation. However one muscle specific gene that is highly up-regulated upon denervation and of potential interest is Embigin (Fig. 2.2).

2.3.2. Validation: PCR verification of transcript expression level

The relative levels of transcripts described in the literature to be up-regulated upon denervation were tested by quantitative RT-PCR on the three innervated and three denervated muscle samples used for RDD. It was confirmed that the mRNAs coding for AChR δ subunit, MyoD and MuSK are strongly up-regulated upon denervation (Fig. 2.3A), as demonstrated previously by Northern Blot (Bowen et al., 1998; Eftimie et al., 1991; Goldman et al., 1988). The up-regulation of mRNAs coding for diffusible factors, GDNF and IGF-1 (Fig. 2.3B), upon denervation is confirmed as demonstrated previously (Krishan and Dhoot, 1996; Lie and Weis, 1998). Therefore the mRNA template used for RDD is validated.

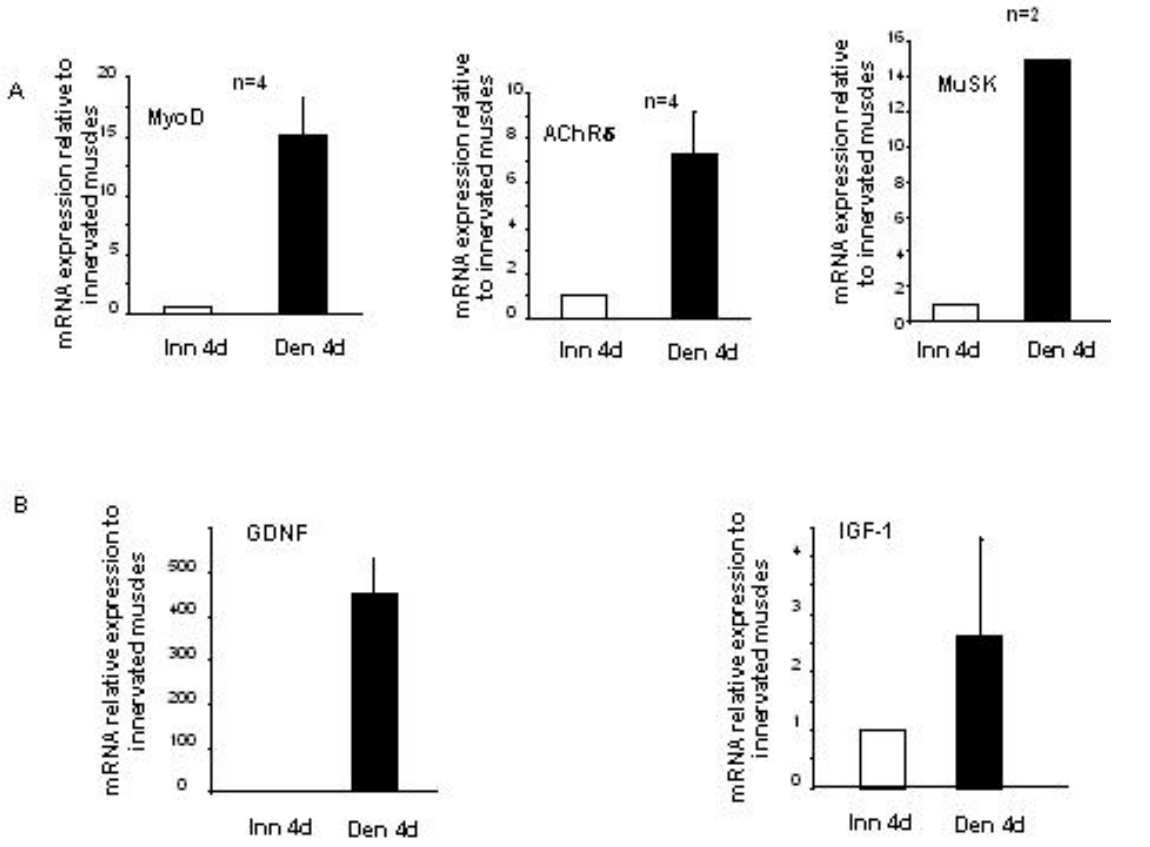


Fig. 2.3. PCR verification of transcript level of synaptic genes and known secreted proteins.
A, Analysis by Real Time PCR of MyoD, AChR δ subunit and MuSK mRNAs expression in soleus rat muscles after 4 days of denervation. Expression of the innervated adult soleus rat muscles was set to 1. Values were normalized to RL8 mRNA (data means±SE). B, Analysis by Real Time PCR of GDNF and IGF-1 mRNAs expression in soleus rat muscles after 4 days of denervation. Expression of the innervated adult soleus rat muscles was set at 1. Values were normalized to RL8 mRNA (data means±SE, n=3 experiments with duplicate values).

The results of the RDD were validated for a number of other genes coding for cytoskeleton proteins, protein degradation proteins, synapse elimination and thrombogenesis proteins, synaptic vesicular transport and unknown protein. In each case, at least, qualitative agreement between the results using the two methods was observed (Table 2).

			Regulation	
Gene	**mRNA expression relative to innervated muscles**	**S.D.**	**Q-PCR**	**RDD**
alpha-tropomyosin	32,77	12,2931	up	up
myosin heavy chain 2B	65,56	17,6459	up	up
myosin light chain	-10,20	1,5100	down	down
smoothelin	-2,72	0,2300	down	down
prosome macropain 28S	13,74	3,9600	up	up
sorting nexin 2	2,73	0,2051	up	up
prothrombin	4,19	0,0318	up	up
synaptic vesicle glycoprotein 2B (Sv2b)	-1,27	0,1520	down	down
LIM and PDZ domain 1 (elfin) (Pdlim1)	-3,66	0,9800	down	down
secreted protein of unknown function	-2,75	0,7400	down	down

Table 2. PCR verification of some transcripts identified by RDD.
Analysis by Real Time PCR using Sybr Green dye of embigin mRNA expression in rat soleus muscles 4 days after denervation used for RDD. Expression of the innervated adult soleus rat muscles was set to 1. Values were normalized to RL8 mRNA (data means±S.D., n=3 animals with duplicate values).

It was also confirmed that the mRNA coding for Embigin is strongly up-regulated upon denervation. Embigin is highly expressed in denervated muscles to attain a maximum level of ≈ 140-fold higher in denervated samples (Fig. 2.4).

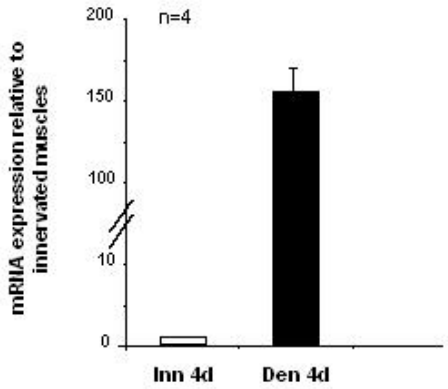


Fig. 2.4. Analysis by Real Time PCR using Sybr Green dye of Embigin mRNA expression in rat soleus muscles 4 days after denervation.
Expression of the innervated adult soleus rat muscles was set to 1. Values were normalized to RL8 mRNA (data means±SE).

2.4. Discussion

RDD allowed the identification of many differentially expressed genes upon denervation. The vast majority of genes are involved in general functions of muscle cells i.e. cytoskeleton, energy metabolism, protein turnover. These genes were generally down-regulated upon denervation as a consequence of muscle atrophy. Only few genes of our interest were isolated. One reason is the technical limitations. 48 primer pairs cover about 71% of total transcripts. Thus several molecules known to be secreted, for example GDNF and CT-1, and molecules known to play key roles at the NMJ, for example AChR, MuSK and MyoD, were not resolved by RDD.

However one gene called Embigin of potential interest has been isolated by RDD. Embigin is a glycoprotein belonging to the IgSF, class of cell adhesion molecules (Ozawa et al., 1988). In Chapter III, experiments will be presented examining the potential involvement of Embigin in NMJ formation.

Chapter III

3. Embigin, a surface molecule in muscle involved in synapse formation?

3.1. Introduction

To define a synaptic cell adhesion molecule three criteria have to be met. First, a high degree of conservation is a hallmark of the key players involved in synaptic cell adhesion activity. N-CAM identified in mammals is conserved to high degree with FasII but it lacks important domain for recognition of PDZ-domain proteins that is present in FasII. Second, the potential synaptic adhesion molecules have to be concentrated at synapses. N-CAM forms are present at presynaptic and postsynaptic membranes (Dityatev et al., 2000) but also elsewhere (Schachner, 1997) and Nectins are found more in the synaptic surroundings (Takai and Nakanishi, 2003). Third, in constitutive- or conditional null-mutants a dramatic synaptic phenotype has to be observed (Missler, 2003). Thus there are many candidates but little evidence: SynCAM and Nectins have been studied only in *in vitro* assays. The molecular nature and the mechanisms governing synaptic cell adhesion molecule are still wide open questions.

The extracellular domain of Embigin shares homologies with the extracellular domain of SynCAM, that has been shown to drive synaptic assembly in the CNS (Biederer et al., 2002) and has a role in cell adhesion (Huang et al., 1993). This led us to investigate the role of Embigin in neuromuscular synapse formation.

3.1.1. Embigin

3.1.1.1. Embigin: a cell adhesion molecule

Embigin is a member of the IgSF, of the Basigin/Embigin/Zov-3 subgroup (see Annexe). It has been identified as a carrier of a developmentally regulated carbohydrate marker found in early embryonic cells (Ozawa et al., 1988). Thus one possible function of Embigin is to play a role in the adhesion leading to formation of a neuromuscular contact. Indeed it has been shown that Embigin enhances adhesion of cultured fibroblasts cells overexpressing Embigin (Huang et al., 1993).

3.1.1.2. Genomic organization

Embigin gene is located in chromosome 13 in mouse. The Embigin gene spans more than 50kb and contains nine exons (Fig. 3.1). All exons contain protein-coding sequences. Each of the two Ig-domains is encoded by two exons and the C-proximal part of the second Ig-domain also encodes the transmembrane domain (Tachikui et al., 1999). There are two potential sites of polyadenylation which result in two transcripts. In mouse, size of the transcripts is 2,1kb and 1,4kb.

The 5'-flanking sequence of Embigin exhibits a promoter activity. The promoter contains a single putative Sp1-binding site but lacks any consensus TATA- and CAAT-boxes (Tachikui et al., 1999). Two putative MyoD-binding sites (E-boxes) have been also identified. They are located at –350bp and –200bp.

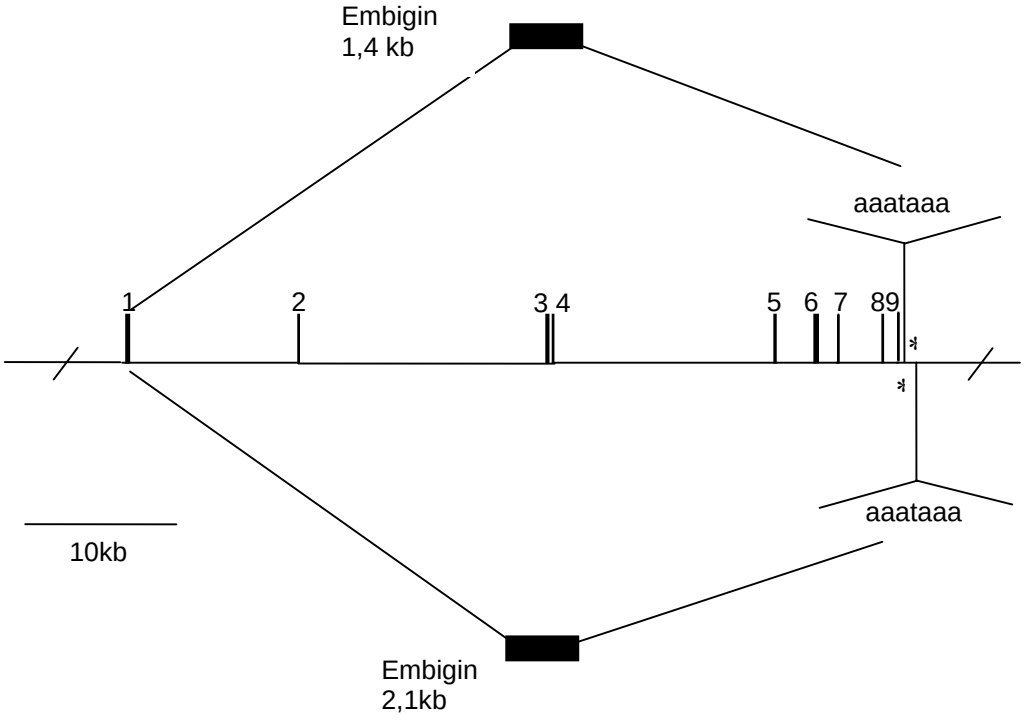


Fig. 3.1. Genomic organization of the mouse Embigin gene.
Two potential sites of polyadenylation result in two transcripts: 1,4 kb and 2,1 kb. This figure has been derived from sequences found in the mouse genome GenBANK database.

3.1.1.3. Protein structure of Embigin

The full-length cDNA for mouse and rat Embigin encodes a protein of 329 aa with a predicted molecular weight of 37kDa. The protein has a predicted 23 aa signal peptide (residues 1-23) and a single transmembrane domain (residues 254-282). Embigin has nine potential sites of N-glycosylation (Fig. 3.2). It is predicted to have one Ig-like domain (88-145) principally folded into multiple β-sheets and a Ig-V-like domain (181-239) near the transmembrane region with cysteines 88, 145 and 181, 239 predicted to be linked by disulfide bonds stabilizing each Ig domain (Ozawa et al., 1988). The cytoplasmic tail is short and composed of 47 aa. Three potential sites of phosphorylation are present in the C-terminal part (Fig. 3.2) with a paucity of any common motifs involved in cell signaling.

Sequence similarities of Embigin in different species were determined (Fig. 3.2). The transmembrane domain is highly conserved between the different species as well as the cytoplasmic part. Embigin is highly conserved during evolution. Homologs of Embigin are present in the genomes of many other species including monkey, mouse, dog, rabbit, chicken, fish and drosophilia (Guenette et al., 1997).

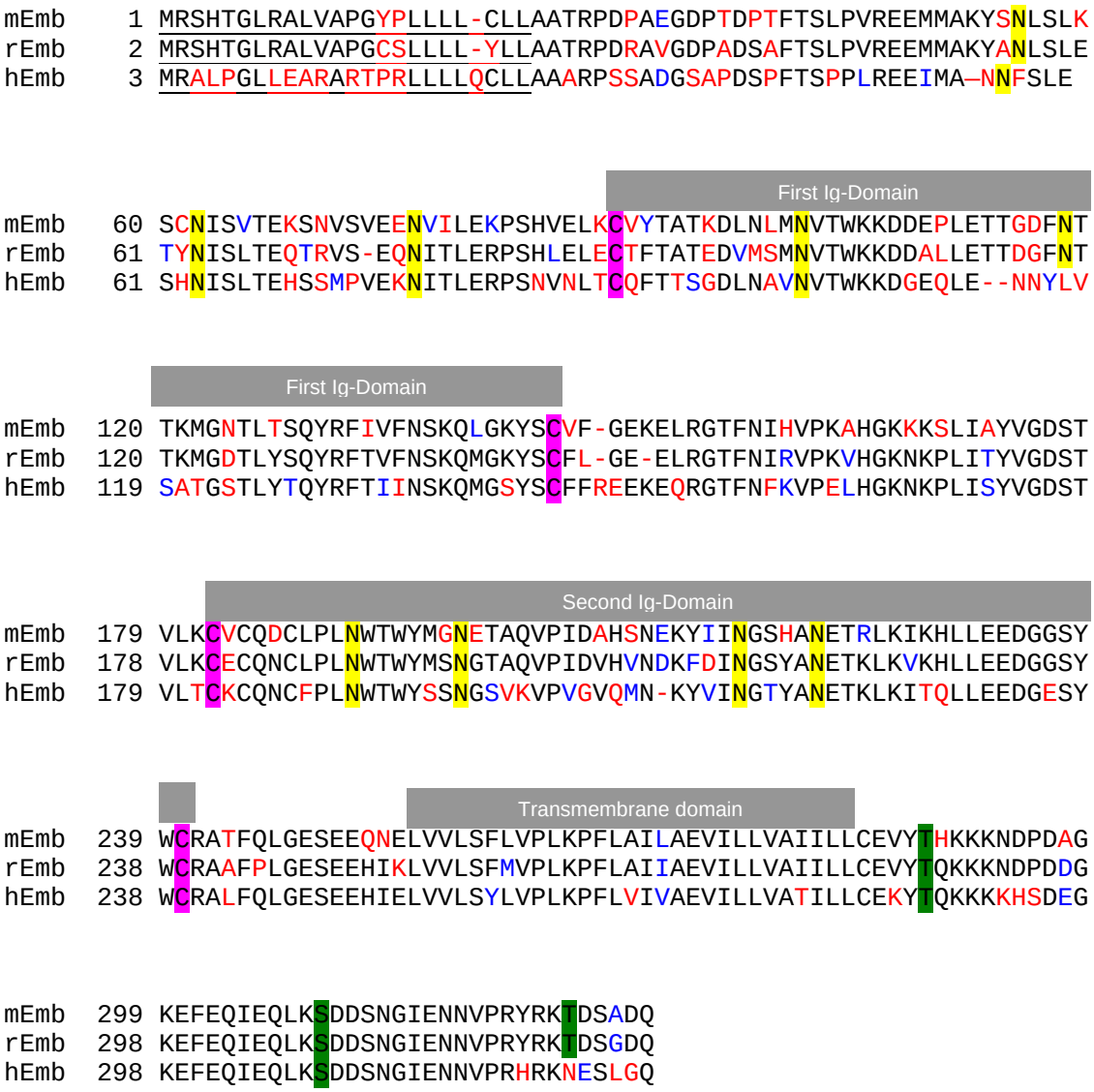


Fig. 3.2. Sequence alignment of Embigin.
Amino acid sequences of mouse, rat and human were aligned by Clustal V multiple alignment. The different domains are indicated. The signal peptide is underlined. Amino acid residues conserved in all three species are shown by black letters. Amino acid residues similar to mouse Embigin are indicated in blue letters and amino acid residues different from mouse Embigin are shown in red letters. Potential N-linked glycosylation sites (identified by ScanProsite) are shown in black letters with yellow background while potential sites of phosphorylation (identified by ScanProsite) in black letters with green background. The conserved cysteine residues are indicated in black letter with a pink background.

3.1.1.4. Developmental and tissue-specific expression of Embigin

Embigin mRNA expression is low in a variety of adult rat and mouse tissues (Guenette et al., 1997; Huang et al., 1993). The abundance of Embigin mRNA appears low in kidney, liver, heart, small intestine and skeletal muscle in mouse and in addition in testis and mammary gland in rat. However in mouse embryos, Embigin mRNA is highly expressed from day 0 to day 9 of gestation (Huang et al., 1990). With in situ hybridization it has been shown that Embigin mRNA is present in higher amount from day 7 to day 9 in the embryo. Embigin mRNA is also present in high amount in embryonal carcinoma (EC) cells. EC cells correspond to cells of embryonic ectoderm of 5- to 6-days gestation (Huang et al., 1990).

