Postnatal expression of aquaporin-4 (AQP4) associated with the functional development of skeletal muscles

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Abstract

Aquaporin-4 (AQP4) water channel is abundant at the sarcolemma of skeletal muscle and presumably plays a substantial role in adapting the rapid changes in fiber volume as well as osmotic pressure during repeated muscle contractions. In this paper we report on the expression of AQP4 and its correlation to the metabolic and functional development of locomotor muscles during the early stage of postnatal development of mouse.

The functional development of hindlimb muscles was analyzed by video recording spontaneous walking of mouse pups in specifically constructed walkway. The expression of AQP4 was examined by using immunofluorescence technique and fiber counting. Metabolic development of the muscles was analyzed by using histochemical staining methods for NADH activity and glycogen phosphorylase activity. According to the results, the number of AQP4 positive fibers increases markedly during the first week after birth. This increase in AQP4 expression is accompanied by rapid functional and metabolic maturation of muscles, such as the gradual decrease in the duration of swing phase and the differentiation of glycolytic activity of the muscle fibers. Importantly, most marked increase in AQP4 expression appeared during the first three postnatal days when also most distinct change in functional and glycolytic potential of muscle fibers took place. However, the general locomotor activity of mouse pups appeared confined until one week after birth. The fiber type dependent expression pattern of AQP4 was evident prior to increased locomotor activity at the second week after birth.

These results showed for the first time that the amount of AQP4 increases in locomotor muscles during early postnatal days in mouse. However, based on the analysis of locomotor movements, it was concluded that the enhanced water permeability may not contribute to muscle performance until the second postnatal week.

Keywords: locomotion, metabolic properties, postnatal development, water channel, water permeability.

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Introduction

The functional development of skeletal muscles towards adult performance involves changes in the expression of ion channels as well as reorganization in contractile and metabolic properties [22, 18]. Analysis of the composition of contractile proteins and metabolic enzymes has been a common way to evaluate the skeletal muscle plasticity associated with development and exercise. However, in order to understand the concept of muscle performance more precisely, several co-phenomena should be considered consistently. One of those is the water transfer capability of the skeletal muscle.

Water movement takes place between capillaries and muscle tissue during contraction. Several consequences have been reported to result from water movement in a muscle. Water neutralizes the osmotic changes associated with repeated activation of a muscle [14, 15]. If not neutralized, increased osmotic pressure leads to dramatic decline in force production [17]. On the other hand, the exercise associated swelling of muscle fibers has been shown to precede the increase of interstitial volume and finally muscle fiber necrosis. These changes have been suggested to result in the swelling induced damages on the sarcolemma [12]. Therefore, in terms of muscle performance, it is important to consider the rate of water transfer across the sarcolemma. In many tissues, the water permeability of plasma membrane is augmented by the aquaporin water channels (for review see [23]). Aquaporins have been localized also at the sarcolemma of fast twitch skeletal muscle fibers [5].

The precise role of aquaporin type-4 (AQP4) in skeletal muscle function has not yet been understood. However, the functional experiments on single muscle fibers and membrane vesicles have revealed a very high intrinsic water permeability of the sarcolemma vesicles containing AQP4 water channels [8]. Functional studies imply that water channels play an important role in rapid neutralization of osmotic changes during repeated muscle contractions. Further support for the functional importance of enhanced sarcolemmal water permeability comes from the findings that the expression of AQP4 is markedly reduced in mice suffering of genetic muscular dystrophy [7]. The symptoms of this disease are complex, but the salient cellular features are the reduced osmotic stability and poor rigidity of the sarcolemma [16].

