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ACUTE AND CHRONIC EFFECTS OF ARTIFICIAL REARING ON RAT
GENIOGLOSSUS MUSCLE

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University

by

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ABSTRACT

ACUTE AND CHRONIC EFFECTS OF ARTIFICIAL REARING ON RAT
GENIOGLOSSUS MUSCLE

By W. Allen Moore, Jr., B.S., MPT

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2005

Major Director: Stephen J. Goldberg, Ph.D., Professor, Department of Anatomy
and Neurobiology

In most mammals, nutritive suckling is critical during the early neonatal period. The genioglossus (GG) muscle in rat plays an important role in protruding the tongue for efficient suckling. The purpose of this study was to examine the contractile properties and myosin heavy chain (MHC) phenotype of the GG following an early period of artificial rearing, which reduced nutritive suckling. Beginning at three days of age Sprague-Dawley rats were fed via implanted gastric cannula until postnatal day 14 (P14). At P14, artificially reared (AR) rat pups were either placed with a lactating dam until the end of the weaning period and allowed to mature until postnatal day 42 (P42), or anesthetized and prepared for physiological experimentation. GG contractile properties at P14 and P42 in AR and dam reared (DR) rats were obtained with a

force transducer and digital recording system through stimulation of the medial branch of the hypoglossal nerve. Following physiological experimentation, muscle samples were removed and stored for MHC analysis. Comparisons were made between AR and DR groups at P14 and P42. At P14 maximum tetanic tension and fatigue index were lower in the AR group than the DR group and no differences were found in MHC distribution. By day 42, AR rats had a higher fatigue index than DR rats and DR rats had a higher percentage of MHCIIa than AR rats. The artificial rearing technique employed in this study was adequate to produce chronic changes in fatigue resistance and MHC distribution in GG muscle. GG muscle of premature human infants requiring early artificial feedings may develop similar changes in their contractile characteristics and MHC phenotype.

Introduction

According to the National Center for Health Statistics pre-term births (births before the completion of the 37th gestational week) accounted for 12.1% of all live births in 2002, up from 11.9% in 2001, and a 29% increase since 1981 (Martin et al., 2003). The increase in pre-term birth rates is not surprising given recent advances in postnatal pre-term care and the rise in twin and triplet/+ birth rate (Martin et al., 2003).

Commonly, pre-term infants are admitted into the neonatal intensive care unit (NICU) until they achieve independent respiratory stability, steady weight gain and are able to manage food from a bottle or nipple; of these tasks, bottle or nipple feeding is considered the most complex (McGrath and Braescu, 2004). Medoff-Cooper et al. (2002) found that gestational age at birth was an important factor in development of oral feeding skills. They found that infants born at 24-29 weeks gestational age took the longest to acquire the necessary feeding skills required to leave the NICU. Additionally, otherwise healthy pre-term infants requiring early nasogastric (NG) tube feeds were found to exhibit delayed feeding behaviors at 11-17 months compared to their age matched full-term counterparts (Dodrill et al., 2004).

In addition to feeding problems associated with pre-term birth, clinicians and researchers are noticing speech and/or language deficits into childhood and beyond in pre-term infants (de Kleine et al., 2003). Jennische and Sedin (2001, 1999 [2], and 1998) found that at 6.5 years of age pre-term children that required NICU placement had more oral motor function deficits and poorer linguistic skills than children born at term who did not require NICU placement. Another study found that at 5 years of age neurologically normal pre-term infants born before 32 weeks gestational age still had language production problems (Luoma et al., 1998). Furthermore, parents of research participants reported that pre-term and full term NICU graduates reached language milestones, such as intelligible speech, later than controls of the same age, and that several of the children born at 23-27 weeks gestation never babbled (Jennisch and Sedin, 1999).

The mammalian tongue plays a vital role in feeding from the first hours of life and later in communication. Humans are able to produce vowel and consonant sounds thanks to intricate interactions between the tongue, lips, pharynx and other oral cavity structures (MacNeilage & Davis, 2000, and Hiemae & Palmer, 2003). This body of research will focus on the development of the genioglossus muscle. The genioglossus is the lone extrinsic protrusor muscle of the tongue and plays a vital role in depression of the tongue during respiration and shaping the tongue during feeding and speech activities. The importance of this ground work investigation into the development of the genioglossus becomes apparent when one considers the increasing rate of pre-

term infants and the combined costs of treating them in the NICU and later through childhood and into adolescence. The aim, at this point, is not to find a cure for feeding and speech disorders associated with pre-term birth, but to enhance our understanding of the tongue's role in this complex system so that one day clinicians can recognize and manage these patients more competently.

Literature Review

Skeletal Muscle

Anatomy

Skeletal muscles are composed of numerous densely packed, elongated, multinucleated cells known as muscle fibers or myofibers. A single myofiber consists of bundles of myofibrils which, when viewed longitudinally through a microscope, display alternating light and dark striations along its length; the light striations are known as I-bands and the dark striations are known as A-bands. Dividing each I-band in half is a Z-disc, and the area from one Z-disc to the next Z-disc is known as a sarcomere. The myofibril and its sarcomeres represent the smallest functional contractile unit in skeletal muscle (McComas, 1996).

The striations seen on myofibrils are due to the regular patterned appearance of two protein filaments: actin and myosin. Actin is the thinner of the two protein filaments and is present in the I-band. Myosin is thicker than actin and is entirely present in the A-band although, actin is also present in all but the center region of the A-band when muscle tissue is relaxed. It is the interaction between actin and myosin within the A-band that is responsible for muscle contraction (McComas, 1996).

In muscle actin is present as a double helix that associates with the two regulatory proteins, troponin and tropomyosin. Myosin is a large protein complex consisting of 2 heavy chains and 2 pairs of light chains. Together, the heavy and the light chains make up a two headed structure with a single long tail. The two heavy chains wrap together to form the tail at one end and divide at the other end to form the two heads. The light chains form part of the heads and play a regulatory role. Myosin heavy chains (MHC) are of particular interest because: 1) they exist in several different structural forms (isoforms) and; 2) they play a significant role in contraction of skeletal muscle (Keynes and Aidley, 2001). The variety of structural myosin isoforms provides the bases for differences in contractile properties among muscles and even within the same muscle.

Contractile Properties

Scientists may use a variety of tools to classify muscles or motor units (a motoneuron and the muscle fibers it innervates) as fast or slow, or fatigable or non-fatigable, for example (Burke, 1981). One of the most direct tools available is the in-situ measurement of specific contractile characteristics when the muscle is under the influence of electrical current applied to a peripheral nerve. On a molecular level, contractile characteristics can be attributed to the specific protein content of the myofibril, enzyme kinetics, anatomical arrangement, and/or muscle preloading (McComas, 1996). The rest of this section will be devoted to defining each of the contractile characteristics measured in the present study.

Twitch tension is the change in amplitude from baseline to peak in response to a single stimulus pulse. Maximum tetanic tension is the change in amplitude from baseline to peak in response to a high frequency train of stimulus pulses; both twitch tension and maximum tetanic tension are measured in grams. Twitch contraction time measures the amount of time, in milliseconds (ms), from the onset of muscle twitch to peak amplitude. Half decay time is the time from peak amplitude until twitch force has decreased by 50%, measured in milliseconds. Fusion frequency (Hz) is the lowest stimulation rate in which individual twitches can no longer be seen. And, fatigue index (FI) is the fractional difference between the first train and the last train in a two minute period of 500 ms/second trains at a given frequency.

Muscle Fiber Types

Myosin heavy chain composition is the most important determinant of contractile performance in skeletal muscle, so although many of the myofibrillar proteins exist in more than one isoform, it makes the most sense to classify skeletal muscle based in its myosin heavy chain complement (Schiaffino & Reggiani, 1994) . Mammalian skeletal muscles are made up of at least 11 different myosin heavy chain isoforms. Some isoforms occur only at certain developmental stages, while others are muscle specific, or even species specific (Pette and Staron, 2000). For added diversity, many muscle fibers exist as hybrids, meaning they consist of more than one myosin heavy chain isoform. It

is this complex organization that allows for the versatility seen among the different muscles.

