Postsynaptic chromatin is under neural control at the neuromuscular junction

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In adult skeletal muscle, the nicotinic acetylcholine receptor (AChR) specifically accumulates at the neuromuscular junction, to allow neurotransmission. This clustering is paralleled by a compartmentalization of *AChR* genes expression to subsynaptic nuclei, which acquire a unique gene expression program and a specific morphology in response to neural cues. Our results demonstrate that neural agrin-dependent reprogramming of myonuclei involves chromatin remodelling, histone hyperacetylation and histone hyperphosphorylation. Activation of *AChR* genes in subsynaptic nuclei is mediated by the transcription factor GABP. Here we demonstrate that upon activation, GABP recruits the histone acetyl transferase (HAT) p300 on the *AChR* ε subunit promoter, whereas it rather recruits the histone deacetylase HDAC1 when the promoter is not activated. Moreover, the HAT activity of p300 is required *in vivo* for *AChR* expression. GABP therefore couples chromatin hyperacetylation and *AChR* activation by neural factors in subsynaptic nuclei.

Introduction

Neurotransmission at synapses requires the conversion of an electrical signal into a chemical signal via neurotransmitter release. In the postsynaptic cell, neurotransmitter receptors convert back this chemical signal into an electrical signal. Correct transmission of the information emitted by the presynaptic neuron requires a highly specialized postsynaptic network. Among the proteins accumulated at the neuromuscular junction (NMJ), the nicotinic acetylcholine receptor (AChR) plays a central role as it binds acetylcholine, the neurotransmitter released by the nerve, and allows the transmission of the nerve influx to the muscle.

The AChR is a heteropentameric cationic channel composed of four subunits, $\alpha_2\beta\gamma\delta$ in the embryo and $\alpha_2\beta\varepsilon$ in adult muscle (Sanes and Lichtman, 2001). The current model stipulates that AChR accumulation at the NMJ results from the combined action of several distinct mechanisms that all depend on the activation of the muscle tyrosine kinase receptor MuSK by the neural factor agrin (Sanes and Lichtman, 1999). The first consequence of MuSK activation is the physical clustering of AChR at the NMJ via the adaptator protein Rapsyn. Besides protein aggregation, the clustering of synaptic proteins is also paralleled by the compartmentalization of gene expression. Upon acetylcholine binding, AChR molecules aggregated at the NMJ trigger membrane depolarization and electrical activity in the muscle cell, thereby inhibiting the expression of the *AChR* α, β, γ and δ subunit genes (Sanes and Lichtman, 1999). Finally, the activation of MuSK, either directly or indirectly via neuregulin, locally triggers the MAPK and JNK intracellular signalling pathways that strongly activate the expression of the genes coding for the components of the NMJ (the synaptic genes) in the nuclei located directly beneath the NMJ (Schaeffer *et al*, 2001; Lacazette *et al*, 2003). The downstream nuclear target of these signalling pathways is the Ets-related transcription factor GABP, which binds the N box present in several synaptic genes, including *AChR* genes (Koike *et al*, 1995; Duclert *et al*, 1996; Fromm and Burden, 1998, 2001; Schaeffer *et al*, 1998; Gramolini *et al*, 1999; Briguet and Ruegg, 2000; de Kerchove *et al*, 2002). The motor neuron thus produces two anterograde signals that control AChR expression, agrin and acetylcholine, which respectively activate *AChR* gene expression in subsynaptic nuclei and inhibit *AChR* gene expression in extrasynaptic nuclei.

In the nucleus, DNA is embedded in chromatin, a repetitive nucleoproteic structure. Nucleosomes are formed by the wrapping of 146 DNA base pairs around a histone octamer and constitute the basic unit of chromatin. The N-terminal tails of histones are subject to numerous post-translational modifications such as phosphorylation, ubiquitination, ADP ribosylation, glycosylation, methylation and acetylation (Fischle *et al*, 2003; Margueron *et al*, 2005). These modifications can facilitate or inhibit transcription, DNA repair, DNA replication, DNA condensation and chromosome segregation. Histone H3 phosphorylation on serine 10 (PH3 S10) is well known to decorate chromosomes during mitosis (Gurley *et al*, 1978; Hsu *et al*, 2000; Murnion *et al*, 2001; Crosio *et al*, 2002), but this modification has also been reported to be associated with the acetylation and activation of the immediate-early gene promoter *cJun* (Sassone-Corsi *et al*, 1999; Thomson *et al*, 1999). Lysine acetylation on H3 and H4 histone tails is the best-characterized histone modification, and hyperacetylated histones are found in active domains of chromatin, whereas hypoacetylated histones are associated to the silent regions (Wolffe and Kurumizaka, 1998). Histone acetylation levels are regulated by two antagonistic enzymatic activities carried by two classes of transcription factors: histone acetyltransferases (HAT) and histone deacetylases (HDAC).

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We have previously demonstrated that the inhibition of *AChR* expression in extrasynaptic nuclei involved the control of muscle chromatin acetylation by electrical activity via HDACs (Mejat *et al*, 2005). In subsynaptic nuclei, *AChR* genes expression is resistant to electrical activity. In the present study, we show that the specific gene expression profile of subsynaptic nuclei correlates with a particular state of chromatin. The observation of NMJ electron micrographs reveals different chromatin organizations in subsynaptic and extrasynaptic nuclei. This implies that the chromatin remodelling machinery is differentially regulated in muscle subsynaptic nuclei. In subsynaptic nuclei, chromatin appeared to be less condensed, a feature that could correlate with increased histone acetylation. We have therefore sought to determine if the particular subsynaptic chromatin organization correlated with increased histone acetylation, and if specific HATs and/or HDACs were involved in the regulation of *AChR* expression at the NMJ and thus participated in the specification of the unique gene expression program of subsynaptic nuclei.

Our results show that chromatin is hyperacetylated and hyperphosphorylated in subsynaptic nuclei, and that the HAT activity of the coactivator p300 is required for *AChR* genes expression at the NMJ. They further demonstrate that histone hyperacetylation on *AChR* promoters is induced by the neural factors agrin and neuregulin. Finally, our results show that histone acetylation on *AChR* genes is due to the loss of HDAC1 and the recruitment of the histone acetyl transferase p300 by GABP, whereas in the absence of activation GABP rather recruits the histone deacetylase HDAC1.

Results

The postsynaptic chromatin structure is different in subsynaptic nuclei

The specific morphology of subsynaptic nuclei was already noticed by Ranvier in 1875, who described them as fundamental nuclei. The observation of muscle nuclei by electron microscopy reveals that in subsynaptic nuclei, chromatin appears much less dense to electrons, thus suggesting that chromatin compaction is lower in these nuclei (Figure 1). Histone acetylation being a marker of relaxed chromatin,

antibodies directed against hyperacetylated histone H3 were used to perform immunofluorescence experiments on isolated muscle fibres. The results showed a strong labelling of subsynaptic nuclei, whereas extrasynaptic nuclei were hardly detected (Figure 2A and B).

Phosphorylation of the serine 10 of H3 was reported to be associated with the acetylation of the neighbouring lysine on immediate-early genes (Cheung *et al*, 2000; Clayton *et al*, 2000; Lo *et al*, 2000). Using an antibody directed against histone H3 both phosphorylated on serine 10 and acetylated on lysine 14 (PAcH3 S10 K14), we indeed observed that histone H3 was strongly phosphoacetylated in subsynaptic nuclei, whereas these modifications were not detected in extrasynaptic nuclei (Figure 2C and D).