Plasticity is a characteristic property of skeletal muscle. Analyzing the expression pattern of molecular components due to changes in muscle use is one tool to evaluate their functional significance. Postnatal development is one good example of skeletal muscle plasticity. Maturation of skeletal muscles involves many complex events which either precede or parallel with development of locomotion. In this study we investigated postnatal expression of AQP4 water channels and its relation to the functional maturation of hind limb muscles. Spontaneous walking of mice pups up to 15-days-old was video recorded by using a specifically designed setup. From the video recordings, both general locomotor behaviour and the duration of step cycles were analyzed. Metabolic maturation of muscle fibers and the expression of AQP4 water channels were investigated by using histochemical and immunohistochemical staining methods. We used a leg muscle, *musculus gastrocnemius,* first because it obviously has a high contribution in force production during walking. Secondly, the red and medial parts of *m. gastrocnemius* are intensively recruited during walking in rodents [13]. The major finding of this study was that the expression of AQP4 increases dramatically during the first three postnatal days and this coincides with the differentiation of glycolytic activity in muscle fibers. However, functional and behavioral analysis of locomotion implies that muscle fibers would not benefit from enhanced water permeability until the second postnatal week.

Materials and methods

Animals, sample taking and preparation

Laboratory mice (*Mus musculus*, CD-1 strain) of age 1, 3, 6 and 15-days (P1, P3, P6 and P15 respectively) were used in all experiments. This study was approved by Animal Care and Use Committee of University of Oulu.

Muscle samples were obtained from mice killed by cervical dislocation. Thereafter the hind limb was frozen with liquid nitrogen and stored at –70 °C until used. For histochemistry 10 µm sections from the whole lower leg of mice up to 6-day-old and from *m. gastrocnemius* of 15-days-old mice were cut with a cryostat microtome (Jung 2800 Frigocut-E, Leica).

Immunofluorescence

In order to compare the immunohistochemically stained sections with the ones stained histochemically, approximately every fourth section was taken for the immunohistochemistry. The sections were air dried and fixed with 3% paraformaldehyde and washed in phosphate buffer. The sections were blocked with 1 % albumin in phosphate buffer (1 % BSA/PBS) for 10 min at room temperature and incubated with 5 µg/ml anti-rabbit AQP4 polyclonal antibody obtained from Chemicon (UK) and TRITCH conjugated phalloidin (Sigma, USA) for 30 min at 37°C. Alternatively, the AQP4 antibody was preincubated with an excess of immunizing peptide (Chemicon, UK) for 50 min prior to application onto the sections. Thereafter the sections were rinsed with high salt (2.7 % NaCl) PBS according to Frigeri *et al.* [5] and incubated with Alexa 488-conjugated secondary antibody (Molecular probes) diluted to 1:50 for 30 min. After the subsequent washes with PBS the sections were covered with Mowiol 4-88 containing 2.5 % 1,4-diazobicyclo-octane to inhibit fading. Laser scanning microscope (LSM 510, Zeiss, Germany) was used for imaging the sections. Fluorescence was excited by the argon laser at 488 nm. Same scanning settings (amplification offset, amplification gain, laser intensity and pinhole) were used for all sections.

From the acquired images total of 400 fibers were counted from medial part of *m. gastrocnemius* by using Image Tool for windows version 3 (UTHSCSA; USA).

Histochemical analysis

Postnatal specialization of muscle fibers was analyzed by using histochemical staining methods based on the activity two enzymes: NADH-tetrazolium reductase (NADH-TR) and glycogen phosphorylase. The oxidative capacity of muscle fibers was assessed by using NADH-TR staining according to Farber *et al.* [4]. The glycolytic capacity was assessed by using staining for glycogen phosphorylase (E.C.2.4.1.1., alfa-1.4-glukan: orthophosphateglukosyltransferase) which is a marker enzyme of glycolytic capacity [3]. The air dried sections were first fixated with cold acetone for 2 min. Thereafter sections were incubated in substrate solution containing 1.3 mM Glucose-1-phosphate, glycogen, 2.1 mM adenosine monophosphate, 21.4 mM NaF, 2.3 mM polyvinylpyrrolidone and few drops of insulin in 0.05 l of acetate buffer. The substrate incubation was carried out at 37 °C for 90 – 103 min. Dehydration of the sections was carried out by consecutive rinsing in 40 %- and absolute ethanol. The formed glycogen was stained with Gram´s iodine solution.

mATPase staining was used to discriminate slow and fast twitch fibers. mATPase staining was performed by applying a method described by Brooke and Kaiser [1]. In mATPase staining the preincubation solution of pH 4.3 was used to discriminate slow and fast twitch fibers.