The denervation of skeletal muscle is thus the first physiological stimulus that induces the expression of Embigin mRNA in adult tissues as it has been shown by RDD previously (Chapter II).

3.2. Materials and methods

3.2.1. Animal handling

For denervation experiments, soleus muscles of 6- to 8-week of Wistar rats and gastrocnemius muscles of 6- to 8-week old BL6/C57 mice were denervated by cutting the sciatic nerve. Rat soleus muscles and mouse gastrocnemius muscles were dissected after different days of denervation. Additionally rat soleus muscles denervated for 5 days were subsequently stimulated for 5 days with intermittent 100Hz stimulation. For innervated control samples, soleus and gastrocnemius muscles from age-matched rats and mice were isolated. Mouse neonatal leg muscles were also dissected at stage P2, P0 being referred to as day of birth.

Diaphragms of 6- to 8-week old Wistar rats were dissected. After section of the diaphragms the whole diaphragm was stained with α-bungarotoxin-rhodamine (Molecular Probes) in Leibowitz 15 (L15) solution (40% of L15 medium from Sigma , 60% of a solution of 140 mM NaCl, 3mM KCl, 37µM $MgCl_2$, 2,5mM $CaCl_2$ with a pH between 7,2 and 7,4; 4mM HEPES, 6,5mM glucose) for 2hrs at 4°C. After a short wash in Ca^{2+} Mg^{+} free PBS (see Molecular Cloning), the extrasynaptic region and the synaptic region of the diaphragm was separated.

Diaphragms of 6- to 8-week old Wistar rats were also denervated for 6 days. After dissection of the diaphragms, the separation between the extrasynaptic region and the synaptic region of the denervated diaphragms was performed as described above using α-bungarotoxin-rhodamine (Molecular Probes).

3.2.2. C2C12 cell culture

C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) low glucose from Sigma, 20% foetal calf serum (Invitromex), 100U/mL streptomycin, 100U/mL penicillin (Sigma), 1x glutamine (Gibco) at 37°C at 5% CO_2 at a confluence of 20%. $8x10^4$ cells were passaged onto 6-well plates that had been coated with 0,003% of gelatine (Fluka). Two days later the growth medium was replaced by the fusion medium DMEM high glucose (Gibco) with 5% horse serum (Invitromex), 100U/mL streptomycin, 100U/mL penicillin (Sigma), 1x glutamine (Gibco). Time course experiment started the day when the medium was switched from the proliferation medium to the differentiation medium and corresponds to T0. The RNA from C2C12 cells were extracted with 500µL of TRIReagent® (Molecular Research Products) after indicated days of differentiation.

3.2.3. Quantitative RT-PCR and Northern Blot

Total RNA from innervated and denervated muscles as well as C2C12 cells were prepared using TRIReagent® according to manufacturers instructions (Molecular Research Products) in green-capped FastPrep™ lysing Matrix D tubes (Bio101, QBiogene) to homogenize the muscle samples. These total RNAs were used in quantitative PCR and Northern Blot. For quantitative PCR, total RNA were first reverse-transcribed with an oligo-dT primer using first strand cDNA synthesis kit for RT-PCR (AMV) (Roche). The cDNAs were then amplified with qPCR™Mastermix Plus for SYBR® Green I (Eurogentec) with the following primer pairs: mouse Embigin forward primer CTC CTC CTT CTA TGT CTT CT, reverse primer TAC ACG CAT TTG AGT TCC AC, rat Embigin forward primer GCC ATC ATC CTG CTT TGT GA, reverse primer CCC CAG AGT CCG TTT TTC TGT, mouse AChR δ subunit

forward primer CTT GTT GTG TGT GCC CTC C, reverse primer GTC TCC TCC ACT TCT TTC AG, rat AChR δ subunit forward primer ACC GTC TCT GCT TGT TTG TG, reverse primer CGC CTG TCC TGT TCA TCA TA, mouse RL8 forward primer ACT GGA CAG TTC GTG TAC TG, reverse primer, GCT TCA CTC GAG TCT TCT TG, rat RL8 forward primer ACT GGA CAG TTC GTG TAC TG, reverse primer GCT TCA CTC GAG TCT TCT TG. Primer sequences used in PCR reactions were chosen based on the sequences available in GenBank. Primers were from Microsynth AG. The size of the fragments for mouse Embigin, rat Embigin, mouse AChR δ subunit, rat AChR δ subunit, mouse RL8 and rat RL8 was respectively 196bp, 151bp, 188bp, 134 bp, 199bp and 201bp. Only primer pairs yielding unique amplification products without primer dimer formation were subsequently used for real-time PCR assays. The internal standard control used in quantitative RT-PCR was ribosomal protein L8 mRNA. The positive control was AChR δ subunit mRNA.

For Northern Blot, the total RNAs were fractioned by electrophoresis on a 2% agarose gel containing 5% formaldehyde. The total RNAs were then transferred onto Zeta-Probe® GT genomics tested blotting membranes (Bio-Rad) in 20xSSC buffer by capillarity for 40 min. The membranes were hybridized in hybridization solution 0,5M NaP pH 7,2 (prepared as followed 2mM Na_2HPO_4 and 0,66mM NaH_2PO_4) 1mM EDTA, 7% SDS overnight. Blots were washed twice in 0,2% SSC, 1% SDS washing solution at room temperature for 15 min. The blots were exposed to X-ray film (Kodak Biomax Film). Blots were quantified on a PhosphorImager (Amersham Biosciences) using ImageQuant tools software (Molecular Dynamics). The cDNAs probes were labelled with ^{32}P using Megaprime DNA labelling kit (Amersham Biosciences) and purified on Mini Quick DNA columns (Roche). The cDNAs probes were diluted in the hybridization buffer at a concentration of 10^6cpm/mL. For normalization, the blots were hybridized with ribosomal protein L8 cDNA probe.

3.2.4. DNA constructs

Mouse Embigin full length was subcloned into pGEM-T easy vector (Promega) using 5'KpnI forward primer GGC GGT ACC ATG CGC TCG CAC ACT GGC and 3'XhoI reverse primer CCG CTC GAG TCA CTG ATC TGC AGA GTC. A pcDNA3 expression vector driven by mouse Embigin full length tagged with myc at the N-terminal part was constructed by initially

subcloning the Kpn I (+105bp) to Xba I (+1097bp) generated fragment into the unique Kpn I/Xba I sites of pcDNA3-basic (Invitrogen). The five-myc-epitope-repeats were subcloned into pGEM-T easy vector (Promega) after digesting the expression vector pcDNA1-myc (Meier et al., 1998a) by Hind III. The five myc epitope repeats were then inserted at +287bp into pcDNA3-Embigin using a generated Hind III restriction site by PCR mutagenesis with the following forward primer TGG CGA AAT ACT CAA AGC TTT CCT TAA AGA GCT G and the reverse primer CAG CTC TTT AAG GAA AGC TTT GAG TAT TTC GCC A. pCAGGS-EGFP is an expression vector constructed by subcloning the Xho I fragment of EGFP from pEGFP-N1 (Clontech) into pCAGGS vector to express EGFP in the whole cell. It was a generous gift from Pr. Bettler and Dr. Gassman. pSCT1-NgCAM is an expression vector constructed by initially subcloning mouse NgCAM full length into pSCT1 expression vector. PSCT1-NgCAM was a generous gift from Pr. Sonderegger.

3.2.5. Protein extracts and western blotting

HEK 293 cells were grown in DMEM high glucose (Sigma), 10% horse serum (Invitromex), 100U/mL streptomycin, 100U/mL penicillin (Sigma), 1x glutamine (Gibco) at 37°C at 5% CO_2. $3x10^5$ cells were passaged onto 6 cm plates and the day after transfected with mouse full length embigin tagged with myc using TransPEI transfection reagent according to manufacturers instructions (Eurogentec). Non transfected and transfected cells were washed 2 times with ice-cold PBS and proteins were extracted with 500µL of 10% SDS. For each sample, the similar amounts of total protein were boiled in SDS gel-loading buffer (50mM Tris.Cl pH 6,8, 100mM dithiothreitol, 2% SDS, 0,1% bromophenol blue, 10% glycerol). Samples were separated on a 10% polyacrylamide gel and transferred to Immobilon-P (Millipore) by semi-dry electroblotting in transfer buffer (48mM Tris base, 39mM glycine, 20% methanol). The membranes were then blocked with a blocking solution (PBS, 5% milk) overnight at 4°C. Embigin was detected by an anti-serum against a twelve aa sequence: PDPAEGDPTDPT designed in the extracellular part of mouse Embigin full length. This anti-serum against Embigin was produced in a sheep by Pineda, Berlin. Anti-serum against Embigin diluted at 1/50 or monoclonal mouse anti-myc clone 9E10 antibody peroxidase conjugated (Santa Cruz Biotechnology) diluted at 1:250 in incubation buffer (PBS, 2,5% milk, 0,1% Tween20) was used to detect embigin on the blots. The membranes were washed

three times in washing solution (PBS, 0,1% Tween20) at room temperature for 5 min. They were then incubated with peroxidase-conjugated anti-sheep antibody (Upstate) diluted at 1/1000 or peroxidase-conjugated anti-mouse antibody (Promega) diluted at 1/1000 in incubation buffer. The membranes were washed again three times in washing solution at room temperature for 5 min. The proteins were visualized followed by electrochemiluminescent western-blotting detection system (ECL) (Amersham Biosciences).

3.2.6. Homophilic binding assay

HEK 293 cells were cultured as described above. They were co-transfected with pCDNA3-Embign-myc and pCAGGS-EGFP or with pSCT1-NgCAM and pCAGGS-EGFP using TransPEI transfection reagent using the manufacturer instructions (Eurogentec). The day after the transfection they were passaged and cultured in suspension in an erlenmeyer with the medium used to culture them supplemented by 10mM HEPES in a volume of 30 mL at a density of 30 000 cells/mL under shaking at 160 rpm for 4hrs. The cells were visualized by GFP fluorescence on a Axioplan microscope (Leica).

3.2.7. Synaptotagmin clustering experiments

Transmembrane-Agrin expressing HEK293 cells were a gift from Dr. G. Bittchner and Dr. M.A. Ruegg (Biozentrum, Switzerland) and were grown as described above for HEK 293 cells. The expression of transmembrane Agrin was induced by incubating the cells overnight with 1µg/mL of tetracycline. COS-7 cells co-transfected with cDNAs encoding mouse full length Embigin and pCAGGS-EGFP using TransPei transfection reagent according to manufacturer instructions (Eurogentec) were cultured in the same medium as described above for HEK 293 cells.

HEK 293 cells (expressing Agrin; untransfected cells) and COS-7 cells (co-expressing Embigin and GFP; untransfected cells) were plated at a density of 3000 cells per 12 mm coverslip (Menzer Glaser) and allowed to settle for 1hr to 2hrs at 37°C. The coverslips were coated overnight with at first poly-L-ornithine 50µg/mL (Sigma) and laminin G 20µg/mL (generous gift from Dr. J. Engel). Fertilized eggs were purchased from Stockli (Zurich, S).

Ciliary ganglion neurons were dissected as described (Bernstein, 2003) and added into the prewarmed 37°C dissection medium MPG (Ca^{2+} Mg^{+} free PBS (see Molecular cloning), 100U/mL of streptomycin and penicillin (Sigma), 0,225% of glucose). The ciliary ganglion neurons were washed once in MPG medium and incubated for 10 min at 37°C. They were then washed twice in the growing medium (MEM Earle's salts (Gibco), 10% horse serum (Invitromex), 100U/mL streptomycin, 100U/mL penicillin (Sigma), 1x glutamine (Gibco), 8% eye extracts from chicken E17 prepared as described by Nishi R. and Berg D.K. (Nishi and Berg, 1981). They were separated by trituration using a pasteur pipette with a reduced diameter. Ciliary ganglion neurons (one ganglion per well) were added to the 24-well plates in ciliary ganglion medium and co-cultured for an additional 48 hrs at 37°C with HEK293 cells or COS-7 cells. The coverslips were then fixed and stained for synaptotagmin as described above. Only cell bodies that had extended processes and were on the poly-L-ornithine/laminin substrate were analyzed for synaptotagmin clusters at points of neurite-HEK 293/COS-7 cells contacts. HEK 293/COS-7 cells were scored for the presence of synaptotagmins clusters. Contacts were scored positive if the relative intensity of the synaptotagmin fluorescent signal was judged significantly greater than surrounding areas of neurites (Bixby and Reichardt, 1985). At least 20 contacts were analyzed in each well and a Student's t test was performed for statistical analysis.

3.2.8. Immunofluorescence

Cells were washed twice in phosphate-buffered saline Ca^{2+}, Mg^{+} free (PBS) then fixed with 4% paraformaldehyde in PBS pH 7,4 for 30 min. Fixed cells were washed twice with PBS and twice with PBS, 20mM glycine. They were incubated for 20 min either in PBS, 1% Triton X-100 and 1% foetal calf serum (FBS) or PBS, 1% FBS. Permeabilized cells were incubated with primary antibody (1:50 dilution of anti-serum sheep anti-Embigin, 1:250 dilution of mouse anti-Myc or 1:500 dilution of anti-Synaptotagmin) and non permeabilized cells were incubated also with primary antibody (1:50 dilution of anti-serum sheep anti-Embigin, 1:250 dilution of mouse anti-Myc, 1/250 dilution of anti-serum goat anti-NgCAM, 1:500 dilution of anti-Agrin in PBS/1% FBS). Coverslips were incubated for 1hr at room temperature. Coverslips were then washed four times with PBS and incubated with Donkey anti-sheep IgG AlexaFluor 488 (Molecular Probes) diluted 1:1000, and Cy3-conjugated anti-

mouse IgG diluted 1:1000 in PBS/FBS/Triton for 1hr at room temperature or Donkey anti-sheep IgG AlexaFluor 488 (Molecular Probes) diluted 1:1000, Cy3-conjugated rabbit anti-goat IgG diluted 1:1000, Cy3-conjugated anti-mouse IgG diluted 1:1000 and goat anti-rabbit IgG AlexaFluor 488 (Molecular Probes) in PBS/FBS. Coverslips were then washed four times with PBS and mounted in Moviol (Calbiochem) for viewing using a 40x objective on a Axioplan microscope (Leica).

3.3. Results

3.3.1. Denervation induces the expression of Embigin and AChR δ subunit transcripts

It has been previously described that denervation intiates the extensive formation of ectopic NMJ by transplanted foreign nerves (Lomo and Slater, 1978). To test whether Embigin could be one of the cues which allows synapse formation in denervated muscle, we examined the changes in muscle content of Embigin mRNA at different time after denervation. We have examined two muscles, rat soleus muscles and mouse gastrocnemius muscles using Northern Blot and Real Time PCR and compared the levels of Embigin mRNA to levels of AChR δ subunit mRNA.

Northern Blot analysis of mRNAs from rat soleus muscles has shown that Embigin transcripts begin to accumulate significantly 5 days after denervation and reach the highest level 5 days after denervation. The two RNA species can be explained by the presence of two putative polyadenylation sites. We confirmed these results also by quantitative RT-PCR. As an internal control, we have also examined the level of AChR δ subunit mRNA. It begins to accumulate after one day of denervation and by day 10 is ≈ 50-fold higher than in innervated muscle (Fig. 3.3).

In mouse gastrocnemius muscles, Embigin mRNA begins to accumulate significantly at 5 days after denervation and peak at 10 days after denervation. AChR δ subunit mRNA increases with the time, beginning accumulation after 1 day of denervation and reaching the highest expression level at day 10 (Fig. 3.4).

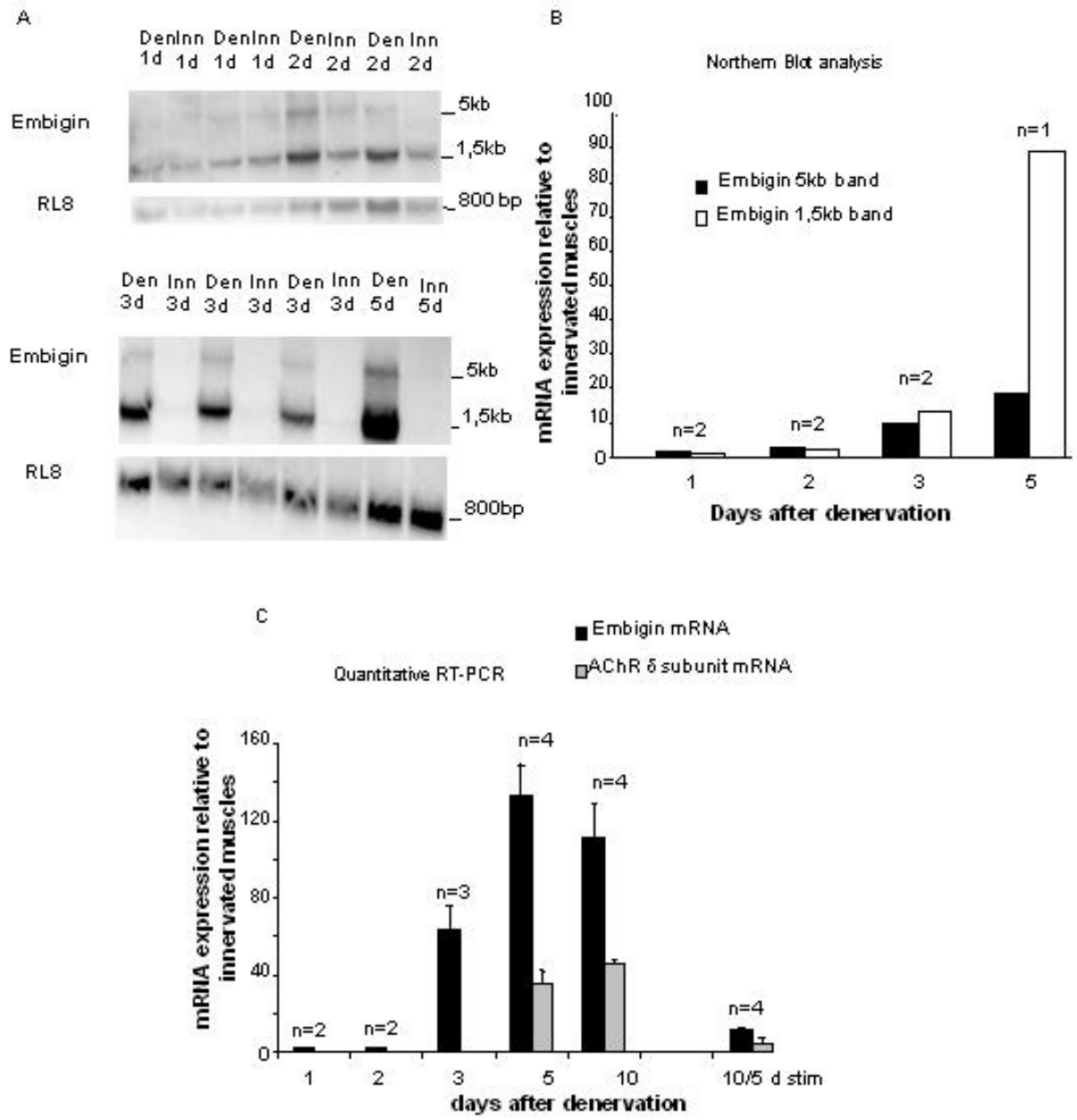


Fig. 3.3.Expression of Embigin mRNA is increased after denervation in rat soleus muscles.
A, Analysis by Northern Blot of Embigin mRNA expression in rat soleus muscles after denervation. A major band of 1,5 kb and a minor band of 5 kb are detected. B, Quantification of Embigin mRNA expression assessed by Northern Blot using Quant tools software from Phosphorimager. Data were normalized to RL8 mRNA. C, Analysis by Real Time PCR of Embigin mRNA expression in rat soleus muscles after denervation relative to levels in innervated muscle. Note that in 10 days denervated muscles, that were chronically stimulated for the last 5 days, Embigin mRNA is down-regulated to almost normal levels as observed in innervated muscle. Expression of the innervated adult soleus rat muscles was set to 1. Values were normalized to RL8 mRNA (data means±SE).