Synaptic gene activation in subsynaptic nuclei depends on MuSK and agrin, and ectopic expression of agrin is sufficient to induce ectopic postsynaptic specialization, with AChR clustering and synaptic gene activation. To determine whether histone hyperacetylation and hyperphosphorylation were also triggered by agrin, ectopic synapses were induced to form in the soleus muscle by injection of purified neural agrin. Two weeks after agrin injection, α-bungarotoxin labelling showed ectopic AChR accumulation. Immunofluorescence experiments performed on these ectopic postsynaptic structures with anti-acetylated histone H3 and anti-phosphoacetylated histone H3 antibodies respectively demonstrated histone hyperacetylation and histone phosphoacetylation in the nuclei located beneath AChR clusters (Figure 3A and B).

Histone H3 acetylation and phosphoacetylation are triggered by neuregulin in cultured myotubes

C2C12 myotubes have been extensively used to dissect the mechanisms of synaptic genes activation in response to neural cues (Schaeffer *et al*, 2001; Mejat *et al*, 2003). However, although neural agrin induced activation of episomic AChR ε reporter constructs in C2C12 myotubes, as previously described, we could not see any significant activation of endogenous *AChR* ε subunit gene expression (data not shown). This prevented the use of agrin to observe histone acetylation at genomic loci in cultured myotubes. Conversely, neuregulin activates both episomic and endogenous *AChR* ε
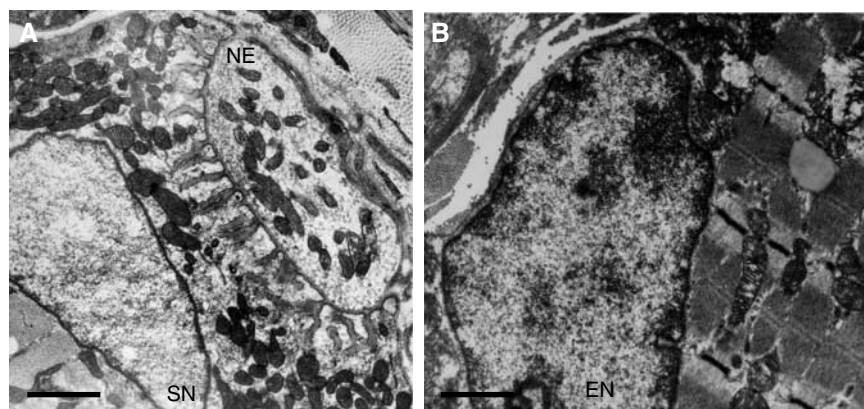
Figure 1 Chromatin is decondensed in subsynaptic nuclei at the NMJ. Electron microscopy of subsynaptic (**A**) and extrasynaptic (**B**) areas of *Tibialis anterior* muscle of 5-week-old OF1 male mice. The electron density of subsynatic nucleus (SN) located just beneath the nerve ending (NE) appears lower than that of the extrasynaptic nucleus (EN). This is directly correlated with the higher chromatin condensation in ES nucleus. Scale bar, 2 μm.
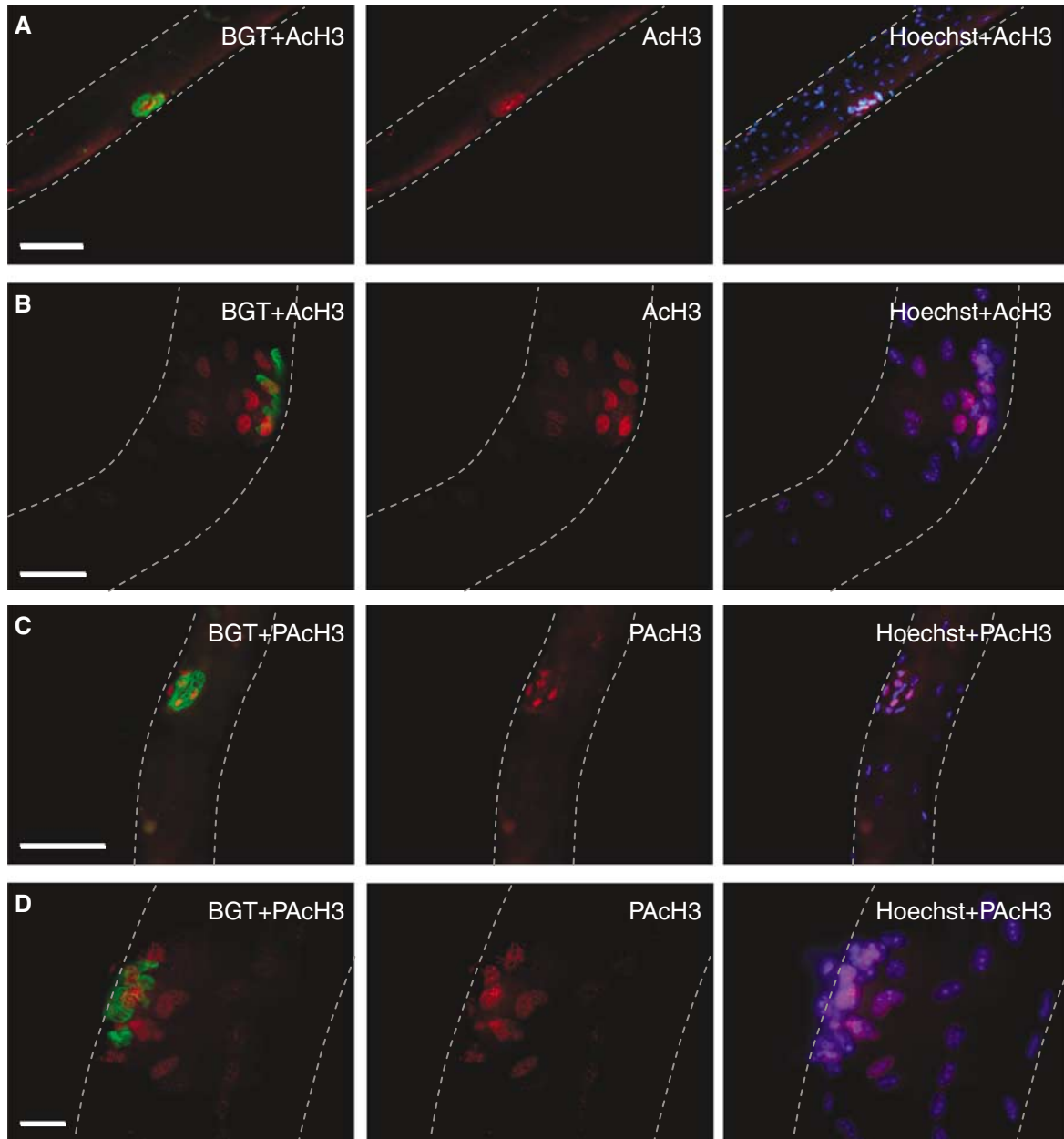
Figure 2 Chromatin is hyperacetylated and hyperphosphoacetylated in subsynaptic nuclei. Immunofluorescence on isolated mouse *Tibialis anterior* muscle fibres using antibodies specific for H3 modifications. (**A**, **B**) AcH3: histone H3 acetylated on K9 and K14. (**C**, **D**) PAcH3: histone H3 phosphorylated on S10 and acetylated on K14. The NMJ was stained with Alexa488 α-Bungarotoxin (BGT) and nuclei with Hoechst 33258. Scale bars, 50 μm (A, C) and 10 μm (B, D). Dashed lines represent the limits of the muscle fibres.

subunit gene expression in C2C12 myotubes (Mejat *et al*, 2003). In addition, similar to agrin, the pathways triggered by neuregulin to activate *AChR* gene expression converge on GABP (Schaeffer *et al*, 1998; Fromm and Burden, 2001; Lacazette *et al*, 2003). Moreover, in cultured myotubes, neuregulin and agrin activate *AChR* expression through a common pathway, neuregulin acting as agrin second messenger, as agrin-dependent *AChR* transcriptional activation is blocked by erbB dominant-negative mutants (Meier *et al*, 1998; Lacazette *et al*, 2003). Neuregulin was therefore used to study histone acetylation upon *AChR* ε gene activation in C2C12 myotubes.

