Contractile properties and myosin heavy chain composition of rat tongue retrusor musculature show changes in early adulthood after 19 days of artificial rearing

J. Chadwick Smith, W. Allen Moore, Stephen J. Goldberg, and Mary S. Shall. Contractile properties and myosin heavy chain composition of rat tongue retrusor musculature show changes in early adulthood after 19 days of artificial rearing. J Appl Physiol 101: 1053–1059, 2006. First published June 29, 2006; doi:10.1152/japplphysiol.00029.2006.—Previously, we showed that artificial rearing using the “pup in a cup” model results in decreased tongue activity and caused some minor alterations in the tongue retrusor musculature. However, the artificial rearing time frame previously chosen was brief (11 days). The purpose of the present investigation was to extend the artificial rearing period from postnatal days 3 to 21 (P21) to determine whether significant alterations occur as a result of this reduced tongue use. Several changes in contractile properties due to the artificial rearing process were observed, which fully recovered by postnatal days 41 to 42 (P41–2). These changes included a shorter twitch contraction time, shorter twitch half-relaxation time, and decreased fatigue resistance. Styloglossus muscle exhibited more neonatal myosin heavy chain (MHC) isoform at P21 for the artificially reared (AR) group. Changes that were persistent at P41–2 were also observed. Maximum tetanic tension was lower for the AR group at P21 and P41–2 compared with their dam-reared counterparts. Twitch tension was also lower by P41–2 in the AR group. At P41–2, the AR group exhibited an increase in MHC IIA and a decrease in MHC IIB for the styloglossus muscle. In addition, the AR group exhibited a decreased MHC IIB for the long head of the biceps brachii at P41–2. Our results are similar to other models of hindlimb immobilization and suspension. By extending our artificial rearing period, this reduced tongue activity induced acute changes and alterations in the tongue retrusor musculature that persisted into early adulthood.

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INFANTS WITH A VARIETY OF health problems are sometimes necessarily fed by nonoral methods. The resulting reduced tongue use may contribute to delays in oromotor development. For example, infants requiring neonatal intensive care display an increased frequency of speech motor disorders (17, 18), and perinatal infants subjected to prolonged parenteral feeds exhibit impaired sucking abilities at term (16). This impaired sucking ability is of particular importance as it can lead to longer hospital stays (27). These outcomes may be due, at least in part, to the disruption of normal development of the hypoglossal motor system by diminished sucking behavior.

We recently reported the use of an artificial rearing model to study the effects of reduced tongue activity during postnatal development (20). The acute changes due to artificial rearing from postnatal days 4 to 14 were lower half-relaxation times, lower fusion frequencies, and a decreased fatigue resistance. After a 1-mo resumption of activity including suckling until weaning (postnatal day 21 (P21)), contractile properties including fatigue resistance recovered fully by postnatal day 42 (P42). Compared with dam-reared controls, a small but significant increase in myosin heavy chain (MHC) IIA expression with a concomitant decrease in MHC IIB was observed at P42 in artificially reared animals. These changes may be associated with not only the modality used but also the duration and specific developmental period in which the artificial rearing process occurred. Differences in growth as measured by body weights between dam-reared and artificially reared animals at P14 may also influence the contractile properties and fatigue characteristics at that age.

In the first 10 days of postnatal development, the neonatal rat brain grows at its maximum rate (10). During this brain growth spurt, the central nervous system is vulnerable to environmental insults such as malnutrition (4) and ethanol exposure (32). In addition, electrotonic coupling has been observed in genioglossal motoneurons up to postnatal day 10 in in vitro brain stem slices (24). Furthermore, there are changes in the firing properties of hypoglossal motoneurons during the first 2 wk of postnatal life (31). Although this time period may be a critical developmental period, our previous study suggests that deprivation of suckling from postnatal day 4 to 14 does not result in chronic alterations of the hypoglossal motor system (20). However, Hall (15) showed that artificially reared animals from postnatal days 1 to 18 took longer to eat standard rat chow, suggesting that a longer time period for artificial rearing may result in dysphagia. The lack of long-term changes within the hypoglossal motor system, in our laboratory’s earlier study (20), may be explained by the 1 wk of normal suckling that followed the 11-day artificial rearing time frame. Therefore, the purpose of this study was to evaluate the acute effects of artificial rearing from postnatal day 3 to P21 (19 days with no normal suckling after artificial rearing) on the contractile properties, fatigue characteristics, and MHC isoform development and to determine what alterations persist into early adulthood.

