Postnatal development of the neuromuscular junction of the masseter muscles in the Japanese field vole, *Microtus montebelli*

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Abstract. The developmental pattern of the neuromuscular junction (NMJ) in masseter muscles of the Japanese field vole, *Microtus montebelli*, was investigated using acetylcholinesterase (AChE) staining and electron microscopy. At birth, intense AChE activity limited to the site of the NMJ where many axon terminals with the cholinergic nature were converging was observed, indicating that cholinergic neuronal activity in the vole masseter muscle begins at this stage. The major morphogenesis of the NMJ such as: AChE staining reaction, concentration of myonuclei at the subneural site, elimination of the axon terminals, formation of the myeline sheath in the intramuscular axons, and the appearance of numerous junctional folds in the postsynaptic membrane was accelerated after postnatal day five, and amended dramatically at day ten with the maturation time of these NMJ components at around day fifteen. From the combination of the present and previous studies, it is clear that both AChE reaction and neuronal structures alter considerably at a time when structural and functional improvements give rise dramatically to muscle fibers. This must be considered in relation to the critical role of the neuronal influence on the differentiation and maturation of the vole masseter muscles that are required for the accomplishment of its own characteristic herbivorous food habits.

Key words: acetylcholinesterase, masseter muscle, neuromuscular junction, postnatal development, ultrastructure.

Among the masticatory muscles of the rodents, the masseter muscle is the largest, and is hence regarded as the most functional muscle during biting. Our recent ontogenical study of a particularly successful herbivorous rodent, the Japanese field vole, *Microtus montebelli* (Sugasawa and Mōri 1997), revealed that vole masseter muscles differentiate abruptly after birth and mature fully before weaning. This developmental pattern of the masseters is distinctly different from that of the masseters of either the rat, *Rattus* spp. or the mouse, *Mus* spp., in which they remain immature even at postnatal day

(PND)23, when the young are weaned from their mothers (Maeda *et al.* 1981, Hurov *et al.* 1992, Miyata *et al.* 1996).

It is well established in both the rat and the mouse, that neuronal actions and factors are significantly involved in the differentiation of developing skeletal muscles in various parts of the body (Ridge 1989, Hall and Sanes 1993, Grinnell 1995). Nevertheless, little is known about the neuromuscular interaction in the ontogenical process of the masseter muscle. Therefore, as the first step towards an understanding of the neuronal influence on the development of the rodent masseter muscle, we examined the developmental pattern of the neuromuscular junction (NMJ) in the vole masseter muscles from birth to PND 15, using histochemistry for acetylcholinesterase (AChE), the enzyme hydrolyzing acetylcholine (ACh) and electron microscopy.

MATERIALS AND METHODS

The Japanese field voles used for this study were obtained from our laboratory colony which originated from wild voles live-trapped in Fukuoka Prefecture. They were kept in cages in an environment-controlled room ($23 \pm 1^\circ\text{C}$, LD 14:10). All the animals were given a herbivorous diet (ZF, Oriental Yeast Co., Ltd., Tokyo), a commercial mouse diet (NMF, Oriental Yeast Co., Ltd., Tokyo) and water *ad libitum*. Twenty four newborn voles of both sexes, which were kept with their mothers in cages, were used in this study. The day of birth (day 21 or 22 of gestation) was regarded as postnatal day 0 (PND 0). The animals were divided by age into the following four groups: PND 0, PND 5, PND 10, and PND 15. For each of the four groups, three individuals were used for AChE histochemistry and electron microscopy.

1. Histochemistry for AChE

The voles were anesthetized with ethyl ether then perfused through the left ventricle with Ringer's solution, followed by 30 ml of ice-cold 4% buffered formaldehyde. The masseter muscles were carefully dissected from the jaws, then postfixed with the same fixative for one hour. They were washed thoroughly with 0.1 M phosphate buffer (PB, pH 7.4), and then immersed sequentially in PB containing 10% and 20% sucrose for two days each at 4°C . For sectioning, the materials were quickly frozen in isopentane chilled with dry ice and sectioned at a thickness of $20\ \mu\text{m}$ in a cryostat. To detect AChE activity, sections were maintained in substrate (acetylcholine iodide, Sigma Chemical, USA), free Karnovsky's medium (Karnovsky and Roots 1964) for 30 min at 4°C , and incubated in the complete medium containing $2 \times 10^{-4}\ \text{M}$ tetraisopropyl pyrophosphoramidate (Sigma Chemical, USA) as an inhibitor of non-specific cholinesterase for one hour at 20°C . These procedures have been described in detail by Andō (1981).