Histochemically stained sections were observed with light microscope (Axiovert 200M, Zeiss, Germany) and selected regions were photographed with digital camera (Axiocam, Zeiss, Germany). Photographed pictures were processed with Adobe Photoshop®.

Video recording and measurements of spontaneous walking

1 (n = 8), 3 (n = 17), 6 (n = 20) and 15 (n = 10)-day-old mice (obtained from three different litters) were studied. Animals were housed in a room with constant temperature (+22 °C) and lighting schedule 12L : 12D. Video recordings of spontaneous locomotion were made during a dark phase of the light dark cycle.

Animals used in the experiment were randomly selected. Ankle joint and 1st toe were marked with black ink in order to locate their position in analysis and to avoid a reselection of the same individuals. The nest temperature (around 32 °C) was measured with infrared thermometer (THI-700, Tasco). This was supposed to be close to the normal activity temperature of mice pups, which have poor endogenous heat production capacity [25]. Before video recordings animals were put into the thermo stated chamber (+33 °C) for 6 min. After temperature acclimatization animals were put into a walkway (30 X 15 cm). The width of walkway was adjusted according to the size of animal. A heat radiator (500 W) was used to adjust the temperature in the walkway around to +33 °C.

A CCD miniature camera (EM-102 pal, ELMO, USA) operating at 25 frames per second was used to obtain pictures of walking. The camera was fixed to a slide rail to allow a smooth horizontal movement along the length of walkway. Camera output was connected to a high quality video tape recorder (type VHS NV-FS100). Every time the animal started to walk, the camera was moved parallel to walking direction. A digital timer with 1/100 second accuracy was placed on the front of the camera in order to add running time to the pictures being recorded. Animals were recorded for 10 min or until no hesitant walking was detected. Recording was performed at a dark room using a red light as a light source.

Recordings were analyzed frame by frame. The duration of an individual frame was determined by calculating the number of frames/second (based on the running time of digital timer). Three to four continuous spontaneous steps of hind limb from each animal filmed were selected for analysis. Nonhesitant and undisturbed walking was set as a criterion.

In analysis one step cycle was divided into two phases, stance and swing. At the onset of walking stance phase was defined to begin when the first sign of rise of the sole (with respect to surface) was detected and to end to the point at which toes were just about to detach from the surface. Swing phase was defined as the next step from the end of stance phase until the ball of the foot stroke again on the ground. During the walking, stance phase was defined as the next step from the end of swing phase. The criteria for the definition of swing phase during walking were the same as described above. For quantitative analysis the duration of each phase was measured.

Walking speed was calculated by measuring the distance traveled by an animal during the three to four continuous steps (same steps as from which the durations of step cycle parameters were measured) and dividing it by the time obtained from the digital timer with an accuracy of ±5 ms. The distance traveled was read with ±2 mm accuracy. The start and the end points of walking were defined on the basis of the position of the 3th and 4th toe.

Statistical analysis

All values are expressed as mean ± S.E.M. Levene´s test was used to test the equality of variances. One-way analysis of variance (ANOVA) was used to evaluate differences in step cycle parameters. $P < 0.05$ was considered statistically significant in all analyses.