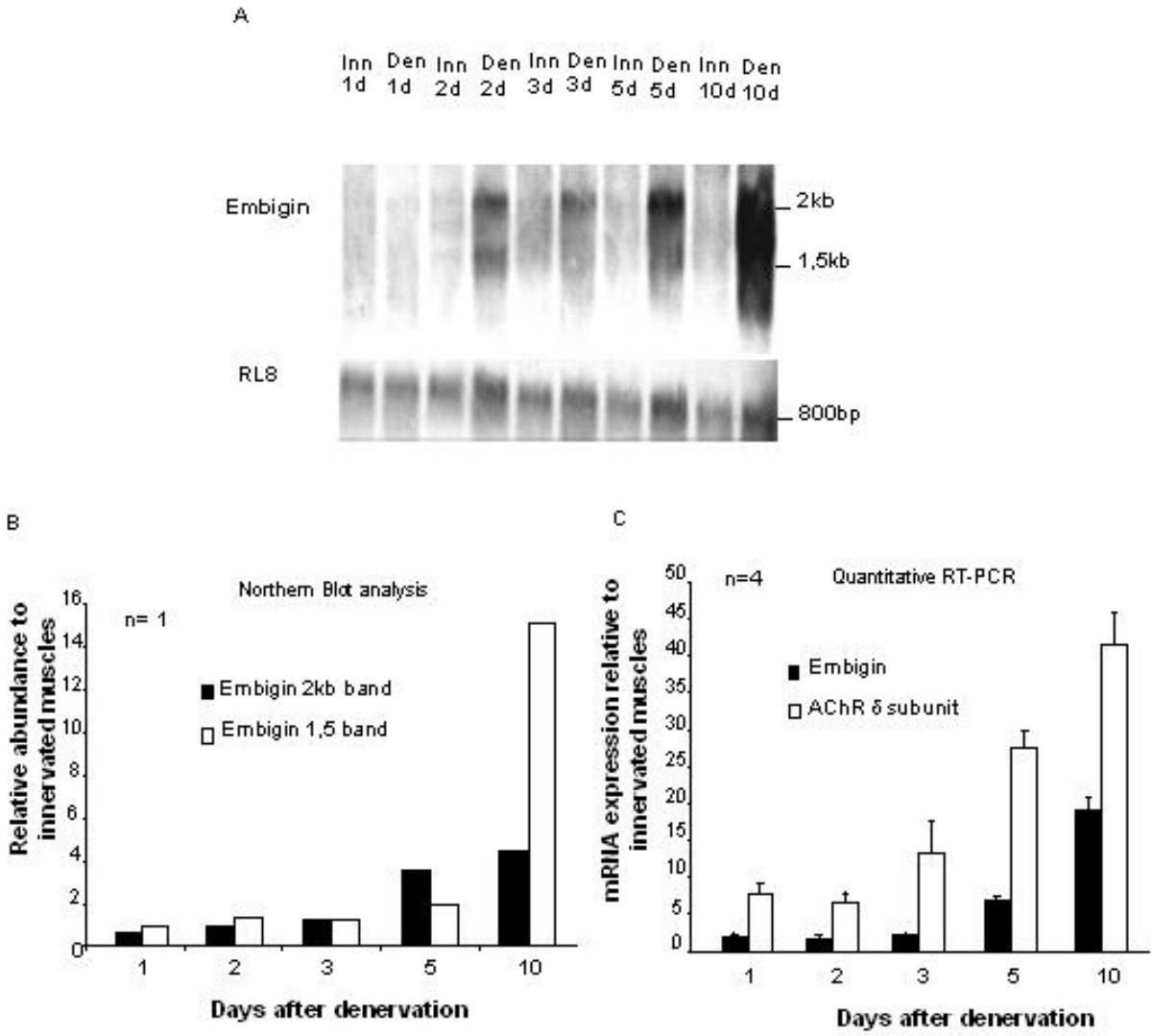


Fig. 3.4. Expression of Embigin mRNA is increased after denervation in gastrocnemius mouse muscles.

A, Analysis by Northern Blot of Embigin mRNA expression in gastrocnemius mouse muscles after denervation. A major band of 2 kb and a minor band of 1,5 kb are detected. B, Quantification of Embigin mRNA expression assessed by Northern Blot using Quant tools software from Phosphorimager. Data were normalized to RL8 mRNA (data means±SE). C, Analysis by Real Time PCR of Embigin and AChR δ subunit mRNAs expression in gastrocnemius mouse muscles after denervation. Expression of the innervated adult gastrocnemius mouse muscles was set to 1. Values were normalized to RL8 mRNA (data means±SE).

3.3.2. Electrical activity suppresses the increase of Embigin and AChR δ subunit transcripts caused by denervation

Previous results showed that electrical muscle stimulation prevented the increase of α-, β-, γ- and δ-subunit specific AChR mRNAs observed in denervated electrically inactive muscles (Goldman et al., 1988). In the present experiment, the effect of electrical activity on Embigin-specific mRNA levels was examined by exogenous stimulation of chronically denervated muscles via implanted electrodes (Lomo et al., 1985). This high frequency stimulation pattern (intermittent 100Hz stimulation) has been found to be highly effective at suppressing extrajunctional denervation supersensitivity (Lomo and Westgaard, 1975). Quantitative RT-PCR analysis has shown that the mRNAs encoding Embigin and AChR δ subunit were strongly reduced by electrical stimulation (Fig. 3.3C) to reach levels close to those observed in innervated muscles. Thus, Embigin mRNA expression is regulated by electrical muscle activity in a similar way as AChR.This is consistent with the role of Embigin in ectopic synapse formation because denervated, but stimulated muscle is not susceptible to ectopic innervation (Lomo and Westgaard, 1975).

3.3.3. Embigin mRNA is induced during differentiation of C2C12 cells

The expression of genes coding for several synaptic proteins including AChR is initially activated during myoblast differentiation and later is strongly down-regulated by innervation (Kues et al., 1995b; Schuetze and Role, 1987). Using quantitative RT-PCR and Northern Blot analysis, we studied the Embigin expression pattern in mouse myogenic cell line C2C12. When proliferation of C2C12 cells is restricted by exposure to medium reduced in serum content, the cells stop dividing and undergo differentiation, producing large amounts of muscle-specific proteins in the process (Chiu and Blau, 1984; Patrick et al., 1977).
Northern Blot analysis of Embigin mRNA showed that Embigin mRNA is expressed at higher amount when C2C12 cells differentiate compared to innervated muscles (Fig. 3.5A). Quantitative RT-PCR showed that the mRNA abundance of Embigin is very low in myoblasts and increases slightly when the cells differentiate (Fig. 3.5B). The mRNA level of AChR δ

subunit used as internal control is also very low before differentiation but then increases dramatically as the C2C12 cells differentiate.

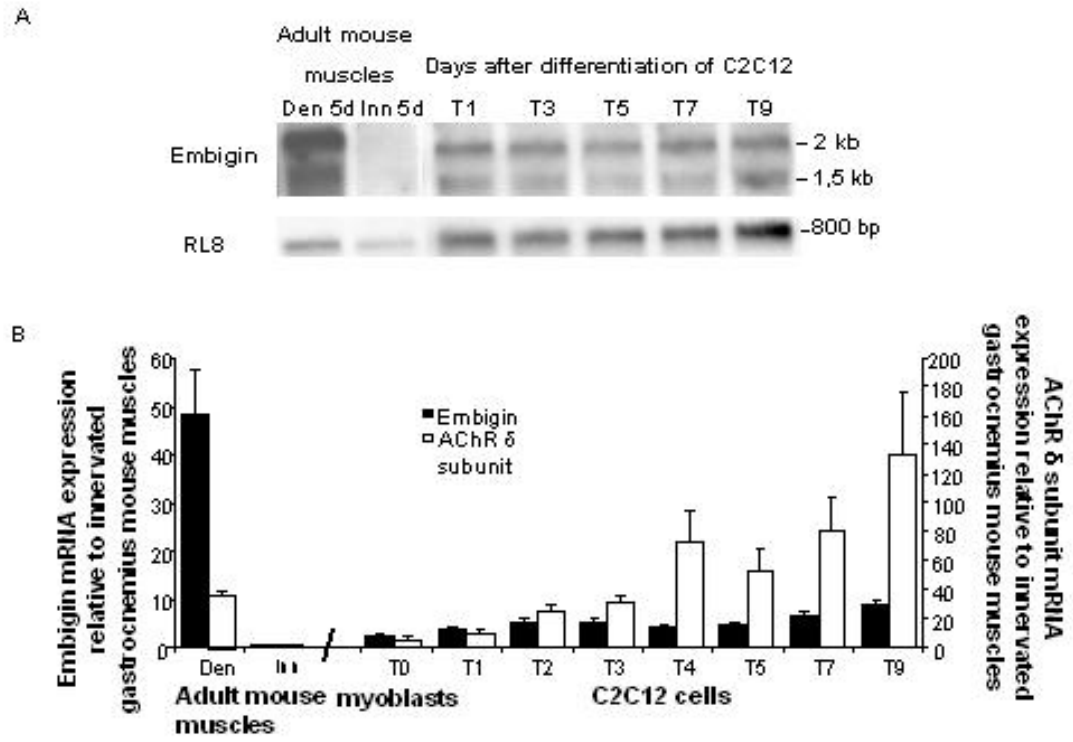


Fig. 3.5. Expression of Embigin mRNA is induced during differentiation of C2C12 cells.
A, Analysis by Northern Blot of Embigin mRNA expression in C2C12 cells during days of differentiation. A major band of 2 kb and a minor band of 1.5 kb are detected. B, Analysis by Real Time PCR of Embigin (n=5 experiments with duplicate samples) and AChR δ subunit (n= 5 experiments with duplicate values) mRNA expression in gastrocnemius mouse muscles after 5 days of denervation and in C2C12 cells during differentiation. Expression level in innervated adult gastrocnemius mouse muscles was set to 1. Values were normalized to RL8 mRNA (data means±SE).

3.3.4. The expression level of Embigin mRNA in neonatal muscles is similar to that in adult muscles

Given that embryonic-like C2C12 cells showed increased levels of Embigin mRNA, we have examined developmental changes in the amount of Embigin and AChR δ subunit mRNAs in mouse neonatal muscles. Assessed by quantitative RT-PCR, the expression level of Embigin mRNA in neonatal muscle exhibits the same expression level as in adult innervated muscle (Fig. 3.6). In contrast, the AChR δ subunit mRNA is present in much higher amount at day 2

after birth than in adult innervated muscle which is in agreement with previous reports (Witzemann et al., 1989a).

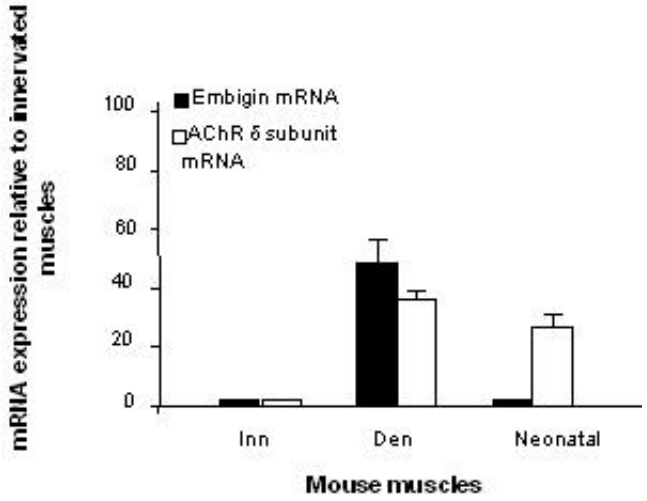


Fig. 3.6. Expression of Embigin mRNA in neonatal mouse muscles is similar to that in innervated adult muscles.
Analysis by Real Time PCR of Embigin and AChR δ subunit mRNAs expression in gastrocnemius mouse muscles and P2 neonatal mouse muscles. Expression of the innervated adult gastrocnemius mouse muscles was set to 1. Values were normalized to RL8 mRNA (data means±SE, n=3 experiments with duplicate values).

3.3.5. The expression level of Embigin mRNA is similar in the extra-synaptic and synaptic regions of diaphragm

Extra-synaptic and synaptic regions of innervated rat diaphragms were dissected to determine whether Embigin transcript is expressed preferentially at the synapse. Quantitative RT-PCR showed that Embigin mRNA level is 1,7-fold higher (Fig. 3.7A) in the extra-synaptic than synaptic regions of the diaphragm but this value is not statistically signicant (2 tailed Ttest, p-value=0,078). As internal standard control, we determined the gene expression pattern of AChR ε subunit. Unlike Embigin mRNA, the level of AChR ε subunit mRNA is markedly increased in synaptic portion of the diaphragm compared to the extra-synaptic part (Fig. 3.7B) as reported previously (Kues et al., 1995a; Kues et al., 1995b).
Extra-synaptic and synaptic regions of denervated rat diaphragms were dissected to determine whether Embigin transcription is differentially regulated after denervation. Quantitative RT-

PCR showed that Embigin mRNA is present in the same amount in the extra-synaptic and synaptic regions of diaphragm after denervation (Fig. 3.7C). In contrast AChR ε subunit mRNA was concentrated at synaptic sites in denervated diaphragms (Fig. 3.7D) as previously demonstrated (Witzemann et al., 1991).

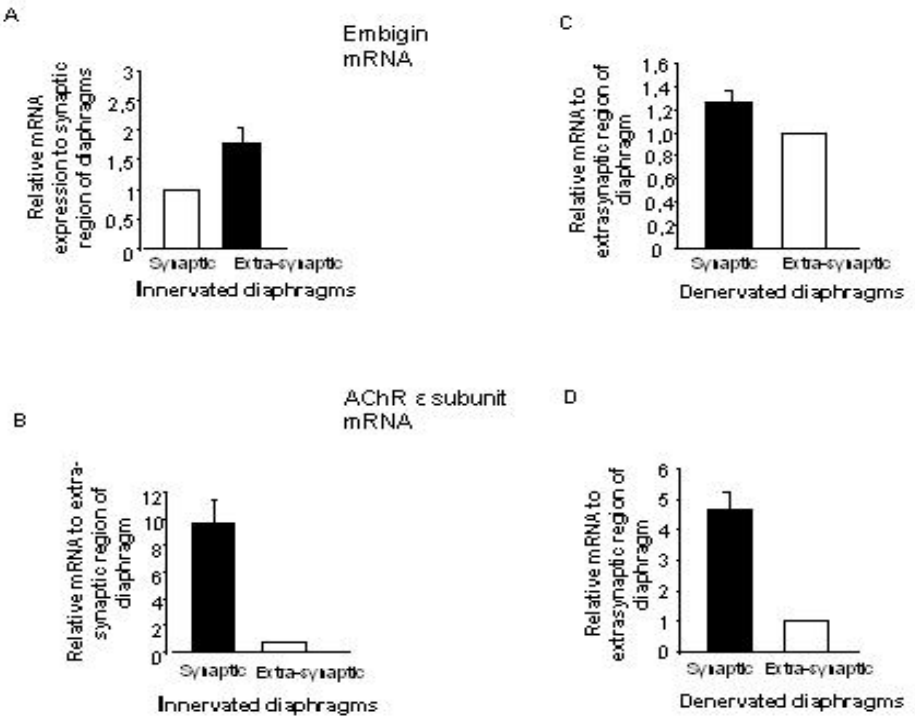


Fig. 3.7. Embigin mRNA is present in similar amounts in extra-synaptic and synaptic regions of diaphragm.
A and B, Analysis by Real Time PCR of Embigin and AChR ε subunit mRNA levels in extra-synaptic region and synaptic region of rat diaphragms (n=5 diaphragms with duplicate values). A, Expression of Embigin in the synaptic region of the diaphragm was set to 1 (data means±SE). B, Expression of AChR ε subunit in the the extra-synaptic region of the diaphragm was set to 1 (data means±SE). Values were normalized to RL8 mRNA (data means±SE). C and D, Analysis by Real Time PCR of Embigin and AChR ε subunit mRNA level in extra-synaptic and synaptic regions of 6 days denervated rat diaphragms (n=2 diaphragms with duplicate values). C, Expression of Embigin in the extra-synaptic region of the diaphragm was set to 1. D, Expression of AChR ε subunit in the extra-synaptic region of the diaphragm was set to 1. Values were normalized to RL8 mRNA.

3.3.6. The anti-serum against Embigin is specific for Embigin protein

In an attempt to investigate the regulation of Embigin at protein level upon denervation, an anti-serum against Embigin was ordered. We tested it first by western blot on cell extracts from HEK293 cells transfected with pcDNA3-Embigin-myc construct.

Using anti-serum anti-Embigin, two bands of 45kDa and of 66 kDa were detected (Fig. 3.8). The 45 kDa band corresponds to the molecular mass of mouse full length Embigin tagged with myc. The 66kDa band could be due to the high carbohydrate content of Embigin. Using anti-myc antibody, two major bands of 55kDa and 66kDa and minor band of 45 kDa were also observed. The additional band of 55 kDa could be due also to the high carbohydrate content of Embigin. As positive control, protein extract of cells expressing pip4KIIβ tagged with Myc which molecular mass is 80kDa is detected with anti-myc as expected.

However we could not detect Embigin protein in innervated and denervated muscle protein extracts. It appears that anti-serum against Embigin does not have enough affinity for endogenous Embigin.