2. Electron microscopy

In order to avoid excessive muscular contraction during direct fixation,

once the voles were fully anesthetized they were decapitated, and the heads were first immersed for 20 min in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate (SC) at pH 7.2. Subsequently, the masseter muscles were dissected out from the jaw in the same fixative, and postfixed for two hours. The materials were washed briefly in 0.1 M SC, and fixed for a further two hours in 1% osmium tetroxide buffered with 0.1 M SC. The tissues were dehydrated in an ethanol series and embedded in Epon 812. Thin sections (~ 60 nm) were cut on a Porter-Blum MT-1 microtome using a glass knife, and doubly stained with lead and uranyl acetate before examination in an Hitachi-H600A electron microscope (75 kV).

RESULTS

1. AChE-activity

In all of the voles examined at birth, AChE activity was observed to be limited to the center of the muscle fibers where the NMJ is formed. The muscular areas stained specifically with AChE were shaped like a button with a diameter of about $3.5\ \mu\text{m}$, and showed a linear profile in the transverse direction (Fig. 1a). The staining reaction at this site did not change signifi-

Fig. 1. AChE reaction of the masseter muscle in the vole at birth (a) and on PND 15 (b). Bar : $50\ \mu\text{m}$.

cantly from PND 0 to PND 5, however, by PND 10, the AChE-positive area had increased to about three times the diameter observed at birth with a rise in the activity of this particular enzyme. The AChE-positive area enlarged further after PND 10, reaching approximately five times the area observed at birth on PND 15. The enzyme activity also became more prominent (Fig. 1b). No detectable difference in the AChE staining properties of the NMJ was observed between the neonates at PND 15 and maternal voles at six months of age.

2. Electron microscopic observations

At birth, many axon terminals covered with Schwann's cells converged at the site of the NMJ, (Fig. 2a). These axon terminals did not contain the cytoskeletal or membranous components indicative of growth cones, but accumulated with small clear vesicles (about 50 nm in diameter) indicating their cholinergic nature (Fig. 2b). At this stage, although the myeline sheath was not yet formed, most of the intramuscular axons were already encircled by Schwann's cells (Fig. 2c). The basal lamina extended over the synaptic cleft in the muscle fibers. On the one hand, no junctional folds or subneural nuclei were seen, nor was an accumulation of mitochondria noted in the soleplate region. On the other hand, the subneural muscle plasma membrane was undercoated with an electron-dense amorphous material, showing a profile similar to the postsynaptic membrane in adults (Fig. 2b).

No appreciable difference in the number of axon terminals in the NMJ was observed between birth and PND 5, however each of the intramuscular axons was surrounded by a thin myeline sheath by PND 5 (Fig. 3a, b, c). After PND 5, some structural specializations in the muscle fibers were observed in the soleplate region. Numerous myonuclei were concentrated at the subneural site, forming the subneural nucleus (Fig. 3a). In parallel with this, the muscle plasma membrane began to invaginate and form junctional folds (Fig. 3c).

By PND 10, the number of axon terminals had decreased markedly (Fig. 4a), and the myeline sheaths of the intramuscular axons became much thicker (Fig. 4b). At this stage, the junctional folds increased greatly in number and grew taller owing to the frequent occurrence of deep invaginations of the plasma membrane. The accumulation of mitochondria, though not so prominent, was also observed in the soleplate regions (Fig. 4a).

By PND 15, the NMJ had only one axon terminal that was filled with small clear vesicles and small mitochondria, which was also furnished with numerous, regularly arranged junctional folds that were very tall and showing structural properties similar to those seen in adult voles (Fig. 5a, b).