In C2C12 cells, the activation of *AChR* expression is a biphasic process, with an early phase corresponding to the activation of the immediate-early genes *cfos* and *cJun*, and a late phase, in which *AChR* genes are activated (Si *et al*, 1999). To determine if *AChR* activation correlated with chromatin acetylation in cultured muscle cells, C2C12 myotubes were treated with recombinant neuregulin, and histone H3 phosphorylation and acetylation were evaluated by Western blot. Figure 4A shows that histone H3 acetylation gradually increased after neuregulin treatment. The analysis of histone H3 serine 10 phosphorylation revealed two peaks of phosphorylation; a first peak 15 min after neuregulin addition and a second peak 4–6 h later (Figure 4A). The two phases of histone modifications occurred concomitantly to the two waves of gene activation previously described by Si *et al* (1999), therefore suggesting that they could affect different
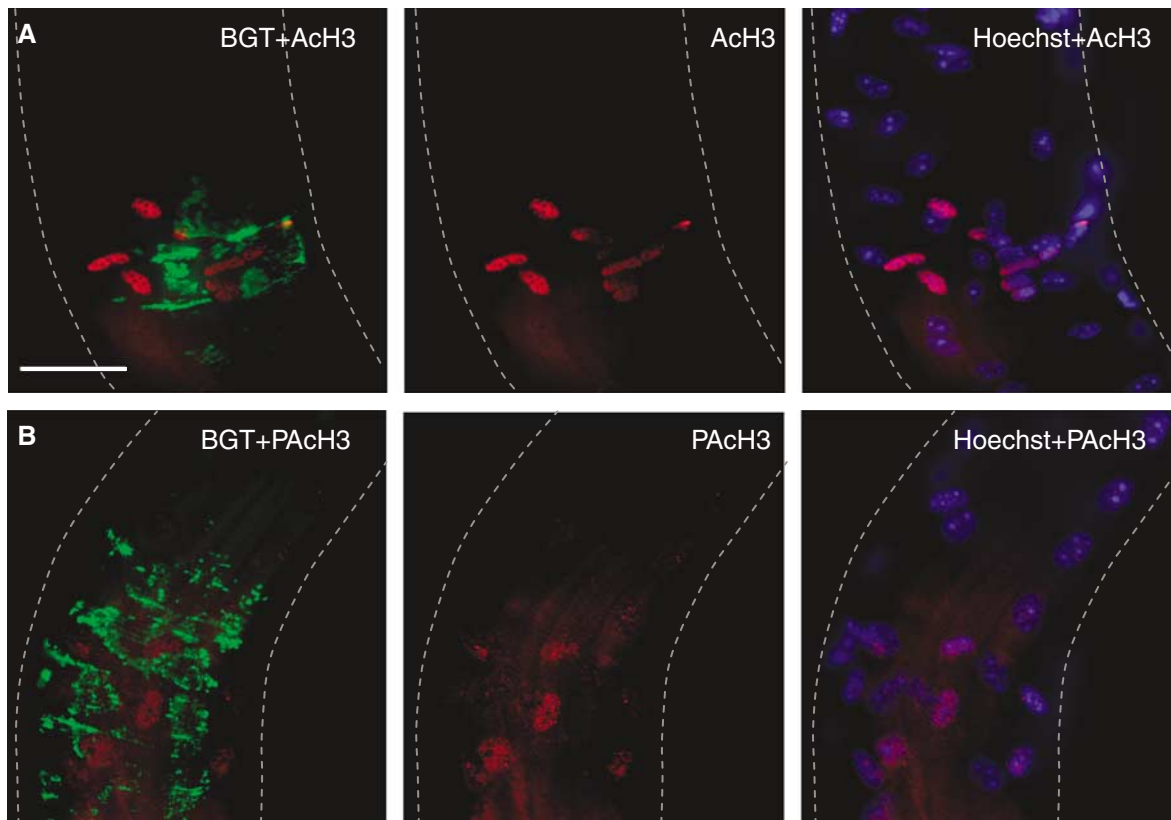
Figure 3 Extrasynaptic expression of neural agrin induces hyperacetylation and hyperphosphoacetylation at ectopic synapses. Ectopic synapses were induced by injecting recombinant agrin into adult mouse *Soleus* muscle fibres. Muscle fibres were isolated 14 days later and histone modification level was analysed by immunofluorescence as described in Figure 2 with antibodies specific for AcH3 (**A**) and PAcH3 (**B**). Scale bar, 20 μm. Dashed lines represent the limits of the muscle fibres.

sets of genes, that is, immediate-early genes and *AChR* genes. The rather high basal level of histone acetylation in C2C12 cells observed in Western blot could mask the presence of two acetylation peaks linked to neuregulin treatment. Chromatin immunoprecipitation (ChIP) was thus used to quantitate the levels of histone acetylation on the promoters of *cJun* and *AChR* ε subunit genes at different times after neuregulin addition to the culture medium. The results showed that on the *cJun* promoter, histone H3 acetylation transiently increased during the first half an hour, whereas on the *AChR* ε promoter histone H3 acetylation increased 4 h later and remained elevated thereafter (Figure 4B). The initial phase of histone acetylation therefore corresponds to the transient activation of immediate-early genes, and the second phase corresponds to the stable activation of *AChR* gene expression.

HDAC1 is recruited on AChR genes

We next sought to identify the histone-modifying activities involved in the neuronal control of postsynaptic chromatin acetylation. In a first attempt, synaptic and extrasynaptic zones of diaphragm muscles were microdissected and the expression levels of several HATs (p300, PCAF and GCN5) and HDACs were measured by quantitative RT–PCR. No differential expression could be detected. The amount of mRNA coding these factors was also quantified in neuregulin-treated or control myotubes, and neuregulin did not affect their expression (data not shown).

Previous studies on class I HDACs report that their regulation is rather post-translational than transcriptional (Zhou *et al*, 2000; Pflum *et al*, 2001; Galasinski *et al*, 2002; Qiu *et al*, 2006; Vashisht Gopal *et al*, 2006). To follow the evolution of class I HDACs protein levels during *AChR* activation, C2C12 myotubes were treated with neuregulin and analysed by immunofluorescence. In the case of HDAC1, neuregulin induced a marked decrease in the immunostaining of the protein, whereas the levels of HDAC2 and HDAC3 were not affected (Figure 5A; data not shown). This suggested that neuregulin could potentially induce a decrease in the amount of HDAC1 in the myotubes. In addition, the reduction of HDAC1 immunostaining was accompanied by a redistribution of the protein in the nucleus, with a dramatic decrease in the number of immunoreactive spots (Figure 5A).

To determine if the reduction of the immunostaining resulted from a reduction of the amount of HDAC1, or a conformational change, or from the masking of its epitope by a new partner, HDAC1 levels were evaluated by Western blot. Consistent with the immunofluorescence results, the amount of HDAC1 started to decrease 4 h after neuregulin addition (Figure 5B).