In general, there are three ways to determine which fiber type(s) are present in a given muscle sample: 1) histochemical staining for myosin ATPase; 2) myosin heavy chain isoform identification through a) immunohistochemical analysis with antimyosin antibodies or b) separation by molecular weight via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); and 3) identification of characteristic metabolic enzymes (Scott et al., 2001 and Pette & Staron, 2000). While several adult isoforms have been identified through these methods, most isoforms are relatively rare, only existing in a few muscles or only at very specific times during development. Four fiber types are widespread in most mammals and warrant discussion at this point. Using histochemical staining, fiber types I, IIA, IID/X and IIB can be delineated in most adult skeletal muscle. These fiber types correspond to the following MHC isoforms (listed in order of contraction speed slowest to fastest): MHCI, MHCIIa, MHCII d/x and MHCIIb. As mentioned earlier, a fiber can exist in a pure form or as a hybrid. Hybrids are most accurately identified by single fiber analysis using the SDS-PAGE method. When hybrids do exist they are typically composed of fibers next to each other on the contraction speed continuum above, for instance, fiber types I and IIA or IIA and IID/X (Pette & Staron, 2000 and Smith et al., 2005).

Several authors have devised techniques to determine how the different fiber types contribute to the contractile characteristics of whole muscle. Sweeney

et al. (1986) forced the conversion of type IIb fibers to type IIa fibers in rabbit anterior tibialis muscle through a prolonged electrical stimulation paradigm. The authors found that type IIb fibers shortened at a greater velocity than type IIa fibers. In a 1985 study, Reiser et al. examined contractile properties of psoas muscle in rabbit at different stages of development and discovered that as fiber types changed from slow types to fast types, shortening velocities increased (Reiser et al., 1985). Using skinned muscle fibers Bottinelli et al. (1991) and Galler et al. (1994) were able to make direct contractile force and speed measurements in skinned muscle fibers of rat. Both groups found that contraction speed was greater in type II fibers compared to type I fibers, but that there was overlap between the various type II fibers. Additionally, Bottinelli et al. showed that type II fibers had a higher maximal power output than type I fibers when cross-sectional area was the same (Bottinelli et al., 1991).

Development

This review will briefly discuss muscle formation and then discuss fiber ontology and fiber transitions in more detail. For a more thorough discussion of muscle formation see McComas (1996). Muscle formation occurs in five steps. The first step begins in the multicellular blastula where the endodermal mass signals adjacent ectodermal cells to develop into mesoderm. In step two, after the blastula expands and invaginates (at this point it is called a gastrula), dorsal mesoderm divides into blocks of tissue called somites; all skeletal muscle of the trunk and limbs is eventually derived from somites (McComas 1996) (somites

that form tongue musculature migrate from the trunk during development) (Borue and Noden, 2004). In the third step, somites proliferate and myogenic regulatory factors transform somites into myoblasts, which continue to proliferate. The myoblasts are uninucleated, spindle shaped cells with an enlarged nucleolus (indicates active RNS synthesis). The myoblasts continue to divide by mitoses until they are signaled to fuse into myotubes at about the seventh gestational week. As more myoblasts fuse with the myotube their nuclei begin to line up to form a chain, cytoplasmic volume increases and polysomes synthesize actin and myosin. Actin and myosin are grouped together and myofibrils begin to form; A and I-bands can be distinguished. Protein synthesis is robust and the myotube enlarges while the sarcoplasmic reticulum and T-tubules become increasingly organized. By the end of the third step, the myotube is a muscle fiber. The fourth step is actually less about muscle development and more about the embryo changing shape. During this step, major body features begin to appear, such as limb buds. The final step occurs once limb buds are present and dorsal and ventral muscle masses appear on the limb buds. The muscle masses are cleaved several times and individual muscles begin to become organized within a connective tissue framework. Soon muscles organize into fascicles and tendons form at each end of the muscle (McComas, 1996).

At birth mammalian muscle is composed primarily of embryonic (MHCemb) and neonatal (MHCneo) myosin heavy chain isoforms. These so called developmental isoforms transform to adult isoforms as the animal matures

(Smith et al., 2005, Agbulut et al., 2003, Geiger et al., 2001 and Bulter-Browne & Whalen, 1984). Progression from developmental isoforms to adult isoforms varies widely depending on the particular muscle and animal analyzed. Bulter-Browne and Whalen (1984) analyzed rat soleus muscle and found that two groups of fibers, based on fiber diameter and each making up about half the fibers in their sample, matured at different rates. The group consisting of the larger fibers contained MHCemb and slow fiber types at one week, but by two weeks consisted only of slow fibers. The other group, consisting of smaller fibers, was made up of MHCemb and MHCneo fibers at one week, and by week 2 or 3 contained some fast fiber types. At around week four this group started to undergo transition from fast fiber types to slow fiber types (Bulter-Browne and Whalen, 1984). By 10 weeks of age, the fiber type composition of soleus resembled its adult form (Bulter-Browne & Whalen, 1984 and Asmussen & Soukup, 1991). Using human diaphragm and genioglossus muscle Lloyd et al. (1996) compared the fiber phenotypes at 16-24 weeks gestational age and at term (40-42 weeks). At 16-24 weeks genioglossus and diaphragm displayed very similar phenotypes, both were almost entirely composed of developmental isoforms. At term, the diaphragm was made up of 15% MHCemb/neo, 32% MHCI, 47% MHCIIA and 6% MCHIIIB compared to genioglossus which was almost entirely MHCIIA. Agbulut et al. (2003) examined fiber type in several muscles in mouse from postnatal day 3 to postnatal day 49. In short, the authors found that postnatal muscle development features two phases of fiber

expression. In phase one, developmental fiber types are down-regulated and eventually eliminated. In phase two, adult fiber types are up-regulated and the mature adult fiber phenotype is realized. The first phase for muscles that are destined to be a slow phenotype takes longer than for muscles destined to be a fast phenotype, although typically less than a week of difference exists between the two groups. Phase 2 varies more widely, once again depending on eventual phenotype, with the fast phenotypes fully maturing to adult form before the slow phenotypes. It has been hypothesized that this disparity between the slow and fast phenotypes may be at least partly due to the slow elimination of poly-neuronal innervation in slow twitch muscles (Agbulut et al., 2003). In summary, normal phenotype transitions begin to take place immediately after birth. These transitions occur at different rates depending on the eventual phenotype and function of the muscle. Multiple factors contribute to these changes including functional demands, innervation, ionic fluxes, aging, and hormonal signals (Agbulut et al., 2003, Pette & Staron, 2001, and Baldwin & Haddad, 2001).

Mutability

Many other factors besides those associated with normal development may cause changes in skeletal muscle; muscle phenotype and ultrastructure may also change based on training protocols and mechanical loading and/or unloading paradigms (altered activity). Several altered activity models intended to induce changes in phenotype and ultrastructure exist in the literature, including muscle unloading by hindlimb suspension (Riley et al., 1990, Asmussen &

Soukup, 1991, McDonald et al., 1994, Huckstorf et al., 2000, Elder & McComas, 1987 and Ohira et al., 2001), casting (D'Antona et al., 2003), altered gravity (Picquet et al., 2005, Adams et al., 2000, Adams et al., 2000, Fitts et al., 2001), and disuse/reduced use models involving masticatory musculature where animals are fed a soft oral diet or fed via gastric cannula directly into the stomach (Kiliaridis et al., 1988, Maeda et al., 1987, Kinirons et al., 2003). Models also exist where muscles have been loaded more than usual (Goldspink, 1999). The effects these models can have on neonatal skeletal muscle are profound.

It seems that the most common altered activity studies involve hindlimb suspension in rats to study the predominately slow-twitch soleus. Studies by Riley et al. (1990), Assmusen and Soukup (1991) and McDonald et al. (1994) found that the soleus muscle atrophied significantly within a range of 4 days to 3 weeks in adult rats, with the greatest atrophy occurring in type I fibers. In addition, McDonald et al. reported that the percentage of type I fibers decreased from 82%-74% following just one week of hindlimb suspension in adult rats, whereas type II fibers increased from 8% in control animals to 26% following 3 weeks of hindlimb suspension (McDonald et al., 1994). In contrast, Assmusen and Soukup found no changes in phenotype following four to six weeks of hindlimb suspension in adult rats (Assmusen and Soukup, 1991). In their 1991 study, Assmusen and Soukup also examined the relatively fast-twitch extensor digitorum longus (EDL) in hindlimb suspended adult rats and found that the EDL did not atrophy following 3 weeks of hindlimb suspension. These authors

concluded that atrophy in the soleus involves mainly type I fibers (Asmussen and Soukup, 1991).

Altered activity via hindlimb suspension has also been utilized in developmental studies using rat pups as young as four days old. The studies reviewed all agree that fiber growth and changes in soleus phenotype are retarded under the influence of hindlimb suspension (Huckstorf et al., 2000, Elder & McComas, 1987, Ohira et al., 2001 and Asmussen & Soukup, 1991).