Results

Video recording of spontaneous walking

Until the 6th postnatal day general locomotor activity was confined. Only some individuals walked continuously with at least 3 to 4 steps: 2 of 20 individuals in P6, 4 of 17 individuals in P3 and 3 of 8 individuals in newborns (P1). In P15 mice were continuously active but walking was predominantly hesitating with characteristically elongated body and hind limb position or periodical with short breaks between steps. Therefore the duration of step cycles were measured from only 5 of 8 individuals. Some individuals in P15 performed also forward directed bounces. In younger age groups onward directed bounces occurred only when disturbed and they resembled uncontrolled hopping rather than quadrupedal running. Qualitatively and quantitatively most marked developmental changes in the use of hind limbs occurred during the first three postnatal days.

The duration of swing phase decreased with development (Fig. 1a). The decrease in duration of swing phase was statistically significant ($F_{3.11} = 9.84$, $p<0.01$). On the other hand, in the duration of stance phase, the measurements revealed distinct differences only between P1 and P3 (Fig. 1b). However, limited number of individuals and inequality of variances did not allow us to make statistical comparisons.

The duration of the whole step cycle was distinctly longer in P1 mice, when compared with the older age groups (Fig. 1c). This cannot, however, be explained

solely by the longer durations of swing and stance phase respectively. The P1 mice as a rule performed a characteristic stepping pattern in which the hindpaws were often rotated upside down at the end of stance phase and were left dragged for a while before the onset of swing phase. The dragging phase lengthened the duration of step cycle and resulted in a clumsy appearance of walking.

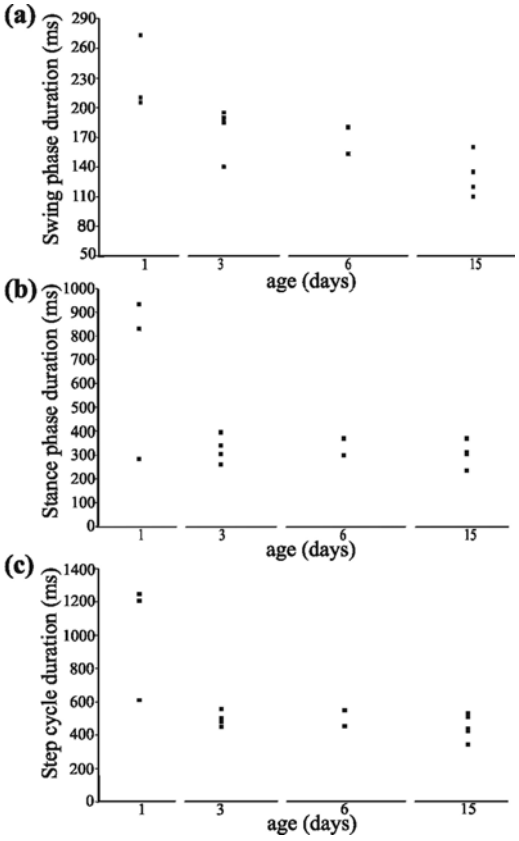

Figure 1. *The parameters measured from the spontaneous non-hesitant walking in mice of different ages. (a) Swing phase duration. (b) Stance phase duration. (c) Step cycle duration. Gray squares represent the average of three to four steps of one individual.*

Since having found out that the durations of swing phase decreased with development, we wanted to find out whether this could be due to the differences in walking speed. Differences in the walking speed between age groups were statistically significant ($F_{3.10} = 6.025$, $p = 0.013$). However, when the duration of swing phase was blotted as a function of walking speed (Fig. 2a) one can see no logical relationship between the mentioned parameters. This implies that the duration of swing phase varies independently of walking speed. The duration of stance phase in turn is clearly related to the walking speed from P1 to P6 (Fig. 2b).