The reduction in the amount of HDAC1 took place 4 h after the addition of neuregulin, precisely when H3 acetylation increased on the *AChR* ε promoter, thus suggesting that HDAC1 could participate in the repression of this gene. To determine if HDAC1 was present on the *AChR* ε promoter, and if the activation of *AChR* expression correlated with

A

B
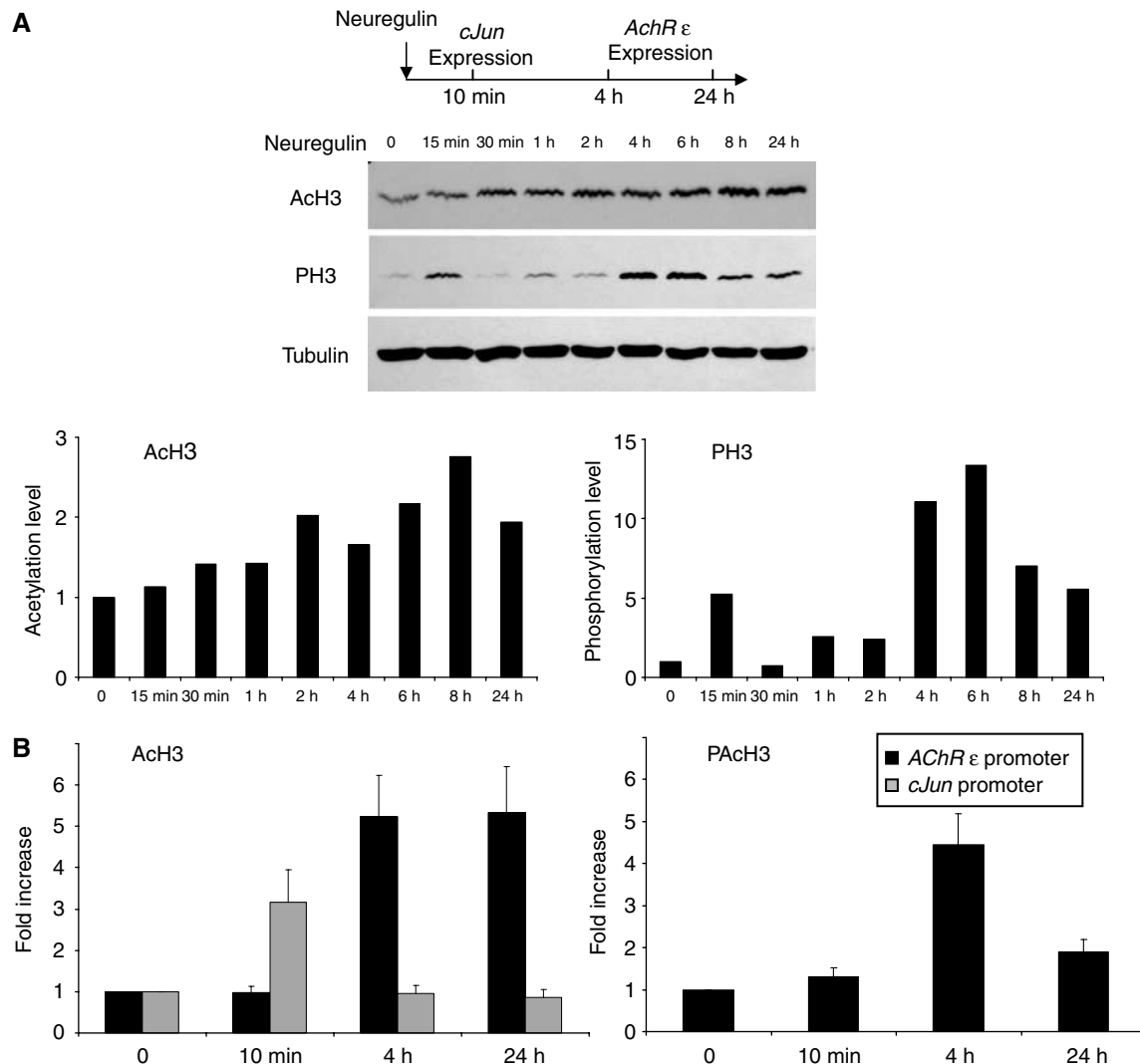
Figure 4 Neuregulin induces histone acetylation and phosphorylation on *AChR* ε gene promoter. (**A**) Neuregulin induces histone acetylation in cultured myotubes. C2C12 myotubes were treated with neuregulin (for indicated time) and 30 μg of whole-cell extracts was analysed by Western blot using antibodies specific for histone H3 modifications (AcH3: histone H3 acetylated on K9 and K14; PH3: histone H3 phosphorylated on S10) and β-tubulin as loading control. Diagrams correspond to Western blot quantification using Image J software. (**B**) Neuregulin induces modification of histone H3 on the *AChR* ε gene promoter. ChIP experiments were performed on C2C12 myotubes treated with neuregulin. Histone modification levels on the *cJun* and *AChR* ε promoter genes were analysed by ChIP using AcH3- and PAcH3- specific antibodies. The data are means $\pm$ s.e.m. of three independent experiments; $P < 0.05$ (*t*-test).

a displacement of HDAC1, ChIP experiments were performed with an anti-HDAC1 antibody. The results indicated that HDAC1 was indeed present on the *AChR* ε subunit promoter, and that neuregulin induced a two-fold decrease in the amount of HDAC1 bound to the promoter (Figure 5C).

HDAC1 thus probably participates in the repression of chromatin acetylation and *AChR* gene expression in the absence of neural factors. However, removal of HDACs is usually not sufficient to obtain histone hyperacetylation, and HAT recruitment is also required.

p300 participates in the activation of AChR genes expression

The best-characterized transcription factor involved in the activation of synaptic genes at the NMJ is GABP (Schaeffer *et al*, 2001; Mejat *et al*, 2003). GABP also activates gene

expression in many non-muscle cells. GABP has been shown to cooperate with the HAT p300 for the activation of IL16 gene in T lymphocytes (Bannert *et al*, 1999), and for the activation of the CD18 gene in myeloid cells (Bush *et al*, 2003; Resendes and Rosmarin, 2006). The recruitment of p300 on the *AChR* ε subunit gene promoter upon activation by neuregulin was therefore evaluated by ChIP. As shown in Figure 6A, p300 was indeed recruited to the *AChR* ε promoter in the presence of neuregulin.