Huckstorf et al. (2000) found delayed expression of type I fibers and less than expected muscle fiber hypertrophy following only nine days of altered activity in soleus starting at postnatal day 8, while Ohira et al. (2001) reported that soleus muscle phenotype recovered fully upon one month resumption of activity in animals under the influence of hindlimb suspension for 20 hours per day from postnatal day 4 to day 21. While soleus is usually the target of the hindlimb suspension studies, several of the studies above also looked at either plantaris or EDL. In contrast to soleus, these two muscles have a relatively high ratio of fast fibers compared to slow fibers and are generally not thought to be major weight bearing muscles. Most of the authors concluded that these two muscles, as a result of their phenotype and function, change very little in terms of fiber size and/or phenotype composition compared to soleus as a result of altered activity (Asmussen & Soukup, 1991, Elder & McComas, 1987 and Huckstorf et al., 2000).

While it seems that hindlimb suspension is the most frequently reported method of altered activity in the literature, other methods have provided valuable insights. Adams et al. (2000) and, by way of review, Fitts et al. (2001) detail skeletal muscle changes that take place in microgravity. Fitts et al. reports that rats undergo a 37% loss in muscle mass, slow twitch fibers more than fast twitch fibers, after as little as a week in space. Adams et al. (2000) found that 16 days of space flight led to reduced expression of MHC I and increased expression of MHC IIx and MHC IIb in neonatal rats. In another altered activity study Kinirons et al. (2003) studied the effects of feeding 4 to 14 day old neonatal rats via gastric cannula, bypassing the mouth and tongue all together. They found that the styloglossus, a tongue retractor, displayed an abnormal ratio of MHC IIa to MHC IIb following a one month resumption of normal eating activity.

In summary, most muscle fibers transition from slow to fast or fast to slow depending on external influences, hormonal inputs, age and innervation (Pette & Staron, 2001). However, by altering normal activity scientists have learned that fiber transitions can be halted in certain muscles at specific developmental stages. Although slow twitch muscles seem the most affected by such alteration, Kinirons et al. (2003) did show that long term phenotype changes may occur in the fast twitch styloglossus as well.

The Tongue

In essence, the mammalian tongue is a group of muscles that play an important role in feeding, communication, and respiration. In respect to feeding, the tongue is active in sucking, ingestion by licking and lapping, and moving food for chewing and swallowing (Hiemae & Palmer, 2003 and Lowe, 1981). In human speech, the tongue changes position and shape to aid in the formation of complex sounds, and in respiration the tongue moves rhythmically within the oral cavity to change the shape and dimensions of the upper airway (Hiemae & Palmer, 2003).

Anatomy

Morphologically it is convenient to divide the musculature making up the tongue into two groups: extrinsic muscles and intrinsic muscles. Extrinsic muscles have a boney origin and insert on the body of the tongue, while intrinsic muscles originate and insert entirely within the body of the tongue (Lowe, 1981). The genioglossus (GG), styloglossus(SG), and hyoglossus (HG) make up the group of hypoglossal innervated extrinsic muscles. The GG originates from the genial tubercle on the mandible and angles superiorly to its broad insertion on the ventromedial surface of the tongue. The SG originates on the styloid process of the temporal bone and angles inferiorly on its way to its insertion along the ventrolateral surface of the base of the tongue. The HG originates from the hyoid bone and angles superiorly toward its insertion on the superior-lateral surface of the base of the tongue (McClung & Goldberg, 2000). The intrinsic muscle group

consists of the vertical, transverse, and longitudinal linguae muscles. The intrinsic muscles are named for their orientation along the body of the tongue rather than their attachment points.

Actions

Grossly, GG protrudes the tongue and SG and HG retract the tongue; in addition, GG and HG depress the base of the tongue and SG elevates the base of the tongue (McClung and Goldberg, 2000). Finer movements of the tongue and movement of the tip of the tongue are managed by the intrinsic muscles via a process described by Kier and Smith in their description of muscular-hydrostats (Kier & Smith, 1985 and McClung & Goldberg, 2000). Briefly, the tongue can be described as a cylinder with a constant volume so that a decrease in any one dimension of the cylinder will result in an increase in a different dimension (Kier & Smith, 1985). For example, contraction of the vertical and transverse lingual muscles will decrease the diameter of the tongue, and as a result the tongue elongates or protrudes. In contrast, contraction of the longitudinal lingual muscles results in shortening and thickening of the body of the tongue and retraction (McClung & Goldberg, 2000).

In reviewing the literature, it is frequently written that “the tongue is a complex structure”. While this is true, it is like saying Mouton-Rothschild’s cabernet sauvignon is merely a good wine; there is more to the story. The tongue plays critical roles in feeding, speech and respiration all of which require very precise movements not easily explained by its observable anatomy,

therefore, the tongue is very complex both structurally and functionally. Like the flavors of a fine wine, the intrinsic and extrinsic muscles must do more than just work to produce individual actions, they must somehow work together as a single unit with integrated actions. Indeed, this is the case in retraction of the tongue, for instance. Smith reported in 1989 that the fibers from the inferior longitudinal muscles and the SG interdigitate within the body of the tongue so that the two muscles may work in series to produce retraction of the tongue (McClung & Goldberg, 2000).

Innervation

Even though other muscles indirectly move the tongue, the muscles discussed in the preceding paragraphs are the primary tongue muscles and are all innervated by motoneurons located in the hypoglossal nucleus. The axons of these motoneurons make up the hypoglossal nerve (cranial nerve XII) which divides about the level of the hyoid bone into its medial and lateral branches. Two subdivisions of the hypoglossal nucleus, ventral and dorsal, have been identified by retrograde labeling studies in rat. Conveniently, the subdivisions are functionally organized such that the dorsal subdivision contributes motoneurons to the lateral branch of the hypoglossal nerve and thus innervates the retractor muscles (SG, HG and longitudinal linguae), and the ventral subdivision contributes motoneurons to the medial branch and innervates the protrusor musculature (GG, transverse linguae and vertical linguae) (McClung & Goldberg, 2000 and Aldes, 1995) .

Adult and Infant MHC Isoforms

The adult rat tongue is made up of four major myosin heavy chain isoforms (MHC): MHCIIb, MHCII d/x, MHCIIa, and MHCI, and their corresponding fiber types: IIB, IID/X, IIA, I. A review of the literature reveals that fiber type distributions vary from one study to the next independent of which muscle is examined, but all reviewed studies agree that the fast fiber types are predominate. In an immunohistochemistry analysis of one adult rat, Sutlive et al. (2000) found that the GG consists of 45% MHCIIA, 28% MHCII B, and 27% MHCII X fibers. In a different rat Sutlive et al. (1999) determined that the SG was composed of 67% MHCIIA, 20% MHCII X, 12% MHCII B and less than 1% slow fibers. In another study, Brozanski et al. (1993) used gel electrophoresis to analyze adult rat GG myosin isoform and found that 45% is MHCII b, 35% is MHCII x/d, 19% is MHCII a with no measurable amounts of any slow isoform. In other adult rat studies by LaFramboise et al. (1992), Vincent et al. (2002), Cobos et al., (2001) and Bar et al. (1988) there is a consensus that the fast MHC isoforms are the most prevalent, but there is no consensus on the order of prevalence (see table 1). In adult human and primate studies, DePaul et al. (1996) and Stål et al. (2003) examined adult intrinsic and found that MHCIIA fiber type was often the dominant type followed by MHCI fibers. Also in humans, it has been reported that the adult GG consists of different percentages of type I and II fibers at the tip of the tongue versus the posterior portion of the tongue; at

the tip 31.3% of the fibers are type I while 68.7% are type II, at the posterior 51% are type I and 49% are type II fibers (Saigusa et al., 2001).

Study of muscle fiber types in neonatal tongue musculature has been studied less frequently. Brozanski et al. (1993) found that at postnatal day four rat GG is composed of only developmental fiber types MHCneonatal (MHCneo) and MHCembryonic (MHC emb) with MHCneo persisting to postnatal day 25 and the muscle achieving its adult MHC isoform composition at postnatal day 30. Kinirons et al. (2003) found that 14 day old rat pup SG muscle consisted of MHCIIa > MHCneo > MHCIIx/d > MHCIIb. At postnatal day 42 MHCneo was no longer present and isoform composition was: MHCIIx/d > MHCIIa > MHCIIb. In addition, artificially reared rats (rats reared without a dam present, usually by feeding a liquid diet through a feeding line that inserts directly into the rat's stomach) from postnatal day 4 to 14 had the long term affect (at postnatal day 42) of increasing MHCIIa and decreasing MHCIIb (Kinirons et al., 2003).