MATERIALS AND METHODS

Virginia Commonwealth University’s Institutional Animal Care and Use Committee approved all procedures and protocols for animal care. All animals were housed on a 12-h light-dark cycle. The dam-reared control animals in this experiment had unlimited access to standard rat chow and water.

Experiments were carried out on 40 Sprague-Dawley rats. Twenty animals were used as dam-reared controls, and the other half were artificially reared from postnatal day 3 to P21. Of these two rearing groups (dam and artificial), half were evaluated at P21 and the other

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half were evaluated at postnatal day 41 or 42 (P41–2). Postnatal day 1 was counted as their day of birth.

**Artificial rearing.** On P3, rat pups intended for the artificial rearing process were taken from their mothers, anesthetized using isoflurane, and had a gastric cannula inserted into their stomachs using methods previously described (15). After the animals recovered from the cannula implantation procedure, they were raised from P3 to P21 using the “pup in a cup” model. Briefly, the pups were placed in a Styrofoam cup containing bedding. A plastic lid with several holes covered the cup and was secured in place with a rubber band to prevent the animal from crawling out. Each cup had a rubber weight mounted to the bottom. The cannula was passed through a central hole in the lid and connected to a feeding line. Each cup floated in a temperature-controlled water bath (38–40°C). To keep the cups in the same approximate location in the water bath, a custom-built frame was placed over the water bath. Every day, the animals were removed from the cups, weighed, cannulae flushed with sterile water, stimulated to urinate and defecate by stroking of the genitorectal area with a moist lint-free tissue paper, and placed back in the cup with clean bedding.

**Milk and feeding schedule.** The substitute milk formula used in this experiment was a modification (32) of the Messer diet (26). Table 1 compares the macronutrient profile of this milk formula with rat milk. The pups were fed via milk-containing syringes using an automated syringe pump system (PHD 2000, Harvard Apparatus, Holliston, MA). The artificially reared animals were fed continuously for 10 min out of every hour in a 24-h period. Feeding volume for each day was determined by the group’s mean body weight for that day (23). Each day, the artificially reared animals received a volume equivalent to 33–42% of their mean body weight. Every 24 h, the syringes were replaced with fresh milk and the feeding lines flushed with sterile water.

**Physiology surgery and measurement of contractile properties.** At the conclusion of the artificial rearing process, animals that did not have their tongue retractor contractile properties assessed on P21 had their tongue retrusor contractile properties assessed on P41 (P41–2). Postnatal day 1 was counted as their day of birth.

**Table 1. Comparison of macronutrient profile for substitute milk formula and rat milk**

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>Substitute Milk Formula (g/100 ml)</th>
<th>Rat Milk (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>9.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Fat</td>
<td>11.3</td>
<td>12.3</td>
</tr>
<tr>
<td>Protein</td>
<td>8.0</td>
<td>9.2</td>
</tr>
</tbody>
</table>

*Data from Dymsza et al. (11).
migration pattern of the developmental rat MHC isoforms using Western blot analysis. Embryonic MHCs have the slowest migration, appearing above the adult MHC IIa isoform. Neonatal MHCs demonstrate an intermediate migration, appearing between the adult MHC IIx and MHC IIb isoforms. In this study, identification of the individual MHC isoform bands was achieved using a rat skeletal muscle standard that contained all six MHC isoforms. The rat skeletal muscle standard was formulated from a mixture of adult and neonatal rat gastrocnemius, plantaris, and soleus muscles prepared as described (see Muscle removal and preparation).