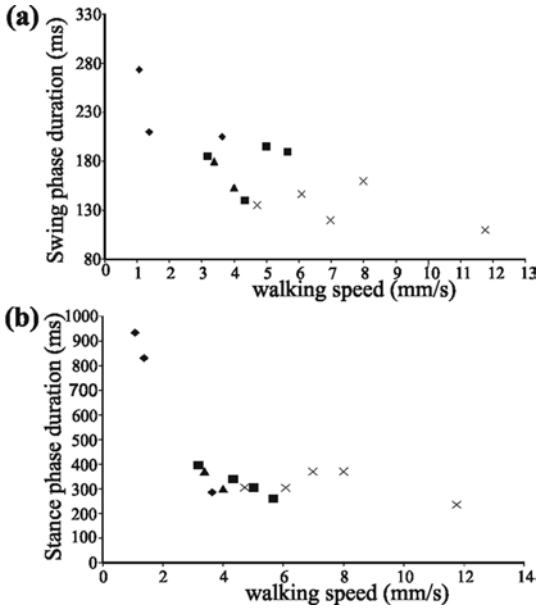

Figure 2. *(a) the relationship between swing phase duration and walking speed. (b) the relationship between stance phase duration and walking speed. ♦ = P1, ■ = P3, ▲ = P6 and **x** = P15.*

Immunohistochemical analysis of AQP4 expression

Incubation of the muscle cross sections with AQP4 antibody revealed staining at the sarcolemma of muscle fibers and to some extent in extracellular structures. Preabsorption of the antibody with an excess of antigen revealed no staining at the sections (Fig. 4e).

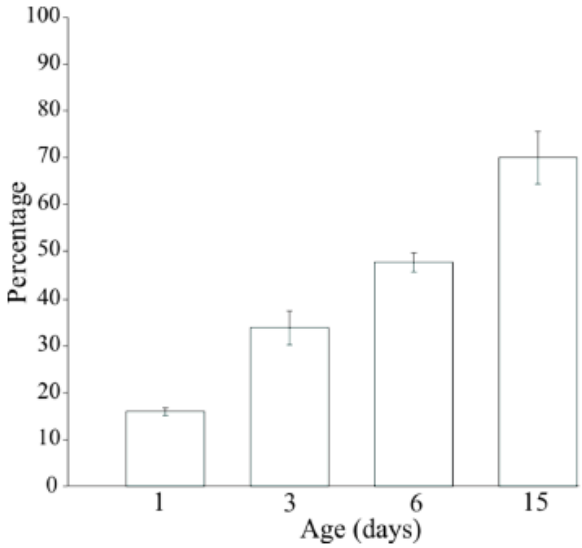

Figure 3. *The proportion of AQP4 positive fibers in the* m. gastrocnemius *in relation to the developmental stage. Values are means ± S.E.M. N = 3 – 4 in each group.*

In order to count the proportion of AQP4 positive fibers from frozen muscle section were doublestained with TRITCH-conjucated phalloidin which specifically attached to f-actin. Phalloidin staining allowed visualization also the fibers which were not stained by AQP4 antibody (Fig 4). According to the fiber counting of the selected regions of *m. gastrocnemius* the proportion of AQP4 positive fibers increased markedly from P1 onwards (Fig. 3). The most dramatic change appeared during the first three postnatal days when the mean proportion of AQP4 positive fibers increased 2.1 fold. In P1 mice majority of muscle fibers appeared to be unstained (Fig. 4a). Furthermore, the fibers, which were stained, showed differences in fluorescence intensity, indicating differences in AQP4 density between the fibers. From the P3 to P6 and P6 to P15 respectively the mean number of AQP4 positive fibers was increased 1.4 fold and 1.5 fold respectively.