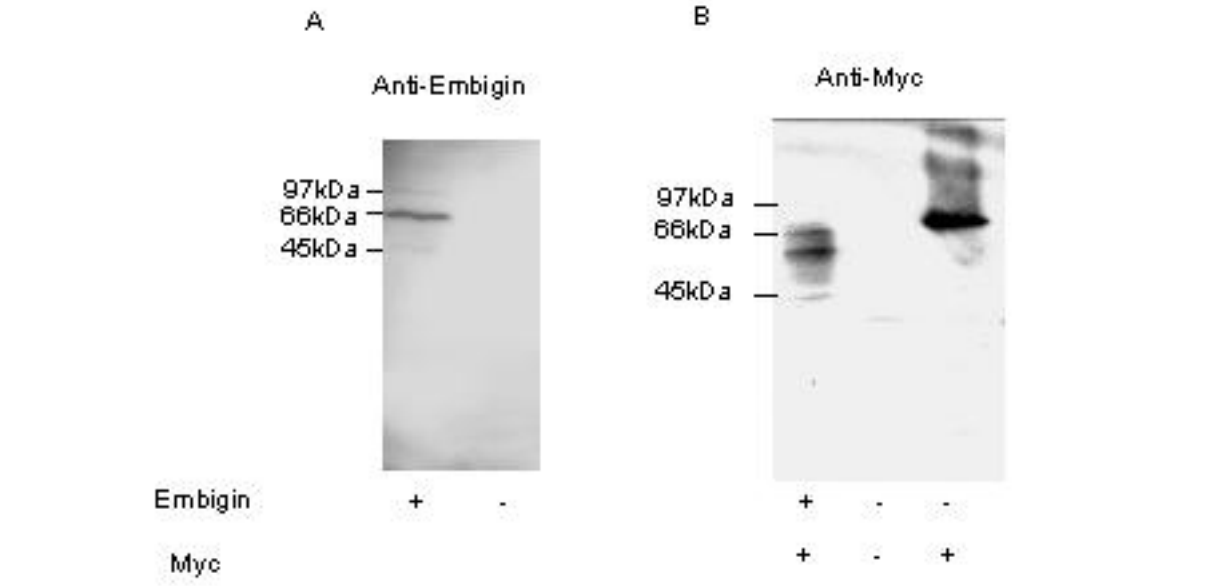


Fig. 3.8. Anti-serum against Embigin is specific for Embigin protein.
a, Immunoblot of Embigin from lysates of HEK cells expressing Embigin (+) or WT cells (-). *b*, Immunoblot of myc from lysates of HEK cells expressing Embigin (+) or WT cells (-). The third line corresponds to a lysate of HEK cells expressing a plasmid control containing myc.

3.3.7. Embigin protein is expressed at the cell surface

In an attempt to investigate the role of Embigin in synapse formation, we co-cultured chick ciliary neurons and untransfected COS-7 cells or COS-7 cells co-expressing Embigin and EGFP. We looked for synaptic-like structures at sites of contacts between chick ciliary neurons and COS-7 cells.

To ensure that COS-7 cells transfected with pcDNA3-Embigin-myc construct were expressing Embigin at the membrane, we co-stained COS-7 cells for Embigin and Myc. COS-7 cells showed strong, homogeneous cell surface Embigin (Fig. 3.9A) and Myc (Fig. 3.9B) staining with prominent membrane staining. In control permeabilized cells co-stained also for Embigin (Fig. 3.9C) and Myc (Fig. 3.9D), an intracellular staining is observed with the diffuse appearance of some puncta revealing the presence of secreting vesicles.

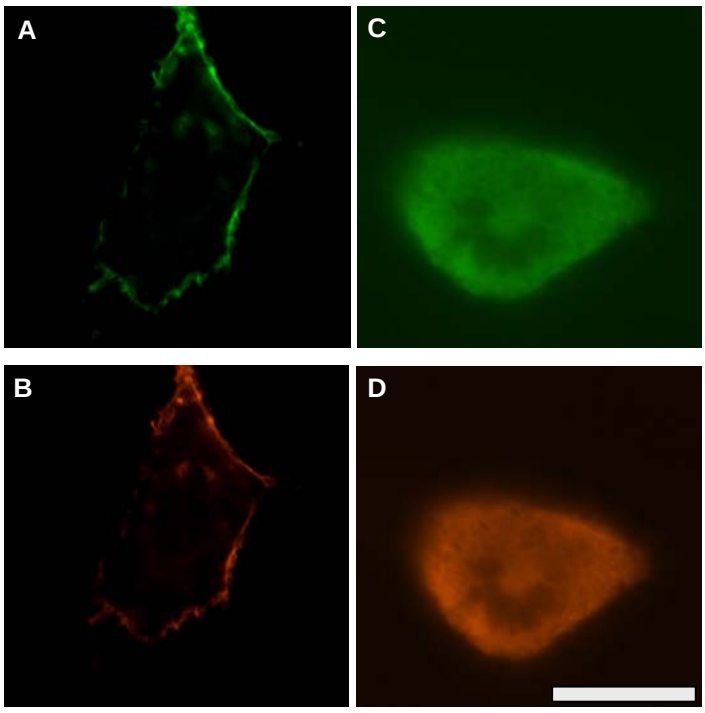


Fig. 3.9. Surface localization of Embigin on COS-7 cells.
COS-7 cells co-transfected with pcDNA3-Embigin-myc and pCAGGS-EGFP constructs were stained with peptide anti-Embigin (A) and (C) and a monoclonal anti-myc (B) and (D). A strong ring-like immunoreactivity typical of surface staining is observed for Embigin (A) and myc (B) staining. In permeabilized cells stained for Embigin (C) and myc (D), an entire cell staining is observed. Bar, 35µm.

3.3.8. Embigin protein is not involved in homophilic interactions

One possible role of Embigin in synapse formation is a role of attraction of motor neurites via homophilic interactions as it has been described for SynCAM at neuron-neuron synapses (Biederer et al., 2002). To test whether Embigin had adhesive function via homophilic interactions between cells expressing Embigin, HEK293 cells were co-transfected with pcDNA3-Embigin-Myc and pCAGGS-EGFP constructs (Fig. 3.11). As internal control, HEK293 cells were transfected with pCAGGS-EGFP construct alone. As positive control, cells were transfected to express NgCAM that has been previously shown to make homophilic interactions (Kunz et al., 1998), and GFP. After exposure to the transfection mixtures overnight, the cells were trypsinized and cultured in suspension for 4hrs. The numbers of Embigin-positive and NgCAM-positive cells were evaluated in aggregates of different cell numbers on a PALM Zeiss microscope with a 40x magnification (Fig. 3.12). The number of cells in aggregates of untransfected cells and of cells expressing Embigin was also examined (Fig. 3.13).

First, a NgCAM immunoreactivity at the cell surface confirmed that HEK293 cells transfected with pSCT1-NgCAM construct were overexpressing NgCAM in their cell membrane (Fig. 3.10).

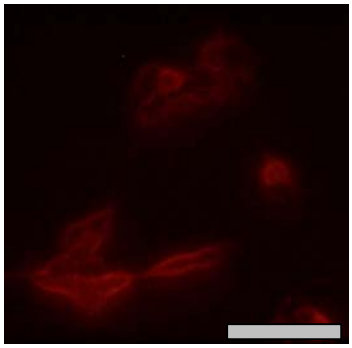


Fig. 3.10. Surface localization of NgCAM.
NgCAM overexpressing cells show a surface staining with prominent membrane staining. Bar, 35µm.

Most cells expressing EGFP and, Embigin/EGFP did not aggregates. In contrast, cells expressing NgCAM/EGFP are present in a cell aggregate composed of numerous NgCAM-positive cells (Fig. 3.11).

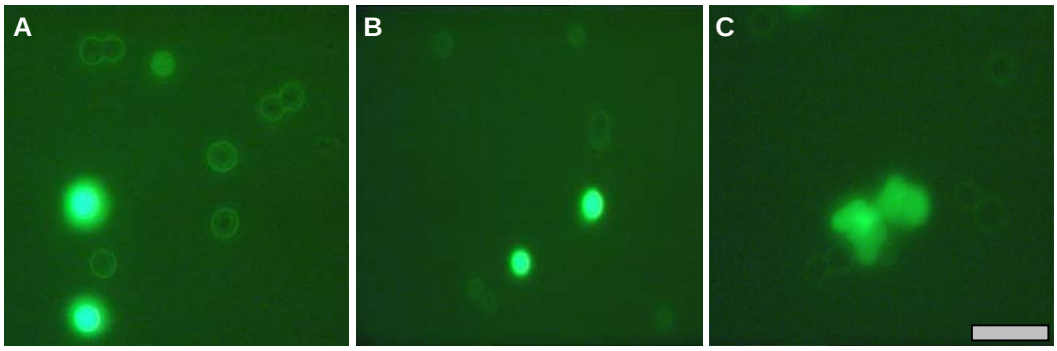


Fig. 3.11. Cells expressing Embigin do not make homophilic binding.
(A) GFP fluorescence of HEK293 cells expressing GFP alone. Most cells expressing GFP do not aggregate. (B) GFP fluorescence of HEK293 cells expressing Embigin and GFP. Most cells do not aggregate. (C) GFP fluorescence of HEK293 cells expressing NgCAM and GFP. NgCAM expressing cells are involved in clusters formed by numerous cells.Bar, 35μm.

The quantification of the data indicated that HEK293 cells expressing Embigin/EGFP are present in majority in cell aggregates composed of 3 and 4 cells (Fig. 3.12). Furthermore the cell aggregates are composed of only ≈ 10% of Embigin/GFP-positive cells (Fig. 3.12). In contrast, HEK293 cells co-transfected with pSCT1-NgCAM and with pCAGGS-EGFP constructs are present in aggregates composed of more than 10 cells to 25 cells (Fig. 3.12). Furthermore the cell aggregates contain ≈ 60% of NgCAM/EGFP-positive cells confirming that cells overexpressing NgCAM are involved in homophlic interactions (Fig. 3.12). Thus, our assay is suited to detect aggregation by homophilic interactions.

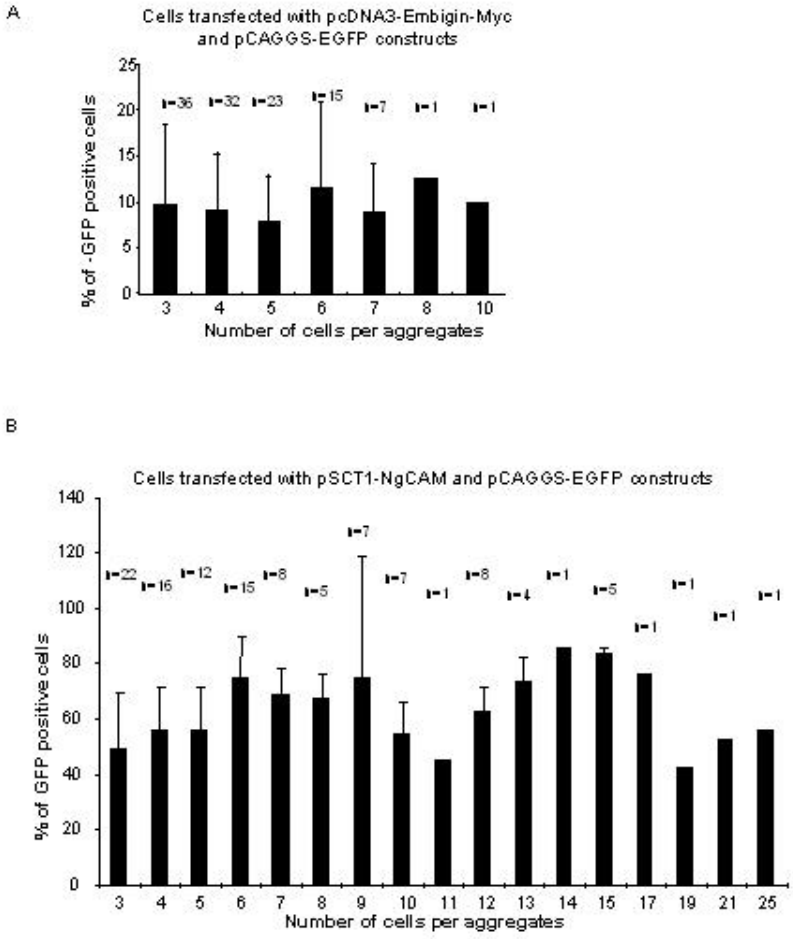


Fig. 3.12. Quantification of cells expressing Embigin and NgCAM present in cell aggregates.
HEK 293 cells were transfected to express Embigin-Myc and EGFP (A), or NgCAM and EGFP (B) and the percentage of EGFP-positive cells in aggregates of different cell numbers was counted by eye. n represents the numbers of aggregates examined. Bars are means±SE. Note the higher percentage of NgCAM-positive cells in aggregates.

To see whether Embigin may promote weak aggregating interactions, we compared the number of cell aggregates in expressing Embigin/EGFP and untransfected cells (Fig. 3.13). Both untransfected cells and Embigin/EGFP expressing cells are mostly present in cell aggregates composed of 3 to 6 cells (Fig. 3.13).

We conclude therefore, that Embigin does not make homophilic interactions. However, this does not exclude that Embigin can make heterophilic interactions as it has been previously shown for other Ig domain proteins (Thiery et al., 1977).

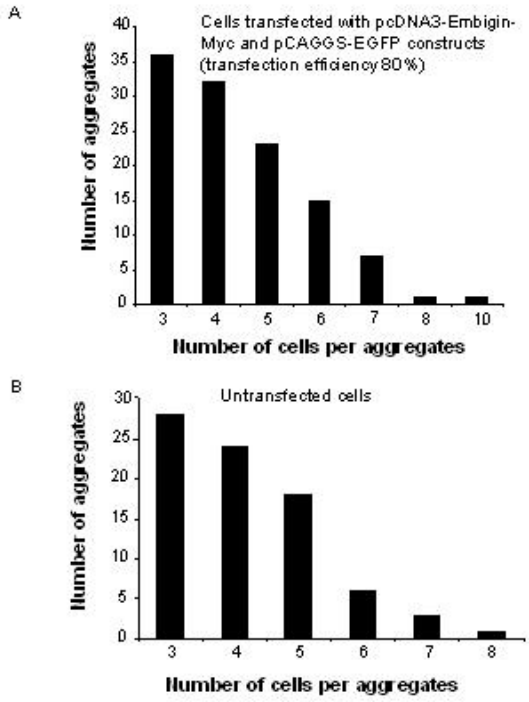


Fig. 3.13. Number of aggregates made by cells expressing Embigin and untransfected cells.
A, HEK 293 cells co-transfected with pcDNA3-Embigin-myc and pCAGGS-EGFP constructs and B, untransfected cells were cultured in suspension for 4hr. A, Cells co-expressing Embigin and EGFP are involved in a vast majority in aggregates of 3 and 4 cells. B, Untransfected cells are also involved in aggregates of 3 and 4 cells.

3.3.9. Embigin does not induce synaptotagmin clusters at sites of contacts with neurons

To investigate whether Embigin might be involved in the development of synaptic contacts, we examined whether chick ciliary ganglion (CG) neurons, which are cholinergic, preferentially make synaptic contacts with COS-7 cells transfected to express Embigin. The expression of the presynaptic marker synaptotagmin at sites of contacts was used as an indication of presynaptic differentiation. Therefore specifically we examined whether contact with COS-7 cells co-transfected with pcDNA3-Embigin-myc and pCAGGS-EGFP constructs could induce the clustering of the synaptic vesicle protein synaptotagmin (syt) in CG neurites. CG neurons and COS-7 were co-cultured for 48hr and analyzed for the presence of synaptotagmin clusters at sites of nerve-COS-7 cells contact.

CG neurites expressed synaptotagmin in co-culture with untransfected COS-7 cells, with HEK293 cells expressing transmembrane Agrin (TM-Agrin), with COS-7 cells expressing Embigin-Myc and EGFP. In contacts with untransfected COS-7 cells and with COS cells expressing Embigin-Myc and EGFP, synaptotagmin clusters were rare (Fig. 3.14). In contrast, neurites contacting TM-Agrin expressing cells (Fig. 3.15) often contained bright spots of synaptotagmin staining as previously shown by Campagna and colleagues (Campagna et al., 1995) (Fig. 3.15). Quantification of the data is shown in Fig. 3.16 which gives percentage of neurite-COS or HEK293 cells contacts that were positive for synaptotagmin clusters. In untransfected cells, 22% of contacts were positive for synaptotagmin clusters (number of contacts examined 135 in 4 experiments). This degree of clustering is not significantly different from that reported for CG neuron-CHO cell contacts (Campagna et al., 1995). In cells expressing Embigin-Myc and EGFP, 27% of contacts were positive for synaptotagmin clusters (number of contacts examined 138 in 4 experiments). However, for contacts between CG neurons and cells expressing TM-Agrin, the clear majority (62%) was associated with synaptotagmin clusters (number of contacts examined 78 in 4 experiments, Ttest p-value<0,001). These data indicate that in contrast to TM-Agrin, Embigin is not capable of clustering synaptotagmin at sites of contact with motoneurons.

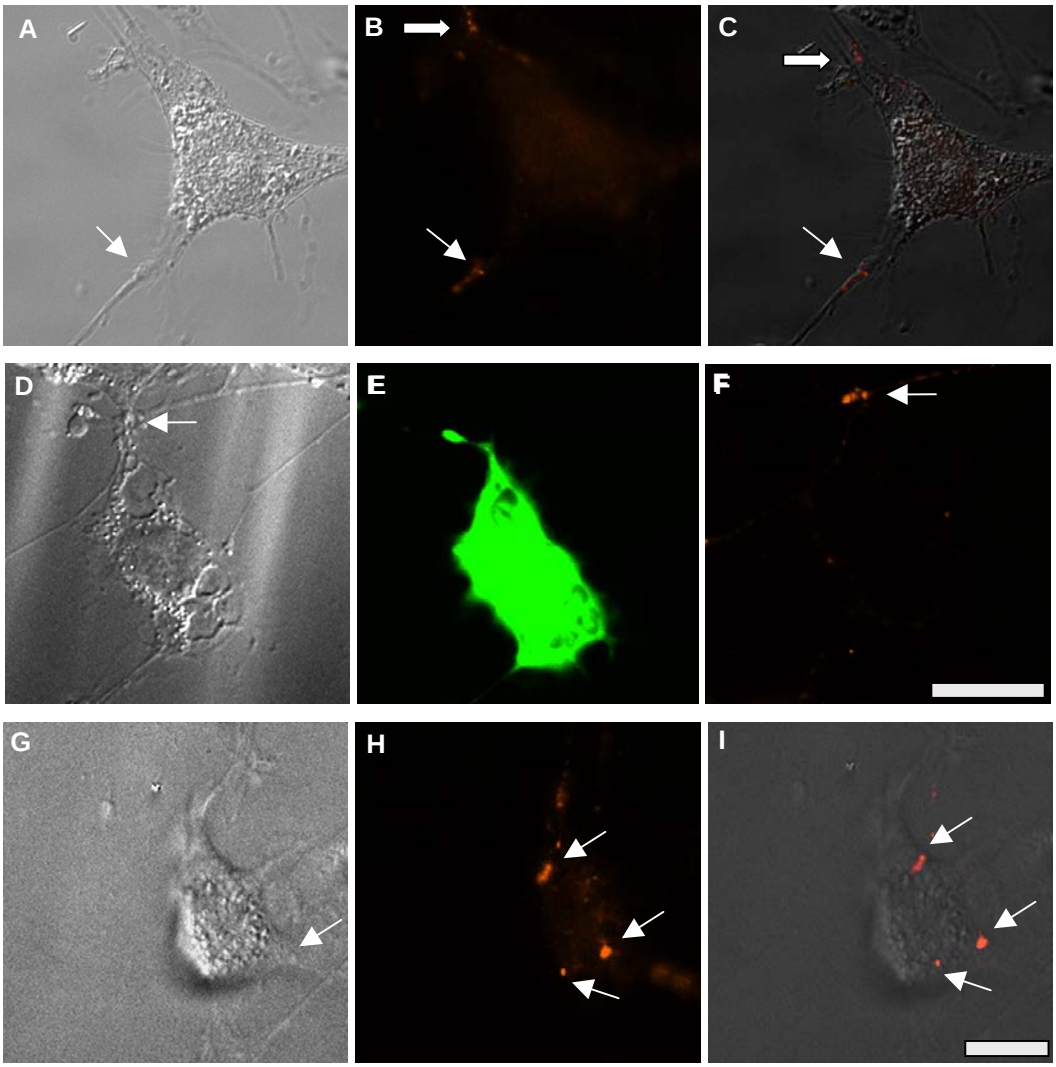


Fig. 3.14. Synaptotagmin accumulates in few CG neurites at sites of contact with untransfected COS-7 cells and COS-7 cells co-expressing Embigin and EGFP contrary to sites of contacts with TM-Agrin expressing cells.
(A) Phase contrast, (B) synaptotagmin fluorescence of CG neuron cultured for 48hr with untransfected COS-7 cells, (C) overlay of A and B. At sites of contact with COS-7 cells, neurites concentrate synaptotagmin (C). (D) Phase-contrast, (F) GFP fluorescence, (E) synaptotagmin fluorescence of CG neuron cultured for 48hr with COS-7 cells co-transfected with Embigin and EGFP. At sites of contact with COS-7 cells indicated with a white arrow, a synaptotagmin cluster appears. Bar, 40μm. (G) Phase contrast and (H) synaptotagmin fluorescence of CG neuron cultured for 48hr with TM-agrin-expressing cells. (I) Overlay of G and H. At sites of contacts with TM-Agrin expressing cells indicated with white arrows, neurites concentrate synaptotagmin. Bar, 15μm. Contact between CG neuron and COS cell indicated with a white block arrow was not counted because it may represent interneuronal contact.

