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Changes in mRNA Expression of Nicotinic Acetylcholine Receptor Subunits during Embryonic Development of Mouse Masseter Muscle

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ABSTRACT—Nicotinic acetylcholine receptors (nAChRs) switch from the embryonic-type ($\alpha_2\beta\gamma\delta$ subunits) to the adult-type ($\alpha_2\beta\epsilon\delta$ subunits), and disappear besides the neuromuscular junctions with the development of trunk and limb skeletal muscles. However, little is known about this process during the embryonic development of masseter muscle. To identify the time course of the nAChR transition from embryonic day (E) 11 to the newborn stage in mouse masseter muscle, we analyzed the expression level of δ , ϵ , and γ subunit mRNAs by competitive polymerase chain reaction in combination with reverse transcription as well as distribution of δ subunit protein by immunohistochemistry. The nAChR δ subunit mRNA was initially detected at E11, showed an approximately 25-fold increase ($p < 0.0001$) between E11 and E17, and plateaued thereafter until the newborn stage. Immunostaining for δ subunit was observed in the whole portions of masseter myofibers at E17 and birth, suggesting that the nAChR elimination does not begin even at the newborn stage. The ϵ subunit mRNA initially appeared at E17, and increased in quantity by 144% ($p < 0.0001$) up to the newborn stage. The quantity of γ subunit mRNA increased by approximately 240% ($p < 0.0001$) between E11 and E17, and then decreased by 22% ($p < 0.05$) from E17 value at the newborn stage. The beginning of the expression of the ϵ subunit mRNA was coincident with the beginning of the decrease in the quantity of the γ subunit mRNA, suggesting that the nAChR subunit switch begins at E17.

Key words: synaptogenesis, nicotinic acetylcholine receptor, masseter muscle, competitive RT-PCR, mouse

INTRODUCTION

The expression level, distribution, and subunit composition of the nicotinic acetylcholine receptor (nAChR) are known to change during the development of skeletal muscle (Brehm and Henderson, 1988; Hall and Sanes, 1993). The embryonic-type nAChR, composed of $\alpha_2\beta\gamma\delta$ subunits, is expressed throughout muscle cells. As the development of skeletal muscle progresses, the γ subunit is replaced by an ϵ subunit to become the adult-type nAChR ($\alpha_2\beta\epsilon\delta$). Outside the neuromuscular junctions, both the adult- and embryonic-types are eliminated, while only the adult-type continues to be expressed at the neuromuscular junctions.

It has been reported that expression of the nAChR subunit genes is controlled by several regulatory elements

including the E box to which the myoD family can bind (Duclet and Changeux, 1995). It has been shown that innervation, denervation, and subsequent electrical stimulation of the denervated muscle induce drastic changes in the expression of the myoD family, which is followed by changes in the expression of the nAChR subunits (Buonanno *et al.*, 1998). These results suggest a close correlation between the myoD family and nAChR subunit expressions. We previously reported that the peak of myoD family expression in masseter muscle appears later than in other skeletal muscle such as the hind limb and tongue muscles (Yamane *et al.*, 2000a), suggesting that the nAChR subunit switch and elimination occur later in masseter muscle than in other types of skeletal muscles.

Masseter muscle has several unique developmental characteristics in comparison with other skeletal muscles such as trunk and limb muscles. Myoblasts of masseter muscle are derived from nonsomitic paraxial head meso-

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derm (Noden *et al.*, 1999), whereas trunk and limb myoblasts originate from somitic mesoderm (Wachtler and Christ, 1992). Pax3/myf5 homozygous mutant mice fail to induce myoD in trunk and limb muscles and therefore do not generate trunk and limb muscles, whereas they generate masseter muscle as well as other craniofacial muscles (Tajbakhsh *et al.*, 1997). Adult human and murine masseter muscles continue to express the neonatal isoforms of myosin heavy chain (D'Albis *et al.*, 1986; Butler-Browne *et al.*, 1988; Soussi-Yanicostas *et al.*, 1990).