In addition to its HAT activity, p300 possesses several activities and functions, and some of its coactivating functions have been shown to be HAT independent (Chan and La Thangue, 2001). Lysine–coenzymeA (LysCoA) has been shown previously to specifically inhibit the HAT activity of p300 (Lau *et al*, 2000; Polesskaya *et al*, 2001). However, the use of this inhibitor has been limited *in vivo* by the fact that it is not cell-permeant. To circumvent this problem, we took
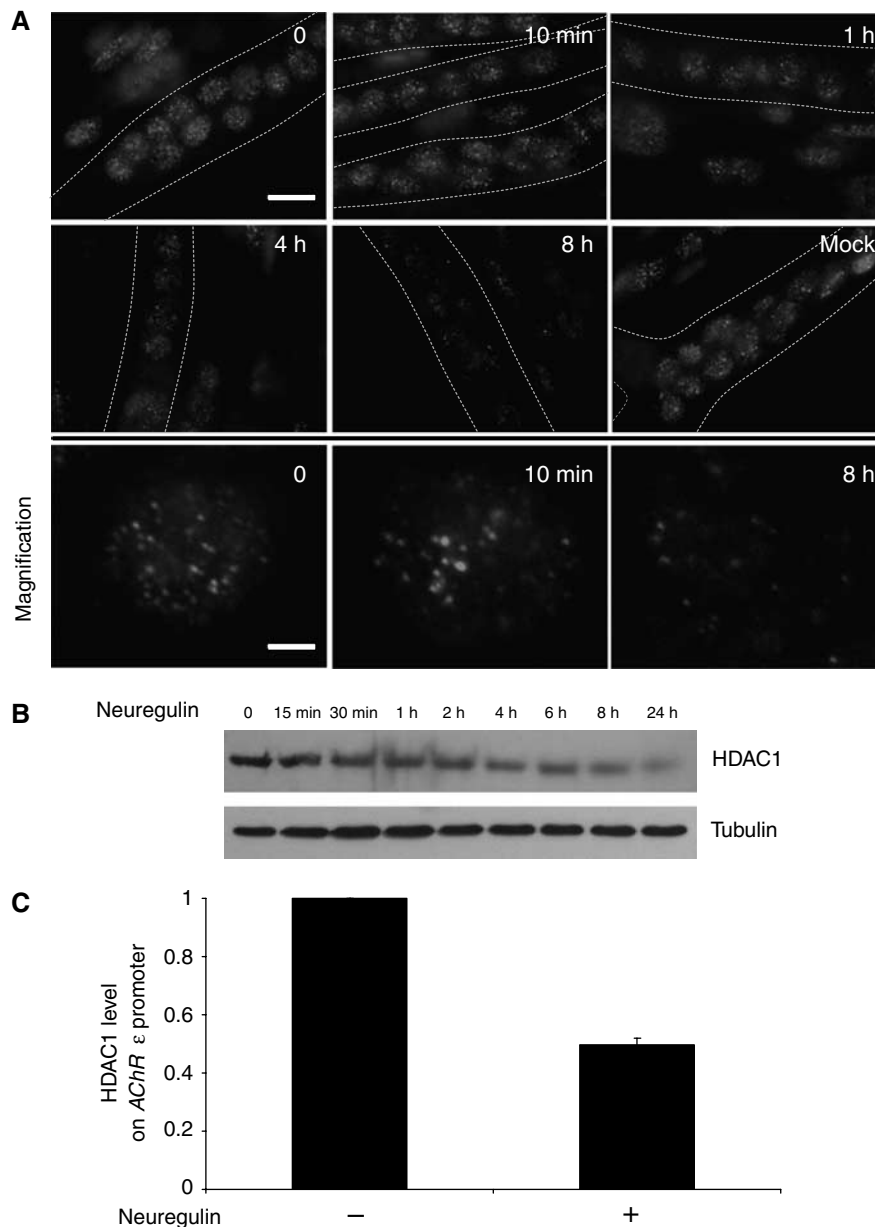
Figure 5 HDAC1 level is reduced in the presence of neuregulin. (**A**) Myotube cultures were treated with neuregulin for the indicated time and were analysed by immunofluorescence using an anti-HDAC1 antibody. Scale bars, 20 and 2.5 μm for magnifications. Dashed lines symbolize the plasma membrane of the myotubes. (**B**) Differentiated myotube cultures were treated with neuregulin (indicated time) and 30 μg of whole-cell extracts was analysed by Western blot using antibodies specific for HDAC1 and β-tubulin as loading control. (**C**) HDAC1 is removed from the *AChR* ε promoter in the presence of neuregulin. ChIP experiments were performed with an anti-HDAC1 antibody on control myotubes and on myotubes treated for 6 h with neuregulin. The data correspond to the means ± s.e.m. of three independent experiments; $P < 0.01$ (*t*-test).

advantage of the susceptibility of skeletal muscle to *in vivo* electroporation. To determine whether the HAT activity of p300 was required for *AChR* ε gene expression, LysCoA was electroporated in *Tibialis anterior* muscles and the expression of the *AChR* ε subunit gene was measured by quantitative RT–PCR. The results were normalized to the effect of the inhibitor on β-actin mRNA levels. Two other genes (*cycloB* and *HPRT*) were also used for normalization and produced similar results (data not shown). In the presence of LysCoA, the *AChR* ε subunit gene mRNA dropped to less than 30% of its original level in 24 (Figure 6B) and 48 h (data not shown), whereas it was not affected by the electroporation of a mixture of Lysine and coenzyme A. In skeletal muscle, the

AChR ε subunit gene is expressed only in subsynaptic nuclei. The reduction of AChR ε mRNA levels thus resulted from the inhibition of the expression of the *AChR* ε subunit gene in subsynaptic nuclei. p300 HAT activity is thus crucial for AChR expression at the NMJ.

To confirm this result, LysCoA was also coelectroporated with a reporter plasmid coding for the luciferase placed under the control of the mouse *AChR* ε promoter. As shown in Figure 6C, the presence of LysCoA strongly inhibited the expression of luciferase at 24 and 48 h. Even with episomic constructs, the HAT activity of p300 is therefore required to activate transcription through the *AChR* ε promoter.

A

B   **C**
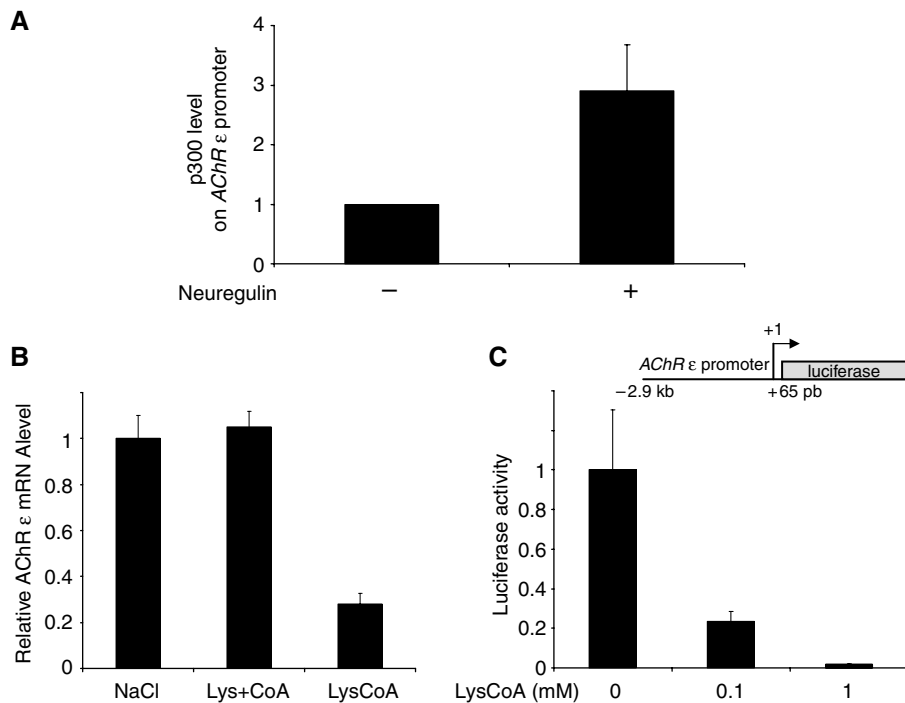
Figure 6 p300 HAT activity is required for the expression of *AChR* ε gene. (**A**) Neuregulin induces the recruitment of p300 on the *AChR* ε promoter in cultured myotubes. The ChIP experiments were performed with an antibody specific for p300 concomitantly to those described in Figure 5C. The data correspond to the means ± s.e.m. of three independent experiments; $P < 0.05$ (*t*-test). (**B**) The CBP/p300 HAT inhibitor LysCoA inhibits the expression of the *AChR* ε gene *in vivo*. NaCl (0.9 %), lysine (1 mM) and CoA (1 mM) or LysCoA (1 mM) were injected and electroporated in *Tibialis anterior* muscles and gene expression was evaluated 1 day later by real-time RT–PCR. The data correspond to the means ± s.e.m. of the results obtained with five mice. $P < 0.05$ (*t*-test). The experiment was reproduced three times. In addition, similar results were obtained when gene expression was measured 2 days after electroporation. (**C**) LysCoA represses *AChR* ε promoter-dependent transcriptional activation. A reporter construct in which the *AChR* ε promoter was placed upstream of the *luciferase* gene was electroporated into *Tibialis anterior* muscle with increasing doses of LysCoA. Whole-muscle luciferase activity was measured 1 day after electroporation. The data correspond to the means ± s.e.m. of the results obtained with three mice. $P < 0.05$ (*t*-test). The experiment was reproduced five times. In addition, similar results were also obtained when luciferase expression was measured 2 and 3 days after electroporation.

GABP selectively interacts with p300 or HDAC1

In the previous sections we have demonstrated that HDAC1 and p300 were differentially recruited on the *AChR* ε promoter in the presence or absence of neuregulin.