Contractile Properties

Measuring contractile properties in tongue musculature has a rather short history. The intrinsic muscles of cat tongue were first assessed in 1981 by Hellstrand and rat tongue by Gilliam and Goldberg in 1995. The 1995 Gilliam and Goldberg study confirmed previous anatomical studies in rat concluding that the medial branch of the hypoglossal nerve drives tongue protrusion and the lateral branch of the hypoglossal nerve drives tongue retraction. Other whole nerve and motor unit studies by Goldberg and colleagues measured single twitch

force, tetanic tension, fusion frequency, contraction time, half decay time and fatigue index in adult rat tongue extrinsic musculature. In general, Goldberg and colleagues showed that tongue retractor and protrusor musculature in normally reared rats was somewhat resistant to fatigue ($FI \geq 0.67$), and had fast contraction times of between 10.94 – 15.68 milliseconds (Kinirons et al., 2003 and Gilliam & Goldberg, 1995). See table 2. A study in 2000 by Sokoloff (2000) was able to ascertain, through examination of single motor units, that the intrinsic longitudinal linguae muscles had similarly fast contraction times and fatigue resistance. In 2003 Kinirons et al. looked at the effects of artificial rearing on contractile properties and concluded that artificial rearing had only short term effects on half decay time, fusion frequency, and fatigue index in 14 day old rat pup retractor musculature. She further demonstrated that contraction time and half decay time decreased as rats aged from 14 to 42 days in both artificial reared and dam reared populations (Kinirons et al., 2003).

Artificial Rearing

Artificial rearing (AR) refers to the care and maintenance of neonatal animals during the pre-weaning period. AR of rats has been around since the mid 1940's when Gustafsson hand fed rat pups a sterilized diet in order to create a germfree colony of rats (Messer et al., 1969 and Pleasants, 1956). The method most commonly used today was originally devised by Messer et al. in the late 1960's to help scientists study the effects of nutritional and social deprivation

(Messer et al., 1969); it was extensively modified by Hall in the mid 1970's (Hall, 1975). Briefly, AR consists of 2 primary steps: 1) placement of a small intragastric cannula in order to feed the rat pup, and 2) maintenance of the rat pup during the pre-weaning period (Patel & Hiremagalur, 1991). A detailed explanation of the artificial rearing technique implemented in this study can be found in the methods section.

A variety of artificial formulas may be made to sustain rats during the AR period. Rat pups in this study received a variation of a formula that was originally created by Messer et al. in 1969 (Messer et al., 1969). The final version of the formula is the result of two modifications in the early and mid 1980's that improved its nutrient content so that the diet is similar to that of rat dam milk (Diaz et al., 1982 and West et al., 1984). This formula closely, but not perfectly, matches the micronutrient and macronutrient content of dam milk and is commonly used today in a variety of behavioral (Lovic & Flemming, 2004 and Gonzalez et al., 2001) and drug studies (Slawecki et al., 2004 and Chen et al., 1999). The modified Messer diet was selected for this study because of the reported low incidence of abnormal abdominal distension and the near normal weight gain of rat pups on the diet (West et al., 1984 and Smart et al., 1984).

Three potential concerns regarding artificial rearing deserve mention: 1) body and organ overgrowth or undergrowth, 2) abnormal brain size and development, and 3) abnormal abdominal distention or "bloating". To this author's knowledge, artificial rearing with all artificial diets has been found to

cause abnormal body and organ growth (Diaz et al., 1982, West et al., 1984, Kinirons et al., 2003, Smart et al., 1984, Smart et al., 1987, Smart et al., 1983, Moore et al., 1990, Dvorak et al., 2000 and Tonkiss et al., 1987). In addition, rat pups artificially reared on dam milk displayed differences in organ and/or body size following 8 and 16 days of artificial rearing on dam milk (Tonkiss et al., 1987). When measured, brains of rat pups raised on artificial formula via AR tended to be of abnormal size and/or development compared to dam reared pups, regardless of which artificial formula was selected (Smart et al., 1984, Diaz et al., 1982, Smart et al., 1983, Moore et al., 1990 and Lasiter & Diaz, 1992). Additionally, Moore et al. (1990) found that artificially reared rat pups raised on dam milk showed significant differences in brain size compared to dam reared controls, and that brainstem size did not differ among pups artificially reared on the modified Messer diet or dam milk. Bloat has been observed with the use of all artificial formulas (Kinirons et al., 2003, Patel & Hiremagalur, 1991, Smart et al., 1984 and Diaz et al., 1982), and even with the long term use of dam milk, but is relatively rare in dam reared pups (Tonkiss et al., 1987). As in the human infant population, feed volumes and frequency must be adjusted when bloating occurs to allow for its dissipation. From the studies reviewed, it is not entirely clear if body, organ and brain size abnormalities and bloating are caused by the formula or by the AR process itself.

Clinical Relevance

Why is a thorough examination of the tongue clinically important? The tongue plays a significant role throughout life in mammals, and is especially important during infancy as a means of obtaining nourishment from a nipple. Healthy full-term infants suckle soon after birth in a rhythmic 2 phase pattern of expression and suction. During the expression phase, the infant uses his tongue to press the nipple against the hard palate so that milk is ejected into the mouth, suction then creates negative pressure to further draw milk into the oral cavity (Lau et al., 2000 and Lau & Schanler, 1996). In healthy full-term infants sucking occurs in coordination with swallowing and breathing processes (Lau et al., 2003, Glass & Wolf, 1994, Lau & Schanler, 1996 and Ross & Browne, 2000). In preterm infants these important skills are often not developed enough for these infants to successfully feed by mouth at birth, and are frequently still not mature when infants are reassessed at 36-40 weeks gestational age (a mature sucking pattern is typically present at 34 weeks gestational age (Lau & Schanler, 1996)) (Hawdon et al., 2000). To complicate matters more, infants requiring respiratory support, infants born with heart defects and/or gastro-intestinal abnormalities may require intravenous or tube feedings throughout early infancy (Jones et al., 2002 and Dodrill et al., 2004). Many of these infants miss a critical period in which it is thought that infants learn to suckle and coordinate sucking with swallowing and breathing (Jones et al., 2002). Once the critical period is missed, acquisition of the skill is more difficult and may not ever be fully mastered

(Rommel et al., 2003 and Illingworth & Lister, 1964). Furthermore, these children may develop oral aversions as a result of prolonged naso-gastric tube placement and/or they may not associate gastric fullness with eating (Jones et al., 2002 and Dodrill et al., 2004).

These infants, having never fully mastered the skills required to eat from a nipple or having mastered these skills after the typical period of attainment, may face delays in normal speech acquisition. According to hypothesis's set forth by Netsell as reviewed by Lau & Schanler (1996) and MacNeilage and Davis (MacNeilage & Davis, 2000 and McNeilage, 1998), early lip, jaw, and tongue movements associated with eating are necessary for the normal development of speech. MacNeilage and Davis hypothesize that the evolutionary and ontological development of speech relies on the ability to properly position oral structures initially learned with eating (MacNeilage & Davis, 2000 and McNeilage, 1998); it is then reasonable to assume that speech may be delayed in infants that acquired eating skills late. Indeed, Jennische and Sedin found that several aspects of speech and language were delayed in 6 1/2 year old preterm children compared to their full-term cohorts (Jennische & Sedin, 1999 and Jennische & Sedin, 1999), while Largo et al. (1990) found that a greater frequency of preterm children stuttered at 9 years of age when compared to healthy full-term children. The consequences of early speech deficits and preterm birth are serious and costly when one comes to realize that such problems are associated with later

reading impairments (Byrne et al., 1993 and Luoma et al., 1998) and failure in school (de Kleine et al., 2003).

Advances in neonatal care over the last several decades have resulted in increased survival rates for preterm infants (de Kleine et al., 2003). The costs associated with the treatment of preterm infants may be significant, however. Preterm infants are at greater risk than their full-term counterparts for developing feeding and nutritional problems (Ross & Browne, 2000 and Dodrill et al., 2004) that may last into the second year of life in otherwise healthy preterm infants (Dodrill et al., 2004). The consequences of feeding problems are profound and may lead to failure to thrive, malnutrition, and behavior problems (Jones et al., 2002). In total, treatment for oral motor disorders may be required into late childhood, therefore, early recognition and appropriate treatment is critical for this population.

This study takes a necessary step in shaping our understanding of oral motor development. A detailed examination of myosin heavy chain isoform content and muscle contractile properties is central to this study. Having knowledge of the deficits displayed by the genioglossus of tube fed animals is an important early step that will allow researchers and clinicians to more precisely focus their attention on developing treatments and therapies for this growing population of infants.