Despite these unique developmental characteristics, the synaptogenesis, including the nAChR elimination and switch, has not been examined during the embryonic development of mouse masseter muscle. We analyzed the mRNA expression and immunolocalization of δ subunit, which is present throughout development, to determine the time course of the nAChR elimination. We also determined the expression levels of ϵ and γ subunit mRNAs to identify the time course of the nAChR subunit switch in mouse masseter muscle from embryonic day (E) 11 to the newborn stage. We compared obtained results with previous reports of limb, trunk and tongue muscles (Missias *et al.*, 1996; Zoubine *et al.*, 1996; Yamane *et al.*, 2001).

MATERIALS AND METHODS

Tissues

Pregnant ICR mice were purchased (Nippon Clea, Tokyo, Japan) and tissues in the middle portion between the otic invagination (ear) and the mouth under the eye of the embryo were removed at embryonic days (E) 11 and E13. Masseter muscle tissue was collected from embryos at E15 and E17, and at birth after the epithelial tissue was carefully removed. The tissues were immediately frozen and stored at -80°C until use. Six samples were collected at each developmental stage, although four preparations at E11 and two at E13 were pooled as one sample due to the small amount of RNA available. The tissues obtained at E15, E17 and newborn mice for immunohistochemistry were immediately fixed in Bouin's solution. All experimental protocols concerning animal handling were reviewed and approved by the Institutional Animal Care Committee of the Tsurumi University School of Dental Medicine.

RNA extraction, reverse transcription, and competitive-polymerase chain reaction (competitive PCR) amplification

Total RNA extraction, reverse transcription, and competitive-PCR amplification were performed as previously described (Yamane *et al.*, 2000b, c). Briefly, total RNA extraction was performed according to the manufacturer's specifications (Rapid total RNA isolation kit, 5 Prime \rightarrow 3 Prime Inc., Boulder, CO, USA). The RNA was treated with 2 units of ribonuclease-free deoxyribonuclease I (Life Technologies, Gaithersburg, MD, USA), and was then reverse transcribed with 200 units of reverse transcriptase (SuperScript II, Life Technologies, Gaithersburg, MD, USA).

In conventional PCR technique, a small difference in the starting amount of target DNA can result in a large change in the yield of the final product because of the exponential nature of the PCR reaction. The plateau effect after many cycles can lead to an inaccurate estimation of final product yield. Furthermore, since the PCR amplification depends on the reaction efficiency, small changes in the efficiency can lead to major differences in the final product yield. To overcome these problems, the competitor (internal standard), which has the same primer sequences with the those of target DNA

Table 1. Sequences of nAChR subunit-specific PCR primers, annealing temperature (temp.), number of cycles, concentration of competitors, and product sizes of target and competitor

nAChR δ subunit	
Forward	5'-CAG CCG TCT ACA GTG GGA TG-3'
Reverse	5'-CTG CCA GTC GAA AGG GAA GTA-3**
Annealing temp. and no. of cycles	35 cycles at 55°C
Concentration of competitor	1×10^{-2} attomoles/ μl
Target product size	235 bp
Competitor product size	291 bp
nAChR ϵ subunit	
Forward	5'-ATT GAA GAG CTT AGC CTG TA-3'
Reverse	5'-TAC ACC TGC AAA ATC GTC CT-3***
Annealing temp. and no. of cycles	35 cycles at 46°C
Concentration of competitor	1×10^{-2} attomoles/ μl
Target product size	222 bp
Competitor product size	340 bp
nAChR γ subunit	
Forward	5'-GAT GCA ATG GTG CGA CTA TCG C-3'
Reverse	5'-GCC TCC GGG TCA ATG AAG ATC C-3**
Annealing temp. and no. of cycles	35 cycles at 55°C
Concentration of competitor	1×10^{-2} attomoles/ μl
Target product size	360 bp
Competitor product size	244 bp