The transcription factor GABP is present both in synaptic and extrasynaptic nuclei, but nevertheless specifically activates the expression of synaptic genes in subsynaptic nuclei (Schaeffer *et al*, 2001; Mejat *et al*, 2003). We therefore examined the possibility that GABP could differentially recruit p300 or HDAC1, depending on the activation/repression context of synaptic genes. For this purpose, co-immunoprecipitation experiments were performed to detect interactions between GABP and p300, or HDAC1. The experiments were first performed with transfected tagged versions of p300 and HDAC1, and the results demonstrated that GABP could indeed interact with either p300 or HDAC1 (Figure 7A). To determine whether the interaction between GABP and p300, or HDAC1, correlated with the activation/repression status of synaptic genes, the co-immunoprecipitation experiments were performed with the endogenous proteins, in control and neuregulin-treated myotubes. The results show that GABP binds p300 more efficiently in the presence of neuregulin, whereas it binds more HDAC1 in the absence of neuregulin (Figure 7B).

Therefore, in conditions where synaptic genes are activated, GABP recruits the HAT p300, whereas in conditions

where they are repressed, GABP recruits the histone deacetylase HDCA1.

Discussion

Altogether, our results demonstrate that chromatin is hyperphosphorylated and hyperacetylated in subsynaptic nuclei. This is consistent with our finding that in cultured cells the activation of the *AChR* ε subunit gene coincides with histone H3 hyperphosphorylation and hyperacetylation on its promoter. We further demonstrated that *AChR* ε activation and hyperacetylation correlate with the recruitment of the HAT p300 by GABP, whereas in the absence of activation, GABP recruits HDAC1. Consistently, *in vivo* electroporation of the p300 HAT activity-specific inhibitor LysCoA reduced the transcriptional activation of the *AChR* ε subunit gene. The histone acetyltransferase activity of p300 is therefore necessary for the activation of the *AChR* ε subunit gene expression at the NMJ.

GABP differentially recruits transcriptional coactivators or repressors on the AChR ε gene promoter

GABP has been shown to regulate the expression of several synaptic proteins, among which are AChR, utrophin and MuSK. Previous studies mainly focused on the upstream signals that triggered GABP to activate the expression of
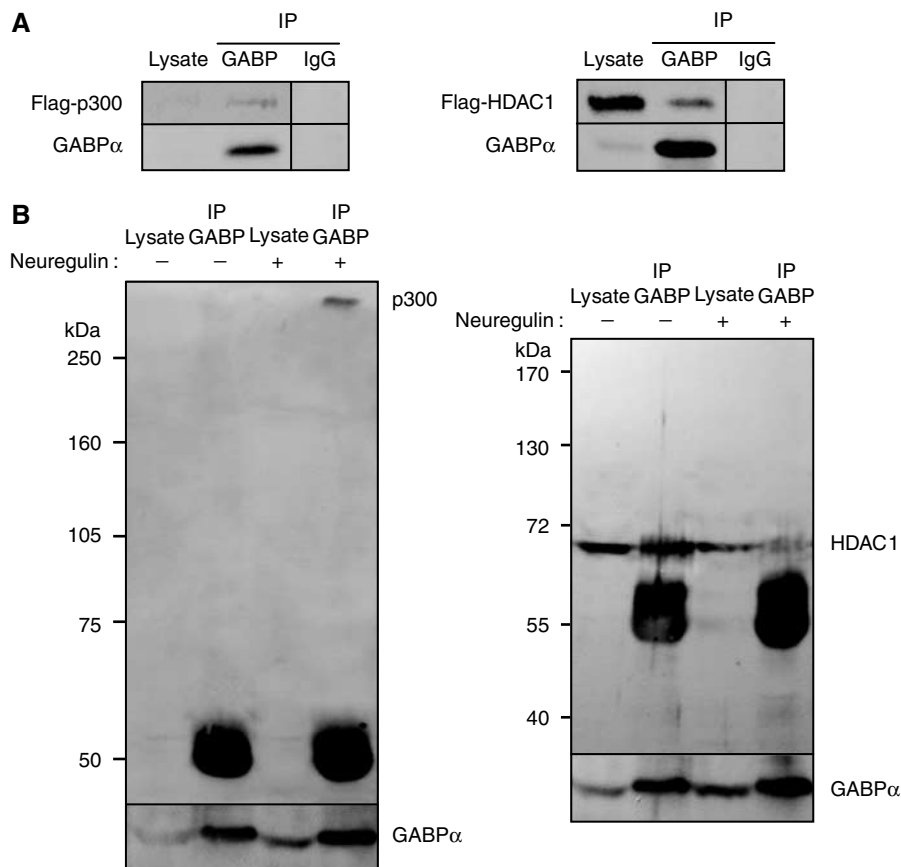
Figure 7 GABP selectively interacts with p300 or HDAC1. (**A**) GABPα interacts with p300 and HDAC1. C2C12 cells were cotransfected with GABPα and Flag-p300 or Flag-HDAC1 expression vectors. Twenty-four hours following transfection, cell lysates were prepared and the immunoprecipitation performed with an anti-GABPα antibody. The fractions were subsequently separated on SDS–PAGE, followed by immunoblotting with anti-Flag and anti-GABPα antibodies. The experiment was repeated three times. (**B**) Endogenous GABPα interacts with endogenous p300 and HDAC1, and neuregulins favour the recruitment of p300. Untreated and neuregulin-treated myotube (6 h) lysates were immunoprecipitated with an anti-GABPα antibody and the immune complex was analysed by immunoblotting with anti-p300 or anti-HDAC1 antibodies. The experiment was repeated three times.

synaptic genes (for review, see Schaeffer *et al*, 2001; Mejat *et al*, 2003), but the molecular mechanisms by which GABP specifically activated transcription in response to neural cues remained elusive. Our results provide a first insight into such mechanisms, as they demonstrate that upon transcriptional activation of the *AChR* ε subunit gene, GABP recruits the coactivator p300, whereas it recruits HDAC1, a well-known repressor, in the absence of *AChR* ε activation (Figure 8).

The decreased interaction between GABP and HDAC1, as well as the reduced recruitment of HDAC1 on the *AChR* ε subunit promoter in the presence of neuregulin could result from the concomitant reduction of the amount of HDAC1 protein in the cells. Such a regulation of HDAC1 is not unprecedented, as it has been shown that quinidin or TNFα treatment induce a diminution of the HDAC1 protein levels in breast cells (Zhou *et al*, 2000; Vashisht Gopal *et al*, 2006).

Trancriptional regulation by GABP involves chromatin acetylation

Both factors recruited by GABP are enzymes that regulate the level of histone acetylation. This suggests that the transcriptional regulation of the *AChR* ε subunit by GABP involves chromatin acetylation, which is consistent with the observa-

tion that histones are hyperacetylated in the nuclei, which express *AChR* genes.

In innervated muscle, the level of chromatin acetylation in myonuclei thus correlates with the expression of the *AChR*. Consistently, in a previous work, we have shown that in response to electrical activity, chromatin acetylation was low in extrasynaptic nuclei (Mejat *et al*, 2005). Upon denervation, a situation in which *AChR* expression is activated throughout the muscle fibres, histone acetylation was strongly increased. Therefore, a relationship exists between the status of histone acetylation and the expression of *AChR* genes in myonuclei.

Moreover, our results demonstrate that in innervated muscle, the HAT activity of p300 is required for *AChR* expression at the NMJ, whereas in the extrasynaptic regions, we previously showed that HDACs repressed *AChR* expression. Histone hyperacetylation is thus not only a marker of the myonuclei, which express *AChR* genes, but also plays a functional role as it is required for *AChR* expression, both at the NMJ in innervated muscles and in extrasynaptic nuclei in denervated muscle.