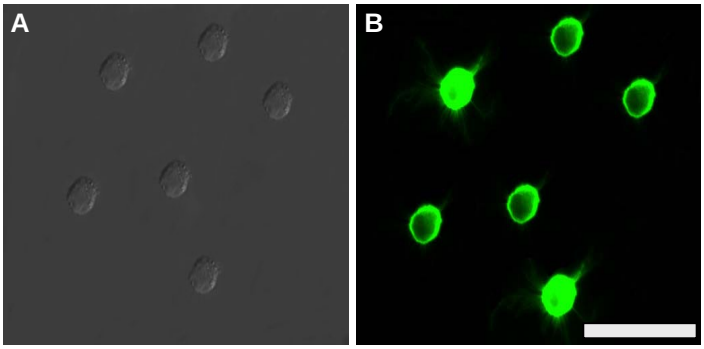


Fig. 3.15. TM-Agrin expressing cells express Agrin at their cell surface.
(A) Phase contrast and (B) Agrin fluorescence of TM-agrin-expressing HEK293 cells. A strong ring-like Agrin immunoreactivity, typical of surface staining is observed after the induction of Agrin expression. Bar, 40μm.

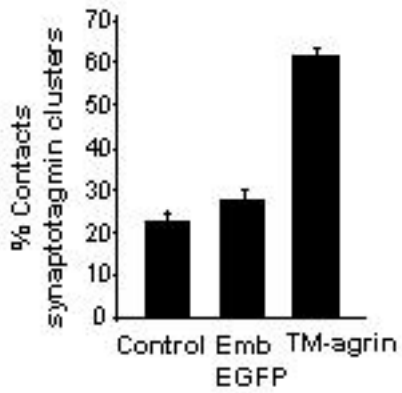


Fig. 3.16. Quantification of synaptotagmin induction by Embigin and by TM-Agrin.
CG neurons were cultured for 48hr with untransfected COS-7 cells, or with cells expressing Embigin and EGFP, or with cells expressing TM-Agrin. The percentage of neurite-COS cell contacts exhibiting synaptotagmin clusters is plotted. The total numbers of contacts examined were 135 on untransfected cells, 138 on Embigin expressing cells, and 78 on TM-Agrin expressing cells (4 cultures each). Cells expressing TM-Agrin but not Embigin induce higher pourcentage of synaptotagmin clusters than control cells (p-value=0,08 an 0,0003 respectively).

Chapter IV

4. General discussion

Direct linkage of pre- and postsynaptic cells via transmembrane adhesion molecules play important roles in synapse formation in the central nervous system. Many of them belong to the Ig superfamily of molecules (Yamagata et al., 2003) which are characterized by extracellular domains containing a varying number of Ig-like domains. Using differential display from innervated and denervated muscles, we have isolated a new protein, Embigin, another member of the Ig superfamily, as a potential candidate in regulating the formation of the neuromuscular synapse.

Very little is known about the biological function of Embigin. Structurally, it belongs to an IgSF subgroup. Based on sequence homologies and gene organization, this subgroup comprises so far Basigin, Embigin, Zov-3 and Gp55/65 (Langnaese et al., 1997; Miyauchi et al., 1990; Ozawa et al., 1988; Saitoh et al., 1993). A sequence comparison is shown in Fig. Annexe (page 100). (Gp65 was not included because unlike the other members which contain two Ig domains, Gp65 contains three). Of these, Embigin and Zov-3 are more closely related based on their Ig-domains and transmembrane structures. The closest similarity among all of them is within and around their transmembrane domains. Four cysteine residues are conserved between all four of them. They may form disulfide bridges leading to the predicted structure of their N-terminal parts, the two Ig-domains. Conspicuously, their transmembrane domains contain a glutamic acid residue that owing to its charge has been proposed to be involved in interactions with other membrane proteins and/or in signal transduction (Williams and Barclay, 1988).

Basigin is involved in neural-glial interactions in the developing avian retina (Fadool and Linser, 1993). Addition of an antibody against Basigin to monolayer cultures of dissociated embryonic retina cells results in alteration in the development of the stereotypic arrangement of neurons and glia characterized by a reduction in the number and complexity of neural extensions upon the glial-derived flat cells. Similarly, addition of antibody against Basigin to dissociated retina cells in rotation-mediated suspension culture significantly reduces retina cell reaggregation (Fadool and Linser, 1993). Basigin has been also shown to enhance tumour invasion by activating metalloprotease (Biswas et al., 1995). It is located on the outer surface of human tumor cells and interacts with fibroblasts to stimulate expression of several matrix metalloproteinases in the fibroblasts (Biswas et al., 1995). Basigin is required for spermatogenesis (Igakura et al., 1998) and female reproduction (Kuno et al., 1998). In an attempt to further clarify the role of Basigin, a mouse mutant lacking Basigin has been produced. The majority of Basigin-null embryos were lost around the time of implantation.

Those surviving into adulthood were infertile, and showed abnormalities in sensory function and behaviour (Naruhashi et al., 1997). The mutant mice showed worse performance than the wild and hetero mice in the Y-maze task, which assesses short-term memory, and in the water finding task, which examines latent learning, without any motor dysfunction. Moreover, the mutant mice showed less acclimation in the habituation task compared with the wild-type mice. The mutant mice were also more sensitive to electric foot-shock (Naruhashi et al., 1997).

Gp 55 and Gp65 are derived from the same gene by alternative mRNA splicing (Langnaese et al., 1997). Gp65 is enriched in the postsynaptic density (PSD) fraction of brain tissue (Hill et al., 1988; Willmott et al., 1992). It is localized to nerve terminals of subsets of neurons predominantly localized in the forebrain (Langnaese et al., 1997). During development, Gp65 is expressed postnatally, its expression level increasing dramatically during the second and third postnatal weeks (Langnaese et al., 1997), when synaptogenesis takes place (Dennis et al., 1981). This suggests an involvement of Gp65 in synapse formation and/or stabilization. In contrast, Gp55 is ubiquitously expressed. In brain, it is expressed early in brain development to reach adult levels by postnatal day 9 (Langnaese et al., 1997).

Two transcript isoforms of Zov-3 have been described in chickens, one ubiquitously expressed and the other being higly specific for brain (Saitoh et al., 1993). However, its function remains unknown.

The experiments presented here are consistent with an involvement of Embigin in neuromuscular synapse formation, but decisive proof of its function is lacking. Specifically, Embigin mRNA expression is up-regulated by muscle denervation, and is down-regulated by electrical muscle activity. Upon denervation, upregulation precedes the formation of ectopic endplates by transplanted foreign nerves by about 1 day, consistent with a role in mediating adhesion of early neuromuscular contacts. Embigin expressed in heterologous cell systems does not induce their aggregation, however, indicating that unlike NgCAM, a role in neuro-muscular adhesion is not mediated by homophilic interactions. Rather, if they occur, they appear to be mediated by interactions with Integrins (Huang et al., 1993). Indeed, a certain Integrin isoform (alpha7beta1) is observed at the neuromuscular synapse, and ablation of Integrin alpha7 gene destabilizes neuromuscular junction in adult mouse soleus muscle (Brenner, unpublished).

On the other hand, unlike the AChR ε-subunit mRNA, an AChR subunit gene induced selectively in subsynaptic nuclei by the nerve (Kues et al., 1995a; Kues et al., 1995b), we did

not observe increased expression levels of Embigin mRNA in the synaptic region of diaphragms. Rather, although not statistically significant, the level of Embigin mRNA expression appeared to be somewhat lower than in non-synaptic muscle segments. Conversely, when the phrenic nerve was cut, synaptic Embigin mRNA was slightly elevated. Spatial resolution of these experiments was limited by the fact that even though endplate bands were dissected carefully under the dissecting microscope, the majority of muscle nuclei in the dissected "endplate band" were non-synaptic, which may obscure the detection of true differences in expression levels between synaptic and non-synaptic nuclei. Qualitatively, the expression pattern of Embigin mRNA in the vicinity of the synapse is similar to the spatiotemporal regulation of the AChR γ-subunit (Kues et al., 1995). This subunit is characteristic of the fetal type of AChR in developing muscle (Mishina et al., 1986) which mediates neuromuscular impulse transmission in neonatal muscle and is subsequently down-regulated by muscle activity (Witzemann et al., 1991). Thus, Embigin may mediate *early neuromuscular adhesion only*, thus promoting initiation of neuromuscular synapse formation; at later stages, it would be down-regulated by electrical muscle activity induced by neuromuscular impulse transmission. Consistent with this down-regulation by impulses, the promoter region contains two so-called E-boxes, which have been shown to bind basic helix loop helix transcription factors of the MyoD family (Santoro et al., 1991). These transcription factors are also contained in AChR subunit genes where they are thought to mediate muscle-specific expression and impulse-dependent inhibition of AChR gene transcription in non-synaptic fiber regions (Berberich et al., 1993; Eftimie et al., 1991; Gilmour et al., 1991; Mendelzon et al., 1994; Numberger et al., 1991; Prody and Merlie, 1991; Prody and Merlie, 1992; Simon and Burden, 1993).

Although the activity-dependence of Embigin mRNA expression in adult muscle is similar as for AChR α, β, γ and δ-subunit genes in non-synaptic muscle regions, the expression pattern is different in cultured muscle cells and in neonatal muscle: expression levels in C2C12 myotubes and in neonatal muscle are lower than for the AChR genes, but like the former, they are dependent on the state of differentiation. The low level of Embigin mRNA levels in cultured myotubes does not exclude a role in early stages of neuromuscular synapse formation, however.

Our attempts to examine expression of Embigin in the synaptic muscle membrane were not successful. Neither a commercial antiserum (even following affinity purification) made against a synthetic 15 aa peptide of the extracellular domain, nor an antiserum against

recombinant extracellular domain (which could detect Embigin overexpressed in heterologous cells) were sufficient to resolve information on the localization of Embigin in the synaptic region of the muscle. The development of higher affinity antibodies and/or of high sensitivity in situ hybridization protocols will be needed to further investigate the potential neural regulation of Embigin expression in the synaptic region of the muscle fiber.

It has been mentioned above that neural Agrin induces postsynaptic structures in muscle fibers in the absence of the nerve (Cohen et al., 1997; Jones et al., 1997; Meier et al., 1997), and that Agrin expressed in COS-7 cells induces neurites of co-cultured ciliary ganglion neurones to differentiate to express the presynaptic marker protein synaptotagmin. In vivo, Agrin induces muscle fibers to express β2-Laminin (Jones et al., 1999) which has been proposed to act as a stop signal on motor neuron growth cones and to initiate presynaptic differentiation (Patton et al., 1997; Porter et al., 1995). Preganglionic parasympathetic ganglion cells can be surgically induced to make synapses on skeletal muscle fibers (Breitschmid and Brenner, 1981). Thus, we tested whether Embigin expressed in the membrane of COS-7 cells induced cocultured neurites to form presynaptic differentiation, monitored by the formation of contact sites rich in the presynaptic marker protein synaptotagmin (Lou and Bixby, 1995). Unlike for Agrin, however, Embigin expressed in the surface of COS cells did not induce presynaptic differentiation of ciliary ganglion neurons contacting them. Again, while this result is disappointing, it does not exclude a role of Embigin in neuromuscular adhesion *per se* (with Embigin merely acting as a stop signal) because the assay used did not allow to quantify the number of neuron-COS cell contacts made.

In summary, when growth cones of axons contact their target cells local interactions regulate the development of synaptic connections (Goodman and Shatz, 1993). Many aspects of transsynaptic signaling have remained obscure, both in the CNS and the PNS. In particular, the mechanisms by which neurons signal to each other the need to differentiate a presynaptic nerve terminal and the molecular glue that holds the pre- and postsynaptic specializations together are poorly understood. Here, we have found strong up-regulation of Embigin mRNA expression by muscle denervation, and strong inhibition in denervated muscle by exogenous stimulation of denervated muscle via implanted electrodes. Muscle activity is the only stimulus to regulate Embigin expression in adult tissue. While this is consistent with a role in neuromuscular synapse formation, positive evidence is still lacking. If such a role is assumed, our data taken together suggest the following model for the function of Embigin in synapse

formation: In vivo, the neural isoform of Agrin containing a Laminin binding domain at its C-terminus is required for the induction of a postsynaptic apparatus (Meier et al., 1997). In contrast, truncated Agrin lacking this domain will not form stable postsynaptic membranes (Cohen et al., 1997). Thus a positionally stable deposit of Agrin may be required for the initiation of a postsynaptic apparatus. This, in turn, implies that motor neuron growth cones, as they grow along a myotube, must be stopped to allow deposition of Agrin sufficient for the postsynaptic membrane induction, including the secretion of reverse signals from the muscle fiber to the growth cone (such as β2-Laminin) to induce presynaptic differentiation. In rodents neuromuscular synapse formation begins at about embryonic day E13 at a time when myoblasts begin to fuse into myotubes (Dennis et al., 1981). At this time an extracellular matrix has not been made to which neural Agrin could be deposited. Thus, growth cones must stop to allow continued interaction of secreted Agrin with the underlying muscle fiber. Our experiments are consistent with the view that Embigin function could be to cause this stop.

REFERENCES

Altiok, N., Bessereau, J. L., and Changeux, J. P. (1995). ErbB3 and ErbB2/neu mediate the effect of heregulin on acetylcholine receptor gene expression in muscle: differential expression at the endplate. EMBO J. *14*, 4258-4266.

Anderson, M. J., and Cohen, M. W. (1977). Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. J Physiol (Lond). *268*, 757-773.

Apel, E. D., Roberds, S. L., Campbell, K. P., and Merlie, J. P. (1995). Rapsyn may function as a link between the acetylcholine receptor and the agrin-binding dystrophin-associated glycoprotein complex. Neuron. *15*, 115-126.

Baines, R. A., Seugnet, L., Thompson, A., Salvaterra, P. M., and Bate, M. (2002). Regulation of synaptic connectivity: levels of fasciclin II influence synaptic growth in the Drosophila CNS. J Neurosci. *22*, 6587-6595.

Barry, J., and Ribchester, R. (1995). Persistent polyneuronal innervation in partially denervated rat muscle after reinnervation and recovery from prolonged nerve conduction block. J Neurosci. *15*, 6327-6339.

Bennett, M. R., and Robinson, J. (1989). Growth and elimination of nerve terminals at synaptic sites during polyneuronal innervation of muscle cells: a trophic hypothesis. Proc R Soc Lond B Biol Sci. *235*, 299-320.

Berberich, C., Durr, I., Koenen, M., and Witzemann, V. (1993). Two adjacent E box elements and a M-CAT box are involved in the muscle-specific regulation of the rat acetylcholine receptor beta subunit gene. Eur J Biochem. *216*, 395-404.

Bernstein, B. W. (2003). Dissection and culturing of chick ciliaryganglion neurons: a system well suited to synaptic study. Methods Cell Biol. *71*, 37-50.

Beumer, K., Matthies, H. J. G., Bradshaw, A., and Broadie, K. (2002). Integrins regulate DLG/FAS2 via a CaM kinase II-dependent pathway to mediate synapse elaboration and stabilization during postembryonic development. Development. *129*, 3381-3391.

Bevan, S., and Steinbach, J. H. (1977). The distribution of alpha-bungarotoxin binding sites of mammalian skeletal muscle developing in vivo. J Physiol (Lond). *267*, 195-213.

Biederer, T., Sara, Y., Mozhayeva, M., Atasoy, D., Liu, X., Kavalali, E. T., and Sudhof, T. C. (2002). SynCAM, a synaptic adhesion molecule that drives synapse assembly. Science. *297*, 1525-1531.

Biswas, C., Zhang, Y., DeCastro, R., Guo, H., Nakamura, T., Kataoka, H., and Nabeshima, K. (1995). The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. Cancer Res. *55*, 434-439.

Bowe, M. A., Deyst, K. A., Leszyk, J. D., and Fallon, J. R. (1994). Identification and purification of an agrin receptor from Torpedo postsynaptic membranes: a heteromeric complex related to the dystroglycans. Neuron. *12*, 1173-1180.

Bowen, D. C., Park, J. S., Bodine, S., Stark, J. L., Valenzuela, D. M., Stitt, T. N., Yancopoulos, G. D., Lindsay, R. M., Glass, D. J., and DiStefano, P. S. (1998). Localization and regulation of MuSK at the neuromuscular junction. Dev Biol. *199*, 309-319.

Braithwaite, A. W., and Harris, A. J. (1979). Neural influence on acetylcholine receptor clusters in embryonic development of skeletal muscles. Nature. *279*, 549-551.

Brand-Saberi, B., Wilting, J., Ebensperger, C., and Christ, B. (1996). The formation of somite compartments in the avian embryo. Int J Dev Biol. *40*, 411-420.

Breitschmid, P., and Brenner, H. R. (1981). Channel gating at frog neuromuscular junctions formed by different cholinergic neurones. J Physiol. *312*, 237-252.

Brenner, H., Herczeg, A., and Slater, C. (1992). Synapse-specific expression of acetylcholine receptor genes and their products at original synaptic sites in rat soleus muscle fibres regenerating in the absence of innervation. Development. *116*, 41-53.

Brenner, H. R., Witzemann, V., and sakmann, B. (1990). Imprinting of acetylcholine receptor messenger RNA accumulation in mammalian neuromuscular synapses. Nature. *344*, 544-547.

Briguet, A., and Ruegg, M. A. (2000). The Ets transcription factor GABP is required for postsynaptic differentiation in vivo. J Neurosci. *20*, 5989-5996.