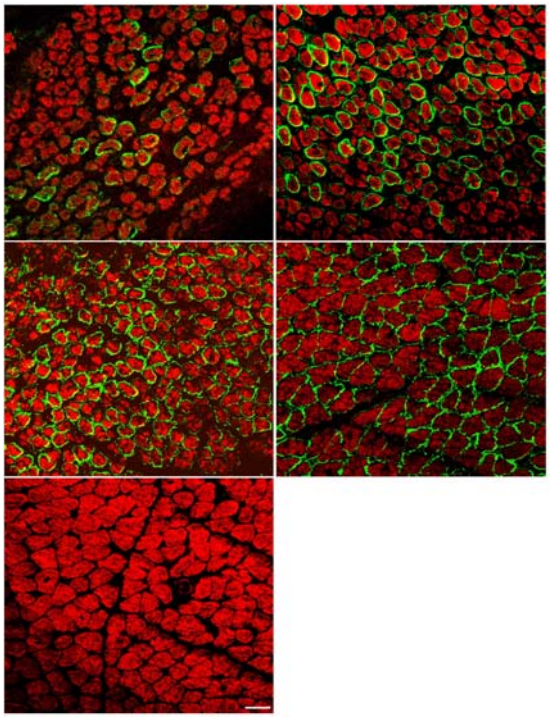

Figure 4. *The immunohistochemical observation of the expression of AQP4 in* m. gastrocnemius *during postnatal development. The muscle cross sections were double stained with TRITCH-conjugated phalloidin (Red) and AQP4 antibody (green). (a) the cross section of* m. gastrocnemius *of P1. (b) the cross section of* m. gastrocnemius *of P3. (c) the cross section of* m. gastrocnemius *of P6. (d) the cross section of* m gastrocnemius *of P15. (e) the cross section of* m. gastrocnemius *of P15 after staining with AQP4 antibody preabsorbed with the peptide used for immunization. Scale bar 20 µm.*

Morphological comparisons of consecutive sections stained for mATPase activity revealed that AQP4 was expressed mainly in fast twitch fibers in P6 and P15 mice, but AQP4 positive staining was present also in some slow twitch fibers (Fig. 5). To investigate whether the observed expression pattern of AQP4 could be related to the development we also stained cross sections of *m. soleus* of adult mouse. As with younger, positive immunostaining was mainly restricted to the fast twitch fibers but lower intensity staining was also found in some slow twitch fibers (Fig. 5 c and d).

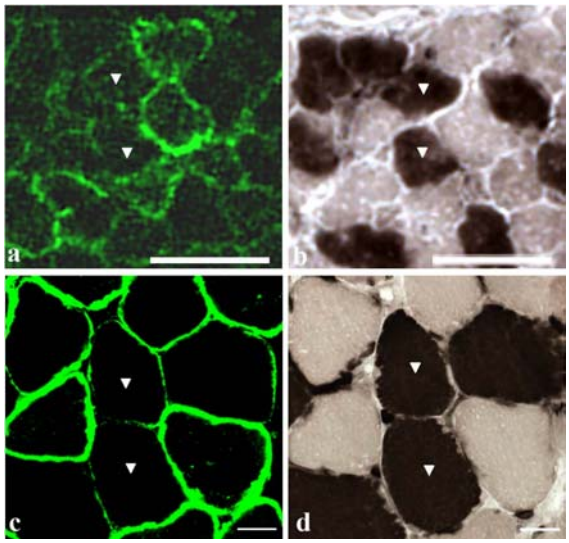

Figure 5. *The fiber type related expression pattern of AQP4. The figures represent the consecutive sections of* m. gastrocnemius *of P6 mouse (a and b) and* m. soleus *of the adult mouse (c and d) stained immunohistochemically by using AQP4 antibody or histochemically for mATPase activity at pH 4.3. In mATPase staining slow twitch fibers are darkly stained. Symbols indicate the slow twitch fibers. Scale bar 20 µm.*

Histochemical staining of fiber types

Histochemical staining of *m. gastrocnemius* for NADH-TR-reductase activity showed no differences between muscle fibers until P6. Clear differences in the shade of staining (and hence in oxidative capacity) was found only in the samples of P15. By contrast, in phosphorylase staining muscle fibers were stained with at least two clearly distinguishable shades from the P3 onwards. In P1 all the fibers were darkly stained indicating a uniform and at least moderate glycolytic capacity. Fig. 6 shows the age dependent differences in metabolic stainings.