* LaPolla *et al.*, 1984

** Buonanno *et al.*, 1989

Rohwedel *et al.*, 1995

bp; base pairs

at 3' and 5' ends, was amplified simultaneously with the target (Gilliland *et al.*, 1990; Siebert and Larrick, 1992; Yamane *et al.*, 1998, 2000c). The competitors constructed according to the manufacturer's instructions of PCR MIMIC Construction Kit (Clontech Laboratory Inc., Palo Alto, CA, USA) were amplified with 50 ng of the total cDNA in the presence of primer pair specific to target genes in a thermal cycler (TP3000, TaKaRa Biochemicals, Shiga, Japan). Table 1 shows the primer sequences and competitive PCR conditions for the δ , ϵ , and γ subunit mRNAs. The amplification products were separated by electrophoresis on an agarose gel containing ethidium bromide. The fluorescent intensities of the bands of the target cDNAs and their respective competitors were measured by an image analyzer (Argus-100, Hamamatsu Photonics K.K., Hamamatsu, Japan). We then calculated the ratios of the fluorescent intensities of the target cDNA bands to those of their respective competitors. The logarithmic value of the fluorescent intensity ratio was used to calculate the amount of endogenous target mRNA based on the line formula derived from a standard curve for each target subunit. The standard curve was generated as described previously (Yamane *et al.*, 2000a, b, c). The quantity of each target mRNA was normalized by the quantity of glyceraldehyde-phosphate dehydrogenase (GAPDH). The resulting ratio value was expressed as a percent value relative to the mean value of each target subunit at the newborn stage.

Immunohistochemistry

Specimens for immunohistochemistry were fixed in Bouin's fixative for two hours at 4°C , immersed in a graduated series of sucrose solutions (5–40% w/v) in phosphate buffered saline (PBS) at 4°C , embedded in Tissue-Tek Oct Compound (Miles Laboratory, Elkhart, IN, USA) and frozen. Sagittal sections of tongues were prepared at a $10 \mu\text{m}$ thickness in a cryostat and air-dried for 1 hr at room temperature. Immunolocalization was analyzed by using Vec-

tor M.O.M. Immunodetection Kit (Vector Laboratories, Inc., Burlingame, CA, USA). Briefly, the frozen sections were post-fixed in acetone at -20°C and incubated with $0.3\%\text{H}_2\text{O}_2$ in methanol to quench endogenous peroxidase for 30 min. They were incubated with M.O.M. Mouse IgG Blocking Reagent for 1 hr to block non-specific immunostaining and incubated with mouse monoclonal antibody against the δ subunit (Affinity Bioreagents, Inc., Golden, CO, USA) for 30 min. They were treated with biotinylated anti-mouse IgG for 10 min. The site of immunoreaction was made visible by incubating the sections with horseradish peroxidase-conjugated streptavidin for 5 min and then with 3-amino-9-ethylcarbazole (AEC) and hydrogen peroxide for 30 min. For control staining, the primary antibody was replaced with normal mouse IgG or PBS.

Statistical Analyses

Scheffe's method was used to compare the mean values between two groups.

RESULTS

Standard curves

Fig. 1 shows the electrophoretic gel pattern (A) of nAChR ϵ subunit cDNA and its competitor after competitive PCR, and the obtained standard curve (B). As the ϵ subunit cDNA concentration increased, the intensity of ϵ subunit bands became progressively strong while that of the competitor bands became weak, showing an inverse relationship