Statistical analysis. A factorial ANOVA was used to assess differences in body weight and contractile characteristics between rearing and age groups. Tukey’s honestly significant difference test was used for post hoc analysis when significant differences were found. Two one-way ANOVAs were used to assess differences for myosin heavy chain composition between rearing groups at P21 and P41–2. Level of significance was set to 0.05. Means and SD are reported.

RESULTS

There was a significant main effect for age on body weights, but there were no differences in body weights between the two rearing groups. The P41–2 age groups weighed more than the P21 age groups. The P21 dam-reared group had a mean body weight of 44.16 g (SD 5.23), and the P21 artificially reared group had a mean body weight of 40.24 g (SD 4.13). The P41–2 dam-reared group had a mean body weight of 148.80 g (SD 24.19), and the P41–2 artificially reared group had a mean body weight of 137.85 g (SD 25.39).

Contractile properties. There was a significant interaction effect for twitch tension (F = 15.880, P < 0.001; Fig. 1). Twitch tension increased with age for the dam-reared (P < 0.001) and artificially reared groups (P < 0.001). In addition, the dam-reared P41–2 group had greater twitch tension than the artificially reared P41–2 group (P = 0.003). There was a significant interaction effect for contraction time, also (F = 8.744, P = 0.005; Fig. 2). At P21, the dam-reared group had a shorter contraction time than the artificially reared group (P = 0.003). Compared with P41–2, the dam-reared and artificially reared groups had a longer contraction time at P21 (P = 0.001, P < 0.001, respectively). A significant interaction effect for half-relaxation time shows a similar pattern (F = 22.513, P < 0.001; Fig. 3). At P21, the dam-reared group had a shorter half-relaxation time compared with their artificially reared counterparts (P < 0.001). Compared with P41–2, the P21 dam-reared and artificially reared groups had longer half-relaxation times (P < 0.001 for both comparisons). There was a tendency for a significant interaction effect for maximum tetanic tension (F = 2.887, P = 0.098; Fig. 4). Maximum tetanic tension significantly increased with age (F = 43.201, P < 0.001) with the artificially reared groups having lower tetanic tension than the dam-reared groups (F = 5.270, P = 0.027). A significant interaction effect was also observed for fatigue index (F = 15.562, P < 0.001; Fig. 5). At P21, the dam-reared group was more fatigue resistant than the artificially reared group (P = 0.001). Across age, the artificially reared group became more fatigue resistant (P < 0.001). However, there was no change in fatigue resistance across age for the dam-reared group. A typical twitch tracing and the
initial and final tetanic tracings during the fatigue protocol are shown in Fig. 6.

**MHC isoform distribution at P21.** Although there were no significant differences in the distribution of MHC isoforms for the hyoglossus muscle between the rearing groups at P21, there was a tendency (although not significant) for the artificially reared group to have more neonatal MHC isoform ($P = 0.066$). The artificially reared group did have more neonatal MHC isoform for the styloglossus muscle ($F = 41.321, P < 0.001$; Fig. 7). Again, there was a tendency for the artificially reared group to have less MHC IIa than their dam-reared counterparts for the styloglossus muscle ($P = 0.084$). At P21, there were no significant differences between the groups for the MHC distribution in the long head of biceps brachii.

**MHC isoform distribution at P41–2.** At P41–2, there were no differences between rearing groups in terms of their MHC isoform distribution for the hyoglossus muscle. For the styloglossus muscle, the artificially reared group had more MHC IIa ($F = 11.194, P = 0.01$) and less MHC IIb ($F = 6.847, P = 0.031$) than their dam-reared counterparts (See Fig. 8). Furthermore, there was a tendency for the artificially reared group to have less MHC IIx ($P = 0.094$) than their dam-reared counterparts. For the long head of the biceps brachii, the artificially reared group had significantly less MHC IIb than their dam-reared counterparts ($F = 10.221, P = 0.013$; Fig. 9).