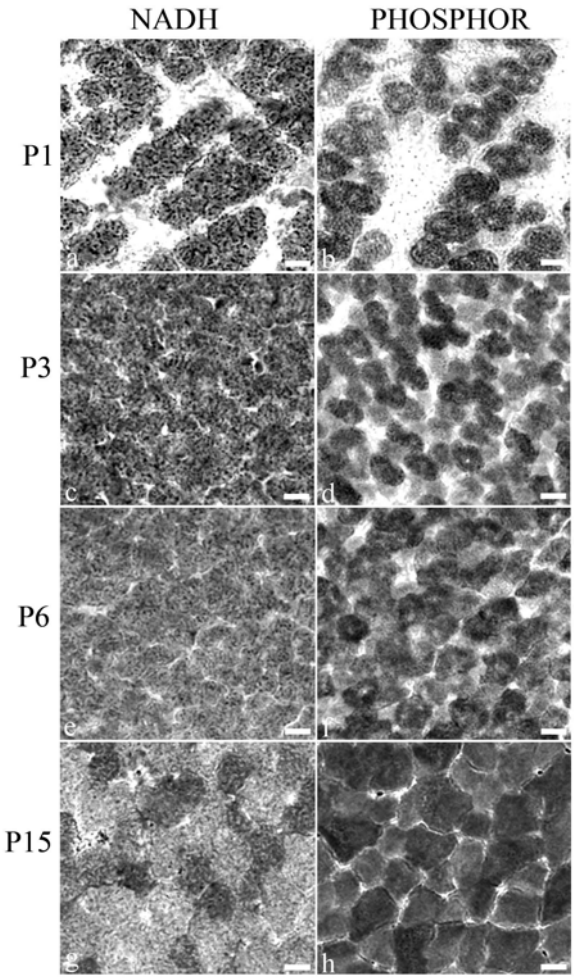

Figure 6. *Metabolic differentiation of muscle fibers in m. gastrocnemius during postnatal development. (a) (c) (e) and (g) NADH-TR stained cross sections of m. gastrocnemius of P1, P3, P6 and P15 respectively. (b) (d) (f) and (h) the glycogen phosphorylase stained cross sections off m. gastrocnemius of P1, P3, P6 and P15 respectively. Note the differentiation of glycolytic phenotype but not oxidative phenotype at third postnatal day. Scale bar 10 µm.*

Discussion

Postnatal development of spontaneous walking and metabolic specialization of fiber types

In this study we analyzed functional development of walking by recording the mean duration of step cycle parameters in mice pups. The results showed a distinct reduction in the duration of swing phase with development. Furthermore, the reduction seemed to be independent of walking speed. Westerga and Gramsbergen [26] noticed a similar trend in the age related reduction of swing phase in rat pups and suggested also that the reduction is independent on the walking speed. They concluded that this might be due to the progressive reduction in contraction time and increase in contraction speed of fast muscles with age. This conclusion is supported by the allometrical constraint, which states that contraction time, if not related to the development, should be shorter in duration in smaller but geometrically similar animals.

The duration of stance phase was clearly related to the walking speed until one-week-old (P6). In P15 the step length appeared to contribute more on walking speed. These results indicate that the contraction speed of extensor muscles can be varied according to walking speed as early as first postnatal day. However, immaturity in motor control systems reflects the stepping performance. This was evident especially in P1 mice in which the hind limb was regularly left dragged before the onset of swing phase thereby increasing the duration of step cycle.

The trend in the functional development parallels with the maturation of some metabolic and excitation-contraction properties observed in the present and some previous studies. In the present study we observed a distinct difference of glycolytic properties from the P3 onwards. This appears consistently with changes in the expression of myosin isoforms which show a gradual shift from embryonic and perinatal mRNA to adult fast type IIB [27]. Furthermore the conduction velocity and the sodium channel density have been shown to increase during the early stages of the postnatal development in mice [11, 13].