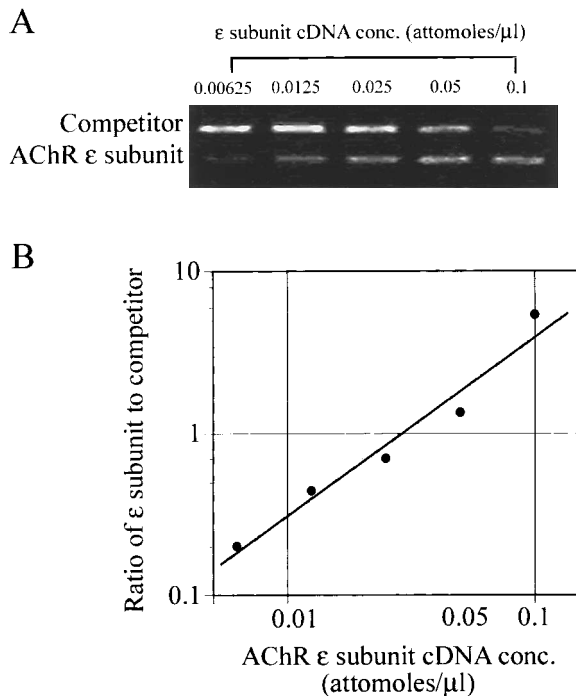


Fig. 1. (A) Electrophoretic gel pattern of the nAChR ϵ subunit and its competitor after competitive PCR for examining the relationship between the amount of PCR products and the concentration of ϵ subunit cDNA. (B) The regression line for the ϵ subunit generated from the result of image analysis of electrophoretic bands in (A). The formula of the regression line is represented by $y=1.110x+1.708$, where y is the logarithmic value of the ratio of the fluorescent intensity in the ϵ subunit band to that in its competitor band and x is the logarithmic value of the concentration of the ϵ subunit cDNA.

Table 2. Formulae for the regression lines and correlation coefficients for the nAChR δ , ϵ , and γ subunits

Target gene	Formula	r	significance
nAChR δ subunit	$y=0.486x + 0.875$	0.999	$p<0.001$
nAChR ϵ subunit	$y=1.110x + 1.708$	0.979	$p<0.001$
nAChR γ subunit	$y=1.004x + 2.221$	0.994	$p<0.001$

y , the logarithmic value of the ratio of the fluorescent intensity in the target gene band to that in its respective competitor band.
 x , the logarithmic value of the concentration of the cDNA standard.
 r , correlation coefficient.

between the intensities of the ϵ subunit and the competitor bands. The formula for the regression line is represented by $y=1.110x+1.708$.

Table 2 contains the formulae for the regression lines and correlation coefficients for the nAChR δ , ϵ , and γ subunits. The correlation coefficients were greater than 0.970 for all the subunits and were statistically significant from zero ($p<0.001$). This result indicates that the quantities of the target cDNAs can be reliably determined from these formulae.

Expression of δ subunit mRNA and immunolocalization of δ subunit protein

The nAChR α , β , and δ subunits are present throughout the entire process of synaptogenesis, but there are sub-

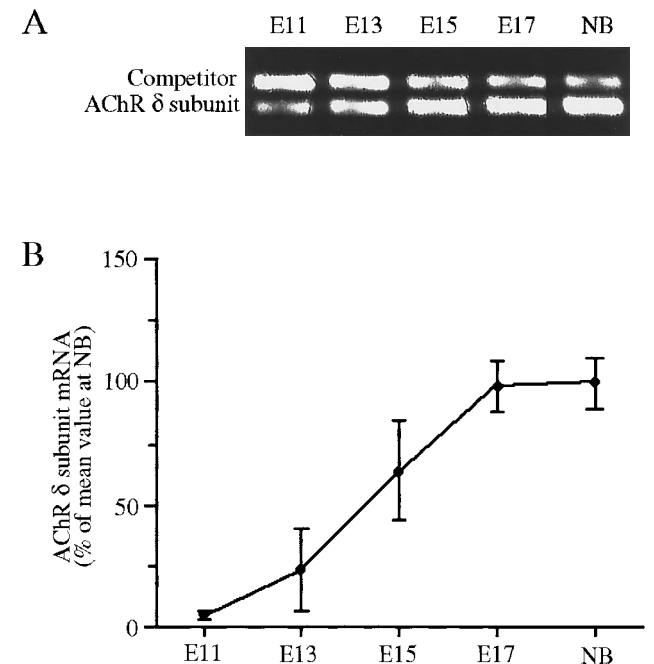


Fig. 2. (A) Representative electrophoretic gel patterns of the PCR products of the nAChR δ subunit and its competitor after competitive RT-PCR amplification. NB: newborn. (B) Relative changes in the mRNA quantity of the nAChR δ subunit in the mouse masseter muscle at E11, 13, 15, 17, and the newborn stage (NB). Each point and its vertical bar represent the mean \pm 1 SD of six samples. The vertical axis is expressed as a percentage of the mean value at the newborn stage.