Histone phosphoacetylation is not restricted to immediate-early genes

ChIP experiments are not yet applicable to subsynaptic nuclei because of the amounts of purified subsynaptic nuclei they
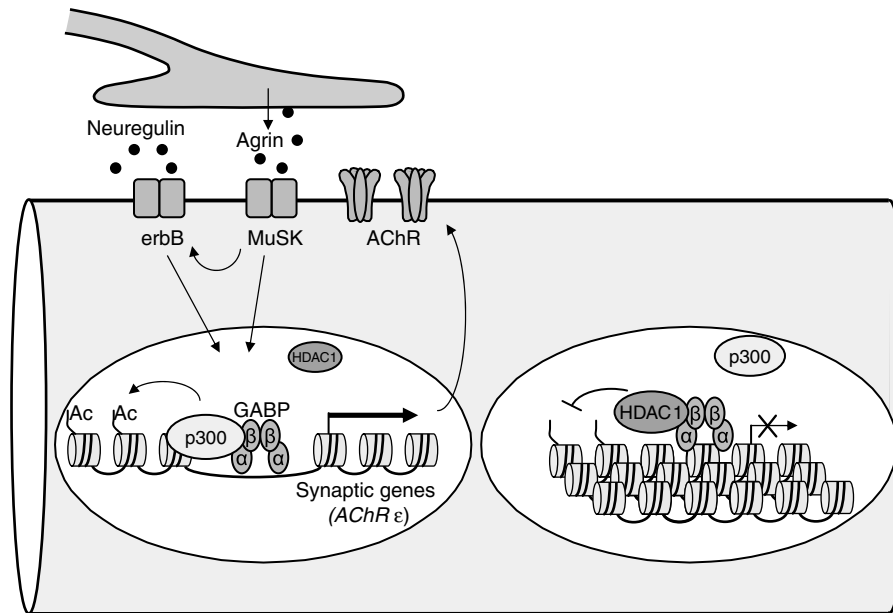
Figure 8 A model for synaptic gene regulation at the NMJ. Agrin and neuregulin are accumulated in the basal lamina of the synaptic cleft and activate their muscle receptor to induce local activation of intracellular signalling pathways, which in turn activate the transcription factor GABP. In addition, they induce histone hyperacetylation and hyperphosphoacetylation, which participate in chromatin decondensation. The recruitment of p300 on synaptic genes by GABP in subsynaptic nuclei favours chromatin hyperacetylation and decondensation. Conversely, in extrasynaptic nuclei, GABP recruits the histone deacetylase HDCA1 on synaptic gene promoters, thereby promoting chromatin compaction.

require. Alternatively, we have tried to perform ChIP experiments on muscles injected with neural agrin, but the amount of nuclei activated by agrin in injected muscles did not allow a sufficient enrichment to see the acetylation of the *AChR* ε promoter raise above background. To visualize histone acetylation by ChIP on the *AChR* ε promoter, we therefore had to use cultured myotubes. The absence of the effect of agrin on endogenous *AChR* ε expression in C2C12 myotubes prevented the possibility to directly demonstrate that agrin induced histone acetylation on the *AChR* ε promoter in cultured myotubes. However, the results obtained with neuregulin can most likely be extrapolated to agrin, as in myotubes, agrin and MuSK activate AChR expression via neuregulin receptors (Meier *et al*, 1998).

In cultured myotubes treated with neuregulin, immediate-early genes are transiently activated before synaptic genes. This transient activation seems to be important for the later activation of synaptic genes, as blocking the action of cJun with a dominant-negative mutant resulted in the inhibition of *AChR* ε subunit activation (Si *et al*, 1999). These observations were made using neuregulin, but could be relevant for agrin and MuSK as they also activate JNK (Lacazette *et al*, 2003) and therefore regulate cJun. Our results show that activation of immediate-early genes by neuregulin correlates with histone H3 hyperphosphoacetylation on the *cJun* promoter. EGF and neuregulin bind to receptors of the erbB family, respectively erbB1 and erbB2, 3 and/or 4, which trigger similar intracellular signalling pathways. This is consistent with the results of the groups of Mahadevan, Allis and Sassone Corsi, who demonstrated that activation of *cJun* expression by EGF was associated with histone H3 phosphoacetylation. These works demonstrated that the phosphorylation of histone H3S10 favoured the acetylation of the neighbouring lysine residues (Cheung *et al*, 2000; Clayton *et al*, 2000; Lo *et al*,

2000). It is therefore predictable that H3S10 phosphorylation upon neuregulin treatment and at the NMJ synergizes with H3 acetylation.

Our observation that H3 phosphoacetylation also affects the *AChR* ε subunit gene 4 h after neuregulin treatment demonstrates that H3 phosphoacetylation is not restricted to the activation of immediate-early genes and can affect other genes. This is corroborated by the immunofluorescence observations at the NMJ, which show that H3 is stably phosphoacetylated at many loci. In future experiments, it will be interesting to determine if the phosphorylation of histone H3 on *AChR* genes is triggered by the same kinases that phosphorylate histone H3 on immediate-early genes (Sassone-Corsi *et al*, 1999; Thomson *et al*, 1999), or if specific kinases are recruited at different genes or in different situations of transcriptional activation.

ErbB receptors are known proto-oncogenes in breast cancer (Linggi and Carpenter, 2006). The finding that erbB signalling induces histone phosphorylation and acetylation on its target genes could also be relevant in this context.

Neuregulin in vitro, agrin in vivo?

Recent publications suggest that although neuregulin and its receptors are critical for the activation of *AChR* expression in cultured myotubes, they only play a marginal role in the activation of synaptic genes *in vivo* that would exclusively rely on a neuregulin-independent pathway triggered by MuSK (Escher *et al*, 2005; Jaworski and Burden, 2006). This conclusion was drawn from the observation that in erbB2/erbB4−/− muscle (Escher *et al*, 2005), as well as in mice lacking neuregulin in motoneurons and muscle (Jaworski and Burden, 2006), NMJ can develop normally. However, these approaches still leave open the possibility that in the normal situation, neuregulin signalling could participate in

AChR activation. Therefore, the hypothesis initially raised by Brenner and co-workers (Lacazette *et al*, 2003), who proposed that agrin would be crucial for NMJ formation and neuregulins would rather participate in its maintenance, could still be valid if one considers the possibility that in the absence of neuregulin signalling, the muscle could adapt to rely solely on the neuregulin-independent pathway triggered by MuSK.

Consistent with a role of neuregulin after agrin action, neuregulin and its receptor accumulate at agrin-induced ectopic synapses (Jones *et al*, 1999). In addition, neuregulins can activate synaptic genes expression *in vivo*, as shown on utrophin in 1999 (Gramolini *et al*, 1999).

Subynaptic chromatin is profoundly remodelled

Neuregulin-induced histone hyperacetylation was detectable in Western blot, suggesting that it affected many loci. The vast number of genes that have to be turned on to generate the postsynaptic scaffold could explain the large distribution of chromatin acetylation in subsynaptic nuclei. Consistently, the ChIP experiments performed with anti-acetylated histone H3 antibodies demonstrated that acetylation occurs on genes activated by neural factors.

The finely punctuated immunolabelling of acetylated and phosphoacetylated histones was distributed all over the nucleoplasm of subsynaptic nuclei, and was reminiscent of the electron micrographs that revealed a global decompaction of chromatin. The hyperacetylation of the muscle genome and synaptic genes activation in subsynaptic nuclei is therefore associated with a broad remodelling of the genome. In addition to chromatin decompaction, such a remodelling likely involves the specification of particular distributions of specific genes to the nucleoplasm.