**DISCUSSION**

A variety of models of disuse or decreased activity have been employed to investigate their effects on slow-twitch and fast-twitch muscles. In general, the slow-twitch muscles will produce more dramatic changes due to disuse compared with the fast-twitch muscles. Obviously, the magnitude of these
changes will depend on the modality and duration used to impose the disuse or decreased activity.

In the present study, we sought to extend the artificial rearing time frame in an attempt to further build on our previous findings (20). This might help explain reports of slower eating in rats (15) or other developmental abnormalities that are seen in human infants necessarily deprived of suckling.

**Contractile properties.** There is ~26% change in contraction time and ~28% change in half-relaxation time between the dam-reared and artificially reared groups at P21. This is similar to percent changes in contraction time and half-relaxation time for extensor digitorum longus in neonatal animals recovering from sciatic nerve injury (14). In addition, our laboratory previously reported a prolonged half-relaxation time immediately after artificially rearing animals from P4 to P14 (20). Alterations in isometric twitch contraction and relaxation times may be related to calcium kinetics associated with the sarcoplasmic reticulum and not speed of shortening (19) or myosin ATPase activity (6). This pattern of prolonged contraction and half-relaxation times has also been observed in fast-twitch muscles involved in hindlimb immobilization in rat (14, 34), which is a more severe model of disuse. Interestingly, no changes in the twitch time course were observed in the rat extensor digitorum longus when using hindlimb suspension for 21 days (25). In the present study, the tongue was allowed to move freely and participate in all of its usual activities except suckling during postnatal days 3 to 21. A full recovery from this impaired isometric twitch time course occurred within 3 wk. Witzmann et al. (35) showed a full recovery for the isometric twitch time course after 6 wk of hindlimb immobilization for the extensor digitorum longus, which contains type IIA and IIB muscle fibers, and the superficial region of the vastus lateralis, which contains type IIB muscle fibers, within 14 and 7 days, respectively.

The lower twitch tension observed in P41–2 artificially reared animals, compared with their dam-reared counterparts, suggests an impaired rate of muscular development. There was ~61% change in the twitch tension of the tongue retrusor musculature from P21 to P41–2 in the dam-reared animals. For the artificially reared animals, there was only ~8% change from P21 to P41–2. Previously, we reported no change in isometric twitch tension between rearing groups at P14 or P42 (20). Therefore, it is readily apparent that the extended artificial rearing process dramatically impairs the age-associated rate of change in isometric twitch tension. If atrophy occurred, its effects were not seen in twitch tension until P41–2. Because the isometric twitch time course has recovered by P41–2, it seems unlikely that this reduced twitch tension is due to alterations in the calcium kinetics associated with the sarcoplasmic reticulum. Therefore, the impaired twitch tension at P41–2 in the artificially reared animals may be due to changes in the contractile proteins, the formation of active cross bridges, or altered myosin ATPase enzymatic activity. This is also the first report of physiological impairment of the hypoglossal motor system induced by the artificial rearing process that is present in early adulthood.

The reduction in maximum tetanic tension for artificially reared groups may be due to a decrease in the number of active interactions between actin and myosin (9). This reduction is probably related to smaller fiber area typically associated with disuse models. Elder and McComas (12) found lower twitch tension and maximum tetanic tension but no change in force per unit of cross-sectional area for the soleus and plantaris muscles in rats undergoing 14, 28, and 206 days of hindlimb suspension. Witzmann et al. (33) also reported a depressed maximum tetanic tension in the extensor digitorum longus of adult rat after 6 wk of hindlimb immobilization but did not measure relative changes in fiber area. Until now, we were unable to elicit a persistent impairment of maximum tetanic tension (20).