types of the α and β subunits that express in neuronal and epithelial tissues (Nguyen *et al.*, 2000; Lindstrom, 2000). Thus, to determine the time course of nAChR elimination in mouse masseter muscle, we analyzed the expression level of δ subunit cDNA. Fig. 2A shows an example of the electrophoretic gel pattern of competitive PCR products for the AChR δ subunit in masseter muscle at E11, 13, 15, 17, and the newborn stage. The lower bands (235 bp) correspond to the amplified δ subunit cDNA and the upper bands (291 bp) to the amplified competitor. From the ratio of the fluorescent intensity of the target gene band to that of its respective

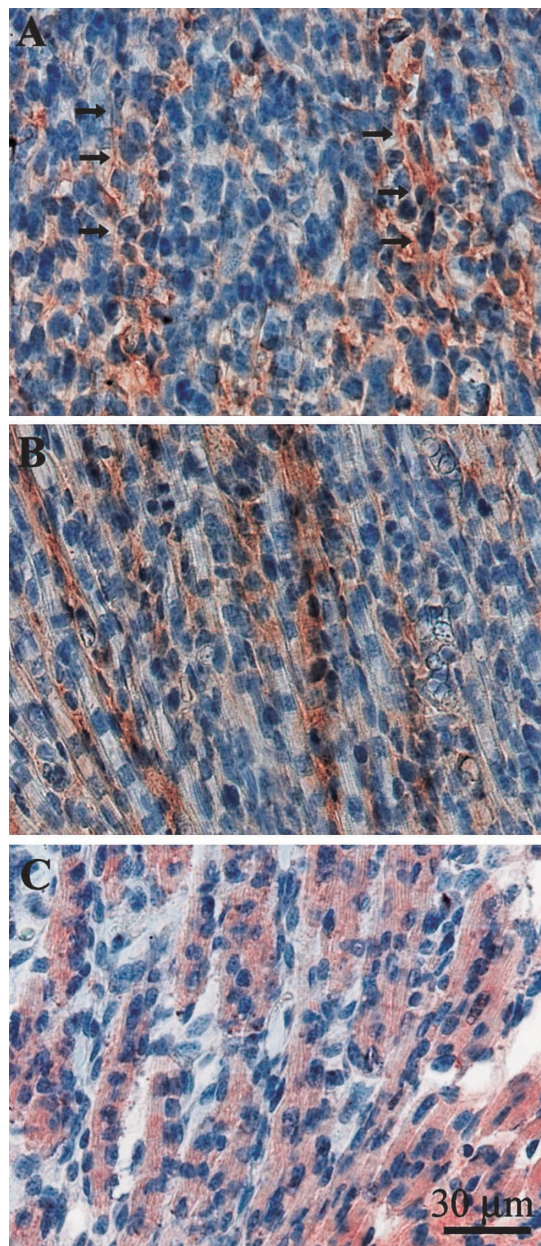


Fig. 3. Immunolocalization of δ subunit in the mouse masseter muscle obtained from E15 (A), E17 (B) and newborn mice (C). Arrows (A) indicate myotubes immunostained for δ subunit. A and B at the same magnification as C.

competitor band in each sample, we determined the concentration of δ subunit cDNA by using the standard curve. The concentration was then normalized by the concentration of GAPDH as described in MATERIALS AND METHODS. Fig. 2B shows the mean ± 1 SD of the normalized values for six samples at each developmental stage. The nAChR δ subunit mRNA in the masseter muscle was detected at E11, and then showed an approximately 25-fold increase ($p < 0.0001$) in the content between E11 and E17. This increase suggests that the number of nAChRs increases with the development of masseter muscle. The content did not change significantly between E17 and the newborn stage, suggesting that nAChR elimination does not begin before birth.