*Table 1: Fiber type distribution in rat tongue.

Muscle	Frequency of Fiber Type	Age	Reference
Genioglossus	IIb > II d/x > IIa > I	Adult	LaFramboise et al. 1992
Genioglossus	IIb (45%) > II d/x (35%) > IIa (19%)	Adult	Brozanski et al. 1993
Genioglossus	IIa (45%) > II b (28%) > II d/x (27%)	Adult	Sutlive et al. 2000
Genioglossus	II d/x > IIa > IIb	Adult	Vincent et al. 2002
Styloglossus	II d/x > IIa > IIb	42 d	Kinirons et al. 2003
Styloglossus	IIa (67%) > II d/x (20%) > IIb (12%) > I (1%)	Adult	Sutlive et al. 2000
Tongue Muscle	IIa (72%) > IIb (20%) > II d/x (8%)	Adult	Bär et al. 1988

*Adopted from Smith et al. 2005

Table 2: Summary of tongue contractile properties in adult rats.

Muscle	Twitch force (g)	Max tension (g)	Contraction time (ms)	½ relaxation time (ms)	Fusion frequency (Hz)	FI	Ref
Whole tongue	11.25		15.68	27.78	80	0.67	Gilliam & Goldberg 1995
retractors	12.02		13.36	20.44	90	0.67	Gilliam & Goldberg 1995
protrusors	1.05	2.15	10.94	18.17		0.76	Gilliam & Goldberg 1995
SG	3.3	19.66	13.81	10.82	109	0.76	Sutlive et al. 1999
longitudinal linguae*	0.035	0.095	15			0.78 to 1.31	Sokoloff 2000
GG	7.02	37.22	14.22	11.68	104	0.72	Sutlive et al. 2000
retractors	11.44	52.84	12.16	12.00	130	0.81	Kinirons et al. 2003

* motor units

Methods

Animals and Design

Three to four pregnant Sprague Dawley rats (Harlan Sprague Dawley, Madison, WI, USA) arrive at VCU's animal care facility during the mid-gestational period. Upon arrival at VCU, rats are placed and maintained in plastic animal cages in light, temperature and humidity controlled rooms with commercial rat chow and water provided ad libitum. Pregnant dams are closely monitored, to ensure accurate delivery date and time, from 2 days prior to the expected delivery date until all rat pups are delivered.

Immediately prior to cannulation, rat pups from any litter are placed into one of two groups: 1) dam reared controls; or 2) artificially reared from postnatal day 3 to 14. On the 14th postnatal day rat pups from each of the two groups are divided into 2 additional subgroups: 1) the short-term subgroup immediately undergoes physiological and anatomical experimentation; 2) animals from the long-term group are dam reared until approximately postnatal day 21, weaned from the dam, and undergo physiological and anatomical experimentation on day 42. Post-natal day 3 was chosen because we can successfully raise most of the rats starting at that time. Postnatal day 14 was selected because rats are large enough to measure physiological parameters and because this is during an

important period of neural development (Nunez, 1994). Postnatal day 42 was selected because the rats are nearly mature having gone through the human equivalent of adolescence (Seminar, January 27 2005, VCU, S.B. Churn).

Overview of Artificial Rearing

Artificial rearing may be divided into 3 distinct tasks: 1) preparation; 2) cannulation; 3) rearing. As overview, animals are taken from the dam on post-partum day 3 and immediately have cannulas placed into their stomachs. Once recovered from the cannulation procedure, rats are housed in Styrofoam cups with bedding that float on a temperature controlled water bath. A feed line of a slightly larger diameter than the cannula is attached to the cannula and pups are fed refrigerated formula directly into their stomachs. Up to 20 pups can be maintained in this way for the entire artificial rearing period.

Preparation for Cannulation

Materials:

Prior to the cannulation procedure, cannulas are made from a 25 cm length of polyethylene tubing with an inside diameter of 0.28 mm (PE-10) and a 1 cm in diameter disc of polyethylene, known as a flange. One end of the 25 cm piece of PE-10 is melted over a Bunsen burner and flattened while maintaining the opening. Once the PE-10 tubing cools, the flange is placed over the unflared end of the PE-10 tubing and is slid down to the flared end of the PE-10 tubing where it is prevented from sliding off the PE-10 by the flared portion of the tube.

The flange is made by punching a hole from a sheet of polyethylene using a standard size paper punch. This flared and flanged end will serve to make a tight and durable seal inside the rat pup's stomach. The cannula is completed at this point.

During routine handling, growth and movement of active pups, the cannula can become dislodged from the stomach. To help prevent this, small washers are friction fitted onto the cannula to position and maintain slack in the cannula. Washers are made by melting and flattening one end of polyethylene tubing with an inside diameter of 0.58 mm (PE-50). Once cut to a length of 2-3 mm, these washers are stored in a sterile Petri dish until they are to be used during the cannulation procedure. These washers fit snugly on PE-10 tubing.

After cannulation, the pups are floated in a warm water bath in 16 ounce Styrofoam beverage containers with matching lids possessing several holes to provide for adequate ventilation. A twenty inch piece of 20 gauge copper wire is thread from small holes near the top of the container on both sides, to the bottom of the container where the copper wire is used to position a weighted rubber stopper. The copper wire, in addition to holding the rubber stopper that keeps the containers floating upright, are looped near the top on the container to provide hooks for elastic bands that help keep the lids in place on the cups. An inch of fresh bedding is placed in the cup immediately prior to putting the rat pups in the containers.

Milk substitute:

The milk formula used in these experiments was selected because: 1) it is very similar to dam milk in terms of calorie density and macronutrient composition; 2) enables growth at a near normal rate; 3) pups raised on this formula have a high postnatal survival rate. The basic formula was invented by Messer in 1969, and was modified by Diez et al. in 1982. The final components of the diet presently used in this research were garnered from a 2001 study by Gonzalez et al. at the University of Toronto. The names and quantities of the ingredients are listed below in table 3.

Table 3: Ingredients and quantities used in preparation of rat pup formula.

Mineral mix:

0.214g	ZnSO ₄
0.12g	CuSO ₄
0.22g	FeSO ₄
2.0g	KCl
2.0g	MgCl

Formula Mix:

1500ml	Carnation Evaporated Milk
450ml	Sterile water
70g	Supro 710 Protein
130ml	Mazola Corn Oil
2.0g	Methionine
1.0g	Tryptophan
10g	Vitamin mix (ICB Biochemicals #02999-999)
11g	Tricalcium Phosphate
0.2g	Deoxychloric Acid
50ml	Mineral mix

The mineral mix is made separately and is dissolved completely in 50 ml of sterile water. All the ingredients of the formula, except for the evaporated milk, are blended together at low speeds in a Waring laboratory blender in short intervals to prevent the formula from getting warm. Once blended, the preparation is transferred to a large plastic container and the evaporated milk is added. After stirring, the formula is poured into 50ml conical bottles

and sealed tightly with lids. Conical containers are placed in 60°C water bath for 30 minutes and then frozen at -20°C for up to 6 weeks.

Cannulation Procedure

Rats are taken from the dam on post-partum day 3 for cannulation. Each animal's weight is recorded immediately prior to cannulation. The cannulation procedure starts by anesthetizing a rat pup with inhaled isoflurane until the pup no longer responds to a paw pinch or other painful stimuli. Once the appropriate amount of anesthesia is administered, the rat's head is held in a slightly extended position between the examiners thumb and index finger, while the animal's body rests on the palmer surface of the experimenter's hand. At this point, a lead tube made from a 6.5 cm length of PE-50, lightly coated in vegetable oil, is carefully slid down the rat's esophagus and into the stomach. Proper positioning is easily confirmed by rotating the rat slightly while extending the rat's torso and palpating for the leading tip of the lead tube in the stomach as slight overpressure is exerted at the exposed tip of the lead tube. Once lead tube position is confirmed, a fine wire stylet made from 31 gauge stainless steel is fed through the lead tube and into the stomach. The experimenter then repositions the animal so that the left abdominal wall is taut and applies pressure to the exposed tip of the wire stylet to pierce the stomach and the abdominal wall. The lead tube is withdrawn while the wire stylet is held in place. The animal is now placed in supine and the unflanged end of a cannula is friction fitted onto the wire stylet. A small amount of vegetable oil is drizzled along the length of the cannula and the experimenter

carefully, pulling the end of the wire stylet that protrudes from the rat's abdomen, threads the cannula down the esophagus and into the stomach. Once in the correct position, the flanged and flared end of the cannula forms a seal with the internal wall of the stomach. Next, a washer is placed on the cannula and slid into its position 2-4 mm from the animal's abdominal wall; this washer will prevent the inside portion of the cannula from moving back into the esophagus. Another washer is slid down the cannula approximately 4 cm from the abdominal wall, at this point the wire stylet is used to pierce the skin on the back of the rat pup's neck and the cannula is pulled through so that the second washer is flush with the rat's skin. Finally, the last washer is placed on the cannula and positioned on the other side of the tuft of neck skin; the last two washers serve to control and adjust the amount of cannula slack between the abdominal wall and the tuft of skin on the neck. The cannulation procedure is complete and the animal begins to awaken. The exit point on the abdomen and the two on the tuft of skin on the neck are swabbed with antiseptic. The animal is then labeled and placed in a heated and oxygenated recovery chamber to await the next phase. The entire cannulation procedure takes approximately 5-10 minutes per animal.