The ultrastructure of muscle subsynaptic nuclei is quite similar to that of central neurons. In central neurons, chromatin acetylation has also been shown to play a crucial role in translating environmental signals into a physiological response. This is, for example, the case for the regulation of *clock* genes expression by the circadian rhythm, in which the transcriptional regulator CLOCK has recently been shown to be a HAT (Doi *et al*, 2006). Histone acetylation has also been shown to be involved in stable modifications of neuronal properties such as long-term potentiation and long-term depression, which involve histone hyper- or hypoacetylation on specific genes involved in these processes via the p300 closely related HAT CBP and HDAC5 (Guan *et al*, 2002).

Materials and methods

Electron microscopy
Tissue samples were fixed in 2% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature, post-fixed in buffered osmium tetroxide and embedded in epoxy resin after ethanol dehydration.

Ultrathin sections were collected on formvar-coated copper grids, contrasted in uranyl acetate and lead citrate and viewed by transmission electron microscopy (Philips CM 120) at the Centre Technologique des Microstructures, Claude Bernard University, Lyon, France.

Constructs and antibodies
The constructs used were pcDNA3-HD1 for human HDAC1 with the Flag epitope (Yang *et al*, 1997), pCI-Flag-p300 (Ogryzko *et al*, 1996), ΔEB GABPα (Schaeffer *et al*, 1998) and the epsilon-luciferase reporter vector (Duclert *et al*, 1993).

The antibodies used were GABPα (Schaeffer *et al*, 1998), Flag M2 and β-tubulin (Sigma), AcH3(K9K14), PAcH3 (K14S10), PH3 (S10), HDAC1 (Upstate Biotechnology), and p300 (sc-584) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell lines, culture conditions and transfections
C2C12 cells were maintained as myoblasts in growth medium (1/2 DMEM and 1/2 HAM F12, Gibco BRL, supplemented with 13% foetal calf serum, HyClone Perbio). Cells were differentiated in differentiation medium (DMEM medium supplemented with 2% horse serum, Bio Media Canada).

The C2C12 myotubes were treated 48 h after being switched to differenciating medium, with neuregulin (recombinant neuregulin-1, EGF domain, Upstate) to induce *AChR* gene expression.

Cell transfections were performed with Lipofectamine Plus (Life Technologies, Gibco BRL) according to the manufacturer's instructions.

In vivo electroporation
Operative procedure was performed using aseptic techniques and according to the local ethical committee recommendations (Comité Rhône Alpes d'Ethique pour l'Expérimentation Animale). Five-week-old OF1 male mice were anaesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) to obtain a deep state of general anesthesia. A 30 μl volume of 0.9% NaCl containing 5 μg of DNA, Lys and CoA or LysCoA was injected into *Tibialis anterior* muscles. Injected muscles were then electroporated with 1 cm^2 plaque electrodes placed on each side of the leg and eight 200 V cm^{-1} pulses of 20 ms applied at 2 Hz (BTX ECM 830).

Ectopic synapse induction
To induce ectopic synapses, 2 μg of purified agrin N25C95-A4B8-His (Scotton *et al*, 2006) was injected into mouse soleus muscles. Two weeks after injection, muscle fibres were isolated under a microscope (SZX 12, Olympus) using ultrafine forceps (Moria, No. 5).

Immunofluorescence
Immunofluorescence analyses were performed as follows. C2C12 cells or isolated muscle fibres were fixed for 5 and 20 min respectively in 3.7% formalin TBS (Tris-buffered saline: 150 mM NaCl in 50 mM Tris, pH 7.4) before permeabilization for 5 or 30 min respectively with 1% Triton in TBS. Cells were incubated with the primary antibodies diluted with 1% BSA in TBS overnight at 4°C, then washed in TBS, and incubated for 1 h with biotinylated anti-rabbit antibodies (Amersham). Biotinylated antibodies were revealed after a 1 h incubation with streptavidin–Texas red (Amersham). The NMJ was stained with α-Bungarotoxin–Alexa488 (Molecular Probes). Nuclei were stained with Hoechst reagent 33258 (1 μg/ml bisbenzimidine, Sigma). Fluorescent images were visualized by microscopy on a Zeiss Axioplan 2 microscope. Images were captured with a Photometrics CoolSNAP fx camera and processed with Photoshop 7 (Adobe Systems).

Co-immunoprecipitation and Western blotting
Whole-cell extracts were prepared from C2C12 cells in NP-40 buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40 and Complete protease inhibitors (Roche)), and precleared with protein-A sepharose (Pharmacia Biotech). 10% of extracts were kept as input sample. Then extracts were incubated with specific antibodies overnight at 4°C, followed by incubation with protein-A sepharose at 4°C for 1 h and were washed three times. The immunoprecipitated material was boiled in 2× loading buffer, electrophoresed by SDS–PAGE and immunodetected.

Chromatin immunoprecipitation
Neuregulin-treated and untreated C2C12 myotubes were fixed in 3.7% formaldehyde in culture medium for 10 min at 37°C. Chromatin was prepared using a kit from Upstate Biotechnology according to the recommendations of the manufacturer, with ten 10-s sonication pulses, which yielded chromatin fragments of an apparent size of 800 bp (as monitored on agarose gels; data not shown). Equivalent amounts of chromatin were immunoprecipitated using specific antibodies. Formaldehyde-induced crosslinking was reversed (4 h at 65°C), and a sequence of the *AChR* ε promoter, *cJun* promoter or *H4* promoter (as an internal control) was detected

by quantitative PCR. The results of the *cJun* and AChR ε promoters quantification were normalized to the levels of histone H4 promoter. The primers used were as follows: for AChR ε (amplified region −161 to +68, 5′-GATGACAGGCCTTGTGGATT-3′ forward and 5′-GACAAGCTTGAGGGAACAGG-3′ reverse); for c-jun (5′-TACT CTCAAGCCCGCTCAAC-3′ forward and 5′-CCGAGAAAGGGCTGAAT GAT-3′ reverse); for *H4* (amplified region −161 to +69, 5′-GA CACCGCATGCAAAGAATAGCTG-3′ forward and 5′-CTTTCCCAAGG CCTTTACCACC-3′ reverse).

RNA preparation, reverse transcription and real-time quantitative PCR

Total mRNA was extracted from homogenized (FastPrep, Bio 101, in RLT buffer, Qiagen) whole *Tibialis anterior* muscles using the RNeasy mini RNA extraction kit (Qiagen) with the additional proteinase K and DNase treatments. First strand cDNA was synthesized from 250 ng of total RNA using the Superscript II (Invitrogen). Gene expression was evaluated by real-time quantitative PCR (qPCR) (LightCycler, Roche) using the LightCycler FastStart DNA Master SYBR Green PCR kit (Roche) according to the manufacturer's instructions. The sequences of the primers were as follows: β-actin forward 5′-CCCTGTATGCCTCTGGTCGT-3′, reverse 5′-ATGGCGTGAGGGAGAGCAT-3′; AChR ε forward 5′-CTTGG TGCTGCTCGCTTACTT-3′, reverse 5′-CGTTGATAGAGACCGTGCATT-3′; ErbB3 forward 5′-CTTACGGGACACAATGCTGA-3′, reverse 5′-GCAT GGCTGGAGTTGGTATT-3′. The measures were normalized to β-actin RNA levels or ErbB3.

Luciferase reporter assay

2 μg of epsilon-luciferase reporter vector and 3 μg of pcDNA3 carrier DNA were co-electroporated in *Tibialis anterior* muscle with increasing dose of LysCoA (produced as described by Lau et al, 2000). 100 μl of muscle homogenates (FastPrep, Bio 101, in passive lysis buffer, Promega) was mixed in an equal volume of luciferase substrate solution (0.8 M tricine pH 7.8, 1.5 M MgCO$_3$, 273 mM MgSO$_4$, 27 mM Coenzyme A, 0.5 M ATP, 10 mM luciferin and 0.5 M EDTA) and placed in a Microplate Luminometer (Veritas Turner BioSystem) to measure light production for 10 s.

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