DISCUSSION

The present study has shown for the first time an age-related change in AChE-activity and in the neuronal elements in the NMJ of the vole masseter muscle. At birth, high AChE activity was localized specifically at the NMJ, where a number of axon terminals with small clear vesicles, typical of a

Fig. 2. Electron micrographs of the NMJ (a, b) and intramuscular axons (c) in the vole masseter muscle at birth. at: axon terminal, bl: basal lamina, im: intramuscular axon, mf: myofiber, ps: postsynaptic membrane, sc=Schwann's cell, sv: small clear vesicle. Bar: 1 μ m (a, c), 0.5 μ m (b).

Fig. 3. Electron micrographs of the NMJ (a, c) and an intramuscular axon (b) in the vole masseter muscle at day 5. at: axon terminal, im: intramuscular axon, jf: junctional fold, mf: myofiber, sc: Schwann's cell, sn: subneural nucleus. Bar: $1\ \mu\text{m}$ (a, b), $0.5\ \mu\text{m}$ (c).

Fig. 4. Electron micrographs of the NMJ (a) and an intramuscular axon (b) in the vole masseter muscle at day 10. at : axon terminal, im : intramuscular axon, jf : junctional fold, m : mitochondrion, mf : myofiber, sc : Schwann's cell, sn : subneural nucleus. Bar = 1 μ m.

cholinergic nature, were concentrated. In addition, the muscle plasma membrane was found to be similar in basic structure to the postsynaptic membrane of adults. These results seem to suggest that neuronal action, provoked by cholinergic transmission (release and hydrolysis of ACh), is operational in the vole masseter muscles at birth, whereas studies of the hind leg muscles of the domestic fowl have indicated that AChE activity, specific for the NMJ, appears some days after the onset of synaptic transmission has been provided (Grinnell 1994).

The present study has further shown that : the major morphogenesis of the NMJ, which is represented by concentration of myonuclei at the subneural site, elimination of the axon terminals, formation of the myeline sheath in the intramuscular axons, and the appearance of numerous junctional folds in the post synaptic membrane, are all accelerated after PND 5 and amended dramatically on PND 10. Likewise, AChE-staining areas enlarged greatly, with a marked rise in enzyme activity, at a stage of development coinciding with the time (PND 10) when young voles start to take solid food, and when the muscle fibers begin to change from an undifferentiated condition into their own specific (fast twitch oxidative) fiber type and to display a potent contractive ability (Sugasawa and Mōri 1997).

By PND 15, the AChE-staining reaction and the neuronal structures in the NMJ attained levels equivalent to the mature pattern observed in six months

old adult voles. This stage coincides with the expression of strong oxidative enzyme activity in the muscle fibers and the commencement of their sustained contraction in adulthood (Sugasawa and Mōri 1997).

As described above, in the ontogenical process of the vole masseter muscle, both AChE activity and the neuronal structures in the NMJ changed considerably at a time when structural and physiological improvements give rise dramatically to muscle fibers with a synchronous maturation of NMJ compo-

Fig. 5. Electron micrographs of the NMJ in the vole masseter muscle at day 15 (A, B). at : axon terminal, jf : junctional fold, m : mitochondrion, mf : myofiber, sc : Schwann's cell, sn : subneuronal nucleus. Bar = 1 μ m (a), 0.5 μ m (b).

nents. Thus, there is good correlation between age-related changes in neuronal and in muscular elements of the vole masseter NMJ. Although no direct evidence, as to which factors and mechanisms participate in the development of the masseter muscles, was provided by the present study, these findings seem to indicate the great significance of the neuromuscular interactions responsible for a chain of ontogenical events on this masticatory muscle. Based on evidence of the functional involvement of cholinergic transmission, particularly its trophic effect, in the starting and advance of muscle ontogeny (Grinnell 1994), such neuronal influence might play a critical role in the differentiation, during each developmental stage, of the vole masseter muscles. This may be closely related to species-specific requirements, for the structural and physiological maturation of this masticatory muscle, which are essential for the accomplishment of the herbivorous food habits so characteristic of the voles.

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