Part 2

nAChR Subunit Elimination and Switch in Tongue Striated Muscles

Results

Expression of AChR α, ε and γ subunit mRNAs

Since α subunit is present throughout the whole process of synaptogenesis, the content of α subunit mRNA seems to reflect the number of nAChR. Thus, to identify the time course of nAChR elimination, I analyzed the expression level of α subunit mRNA (Figures 10 A and B). Figure 10A shows an example of the electrophoretic gel pattern of the AChR α subunit competitive PCR products in the tongue muscle. The lower bands (245 bp) correspond to the amplified α subunit mRNA and the upper bands (348 bp) to the amplified competitor.

AChR α subunit mRNA in the tongue and hind limb muscles was detected at E11, and began to increase thereafter (Figure 10B). In the tongue muscle, the quantity of α subunit mRNA increased by 126% ($p<0.01$) between E11 and E13, and plateaued between E13 and E15. After E15, the quantity decreased and became less than 50% of the E15 value ($p<0.001$) at the newborn stage. In the hind limb muscle, the quantity of α subunit mRNA increased by 178% ($p<0.0001$) up to E15, and plateaued between E15 and E17. The quantity began to decrease at E17, but more than 60% of the E17 value ($p<0.05$) was still expressed at the newborn stage. The beginning of the decrease in the content of α subunit mRNA (Figure 10B) suggests that the nAChR elimination begins at E15 in the tongue muscle and at E17 in the hind limb muscle.

I determined the expression level of ε (Figure 10C) and γ (Figure 10D) subunit mRNAs to identify the time course of the nAChR subunit switch. The ε

subunit mRNA initially appeared at E15 in the tongue and at E17 in the hind limb. At E17, the expression levels in both the muscles were almost identical and then increased (approximately 40%, $p<0.0001$) by birth (Figure 10C).

In both muscle types, γ subunit mRNA was initially detected at E11, and increased in quantity thereafter (Figure 10D). In the tongue muscle, the quantity increased by 114% ($p<0.001$) up to E13, and plateaued between E13 and E15. After E15, the quantity began to decrease and became less than 30% of the E15 value ($p<0.0001$) at birth. In the hind limb muscle, the quantity of γ subunit mRNA increased by 274% ($p<0.0001$) between E11 to E15, and plateaued between E15 and E17. After E17, the quantity decreased but remained greater than 70% of the E17 value ($p<0.01$) at the newborn stage.

The beginning of ε subunit mRNA expression (Figure 10C) and of the decrease in the quantity of γ subunit mRNA (Figure 10D) indicates that the nAChR switch begins at E15 in the tongue muscle and at E17 in the hind limb muscle. The predominance of ε subunit mRNA over γ subunit mRNA in the tongue muscle at birth (Figures 10C and 10D) indicates that the nAChR subunit switch is close to the termination at birth.

Immunolocalization of AChR δ subunit in the tongue

Since δ subunit is present throughout the whole process of synaptogenesis as the α subunit. Thus, to understand the regional difference of the nAChR elimination in the mouse tongue, I examined immunolocalization of δ subunit. Figure 11 shows the immunolocalization at E13 (A and B), E15 (C and D), E17 (E

and F), and at birth (G and H). At E13, faint immunostaining for the δ subunit was found in the anterior portion of the transverse and vertical tongue muscles and in the whole portion of the superior longitudinal tongue muscle (Figures 11A and 11B). At E15, the intensity of immunostaining became strong in the whole portion of the tongue muscles (Figures 11C and 11D). After E15, the intensity of immunostaining in the tongue muscle weakened rapidly. At E17, the immunostaining was observed only in the anterior portion of the transverse and vertical muscles (Figure 11E) and disappeared in the middle (Figure 11F) and posterior (data not shown) portions of the transverse and vertical muscles, and in the whole portion of the longitudinal muscles (data not shown). This suggests that the nAChR elimination occurs later in the anterior portions of the transverse and vertical muscles than in other portions. At birth, spot-shaped immunostaining for the δ subunit appeared sparsely throughout the whole portion of the tongue muscle (Figures 11G and 11H). In conjunction with the PCR result that the content of α subunit mRNA did not change between E17 and newborn (Figure 11B), the immunolocalization for the δ subunit at birth suggests that the nAChR elimination is nearly complete at birth in the mouse tongue muscle.