Rearing

Artificially reared rats are housed in 16 ounce Styrofoam beverage containers floated on a temperature controlled water bath with a 12 hour light cycle and a 12 hour dark cycle. Although attached to a feed line via a cannula, rats are allowed freedom of movement within the 16 ounce beverage containers.

The Styrofoam containers are kept apart and in place by a rigid plastic grid that sits on top of the water bath. Within this grid cups are allowed to tilt slightly and rotate, but they cannot come in contact with each other. The cannulas exit the container through a hole in the center of the lid, so that the cannulas meet with a feed line approximately 10-15 cms above the cup; cannulas are friction fitted to the feed lines. Feed lines, made of lengths of PE-50, connect directly to syringes containing the rat's daily supply of formula. Syringe pumps holding the formula syringes are stored between 34 and 40 degrees in a refrigerator, this ensures that rats receive formula that is not spoiled. A small opening was drilled into the side of the refrigerator immediately adjacent to the water bath where the animals are raised, from this hole, the feed lines exit the refrigerator and travel along a steel grate until they are above the container with the pup that that line is intended to feed. Once above the pup, the line is directed through a small hole in the grate so that it can connect with the cannula. Our system is set up to feed 20 pups at one time. All feed lines are replaced after a group of rats completes their artificial rearing cycle. Formula volume is calculated daily, based on the average weight of all the rats, and caloric needs to maintain growth at an adequate rate.

Daily pup maintenance:

Every morning, a routine is carried out to ensure the health, comfort and safety of all the rat pups. Rats are individually removed from their cups, weighed, and fresh bedding replaces the old bedding in the containers. The genito-rectal area is lightly stroked with a moistened lint-free wipe to stimulate

urination and defecation, and cannulas are cleared of formula by injecting a small volume of sterile water into the cannula. For the 3 days post surgery, wounds are swabbed with antiseptic. The feed lines are cleaned by forcing sterile water through them. Fresh formula is removed from the freezer and allowed to thaw at room temperature; the formula is then mixed to ensure uniformity and loaded into sterile syringes which are then attached to the individual feed lines. Gas bubbles are forced from the syringes and the syringes are then placed into slots on automated syringe pumps. After the pumps are primed, to ensure no gas bubbles will be introduced to the rat pup's stomach, daily feed volume is calculated based on the average weight of the pups, and the pumps are allowed to begin feeding at the next scheduled feed time.

Physiology

On day 14, rats are removed from their cups and either placed back with a lactating dam whose pups are a similar postnatal age or they are prepared for physiological experimentation. Rats used for physiological experimentation are anesthetized by an initial dose of urethane at 1.3 g/kg body weight given via IP injection. Subsequent doses at 300 mg/kg body weight are given every 25-30 minutes until the animal is adequately anesthetized (no response to paw pinch). The anesthetized rat is placed in a supine position on a heated gel pack, to maintain body temperature, and a midline ventral incision is made from the mandible to approximately the top of the sternum. Several layers of tissue are

carefully removed so that the distal hypoglossal nerve trunk and both the lateral and medial branch of the hypoglossal nerve and the entire genioglossus muscle are exposed. The lateral branch of the hypoglossal nerve is carefully cut and a large section of the branch is removed from the field. Next, the mandibular origin of the genioglossus is freed from its bony insertion and a length of 7-0 Ethilon (for 14 day old rats; 6-0 silk for 42 day old rats) is attached to the detached end of the genioglossus muscle and then tied to form a loop. The tongue is glued to the hard palate of the rat's mouth with cyanoacrylate to ensure that shortening from a contracting genioglossus is accurately measured. The animal is carefully moved to an elevated surgical platform where body temperature is maintained via a water circulating heating pad. The rat's torso is secured to the platform with adhesive strips and a length of 0-0 silk, secured to the platform on either side of the animal's mouth, runs through the open mouth of the rat and holds the rat's head in place. The afore mentioned loop of 7-0 Ethilon (or 6-0 silk) is attached to an ADInstruments MLT 050D strain gauge and the position of the strain gauge is adjusted precisely in 3 dimensions to ensure similar alignment between all test animal. A bipolar insulated stainless steel electrode with the last 2 mms of insulation removed is positioned touching the trunk of the hypoglossal nerve and delivers the stimulus to the medial branch of the hypoglossal nerve. Warm mineral oil is applied to the area to maintain moisture and temperature and to prevent current spread.

The stimulation paradigm, consisting of 0.1 ms pulses at 1 Hertz (Hz), 200 ms bursts at 20-160 Hz for 14 day old rats and 50-230 Hz for 42 day old rats, and a fatigue protocol consisting of 500ms bursts/second for 2 minutes at 50 Hz for 14 day old rats and 90Hz for 42 day old rats was loaded into an A.M.P.I. Master-8 cp stimulator (A.M.P.I., Jerusalem, Israel). The fatigue protocol is similar to the one employed by Kinirons et al. in 2003 and Sutlive et al. in 2000. The stimulator, in combination with a World Precision Instruments A360 high voltage stimulus isolator (W.P.I. Inc., Sarasota, Florida), was used to deliver the stimulus. Information garnered from single twitches included maximal isometric twitch tension, contraction time and half decay time. Information from trains included maximum tension, fusion frequency and fatigue index (please see literature review for a discussion on each).

Anatomy

Tissue Collection

Following collection of physiology data, muscle tissue samples from the long head of the biceps brachii and the genioglossus were isolated and removed while the animal was still deeply anesthetized. The samples were immediately placed in small cryostable conical tubes and flash frozen in liquid nitrogen. Samples were stored at -70° Celsius until analyzed for myosin heavy chain content and concentration. The long head of the biceps brachii was selected to detect any systemic effects of artificial rearing for two primary reasons. One, the

long head of the biceps brachii is composed of mostly fast fiber types, similar to the genioglossus (Fuentes et al., 1998). And two, the long head of the biceps brachii is a forelimb flexor, not a primary postural muscle, making it less prone to suffering the effects of long term activity changes potentially brought on by artificial rearing.

Tissue Preparation for Electrophoresis

On the day the muscle samples were to begin the process of analyzing for myosin heavy chain content and concentration, frozen samples were dehydrated in a vacuum, weighed and trimmed so that samples weighing between 1 and 3 milligrams were preserved in microcentrifuge tubes. The individual samples were carefully minced and reacted with half a milliliter of ice-cold myosin heavy chain extraction buffer (0.3 M NaCl, 0.15 M Na₂HPO₄ and 10mM EDTA at a pH of 6.5) for every milligram of muscle. Sample and buffer were placed in cold storage (3-4° C) for approximately an hour undergoing frequent agitation. After an hour, the microcentrifuge tubes containing each sample were centrifuged for 10 minutes at 12,000 revolutions per minute.

Following centrifugation protein concentration of each sample was assessed using the Bio-Rad protein assay for microtiter plates. A Sunrise microplate reader (Tecan, Hombrechtikon, Switzerland) measured absorbance at 595 nm. Ten micro liters of each sample was pipetted into its own individual well on a microplate and combined with 200 micro liters of diluted dye reagent (Bio-Rad Laboratories). Samples were compared to known protein concentrations of

bovine serum albumin and sample protein concentration values were calculated based on the reported optical density. Once protein concentration had been calculated, one hundred microliters of each sample was transferred to a clean microcentrifuge tube and combined with myosin heavy chain extraction buffer to adjust the protein concentration to approximately 25mg/ml. These samples were stored at -70° C until they were needed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoresis

SDS-PAGE allows for the separation of different myosin heavy chain isoforms based on molecular weight. Separation occurs within a 0.75 mm thick gel slab made up of a 4 cm stacking gel which sits atop a 13.5 cm separating gel. The separating gel and the stacking gel are made from the same stock of electrophoresis grade chemicals. The separating gel is made up of 30% glycerol, 8% acrylamide-N, N'-methylene-bis acrylamide (bis), 0.2 M Tris (pH 8.8), 0.1 M glycine and 0.4% SDS. The stacking gel is made up of 30% glycerol, 4% acrylamide-bis, 70 mM Tris (pH 6.8), 4 mM EDTA and 0.4% SDS. Polymerization was initiated with 0.1% ammonium persulfate and 0.05% N, N, N', N',-tetramethylethylenediamine (Talmadge and Roy, 1993).