Brown, M. C. (1984). Sprouting of motor nerves in adult muscles: a recapitulation of ontogeny. Trends Neurosci. *378*, 10-14.

Brown, M. C., Holland, R. L., Hopkins, W. G., and Keynes, R. J. (1981). An assessment of the spread of the signal for terminal sprouting within and between muscles. Brain Res. *210*, 145-151.

Brown, M. C., Hopkins, W. G., and Keynes, R. J. (1982). Short- and long-term effects of paralysis on the motor innervation of two different neonatal mouse muscles. J Physiol. *329*, 439-450.

Buonanno, A., Edmondson, D. G., and Hayes, W. P. (1993). Upstream sequences of the myogenin gene convey responsiveness to skeletal muscle denervation in transgenic mice. Nucleic Acids Res. *21*, 5684-5693.

Buonanno, A., and Fischbach, G. D. (2001). Neuregulin and ErbB receptor signaling pathways in the nervous system. Curr Opin Neurobiol. *11*, 287-296.

Burden, S., Sargent, P., and McMahan, U. (1979). Acetylcholine receptors in regenerating muscle accumulate at original synaptic sites in the absence of the nerve. J Cell Biol. *82*, 412-425.

Burden, S. J. (1998). The formation of neuromuscular synapses. Genes Dev. *12*, 133-148.

Burgess, R. W., Nguyen, Q. T., Son, Y.-J., Lichtman, J. W., and Sanes, J. R. (1999). Alternatively spliced isoforms of nerve- and muscle-derived agrin: their roles at the neuromuscular junction. Neuron. *23*, 33-44.

Burgess, R. W., Skarnes, W. C., and Sanes, J. R. (2000). Agrin isoforms with distinct amino termini: differential expression, localization, and function. J Cell Biol. *151*, 41-52.

Campagna, J. A., Ruegg, M. A., and Bixby, J. L. (1995). Agrin is a differentiation-inducing "stop signal" for motoneurons in vitro. Neuron. *15*, 1365-1374.

Campanelli, J. T., Roberds, S. L., Campbell, K. P., and Scheller, R. H. (1994). A role for dystrophin-associated glycoproteins and utrophin in agrin-induced AChR clustering. Cell. *77*, 663-674.

Caroni, P., and Becker, M. (1992). The downregulation of growth-associated proteins in motoneurons at the onset of synapse elimination is controlled by muscle activity and IGF1. J Neurosci. *12*, 3849-3861.

Caroni, P., and Schneider, C. (1994). Signaling by insulin-like growth factors in paralyzed skeletal muscle: rapid induction of IGF1 expression in muscle fibers and prevention of interstitial cell proliferation by IGF-BP5 and IGF-BP4. J Neurosci. *14*, 3378-3388.

Cartaud, A., Coutant, S., Petrucci, T. C., and Cartaud, J. (1998). Evidence for in situ and in vitro association between beta -dystroglycan and the subsynaptic 43K rapsyn protein. Consequence for acetylcholine receptor clustering at the synapse. J Biol Chem. *273*, 11321-11326.

Chen, J., Butowt, R., Rind, H. B., and von Bartheld, C. S. (2003). GDNF increases the survival of developing oculomotor neurons through a target-derived mechanism. Mol Cell Neurosci. *24*, 41-56.

Chiu, C. P., and Blau, H. M. (1984). Reprogramming cell differentiation in the absence of DNA synthesis. Cell. *37*, 879-887.

Cohen, I., Rimer, M., Lomo, T., and McMahan, U. J. (1997). Agrin-induced postsynaptic-like apparatus in skeletal muscle fibers in vivo. Mol Cell Neurosci. *9*, 237-253.

Cohen, M. W., Hoffstrom, B. G., and DeSimone, D. W. (2000). Active zones on motor nerve terminals contain alpha 3beta 1 Integrin. J Neurosci. *20*, 4912-4921.

Costanzo, E. M., Barry, J. A., and Ribchester, R. R. (1999). Co-regulation of synaptic efficacy at stable polyneuronally innervated neuromuscular junctions in reinnervated rat muscle. J Physiol (Lond). *521*, 365-374.

Couteaux, R. (1973). Motor endplate structure. In The structure and function of muscle, G. H. Bourne, ed. (New York: Academic), pp. 483-530.

Daggett, D. F., Cohen, M. W., Stone, D., Nikolics, Karoly, Rauvala, H., and Peng, H. B. (1996). The role of an agrin-growth factor interaction in ACh receptor clustering. Mol Cell Neurosci. *8*, 272-285.

Dai, Z., Luo, X., Xie, H., and Peng, H. B. (2000). The actin-driven movement and formation of acetylcholine receptor clusters. J Cell Biol. *150*, 1321-1334.

Dann, C. E., Hsieh, J.-C., Rattner, A., Sharma, D., Nathans, J., and Leahy, D. J. (2001). Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. Nature. *412*, 86-90.

DeChiara, T. M., Bowen, D. C., Valenzuela, D. M., Simmons, M. V., Poueymirou, W. T., Thomas, S., Kinetz, E., Compton, D. L., Rojas, E., and Park, J. S. (1996). The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell. *85*, 501-512.

Dennis, M., J,, Ziskind-Conhaim, L., and Harris, A. J. (1981). Development of neuromuscular junctions in rat embryos. Dev Biol. *81*, 266-279.

Dennis, M. J. (1981). Development of the neuromuscular junction: inductive interactions between cells. Annu Rev Neurosci. *4*, 43-68.

Denzer, A., Gesemann, M., Schumacher, B., and Ruegg, M. (1995). An amino-terminal extension is required for the secretion of chick agrin and its binding to extracellular matrix. J Cell Biol. *131*, 1547-1560.

Denzer, A. J., Brandenberger, R., Gesemann, M., Chiquet, M., and Ruegg, M. A. (1997). Agrin binds to the nerve-muscle basal lamina via laminin. J Cell Biol. *137*, 671-683.

Diffee, G. M., Caiozzo, V. J., Herrick, R. E., and Baldwin, K. M. (1991). Contractile and biochemical properties of rat soleus and plantaris after hindlimb suspension. Am J Physiol Cell Physiol. *260*, C528-534.

Dityatev, A., Dityateva, G., and Schachner, M. (2000). Synaptic strength as a function of post- versus presynaptic expression of the neural cell adhesion molecule NCAM. Neuron. *26*, 207-217.

Duclert, A., Savatier, N., Schaeffer, L., and Changeux, J.-P. (1996). Identification of an element crucial for the sub-synaptic expression of the acetylcholine receptor epsilon -subunit gene. J Biol Chem. *271*, 17433-17438.

Eftimie, R., Brenner, H. R., and Buonanno, A. (1991). Myogenin and MyoD join a family of skeletal muscle genes regulated by electrical activity. Proc Natl Acad Sci USA. *88*, 1349-1353.

English, A. W., and Schwartz, G. (1995). Both basic fibroblast growth factor and ciliary neurotrophic factor promote the retention of polyneuronal innervation of developing skeletal muscle fibers. Dev Biol. *169*, 57-64.

Ervasti, J. M., and Campbell, K. P. (1991). Membrane organization of the dystrophin-glycoprotein complex. Cell. *66*, 1121-1131.

Fadool, J. M., and Linser, P. J. (1993). 5A11 antigen is a cell recognition molecule which is involved in neuronal-glial interactions in avian neural retina. Dev Dyn. *196*, 252-262.

Fallon, J., and Gelfman, C. (1989). Agrin-related molecules are concentrated at acetylcholine receptor clusters in normal and aneural developing muscle. J Cell Biol. *108*, 1527-1535.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S., and Fischbach, G. D. (1993a). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell. *72*, 801-815.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S., and Fischbach, G. D. (1993b). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. Cell. *72*, 801-815.

Fambrough, D. M., and Hartzell, H. C. (1972). Acetylcholine receptors: number and distribution at neuromuscular junctions in rat diaphragm. Science. *176*, 189-191.

Ferns, M., Hoch, W., Campanelli, J. T., Rupp, F., Hall, Z. W., and Scheller, R. H. (1992). RNA splicing regulates agrin-mediated acetylcholine receptor clustering activity on cultured myotubes. Neuron. *8*, 1079-1086.

Finn, A. J., Feng, G., and Pendergast, A. M. (2003). Postsynaptic requirement for Abl kinases in assembly of the neuromuscular junction. Nat Neurosci. *6*, 717-723.

Fischbach, G. D., and Cohen, S. A. (1973). The distribution of acetylcholine sensitivity over uinnervated and innervated muscle fibers grown in cell culture. Dev Biol. *31*, 147-162.

Fontaine, B., Klarsfeld, A., and Changeux, J. (1987). Calcitonin gene-related peptide and muscle activity regulate acetylcholine receptor alpha-subunit mRNA levels by distinct intracellular pathways. J Cell Biol. *105*, 1337-1342.

Frank, E., and Fischbach, G. (1979). Early events in neuromuscular junction formation in vitro: induction of acetylcholine receptor clusters in the postsynaptic membrane and morphology of newly formed synapses. J Cell Biol. *83*, 143-158.

Fromm, L., and Burden, S. J. (1998). Synapse-specific and neuregulin-induced transcription require an Ets site that binds GABPalpha /GABPbeta. Genes Dev. *12*, 3074-3083.

Fuhrer, C., Sugiyama, J. E., Taylor, R. G., and Hall, Z. W. (1997). Association of muscle-specific kinase MuSK with the acetylcholine receptor in mammalian muscle. EMBO J. *16*, 4951-4960.

Garratt, A. N., Britsch, S., and Birchmeier, C. (2000). Neuregulin, a factor with many functions in the life of a schwann cell. Bioessays. *22*, 987-996.

Gatchalian, C., Schachner, M., and Sanes, J. (1989). Fibroblasts that proliferate near denervated synaptic sites in skeletal muscle synthesize the adhesive molecules tenascin(J1), N-CAM, fibronectin, and a heparan sulfate proteoglycan. J Cell Biol. *108*, 1873-1890.

Gautam, M., Noakes, P. G., Moscoso, L., Rupp, F., Scheller, R. H., Merlie, J. P., and Sanes, J. R. (1996). Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell. *85*, 525-535.

Gautam, M., Noakes, P. G., Mudd, J. J., Nichol, M., Chu, G. C., Sanes, J. R., and Merlie, J. P. (1995). Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. Nature. *377*, 232-236.

Gee, S. H., Montanaro, F., Lindenbaum, M. H., and Carbonetto, S. (1994). Dystroglycan-alpha, a dystrophin-associated glycoprotein, is a functional agrin receptor. Cell. *77*, 675-686.

Gesemann, M., Denzer, A., and Ruegg, M. (1995). Acetylcholine receptor-aggregating activity of agrin isoforms and mapping of the active site. J Cell Biol. *128*, 625-636.

Gesemann, M., V., C., Denzer, A. J., Brancaccio, A., Schumacher, B., and Ruegg, M. A. (1996). Alternative splicing of agrin alters its binding to heparin, dystroglycan, and the putative agrin receptor. Neuron. *16*, 755-767.

Gilmour, B., Fanger, G., Newton, C., Evans, S., and Gardner, P. (1991). Multiple binding sites for myogenic regulatory factors are required for expression of the acetylcholine receptor gamma-subunit gene. J Biol Chem. *266*, 19871-19874.

Glass, D. J., Bowen, D. C., Stitt, T. N., Radziejewski, C., Bruno, J., Ryan, T. E., Gies, D. R., Shah, S., Mattsson, K., and Burden, S. J. (1996). Agrin acts via a MuSK receptor complex. Cell. *85*, 513-523.

Godfrey, E., Nitkin, R., Wallace, B., Rubin, L., and McMahan, U. (1984). Components of Torpedo electric organ and muscle that cause aggregation of acetylcholine receptors on cultured muscle cells. J Cell Biol. *99*, 615-627.

Goldman, D., Brenner, H. R., and Heinemann, S. (1988). Acetylcholine receptor alpha-, beta-, gamma-, and delta-subunit mRNA levels are regulated by muscle activity. Neuron. *1*, 329-333.

Goll, D. E., Thompson, V. F., Taylor, R. G., and Ouali, A. (1998). The calpain system and skeletal muscle growth. Can J Anim Sci. *78*, 503-512.

Goodearl, A., Yee, A., Sandrock, A., Jr, Corfas, G., and Fischbach, G. (1995). ARIA is concentrated in the synaptic basal lamina of the developing chick neuromuscular junction. J Cell Biol. *130*, 1423-1434.

Goodman, C. S., and Shatz, C. J. (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. Cell. *72*, Suppl 77-98.

Grady, R. M., Zhou, H., Cunningham, J. M., Henry, M. D., Campbell, K. P., and Sanes, J. R. (2000). Maturation and maintenance of the neuromuscular synapse: genetic evidence for roles of the dystrophin--glycoprotein complex. Neuron. *25*, 279-293.

Grenningloh, G., Bieber, A. J., Rehm, E. J., Snow, P. M., Traquina, Z. R., Hortsch, M., Patel, N. H., and Goodman, C. S. (1990). Molecular genetics of neuronal recognition in Drosophila: evolution and function of immunoglobulin superfamily cell adhesion molecules. Cold Spring Harb Symp Quant Biol. *55*, 327-340.

Grenningloh, G., Rehm, E. J., and Goodman, C. S. (1991). Genetic analysis of growth cone guidance in Drosophila: fasciclin II functions as a neuronal recognition molecule. Cell. *67*, 45-57.

Guenette, R. S., Sridhar, S., Herley, M., Mooibroek, M., Wong, P., and Tenniswood, M. (1997). Embigin, a developmentally expressed member of the immunoglobulin super family, is also expressed during regression of prostate and mammary gland. Dev Genet. *21*, 268-278.

Gundersen, K., Sanes, J., and Merlie, J. (1993). Neural regulation of muscle acetylcholine receptor epsilon- and alpha- subunit gene promoters in transgenic mice. J Cell Biol. *123*, 1535-1544.

Gundersen, R. W. (1987). Response of sensory neurites and growth cones to patterned substrata of laminin and fibronectin in vitro. Dev Biol. *121*, 423-431.

Haase, G., Dessaud, E., Garces, A., de Bovis, B., Birling, M.-C., Filippi, P., Schmalbruch, H., Arber, S., and deLapeyriere, O. (2002). GDNF acts through PEA3 to regulate cell body positioning and muscle innervation of specific motor neuron pools. Neuron. *35*, 893-905.

Habas, R., Kato, Y., and He, X. (2001). Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel formin homology protein Daam1. Cell. *107*, 843-854.

Harris, B. Z., and Lim, W. A. (2001). Mechanism and role of PDZ domains in signaling complex assembly. J Cell Sci. *114*, 3219-3231.

Henderson, C. E., Huchet, M., and Changeux, J. P. (1983). Denervation increases a neurite-promoting activity in extracts of skeletal muscle. Nature. *302*, 609-611.

Henderson, C. E., Phillips, H. S., Pollock, R. A., Davies, A. M., Lemeulle, C., Armanini, M., Simmons, L., Moffet, B., Vandlen, R. A., and Simpson, L. C. (1994). GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science. *266*, 1062-1064.

Hill, I. E., Selkirk, C. P., Hawkes, R. B., and Beesley, P. W. (1988). Characterization of novel glycoprotein components of synaptic membranes and postsynaptic densities, gp65 and gp55, with a monoclonal antibody. Brain Res. *461*, 27-43.

Hoch, W., Ferns, M., Campanelli, J. T., Hall, Z. W., and Scheller, R. H. (1993). Developmental regulation of highly active alternatively spliced forms of agrin. Neuron. *11*, 479-490.

Hopf, C., and Hoch, W. (1996). Agrin binding to alpha-dystroglycan. J Biol Chem. *271*, 5231-5236.

Huang, C. F., Flucher, B. E., Schmidt, M. M., Stroud, S. K., and Schmidt, J. (1994). Depolarization-transcription signals in skeletal muscle use calcium flux through L channels, but bypass the sarcoplasmic reticulum. Neuron. *13*, 167-177.

Huang, C. F., Tong, J., and Schmidt, J. (1992). Protein kinase C couples membrane excitation to acetylcholine receptor gene inactivation in chick skeletal muscle. Neuron. *9*, 671-678.

Huang, R. P., Ozawa, M., Kadomatsu, K., and Muramatsu, T. (1990). Developmentally regulated expression of embigin, a member of the immunoglobulin superfamily found in embryonal carcinoma cells. Differentiation. *45*, 76-83.

Huang, R.-P., Ozawa, M., Kadomatsu, K., and Muramatsu, T. (1993). Embigin, a member of the immunoglobulin superfamily expressed in embryonic cells, enhances cell-substratum adhesion. Dev Biol. *155*, 307-314.

Igakura, T., Kadomatsu, K., Kaname, T., Muramatsu, H., Fan, Q.-W., Miyauchi, T., Toyama, Y., Kuno, N., Yuasa, S., and Takahashi, M. (1998). A null mutation in basigin, an immunoglobulin superfamily member, indicates its important roles in peri-implantation development and spermatogenesis. Dev Biol. *194*, 152-165.

Jennings, C., Dyer, S., and Burden, S. (1993). Muscle-specific trk-related receptor with a kringle domain defines a distinct class of receptor tyrosine kinases. Proc Natl Acad Sci USA. *90*, 2895-2899.

Jessell, T. M., Siegel, R. E., and Fischbach, G. D. (1979). Induction of acetylcholine receptors on cultured skeletal muscle by a factor extracted from brain and spinal cord. Proc Natl Acad Sci USA. *76*, 5397-5401.

Jones, G., Herczeg, A., Ruegg, M. A., Lichtsteiner, M., Kroger, S., and Brenner, H. R. (1996). Substrate-bound agrin induces expression of acetylcholine receptor varepsilon -subunit gene in cultured mammalian muscle cells. Proc Natl Acad Sci USA. *93*, 5985-5990.

Jones, G., Meier, T., Lichtsteiner, M., Witzemann, V., Sakmann, B., and Brenner, H. R. (1997). Induction by agrin of ectopic and functional postsynaptic-like membrane in innervated muscle. Proc Natl Acad Sci USA. *94*, 2654-2659.

Jones, G., Moore, C., Hashemolhosseini, S., and Brenner, H. R. (1999). Constitutively active MuSK is clustered in the absence of agrin and induces ectopic postsynaptic-like membranes in skeletal muscle fibers. J Neurosci. *19*, 3376-3383.

Jordan, C. L. (1996). Ciliary neurotrophic factor may act in target musculature to regulate developmental synapse elimination. Dev Neurosci. *18*, 185-198.

Jordan, C. L., Watamura, S., and Arnold, A. P. (1995). Androgenic, not estrogenic, steroids alter neuromuscular synapse elimination in the rat levator ani. Brain Res Dev Brain Res. *84*, 215-224.

Kandel, E. R., Schartz, J. H., and Jessell, T. M. (2000). Nerve cells and behavior. In Principles of neural science, J. Butler, and H. Lebowitz, eds. (McGraw-Hill), pp. 19-35.

Kasthuri, N., and Lichtman, J. W. (2003). The role of neuronal identity in synaptic competition. Nature. *424*, 426-430.