To verify if the nAChR elimination does not begin before birth, we analyzed immunolocalization of δ subunit protein in the mouse masseter muscle at E15 (Fig. 3A), E17 (Fig. 3B) and newborn stage (Fig. 3C). We observed the entire portions of masseter muscle. Here, we show the photographs of the areas where well differentiated myotubes and well

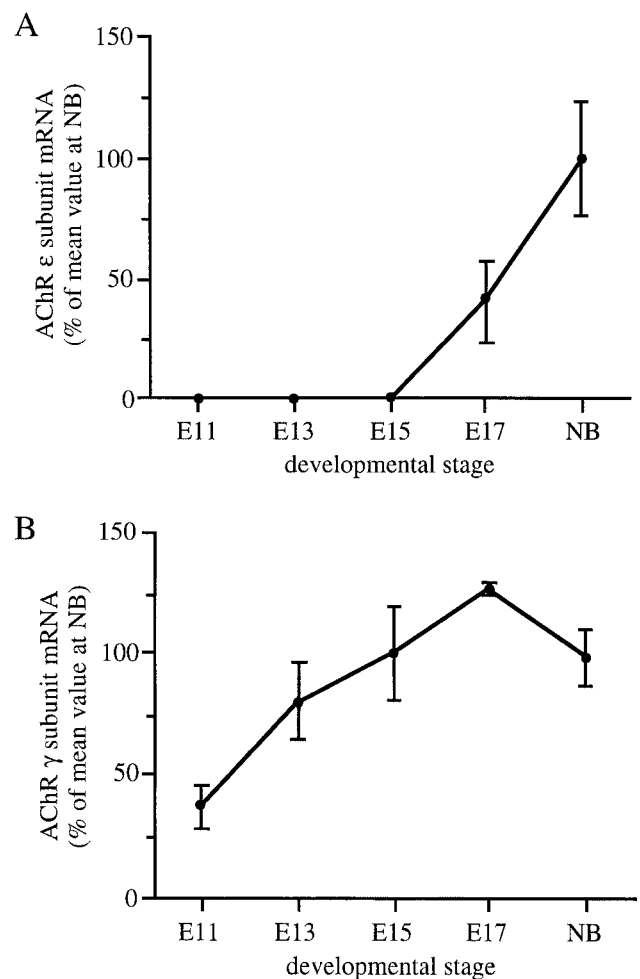
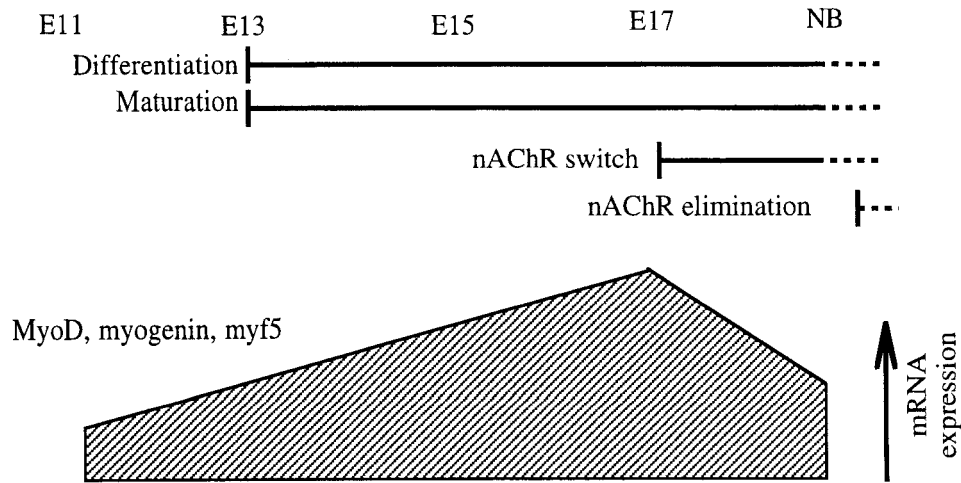
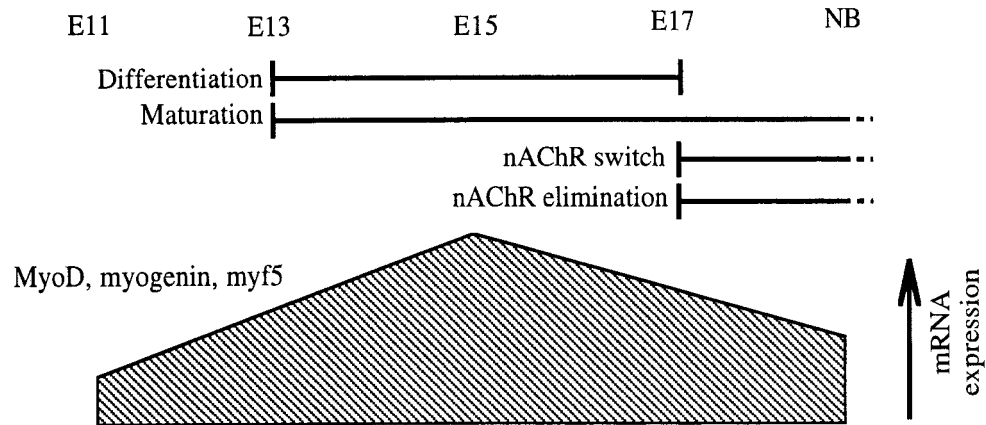