While the gel polymerizes at room temperature, the previously prepared muscle samples are allowed to thaw. The protein concentration of the thawed samples are again reduced to 0.125 mg/ml with the addition of 2X Laemmli sample buffer (0.125 M Tris, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and

0.002% bromophenol blue). Samples are then boiled for 5 minutes to denature the protein and allowed to cool to room temperature. Once the gel is suitably polymerized, samples containing approximately 2.5 micrograms of protein are carefully loaded into their individual lanes in the stacking portion of the total gel. After the samples are loaded, the gel slab is positioned in a vertical slab gel unit (Protean II xi Cell, Bio Rad Labs) and moved to a cold storage unit maintained at approximately 4° C. Cold tris-glycine-SDS running buffers are poured into place around the gel and a constant 275 volt, 20 mAmp/gel current is applied to the apparatus for 1 hour and 30 minutes. After 1 hour and 30 minutes, the current is adjusted to 15 mAmps/gel for 29 hours.

Following electrophoresis for 30.5 hours, the separating gel is silver stained using the Silver Stain Plus Kit by Bio Rad Laboratories. Prior to staining, the gel is placed in a fixative solution (50% methanol, 10% acetic acid, 10% fixative enhancer concentrate and 30% deionized water) for 20 minutes followed by 2 ten minute rinses in deionized water. Following silver staining the gel is immediately placed in a 5% solution of acetic acid for 15 minutes to stop the staining process and then rinsed in deionized water for 5 minutes.

Once adequately stained and rinsed, the gel is scanned using an AGFA Duoscan HiD scanner (AGFA Corp., Ridgefield Park, New Jersey, USA). Relative amounts of myosin heavy chain isoforms are assessed using Gel-Pro Analyzer (Media Cybernetics, Silver Spring, Maryland, USA), image analysis software. The particular migration pattern of adult myosin was delineated by Bar

and Pette (1988), Talmadge and Roy (1993) and Termin et al (1989). Migration patterns of developmental isoforms were observed later by Adams et al. (1999). From these studies, it is now known that embryonic isoforms separate first followed by IIa, IIx/d, neonatal, IIb and I. In this study two measures were taken to make sure bands were correctly identified. First, a Broad Range SDS-PAGE Standard (Bio Rad Laboratories) was run along with the test samples so that the region containing myosin isoforms could be correctly identified. Second, a standard containing all six rat myosin heavy chain isoforms was made using adult and neonatal gastrocnemius, plantaris and soleus. This standard was run on the gels along with the various unknown samples.

Statistics

Data from physiology and anatomy experiments were analyzed using SPSS statistical software. Contractile properties were compared using one factor analysis of variance (ANOVA) in postnatal 14 day groups and postnatal 42 day groups. Myosin heavy chain profiles were compared in the same fashion. Statistical significance was set at $P < 0.05$ unless otherwise stated.

Results

These results are from data collected between May 6, 2004 and April 29, 2005. Results presented concerning artificially reared rats at day 14 and 42 came from 6 groups of rats raised artificially from postnatal day 3 to 14. Dam reared rats came from separate litters ordered at the same time as the litters used for artificial rearing. All data is expressed as mean +/- standard deviation unless otherwise noted. Statistical significance was set at $p < 0.05$.

Artificial Rearing

Survival

We were able to raise as many as 20 rats at a time using our artificial rearing system. A total of 8 trials were run. A mean of 18 +/- 3.8 rats started the artificial rearing process with an effort to keep the trial size at 20 by cannulating rats to replace rats that died within the 12 hour period immediately following cannulation. Overall, 53% of all rats that were cannulated survived to day 14; of the 47% that died before day 14, 38% died in the first 24 hours as a result of trauma during cannulation. Rats surviving past the first 24 hours typically did well. The ones that did not survive, died from respiratory problems resulting from excessive abdominal distension. The data contained within this dissertation

comes from 20 artificially reared rats (ten 14 day old rats and ten 42 day old rats).

Weight

Figure 1 shows weight gain from postnatal day 3 to 14 for all rats artificially reared from day 3 to 14 compared to 4 groups of dam reared rats weighed daily at approximately the same time as their artificially reared counterparts. Initially, the rats that were destined for dam rearing weighed 4.5% more than the ones destined for artificial rearing. By day 14 the dam reared rats weighed 15.7% more than their artificially reared counterparts.

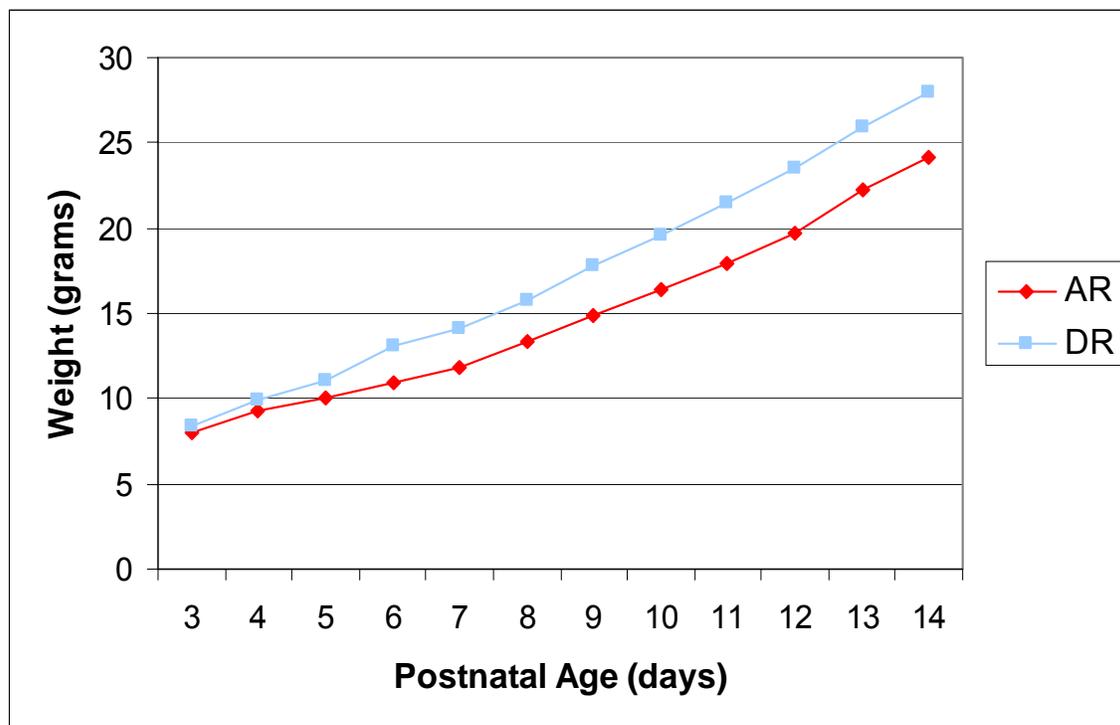


Figure 1: Body weight comparison postnatal day 3 to 14 in artificially reared (AR) and dam reared (DR) rats. Approximately 40 DR rats and 45 AR rats were used in this comparison.

Weight at 14 days and 42 days was significantly different for artificially reared and dam reared rats used in physiology testing ($p = 0.008$ and 0.001 respectively). See table 4.

Table 4: Comparison of dam reared and artificial reared rat weights at postnatal days 14 and 42 immediately prior to surgery.

Group (n)	Postnatal Day 14 (g)	Postnatal Day 42 (g)
Dam Reared (20)	27.6 +/- 5.4	160 +/- 18.6
Artificially Reared (20)	22.1 +/- 2.0	133 +/- 12.4

Physiology

Postnatal day 14 rat genioglossus muscle

Although body weight differences existed between artificial and dam reared groups at postnatal day 14, twitch force, contraction time, half decay time, fusion frequency, frequency at maximum tension and preload in genioglossus muscle were not statistically different between groups. Differences did exist between artificial and dam reared groups at postnatal day 14 in maximum tetanic tension ($p = 0.009$) and fatigue index (0.002). Frequency at maximum tension ($p=0.07$) and twitch force ($p=0.074$) approached the level of significance. Table 5 shows postnatal day 14 contractile characteristics in artificially reared and dam reared rat genioglossus.