However, despite of distinct functional maturation of hind limb muscles the general locomotor activity remained confined until one week old. The confined locomotor activity correlates with the immaturity of the properties of skeletal muscles involved in locomotion [20], but it is not the only explanative factor. In young animals the small, body size with small reserves of energy and high resting metabolic rate limits their locomotor activity [2]. Also, the lacks of experience about the environment and about the capabilities of animal's own motor system are the limiting factors of locomotor activity [2]. In this study environmental variation was reduced as small as possible; i.e. by adjusting the temperature in the walkway close to that in the nest and by using red light illumination in order to suppress the light factors on locomotor behaviour.

4.2. Postnatal expression of AQP4 water channel protein and its correlation with fiber type specialization and development of spontaneous walking

The expression of AQP4 in muscle fibers showed a similar trend with many other previously mentioned parameters. The expression increased most markedly (2.1 fold) during the first three postnatal days which coincides with the molecular changes in glycolytic enzyme activity and myosin heavy chain expression [27]. The increase of expression appeared almost linear

up to P15 at which age metabolic differentiation (oxidative and glycolytic properties) of muscle fibers was fully achieved. Moreover, skeletal muscles in P15 appeared to reach the stage of functional maturity, which allowed them to perform various locomotor tasks, from sustained quadrupedal walking to high speed bursts.

It is evident that muscle fibers incur a direct benefit from enhanced water permeability. First, because osmotic conditions change very rapidly in working muscles [9]. Secondly, the swelling of muscle fibers associated with activity has been proposed to exert a strong mechanical stretch on the sarcolemma, which may finally lead to the ruptures in sarcolemma [12]. In that respect, water channels may also mediate rapid outflow of water due to the hydrostatic pressure gradients. In both cases, the enhanced water permeability of the sarcolemma would improve the performance of the skeletal muscle.

However, despite rapid acceleration of AQP4 expression at early stage of postnatal development, we consider it unlikely that water channels would provide a direct benefit of enhanced sarcolemmal water permeability for skeletal muscle function. Accordingly, although some functional and metabolic maturation of skeletal muscles evidently takes place during the first postnatal week, the general locomotor behaviour clearly limits the performance of the whole animal. Rather, we find it more likely that the development of water permeability properties together with many other functional properties precede the increased activity of skeletal muscles, which in the present study was evident at P15.

AQP4 water channels in adult rats, has been shown to be distributed only in fast twitch fibers which experiences the highest osmotic load during exercise [5]. However, in contrast to rat, muscle fibers rich in mitochondria in mouse has been showed to express some amount of AQP4 [28]. More recently AQP4 was found in the slow twitch fibers of transgenic mice overexpressing AQP4 but not in wild mouse [24]. In the present study morphological comparison of muscle sections stained either with AQP4 antibody or histochemically for mATPase activity revealed AQP4 staining being restricted mainly to the fast twitch fibers, but lower intensity staining was found also in some slow twitch (type I) fibers (Fig. 5). In mice pups the described staining pattern of AQP4 was found first time as early as one week old.

It has been shown in previous studies that the expression of AQP4 is modified along with changes in muscle loading [6] and the expression is essentially dependent on nerve supply in regenerating muscles [10]. It is unclear, however, whether the effect of nerve innervation is mediated by the electrical activity of motoneurons or by nerve-supplied trophic factors. Both of these factors have been shown to effect on skeletal muscle properties [21]. We find it unlikely that the electrical activity of skeletal muscles plays a significant role in the regulation of the AQP4 expression at the early stage of postnatal development. We base this assumption on the low locomotor activity and thereby low contraction frequency of the skeletal muscles.

Altogether, the results indicate that the expression of AQP4 increases markedly during first postnatal week and this concurs with the rapid maturation of certain functional and metabolic properties in hind limb muscles. However, both functional and behavioral constraints limit the locomotor performance at early stage of postnatal development. In light of that, the results of the present study point out that water channels must be present in the muscle fibers prior to frequent activation of muscles. The functional role of AQP4 water channels is plausible linked to the rapid balancing of osmotic and associated volume changes in working muscles, although not completely understood.

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