Fig. 4. Relative changes in the mRNA quantity of the nAChR ϵ (A) and γ (B) subunits in the mouse masseter muscle at E11, 13, 15, 17, and the newborn stage (NB).

Mouse masseter muscle



Mouse hind limb muscle



Mouse tongue muscle

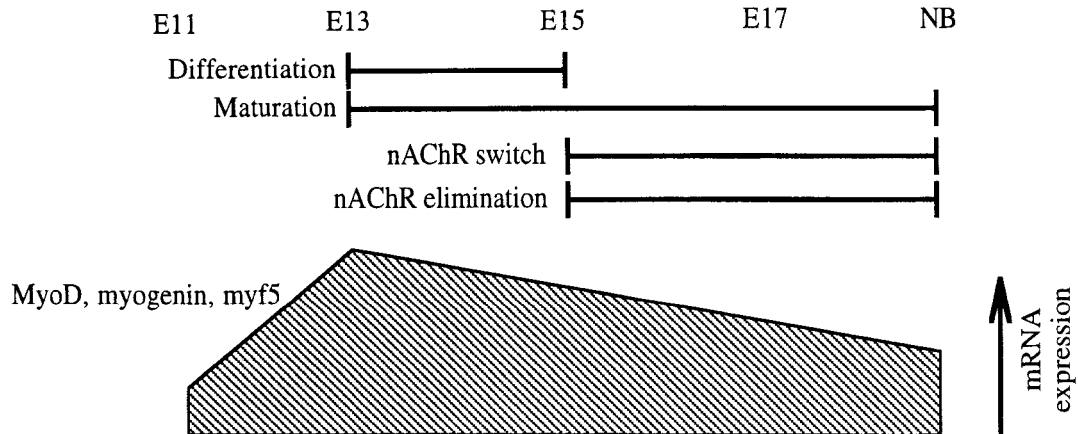


Fig. 5. The synaptogenesis, myogenesis, and expression of the myoD family mRNA in mouse masseter (upper), hind limb (middle) and tongue (lower) muscles. Data for the tongue and hind limb muscles, and for the myogenesis and expression of the myoD family mRNA in masseter muscle are from our previous studies (Yamane *et al.*, 2000a, c, 2001).

matured myofibers were observed. At E15, immunostaining for δ subunit was found in multi-nucleated myotubes (arrows in Fig. 3A). At E17, the immunostaining was distributed in the whole portion of immature myofibers. At birth, myofibers in the masseter muscle appeared to become thick, but still appeared to be immature (Fig. 3C). The immunostaining for δ subunit was observed in the whole portion of myofibers. The immunolocalization of δ subunit at E17 and birth seems to indicate that nAChR elimination does not begin before birth, in concordance with the PCR results.