In the normally developing rat, the tongue retrusor musculature has its fatigue resistance determined by P21. The severe limitation of suckling experience, through the use of artificial rearing, decreases the fatigue resistance of these muscles. Metabolic changes associated with disuse have been well documented. Possible changes in oxidative capacity associated with inactivity (13) may explain the decreased fatigue resistance (7). Because our milk formula has evaporated cow’s milk...
as its base and rat’s milk contains various hormones, there is a possibility that our formula could alter normal macronutrient metabolism. However, Yeh and colleagues (36, 37) found no difference in growth hormone, corticosterone, and thyroxine concentrations between animals that were artificially fed cow’s milk and dam-reared rats at postnatal days 13 and 16. This suggests that our milk formula would not alter macronutrient metabolism.

**MHC isomorph distribution.** By the end of the first 30 days of postnatal life, the amount of neonatal MHC isoform has diminished, and the amount of adult MHC isoforms has increased with some variability between muscles in the rat. By P21, almost all of the neonatal MHC isoform was no longer present with subsequent increases in the adult MHC isoforms in the hyoglossus and styloglossus muscles in the rat. The artificial rearing process slows the decrease of the neonatal MHC isoform at P21 in styloglossus and possibly hyoglossus muscles. However, the order of MHC predominance may be changed in P21 animals with the dam-reared demonstrating an IIb > IIX > IIA > neonatal order, whereas the artificially reared animals demonstrate an IIB > IIX > neonatal > IIA order. It should be noted that the percent distribution of IIA and neonatal MHC isoforms are very similar in the artificially reared P21 animals. By P42, the hyoglossus muscle recovered from any possible disuse effects, suggesting that the possible elevated neonatal MHC isoform present at P21 has now diminished with a concomitant increase in the typical adult MHC isoforms that is consistent with the dam-reared hyoglossus muscles. In the styloglossus muscle, a possible decrease in MHC type IIA coincided with a significant increase in neonatal MHC isoform in the artificially reared group at P21. The order of MHC predominance was IIA > IIX > IIB > neonatal for the dam-reared group whereas the artificially reared group had an IIX > IIA > neonatal > IIB order of predominance. Previously, animals artificially reared from P4 to P14 demonstrated no significant immediate alterations in the MHC phenotype of the styloglossus muscle (20). The styloglossus muscle exhibited some changes in the MHC phenotype that persisted 3 wk beyond the artificial rearing process. The increased MHC IIA and decreased MHC IIB in our artificially reared animals at P41–2 is consistent with changes we previously reported in animals artificially reared from P4 to P14 (20). However, the tendency for a decrease in MHC IIX as well as more dramatic changes in the percent distribution of the MHC phenotype suggest that the extended artificial rearing time frame induced a more significant impairment of the tongue retractor musculature. No change in the percent distribution of the MHC phenotype between dam-reared and artificially reared animals at P21 for the long head of the biceps brachii suggests that the artificial rearing model did not have any immediate systemic effects due to undernourishment or stress. However, by P41–2 there was a significant decrease in MHC IIB in the artificially reared animals. Perhaps the reintegration of the artificially reared animals into a cage with other rats after prolonged social isolation produced some systemic effect. Open field tests on adolescent animals that were artificially reared have revealed that these animals display an increased activity pattern compared with dam-reared animals (22).

In conclusion, our results now show that extending the artificial rearing time frame resulted in significant alterations within the tongue retractor musculature. This new extended artificial rearing model is closer to the conditions faced by infants necessarily deprived of suckling. And the changes we observed were as severe as those seen with hindlimb immobilization and hindlimb suspension. In addition, our results underscore the importance of activity, especially suckling, in the normal development of the tongue retractor musculature. Because primates have a large percentage of type I fibers in the extrinsic tongue muscles (28) and the effects of disuse are more dramatic for these slow-twitch fibers, the results we report may be more dramatic for those infants that are necessarily deprived of suckling for extended periods during postnatal and perinatal development.

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**REFERENCES**