Keller-Peck, C. R., Feng, G., Sanes, J. R., Yan, Q., Lichtman, J. W., and Snider, W. D. (2001). Glial cell line-derived neurotrophic factor administration in postnatal life results in motor unit enlargement and continuous synaptic remodeling at the neuromuscular junction. J Neurosci. *21*, 6136-6146.

Knaack, D., Shen, I., Salpeter, M., and Podleski, T. (1986). Selective effects of ascorbic acid on acetylcholine receptor number and distribution. J Cell Biol. *102*, 795-802.

Koike, S., Schaeffer, L., and Changeux, J. (1995). Identification of a DNA element determining synaptic expression of the mouse acetylcholine receptor {delta}-subunit gene. Proc Natl Acad Sci USA. *92*, 10624-10628.

Krishan, K., and Dhoot, G. K. (1996). Changes in some troponin and insulin-like growth factor messenger ribonucleic acids in regenerating and denervated skeletal muscles. J Muscle Res Cell Motil. *17*, 513-521.

Kues, W., Brenner, H., Sakmann, B., and Witzemann, V. (1995a). Local neurotrophic repression of gene transcripts encoding fetal AChRs at rat neuromuscular synapses. J Cell Biol. *130*, 949-957.

Kues, W. A., Sakmann, B., and Witzemann, V. (1995b). Differential expression patterns of five acetylcholine receptor subunit genes in rat muscle during development. Eur J Neurosci. *7*, 1376-1385.

Kummer, T. T., Misgeld, T., Lichtman, J. W., and Sanes, J. R. (2004). Nerve-independent formation of a topologically complex postsynaptic apparatus. J Cell Biol. *164*, 1077-1087.

Kuno, N., Kadomatsu, K., Fan, Q.-W., Hagihara, M., Senda, T., Mizutani, S., and Muramatsu, T. (1998). Female sterility in mice lacking the basigin gene, which encodes a transmembrane glycoprotein belonging to the immunoglobulin superfamily. FEBS Letters. *425*, 191-194.

Kunz, S., Spirig, M., Ginsburg, C., Buchstaller, A., Berger, P., Lanz, R., Rader, C., Vogt, L., Kunz, B., and Sonderegger, P. (1998). Neurite fasciculation mediated by complexes of axonin-1 and Ng cell adhesion molecule. J Cell Biol. *143*, 1673-1690.

Kwon, Y. W., Abbondanzo, S. J., Stewart, C. L., and Gurney, M. E. (1995). Leukemia inhibitory factor influences the timing of programmed synapses withdrawal from neonatal muscles. J Neurobiol. *28*, 35-50.

Kwon, Y. W., and Gurney, M. E. (1996). Brain-derived neurotrophic factor transiently stabilizes silent synapses on developing neuromuscular junctions. J Neurobiol. *29*, 503-516.

Lacazette, E., Le Calvez, S., Gajendran, N., and Brenner, H. R. (2003). A novel pathway for MuSK to induce key genes in neuromuscular synapse formation. J Cell Biol. *161*, 727-736.

Langnaese, K., Beesley, P. W., and Gundelfinger, E. D. (1997). Synaptic membrane glycoproteins gp65 and gp55 are new members of the immunoglobulin superfamily. J Biol Chem. *272*, 821-827.

Lanier, L. M., and Gertler, F. B. (2000). Actin cytoskeleton: thinking globally, actin' locally. Curr Biol. *10*, R655-R657.

LaRochelle, W., and Froehner, S. (1986). Determination of the tissue distributions and relative concentrations of the postsynaptic 43-kDa protein and the acetylcholine receptor in Torpedo. J Biol Chem. *261*, 5270-5274.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C., and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. Nature. *378*, 394-398.

Letinsky, M. S., Fischbeck, K. H., and McMahan, U. J. (1976). Precision of reinnervation of original postsynaptic sites in frog muscle after a nerve crush. J Neurocytol. *5*, 691-718.

Liang, P., and Pardee, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science. *257*, 967-971.

Lie, D. C., and Weis, J. (1998). GDNF expression is increased in denervated human skeletal muscle. Neurosci Lett. *250*, 87-90.

Loeb, J., and Fischbach, G. (1995). ARIA can be released from extracellular matrix through cleavage of a heparin-binding domain. J Cell Biol. *130*, 127-135.

Lomo, T., Massoulie, J., and Vigny, M. (1985). Stimulation of denervated rat soleus muscle with fast and slow activity patterns induces different expression of acetylcholinesterase molecular forms. J Neurosci. *5*, 1180-1187.

Lomo, T., and Rosenthal, J. (1972). Control of ACh sensitivity by muscle activity in the rat. J Physiol. *221*, 493-513.

Lomo, T., and Slater, C. (1978). Control of acetylcholine sensitivity and synapse formation by muscle activity. J Physiol (Lond). *275*, 391-402.

Lomo, T., and Westgaard, R. H. (1975). Further studies on the control of ACh sensitivity by muscle activity in the rat. J Physiol. *252*, 603-626.

Lou, X. J., and Bixby, J. L. (1995). Patterns of presynaptic gene expression define two stages of synaptic differentiation. Mol Cell Neurosci. *6*, 252-262.

Luo, Z. G., Wang, Q., Zhou, J. Z., Wang, J., Luo, Z., Liu, M., He, X., Wynshaw-Boris, A., Xiong, W. C., Lu, B., and Mei, L. (2002). Regulation of AChR clustering by Dishevelled interacting with MuSK and PAK1. Neuron. *35*, 489-505.

Marchionni, M. A., Goodearl, A. D., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., and al, e. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature. *362*, 312-318.

Marshall, L. M., Sanes, J. R., and Mc Mahan, U. J. (1977). Reinnervation of original synaptic sites on muscle fiber basement membrane after disruption of the muscle cells. Proc Natl Acad Sci USA. *74*, 3073-3077.

Martin, P., and Sanes, J. (1997). Integrins mediate adhesion to agrin and modulate agrin signaling. Development. *124*, 3909-3917.

Martin, P. T., and Sanes, J. R. (1995). Role for a synapse-specific carbohydrate in agrin-induced clustering of acetylcholine receptors. Neuron. *14*, 743-754.

Martinou, J., Falls, D., Fischbach, G., and Merlie, J. (1991). Acetylcholine receptor-inducing activity stimulates expression of the {varepsilon}-subunit gene of the muscle acetylcholine receptor. Proc Natl Acad Sci USA. *88*, 7669-7673.

Masiakowski, P., and Yancopoulos, G. D. (1998). The Wnt receptor CRD domain is also found in MuSK and related orphan receptor tyrosine kinases. Curr Biol. *8*, R407.

Matsumura, K., Ervasti, J. M., Ohlendieck, K., Kahl, S. D., and Campbell, K. P. (1992). Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. Nature. *360*, 588-591.

Mc Mahan, U. J. (1990). The agrin hypothesis. Cold Spring Harb Symp Quant Biol. *55*, 407-418.

McDonald, K. S., Blaser, C. A., and Fitts, R. H. (1994). Force-velocity and power characteristics of rat soleus muscle fibers after hindlimb suspension. J Appl Physiol. *77*, 1609-1616.

McMahan, U., and Slater, C. (1984). The influence of basal lamina on the accumulation of acetylcholine receptors at synaptic sites in regenerating muscle. J Cell Biol. *98*, 1453-1473.

Meier, T., Hauser, D. M., Chiquet, M., Landmann, L., Ruegg, M. A., and Brenner, H. R. (1997). Neural agrin induces ectopic postsynaptic specializations in innervated muscle fibers. J Neurosci. *17*, 6534-6544.

Meier, T., Marangi, P. A., Moll, J., Hauser, D. M., Brenner, H.-R., and Ruegg, M. A. (1998a). A minigene of neural agrin encoding the lamininbinding and acetylcholine receptoraggregating domains is sufficient to induce postsynaptic differentiation in muscle fibres. Eur J Neurosci. *10*, 3141-3152.

Meier, T., Masciulli, F., Moore, C., Schoumacher, F., Eppenberger, U., Denzer, A. J., Jones, G., and Brenner, H. R. (1998b). Agrin can mediate acetylcholine receptor gene expression in muscle by aggregation of muscle-derived neuregulins. J Cell Biol. *141*, 715-726.

Mendelzon, D., Changeux, J. P., and Nghiem, H. O. (1994). Phosphorylation of myogenin in chick myotubes: regulation by electrical activity and by protein kinase C. Implications for acetylcholine receptor gene expression. Biochemistry. *33*, 2568-2575.

Meyer, D., and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. Nature. *378*, 386-390.

Mishina, M., Takai, T., Noda, M., Takahashi, T., Numa, S., Methfessel, C., and Sakmann, B. (1986). Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature. *321*, 406-411.

Missler, M. (2003). Synaptic cell adhesion goes functional. Trends Neurosci. *26*, 176-178.

Miyauchi, T., Kanekura, T., Yamaoka, A., Ozawa, M., Miyazawa, S., and Muramatsu, T. (1990). Basigin, a new, broadly distributed member of the immunoglobulin superfamily, has strong homology with both the immunoglobulin V domain and the beta-chain of major histocompatibility complex class II antigen. J Biochem. *107*, 313-323.

Mizoguchi, A., Nakanishi, H., Kimura, K., Matsubara, K., Ozaki-Kuroda, K., Katata, T., Honda, T., Kiyohara, Y., Heo, K., Higashi, M., *et al.* (2002). Nectin: an adhesion molecule involved in formation of synapses. J Cell Biol. *156*, 555-565.

Mook-Jung, I., and Gordon, H. (1995). Acetylcholine receptor clustering in C2 muscle cells requires chondroitin sulfate. J Neurobiol. *28*, 482-492.

Moore, C., Leu, M., Muller, U., and Brenner, H. R. (2001). Induction of multiple signaling loops by MuSK during neuromuscular synapse formation. Proc Natl Acad Sci USA. *98*, 14655-14660.

Moscoso, L. M., Chu, G. C., Gautam, M., Noakes, P. G., Merlie, J. P., and Sanes, J. R. (1995). Synapse-associated expression of an acetylcholine receptor-inducing protein, ARIA/Heregulin, and its putative receptors, ErbB2 and ErbB3, in developing mammalian muscle. Dev Biol. *172*, 158-169.

Mueller, B. K. (1999). Growth cone guidance: First steps towards a deeper understanding. Annu Rev Neurosci. *22*, 351-388.

Naruhashi, K., Kadomatsu, K., Igakura, T., Fan, Q.-W., Kuno, N., Muramatsu, H., Miyauchi, T., Hasegawa, T., Itoh, A., Muramatsu, T., and Nabeshima, T. (1997). Abnormalities of sensory and memory functions in mice lacking Bsg gene. Biochem Biophys Res Commun. *236*, 733-737.

Neumann, F. R., Bittcher, G., Annies, M., Schumacher, B., Kroger, S., and Ruegg, M. A. (2001). An alternative amino-terminus expressed in the central nervous system converts agrin to a type II transmembrane protein. Mol Cell Neurosci. *17*, 208-225.

Nguyen, Q. T., Parsadanian, A. S., Snider, W. D., and Lichtman, J. W. (1998). Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. Science. *279*, 1725-1729.

Nishi, R., and Berg, D. K. (1981). Two components from eye tissue that differentially stimulate the growth and development of ciliary ganglion neurons in cell culture. J Neurosci. *1*, 505-513.

Nitkin, R., Smith, M., Magill, C., Fallon, J., Yao, Y., Wallace, B., and McMahan, U. (1987). Identification of agrin, a synaptic organizing protein from Torpedo electric organ. J Cell Biol. *105*, 2471-2478.

Noakes, P. G., Gautam, M., Mudd, J., Sanes, J. R., and Merlie, J. P. (1995). Aberrant differentiation of neuromuscular junctions in mice lacking s-laminin/laminin beta 2. Nature. *374*, 258-262.

Noakes, P. G., Phillips, W. D., Hanley, T. A., Sanes, J. R., and Merlie, J. P. (1993). 43K protein and acetylcholine receptors colocalize during the initial stages of neuromuscular synapse formation in vivo. Dev Biol. *155*, 275-280.

Numberger, M., Durr, I., Kues, W., Koenen, M., and Witzemann, V. (1991). Different mechanisms regulate muscle-specific AChR gamma- and epsilon-subunit gene expression. EMBO J. *10*, 2957-2964.

Oh, T. H., and Markelonis, G. J. (1982). Chicken serum transferrin duplicates the myotrophic effects of sciatin on cultured muscle cells, pp. 535-545.

O'Toole, J. J., Deyst, K. A., Bowe, M. A., Nastuk, M. A., McKechnie, B. A., and Fallon, J. R. (1996). Alternative splicing of agrin regulates its binding to heparin, alpha -dystroglycan, and the cell surface. Proc Natl Acad Sci USA. *93*, 7369-7374.

Ozawa, M., Huang, R., Furukawa, T., and Muramatsu, T. (1988). A teratocarcinoma glycoprotein carrying a developmentally regulated carbohydrate marker is a member of the immunoglobulin gene superfamily. J Biol Chem. *263*, 3059-3062.

Patrick, J., McMillan, J., Wolfson, H., and O'Brien, J. C. (1977). Acetylcholine receptor metabolism in a nonfusing muscle cell line. J Biol Chem. *252*, 2143-2153.

Patton, B. L., Miner, J. H., Chiu, A. Y., and Sanes, J. R. (1997). Distribution and function of laminins in the neuromuscular system of developing, adult, and mutant mice. J Cell Biol. *139*, 1507-1521.

Pendergast, A. M. (2002). The Abl family kinases: mechanisms of regulation and signaling. Adv Cancer Res. *85*, 51-100.

Peng, H. B., Baker, L. P., and Chen, Q. (1991). Induction of synaptic development in cultured muscle cells by basic fibroblast growth factor. Neuron. *6*, 237-246.

Perrimon, N., and Mahowald, A. P. (1987). Multiple functions of segment polarity genes in Drosophila. Dev Biol. *119*, 587-600.

Plattner, R., Kadlec, L., DeMali, K. A., Kazlauskas, A., and Pendergast, A. M. (1999). c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF. Genes Dev. *13*, 2400-2411.

Porter, B. E., Weis, J., and Sanes, J. R. (1995). A motoneuron-selective stop signal in the synaptic protein S-laminin. Neuron. *14*, 549-559.

Prody, C., and Merlie, J. (1991). A developmental and tissue-specific enhancer in the mouse skeletal muscle acetylcholine receptor alpha-subunit gene regulated by myogenic factors. J Biol Chem. *266*, 22588-22596.

Prody, C. A., and Merlie, J. P. (1992). The 5'-flanking region of the mouse muscle nicotinic acetylcholine receptor beta subunit gene promotes expression in cultured muscle cells and is activated by MRF4, myogenin and myoD. Nucleic Acids Res. *20*, 2367-2372.

Raftery, M. A., Hunkapiller, M. W., Strader, C. D., and Hood, L. E. (1980). Acetylcholine receptor: complex of homologous subunits. Science. *208*, 1454-1456.

Rafuse, V. F., Polo-Parada, L., and Landmesser, L. T. (2000). Structural and functional alterations of neuromuscular junctions in NCAM-deficient mice. J Neurosci. *20*, 6529-6539.

Rassendren, F., Bloch-Gallego, E., Tanaka, H., and Henderson, C. (1992). Levels of mRNA coding for motoneuron growth-promoting factors are increased in denervated muscle. Proc Natl Acad Sci USA. *89*, 7194-7198.

Reynolds, M. L., and Woolf, C. J. (1992). Terminal Schwann cells elaborate extensive processes following denervation of the motor endplate. J Neurocytol. *21*, 50-66.

Rind, H. B., and von Bartheld, C. S. (2002). Target-derived cardiotrophin-1 and insulin-like growth factor-I promote neurite growth and survival of developing oculomotor neurons. Mol Cell Neurosci. *19*, 58-71.

Ruegg, M. A., Tsim, K. W. K., Horton, S. E., Kroger, S., Escher, G., Gensch, E. M., and Mc Mahan, U. J. (1992). The agrin gene codes for a family of basal lamina proteins that differ in function and distribution. Neuron. *8*, 691-699.

Rupp, F., Payan, D. G., Magill-Solc, C., Cowan, D. M., and Scheller, R. H. (1991). Structure and expression of a rat agrin. Neuron. *6*, 811-823.

Saitoh, Y., Ogawa, A., Hori, T., Kunita, R., and Mizuno, S. (1993). Identification and localization of two genes on the chicken Z chromosome: implication of evolutionary conservation of the Z chromosome among avian species. Chromosome Res. *1*, 239-251.

Salpeter, M., Marchaterre, M., and Harris, R. (1988). Distribution of extrajunctional acetylcholine receptors on a vertebrate muscle: evaluated by using a scanning electron microscope autoradiographic procedure. J Cell Biol. *106*, 2087-2093.

Salpeter, M. M., and Loring, R. H. (1985). Nicotinic acetylcholine receptors in vertebrate muscle: properties, distribution and neural control. Prog Neurobiol. *25*, 297-325.

Sandrock, A. W., Jr., Dryer, S. E., Rosen, K. M., Gozani, S. N., Kramer, R., Theill, L. E., and Fischbach, G. D. (1997). Maintenance of acetylcholine receptor number by neuregulins at the neuromuscular junction in vivo. Science. *276*, 599-603.

Sanes, J., Marshall, L., and McMahan, U. (1978). Reinnervation of muscle fiber basal lamina after removal of myofibers. Differentiation of regenerating axons at original synaptic sites. J Cell Biol. *78*, 176-198.

Sanes, J., Schachner, M., and Covault, J. (1986). Expression of several adhesive macromolecules (N-CAM, L1, J1, NILE, uvomorulin, laminin, fibronectin, and a heparan sulfate proteoglycan) in embryonic, adult, and denervated adult skeletal muscle. J Cell Biol. *102*, 420-431.

Sanes, J. R., and Lichtman, J. W. (1999). Development of the vertebrate neuromuscular junction. Annu Rev Neurosci. *22*, 389-442.

Santoro, I. M., Yi, T. M., and Walsh, K. (1991). Identification of single-stranded-DNA-binding proteins that interact with muscle gene elements. Mol Cell Biol. *11*, 1944-1953.

Sapru, M. K., Florance, S. K., Kirk, C., and Goldman, D. (1998). Identification of a neuregulin and protein-tyrosine phosphatase response element in the nicotinic acetylcholine receptor varepsilon subunit gene: Regulatory role of an Ets transcription factor. Proc Natl Acad Sci USA. *95*, 1289-1294.

Schachner, M. (1997). Neural recognition molecules and synaptic plasticity. Curr Opin Cell Biol. *9*, 627-634.

Schaeffer, L., Duclert, N., Huchet-Dymanus, M., and Changeux, J. P. (1998). Implication of a multisubunit Ets-related transcription factor in synaptic expression of the nicotinic acetylcholine receptor. EMBO J. *17*, 3078-3090.

Schuetze, S. M., and Role, L. W. (1987). Developmental regulation of nicotinic acetylcholine receptors. Annu Rev Neurosci. *10*, 403-457.

Si, J., Luo, Z., and Mei, L. (1996). Induction of acetylcholine receptor gene expression by ARIA requires activation of mitogen-activated protein kinase. J Biol Chem. *271*, 19752-19759.