Expression of ϵ and γ subunit mRNAs

We determined the expression level of ϵ (Fig. 4A) and γ (Fig. 4B) subunit mRNAs to determine the time course of the nAChR subunit switch. The ϵ subunit mRNA was initially detected in the masseter muscle at E17, and increased by 144% ($p < 0.0001$) up to the newborn stage. The γ subunit mRNA was initially detected at E11, and increased by approximately 240% ($p < 0.0001$) between E11 and E17. After that, it decreased by 22% ($p < 0.05$) from E17 value at the newborn stage. The beginning of the expression of ϵ subunit mRNA was coincident with the beginning of the decrease in the quantity of γ subunit mRNA, suggesting that the nAChR subunit switch begins at E17 in masseter muscle.

DISCUSSION

In the present study, we determined the time course of the nAChR elimination and subunit switch during the development of mouse masseter muscle. We compared the results with those of tongue, limb and trunk muscles previously reported (Missias *et al.*, 1996; Zoubine *et al.*, 1996; Yamane *et al.*, 2001). Furthermore, we related our present findings to the expression profiles of the myoD family and the myogenesis reported previously, as schematically shown in Fig. 5 (Yamane *et al.*, 2000a, c, 2001).

The expression profiles of the δ mRNAs and immunolocalization for δ subunit in the present study suggest that the nAChR elimination does not begin before birth in the mouse masseter muscle. Saito *et al.* (submitted for publication) already observed that in rat masseter muscle the δ subunit mRNA showed a marked decrease between 7 and 28 days of age after birth, implying that the nAChR elimination in murine masseter muscle primarily occurs between 7 and 28 days of age. It was already reported that the nAChR elimination occurs between E15 and newborn stage in mouse tongue muscle (Yamane *et al.*, 2001) and that it begins just before birth and ends at 18 days of age after birth in mouse hind limb muscle (Zoubine *et al.*, 1996; Yamane *et al.*, 2001). Thus the nAChR elimination seems to occur later in murine masseter muscle than in murine tongue and hind-limb muscles.

The beginnings of the decrease in γ subunit mRNA and of the expression of ϵ subunit mRNA suggest that the nAChR subunit switch from embryonic- to adult types begins at E17 in mouse masseter muscle. Saito *et al.* (submitted for

publication) already observed that in rat masseter muscle the quantity of γ subunit mRNA decreased markedly in association with an increase in the amount of ϵ subunit mRNA between 1 and 21 days of age after birth. Taken together, these results suggest that the nAChR subunit switch in murine masseter muscle begins just before birth and ends at 21 days of age. We previously reported that the nAChR subunit switch mainly occurs between E15 and newborn stage in mouse tongue muscle (Yamane *et al.*, 2001). This strongly suggests that the nAChR subunit switch seems to occur later in masseter muscle than in the tongue muscle. Additionally, the nAChR subunits are reported to switch between 1 and 9 days of age in mouse tibialis anterior, extensor digitorum longus, sternomastoid, and diaphragm muscles (Missias *et al.*, 1996). Thus, the nAChR subunit switch in masseter muscle seems to begin at an approximately same stage (perinatal stage) as, but end at a later stage than that in these trunk and limb muscles.

We previously observed that the quantities of myoD, myogenin, and myf5 mRNAs peak later in mouse masseter muscle (E17) than in mouse tongue (E13) and hind limb muscles (E15), as schematically shown in Fig. 5 (Yamane *et al.*, 2000a, c). It was previously reported that the myoD family regulates the transcription of the δ , ϵ , and γ subunit genes by binding to E boxes in the regulatory region of the genes (Duclert and Changeux, 1995). Thus, the relatively later expression of myoD, myogenin, and myf5 in mouse masseter muscle seems to be related to the later nAChR elimination and subunit switch in comparison with tongue and hind limb muscles.

In the present study, we observed that synaptogenesis in the mouse masseter muscle was still in an immature state at birth. We previously reported that myogenesis in the mouse masseter muscle does not finish at birth, as schematically shown in Fig. 5 (Yamane *et al.*, 2000a). The masseter muscle mainly functions in the biting movement of the jaw and mouse feeding behavior switches from suckling to biting between 2 and 3 weeks after birth (Maeda *et al.*, 1981; Kubota *et al.*, 1988). Thus, we assume that both synaptogenesis and myogenesis in mouse masseter muscle may be genetically programmed to finish by the time when the biting movement begins after birth.

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