Discussion

In the present study, I identified the time course of the nAChR elimination and subunit switch in the mouse tongue muscle. I compared the present results with those in other striated muscles, including the hind limb muscle, and related findings to the expression profiles of the myoD family and the myogenesis previously reported (Yamane *et al.*, 2000a, Figure 12).

I showed that the elimination of superfluous nAChR began at E15 in the tongue muscle and at E17 in the hind limb muscle. It was previously reported that the elimination of superfluous nAChR begins after E18 in the rat internal intercostal muscle (Dennis *et al.*, 1981) and after birth in rat sternomastoid (Steinbach, 1981) and mouse gastrocnemius (Zoubine *et al.*, 1996) muscles. Thus, the elimination of nAChR seems to occur earlier in the tongue muscle than in other skeletal muscles including the hind limb muscle (Figure 12).

I demonstrated that the nAChR switch began at E15 in the tongue muscle and at E17 in the hind limb muscle. It was previously reported that the nAChR switch begins after E17 in the mouse tibialis anterior, extensor digitorum longus, sternomastoid, diaphragm and soleus muscles (Brenner *et al.*, 1994; Missias *et al.*, 1996). The nAChR switch appears to begin at an earlier stage in the tongue muscle compared with other skeletal muscles including hind limb (Figure 12).

I previously reported that the decrease in myoD and myogenin mRNA, and the expression of MRF4 mRNA, begin at E13 in the tongue muscle and at E15 in the hind limb muscle (Figure 12, Yamane *et al.*, 2000a). These stages precede those for the beginning of the decrease in AChR α and γ subunit mRNAs, and the beginning of the expression of ε subunit mRNA by 2 days in both the tongue and

hind limb muscles. It was previously reported that transcription of these nAChR subunit genes is regulated by several promoters including E box to which the myoD family can bind (Duclert and Changeux, 1995). Thus, the decrease in myoD and myogenin mRNA, and the expression of MRF4 mRNA, may be closely related with the decrease in nAChR α and γ subunit mRNA, and the expression of ε subunit mRNA, respectively. The 2 day time difference between myoD family and nAChR expressions seems to be due to the time period during which myoD family mRNAs are translated and the translated myoD family proteins are transferred into the nucleus of cells and bind to the regulatory region of nAChR genes. It was shown that innervation, denervation and subsequent electrical stimulation of the denervated muscle induce drastic changes in the expression of myoD family followed by those in the expression of nAChR subunit (Reviewed by Buonanno *et al.*, 1998). These results seem to support the present view.

I found that the nAChR switch from the embryonic to the adult type began at E15 in the tongue muscle and at E17 in the hind limb muscle. These stages are consistent with those at which myoblast differentiation terminates and myofiber maturation actively proceeds in both the tongue and hind limb muscles. The nAChR switch is reported to cause a change in the channel properties of nAChR from slow-gated and low conductance to fast-gated and high conductance channels (Brehm and Henderson, 1988). This change in the channel properties of nAChR seems to be related to the termination of differentiation and the progress of maturation in tongue and hind limb muscle cells.

In this part, I suggest that the nAChR elimination and the subunit switch are nearly complete at birth in the mouse tongue muscle. Recently, I reported that

myogenesis in the mouse tongue is also completed at birth (Yamane *et al.*, 2000a). Since tongues are necessary for suckling and swallowing immediately after birth, the completion of synaptogenesis as well as myogenesis at birth in the mouse tongue muscle are probably due to these early functional requirements.