Simon, A. M., and Burden, S. J. (1993). An E box mediates activation and repression of the acetylcholine receptor delta-subunit gene during myogenesis. Mol Cell Neurosci. *13*, 5133-5140.

Slack, J. R., and Pockett, S. (1981). Terminal sprouting of motoneurones is a local response to a local stimulus. Brain Res. *217*, 368-374.

Son, Y.-J., Patton, B. L., and Sanes, J. R. (1999). Induction of presynaptic differentiation in cultured neurons by extracellular matrix components. Eur J Neurosci. *11*, 3457-3467.

Son, Y. J., and Thompson, W. J. (1995a). Nerve sprouting in muscle is induced and guided by processes extended by Schwann cells. Neuron. *14*, 133-141.

Son, Y. J., and Thompson, W. J. (1995b). Schwann cell processes guide regeneration of peripheral axons. Neuron. *14*, 125-132.

Springer, J. E., Seeburger, J. L. H. J., Gabrea, A., Blankenhorn, E. P., and Bergman, L. W. (1995). cDNA sequence and differential mRNA regulation of two forms of glial cell line-derived neurotrophic factor in Schwann cells and rat skeletal muscle. Exp Neurol. *131*, 47-52.

Stein, J., and Liang, P. (2002). Differential display technology: a general guide. Cell Mol Life Sci. *59*, 1235-1240.

Steljes, T. P., Kinoshita, Y., Wheeler, E. F., Oppenheim, R. W., and Von Bartheld, C. S. (1999). Neurotrophic factor regulation of developing avian oculomotor neurons: differential effects of BDNF and GDNF. J Neurobiol. *41*, 295-315.

Storms, S. D., Kim, A. C., Tran, B. H., Cole, G. J., and Murray, B. A. (1996). NCAM-mediated adhesion of transfected cells to agrin. Cell Adhes Commun. *3*, 497-509.

Sugiyama, J., Bowen, D. C., and Hall, Z. W. (1994). Dystroglycan binds nerve and muscle agrin. Neuron. *13*, 103-115.

Suzuki, H., Hase, A., Yoon Kim, B., Miyata, Y., Ikuya Nonaka, Arahata, K., and Akazawa, Chihiro (1998). Up-regulation of glial cell line-derived neurotrophic factor (GDNF) expression in regenerating muscle fibers in neuromuscular diseases. Neurosci Letters. *257*, 165-167.

Suzuki, K., Grinnell, A. D., and Kidokoro, Y. (2002). Hypertonicity-induced transmitter release at Drosophila neuromuscular junctions is partly mediated by integrins and cAMP/protein kinase A. J Physiol (Lond). *538*, 103-119.

Tachikui, H., Kurosawa, N., Kadomatsu, K., and Muramatsu, T. (1999). Genomic organization and promoter activity of embigin, a member of the immunoglobulin superfamily. Gene. *240*, 325-332.

Takai, T., Noda, M., Mishina, M., Shimizu, S., Furutani, Y., Kayano, T., Ikeda, T., Kubo, T., Takahashi, H., Takahashi, T., and al., e. (1985). Cloning, sequencing and expression of cDNA for a novel subunit of acetylcholine receptor from calf muscle. Nature. *315*, 761-764.

Takai, Y., and Nakanishi, H. (2003). Nectin and afadin: novel organizers of intercellular junctions. J Cell Sci. *116*, 17-27.

Tam, S. L., Archibald, V., Jassar, B., Tyreman, N., and Gordon, T. (2001). Increased neuromuscular activity reduces sprouting in partially denervated muscles. J Neurosci. *21*, 654-667.

Tansey, M., Chu, G., and Merlie, J. (1996). ARIA/HRG regulates AChR epsilon subunit gene expression at the neuromuscular synapse via activation of phosphatidylinositol 3-kinase and Ras/MAPK pathway. J Cell Biol. *134*, 465-476.

Taraviras, S., Marcos-Gutierrez, C., Durbec, P., Jani, H., Grigoriou, M., Sukumaran, M., Wang, L., Hynes, M., Raisman, G., and Pachnis, V. (1999). Signalling by the RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system. Development. *126*, 2785-2797.

Tepass, U., Truong, K., Godt, D., Ikura, M., and Peifer, M. (2000). Cadherins in embryonic and neural morphogenesis. Nat Rev Mol Cell Biol. *1*, 91-100.

Thiery, J. P., Brackenbury, R., Rutishauser, U., and Edelman, G. M. (1977). Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. J Biol Chem. *252*, 6841-6845.

Thomason, D. B., and Booth, F. W. (1990). Atrophy of the soleus muscle by hindlimb unweighting. J Appl Physiol. *68*, 1-12.

Thompson, W. J. (1985). Activity and synapse elimination at the neuromuscular junction. Cell Mol Neurobiol. *5*, 167-182.

Tsay, H., and Schmidt, J. (1989). Skeletal muscle denervation activates acetylcholine receptor genes. J Cell Biol. *108*, 1523-1526.

Tsen, G., Halfter, W., Kröger, S., and Cole, G. J. (1995). Agrin is a heparan sulfate proteoglycan. J Biol Chem. *270*, 3392-3399.

Unwin, N. (1993). Neurotransmitter action: opening of ligand-gated ion channels. Cell. *72*, Suppl 31-41.

Unwin, N. (2000). The Croonian Lecture 2000. Nicotinic acetylcholine receptor and the structural basis of fast synaptic transmission. Philos Trans R Soc Lond B Biol Sci. *355*, 1813-1829.

Usdin, T., and Fischbach, G. (1986). Purification and characterization of a polypeptide from chick brain that promotes the accumulation of acetylcholine receptors in chick myotubes. J Cell Biol. *103*, 493-507.

Valenzuela, D. M., Stitt, T. N., DiStefano, P. S., Rojas, E., Mattsson, K., Compton, D. L., Nunez, L., Park, J. S., Stark, J. L., Gies, D. R., and al, e. (1995). Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. Neuron. *15*, 573-584.

Vogel, Z., Christian, C., Vigny, M., Bauer, H., Sonderegger, P., and Daniels, M. (1983). Laminin induces acetylcholine receptor aggregation on cultured myotubes and enhances the receptor aggregation activity of a neuronal factor. J Neurosci. *3*, 1058-1068.

Wallace, B. (1989). Agrin-induced specializations contain cytoplasmic, membrane, and extracellular matrix-associated components of the postsynaptic apparatus. J Neurosci. *9*, 1294-1302.

Walsh, F. S., and Doherty, P. (1997). Neural cell adhesion molecules of the immunoglobulin superfamily: Role in axon growth and guidance. Annu Rev Cell Dev Biol. *13*, 425-456.

Walsh, M. K., and Lichtman, J. W. (2003). In vivo time-lapse imaging of synaptic takeover associated with naturally occurring synapse elimination. Neuron. *37*, 67-73.

Weston, C., Gordon, C., Teressa, G., Hod, E., Ren, X.-D., and Prives, J. (2003). Cooperative regulation by Rac and Rho of agrin-induced acetylcholine receptor clustering in muscle cells. J Biol Chem. *278*, 6450-6455.

Weston, C., Yee, B., Hod, E., and Prives, J. (2000). Agrin-induced acetylcholine receptor clustering is mediated by the small guanosine triphosphatases Rac and Cdc42. J Cell Biol. *150*, 205-212.

Willmott, T., Skitsa, I., Hill, I., Mummery, R., and Beesley, P. W. (1992). Molecular characterisation and structural relationship of the synapse-enriched glycoproteins gp65 and gp55. J Neurochem. *58*, 2037-2043.

Witzemann, V., Barg, B., Criado, M., Stein, E., and Sakmann, B. (1989a). Developmental regulation of five subunit specific mRNAs encoding acetylcholine receptor subtypes in rat muscle. In FEBS Letters, pp. 419-424.

Witzemann, V., Barg, B., Criado, M., Stein, E., and Sakmann, B. (1989b). Developmental regulation of five subunit specific mRNAs encoding acetylcholine receptor subtypes in rat muscle. FEBS Lett. *242*, 419-424.

Witzemann, V., Brenner, H., and Sakmann, B. (1991). Neural factors regulate AChR subunit mRNAs at rat neuromuscular synapses. J Cell Biol. *114*, 125-141.

Xu, Y. K., and Nusse, R. (1998). The Frizzled CRD domain is conserved in diverse proteins including several receptor tyrosine kinases. Curr Biol. *8*, R405-406.

Yamagata, M., Sanes, J. R., and Weiner, J. A. (2003). Synaptic adhesion molecules. Curr Opin Cell Biol. *15*, 621-632.

Yan, Q., Matheson, C., and Lopez, O. T. (1995). In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. Nature. *373*, 341-344.

Yang, J. S., Bennett, P. B., Makita, N., George, A. L., and Barchi, R. L. (1993). Expression of the sodium channel beta 1 subunit in rat skeletal muscle is selectively associated with the tetrodotoxin-sensitive alpha subunit isoform. Neuron. *11*, 915-922.

Yang, X., Arber, S., William, C., Li, L., Tanabe, Y., Jessell, T. M., Birchmeier, C., and Burden, S. J. (2001). Patterning of Muscle Acetylcholine Receptor Gene Expression in the Absence of Motor Innervation. Neuron. *30*, 399-410.

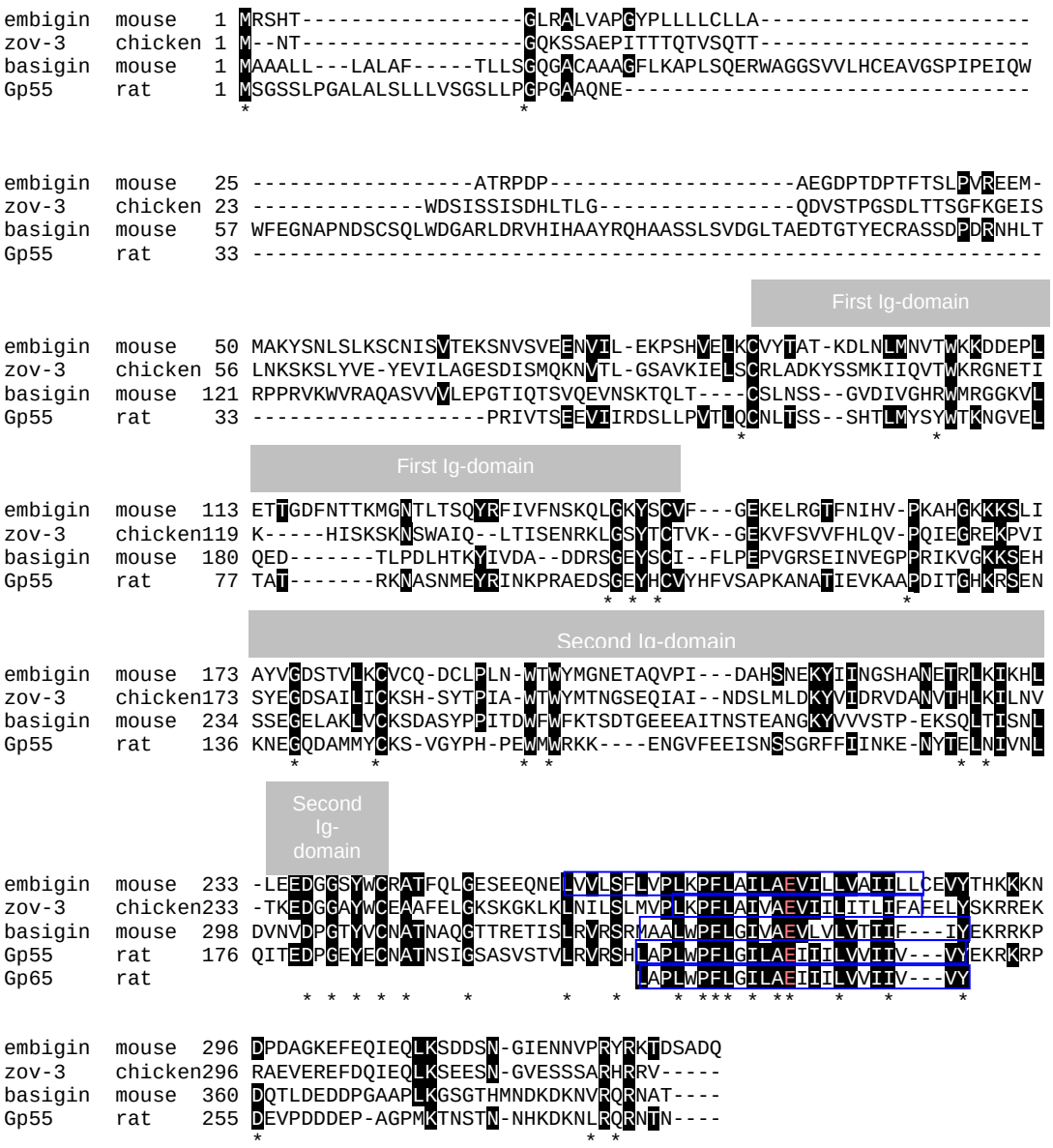
Yang, X., Li, W., Prescott, E. D., Burden, S. J., and Wang, J. C. (2000). DNA topoisomerase II and neural development. Science. *287*, 131-134.

Zhou, H., Muramatsu, T., Halfter, W., Tsim, K. W. K., and Peng, H. B. (1997). A Role of midkine in the development of the neuromuscular junction. Mol Cell Neurosci. *10*, 56-70.

Zhu, X., Lai, C., Thomas, S., and Burden, S. (1995). Neuregulin receptors, erbB3 and erbB4, are localized at neuromuscular synapses. EMBO J. *14*, 5842-5848.

Zwick, M., Teng, L., Mu, X., Springer, J. E., and Davis, B. M. (2001). Overexpression of GDNF induces and maintains hyperinnervation of muscle fibers and multiple end-plate formation. Exp Neurol. *171*, 342-350.

ANNEXE



Embigin has
29,5% similarity with zov-3
17% similarity with basigin
21,5% similarity with Gp55

Fig. Annexe. Comparison of Embigin structure with those of related IgSF members.
Putative Ig domains are indicated and transmembrane domain are boxed in blue boxes. Amino acid residues identical to mouse embigin are shown by white letters with black background and amino acid residues conserved in all six proteins are furthermore marked with an asterisk. The conserved glutaminic acid residue determining this group of IgSF is shown by in red with black background and marked with an asterisk. Only the putative transmembrane domain of Gp65 was aligned. All five sequences were aligned using Clustal V multiple alignment.

ACKNOWLEDGMENTS

First of all, I would like to express my gratitude to Prof. Dr. Hans-Rudolf Brenner for giving me the opportunity to join his lab for doing my thesis.

I am very grateful also to Prof. Dr. Heinrich Reichert and Prof. Dr. Markus Ruegg to join my thesis committee.

I am very grateful to previous members and members of "Brenner-lab" for their help, fruitful discussions and their friendship including Dr. Eric Lacazette, Valérie Crotet, Annick Werner, Dr. Sophie Lecalvez, Michèle Courtet, Dr. Agnieszka Sadowska, Dr. Enzo Lain, Dr. Alex Blindenbacher and Dr. Pascal Escher. Especially I would like to thank first Dr. Eric Lacazette for his humour, his friendship, the fruitful scientific and non-scientific discussions, and his moral support and also Valérie Crotet for her technical assistance, her moral support and, furthermore her humour and friendship in and out of the lab. I am very grateful to Annick Werner for her technical assistance, her moral support and friendship.

I would like to thank all people at the Biozentrum and Pharmazentrum I had the pleasure to meet and to interact with at work and in private life.

Finally I would like to thank all my friends for their friendship, their humour and moral support including Dr. Samuel Frere, Mrs. and Mr. Bonenfant Débora and Chris, Dr. Réjan Vigot, Dr. Patrick Scotton, Ainhoa Mielgo.

Last but not least I would like to thank my parents, my sister, my brother, my grand-parents and Jean-François for their love, patience and support they gave me during my thesis.

CURRICULUM VITAE

Soizic CARNEJAC
Nationality: French
Date of birth: 10th January 1978
Marital status: Single

Profile : Chemist-biochemist-biologist

FORMATION

2002-2005 **PhD Thesis at the institute of Physiology at the University of Basel, Switzerland, Pr. Brenner**

-Identification and characterization of new factors involved in neuromuscular junction formation

1998-2001 **Ingénieur (equivalent to a Master) in chemistry at the Ecole Nationale Supérieure de Chimie de Montpellier (ENSCM), France** specialized in biochemistry-medicinal chemistry option: chemistry-biology-health

2001 **DEA (equivalent to a Master) at the interface chemistry-biology: molecular systems with therapeutic aiming** option: pharmacology

Sanofi-Aventis Research in Labège, France, Mrs Walker-Aubry (7 months): Localization of mRNA targets of Sanofi-Aventis with development of non isotopic in situ hybridization methods

Laboratory of Pharmaceutical Chemistry at the University of Pharmaceutical Sciences in Toulouse, France (Pr. Payard) Pr. Baziard-Mouysset (2 months) : Conceive and development of the synthesis of imidazolins and analogs for the diabetes type 2 treatment

1996-1998 **DEUG (equivalent to the first 2 years of a Bachelor) at the University Paul Sabatier in Toulouse, France**

SKILLS

Techniques in biology

- Molecular biology: PCR, Real Time PCR, Northern Blot
- Cell culture: animal cell culture (primary cultures and cell lines)
- Cell biology and biochemistry: immunoprecipitation, Western Blot, immunohistochemistry, in situ hybridization

Techniques in chemistry

- Organic synthesis: macro and micro-synthesis
- Purification techniques of organic compounds: recristallization, chromatography on column, extraction
- Analytical techniques of organic compounds: Infra-red, NMR ^{1}H and ^{13}C, melting point

- Knowledge: HPLC, GPC, DSC, mass spectrometry

Informatics

- Windows office (Word, Excel, Power Point, Adobe Photoshop, Access)
- Internet
- Data bases in chemistry and in biology
- Molecular modeling: Mopac and Hyperchem

Language skills

French: mother tongue English: fluently Spanish: Fairly good

CENTERS OF INTERESTS

Responsible for humanitarian project for Restos du Cœur in France in 1996
Enthousiastic for tennis, cinema and excursions

COMMUNICATIONS

"Mechanisms of synapse formation at the neuromuscular junction"
Carnejac S, Escher P, Lacazette E, Brenner HR
Poster presented at the meeting From Bench to Bedside, 2003, Basel, Switzerland

"First Part: Transcriptional changes in rat soleus muscle upon denervation analyzed by mRNA Differential Display
Second Part: Expressional changes in functional gene units elicited by denervation in rat soleus muscle"
Carnejac S
Oral communication at the International meeting on neuromuscular junction, 2002, Sils Maria, Switzerland

"Embigin, a surface molecule in muscle involved in neuromuscular synapse formation?"
Carnejac S
Oral communication at the International meeting on neuromuscular junction, 2004, Sils Maria, Switzerland

REFERENCES

Mrs. Nancy Walker-Aubry
Email: Nancy.Walker@sanofi-aventis.com

Pr. Geneviève Baziard-Mouysset
Email: chimphar@cict.fr

Pr. Hans-Rudolf Brenner
Email: hans-rudolf.brenner@unibas.ch