Table 5: Summary of genioglossus contractile properties in AR and DR rats at postnatal day 14. Significant results are indicated by (*). Significance is where $p < 0.05$. Results are listed as means \pm SD. N=10 in both the DR and AR groups.

Parameter	Artificial Reared	Dam Reared
preload (g)	1.48 \pm 0.418	1.37 \pm 0.526
twitch force (g)	3.07 \pm 0.748	3.62 \pm 0.508
contraction time (ms)	30.3 \pm 0.432	28.1 \pm 0.37
half decay time (ms)	24.5 \pm 0.49	23.8 \pm 0.47
fusion frequency (hz)	86 \pm 7.0	87 \pm 10.6
maximum tension (g)	6.02 \pm 1.63*	8.3 \pm 1.85*
frequency at maximum tension (hz)	57 \pm 10.6	73 \pm 24.1
fatigue index	0.387 \pm 0.058*	0.502 \pm 0.079*

Figures 2 - 4 depict the different contractile characteristics measured in this study. Figure 2 compares a single whole genioglossus muscle twitch in dam reared and artificial reared rats at postnatal day 14. No differences were observed in contraction time or half decay time, but difference in force approached the significance level ($p=0.074$). The curves in figure 3 are representative trains at 3 different frequencies in a dam reared rat and an artificially reared rat. As previously mentioned, significant differences in maximum force existed between groups; differences approaching significance ($p=0.07$) also existed in frequency at which maximum force occurred. No differences between force at fusion or fusion frequency were noticed. Figure 4 shows the first and last force curves in an artificially reared rat and a dam reared rat in the fatigue paradigm utilized in this study.

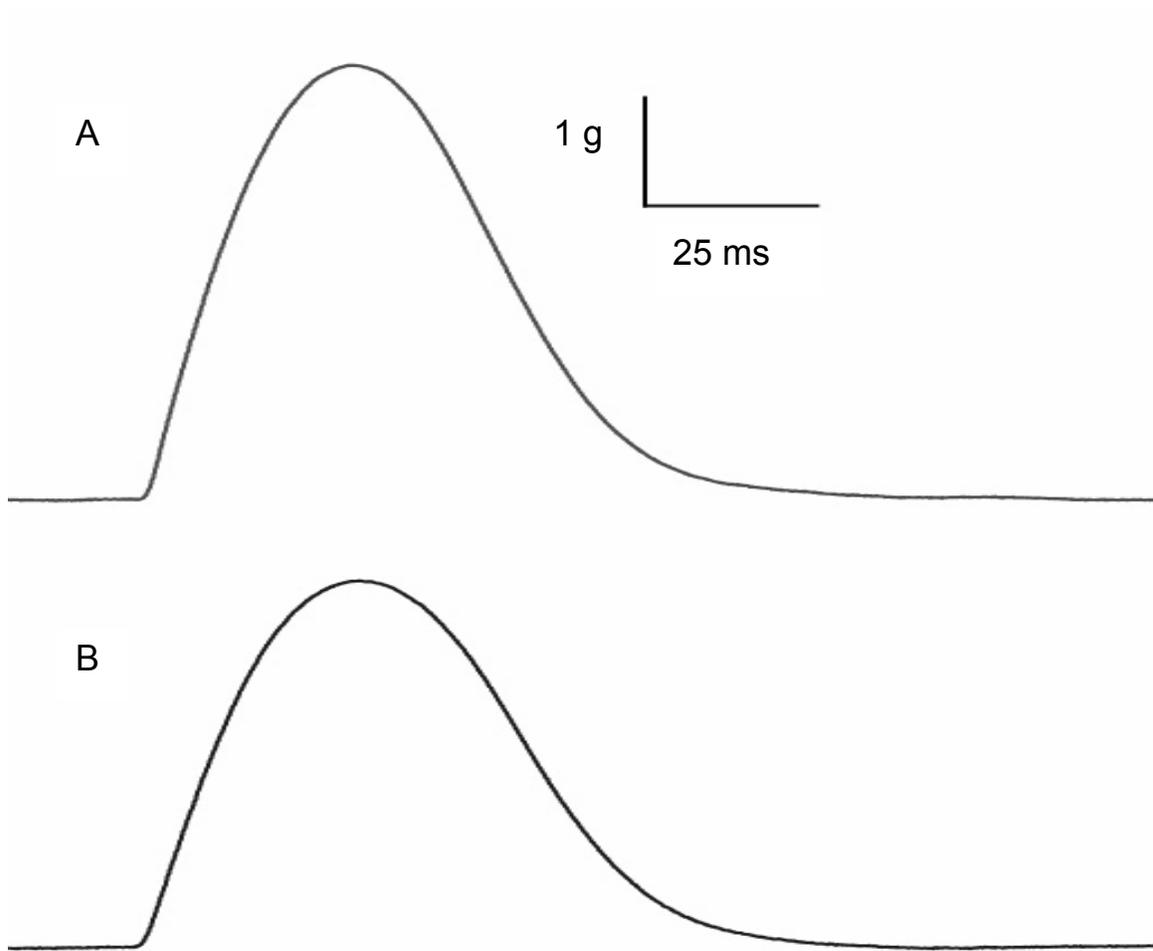


Figure 2: Comparison of single whole muscle genioglossus twitch in a dam reared rat (A) and an artificially reared rat (B). Mean dam reared force is 3.62 grams and mean artificially reared force is 3.07 grams.

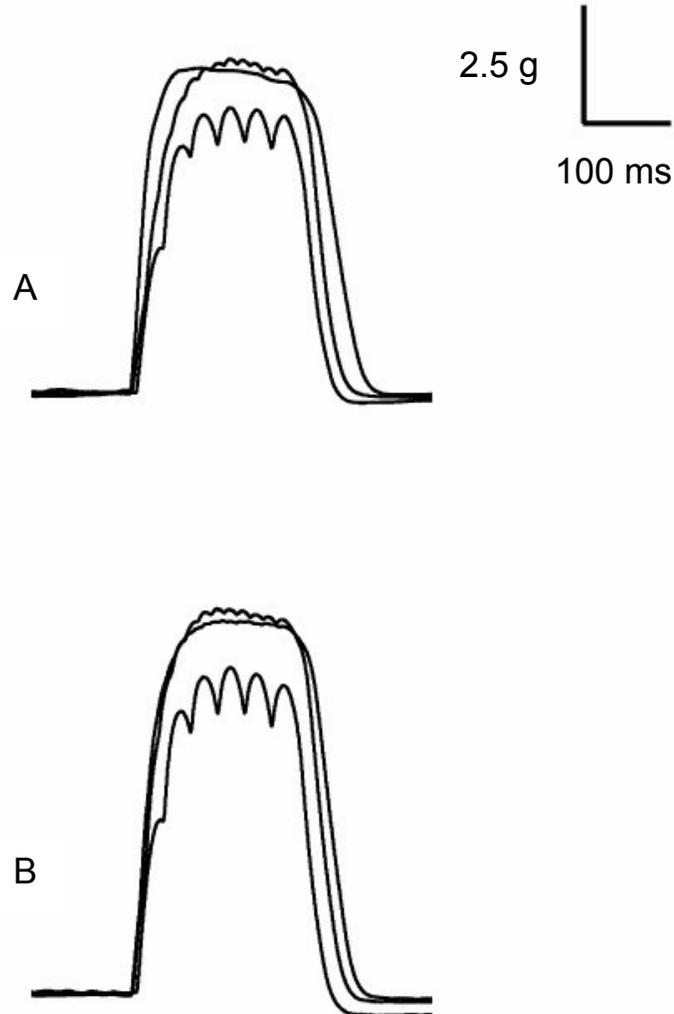


Figure 3: Comparison between force at frequency milestones in artificially reared (A) and dam reared (B) rat genioglossus muscle. All trains are 200 ms in length. In the artificially reared rat the frequencies shown, from least to most force, are: 30 Hz, 90 Hz (fusion) and 60 Hz (maximum tension). In the dam reared rat the frequencies shown, from least to most force, are: 30 Hz, 90 Hz (fusion), 70 Hz (maximum force). The frequencies for fusion and maximum tension reported here represent the means for their groups (dam reared and artificially reared). Mean maximum tension, the only significant difference represented here, in the dam reared group is 8.3 grams while the mean force in the artificially reared group is 6.03 grams.

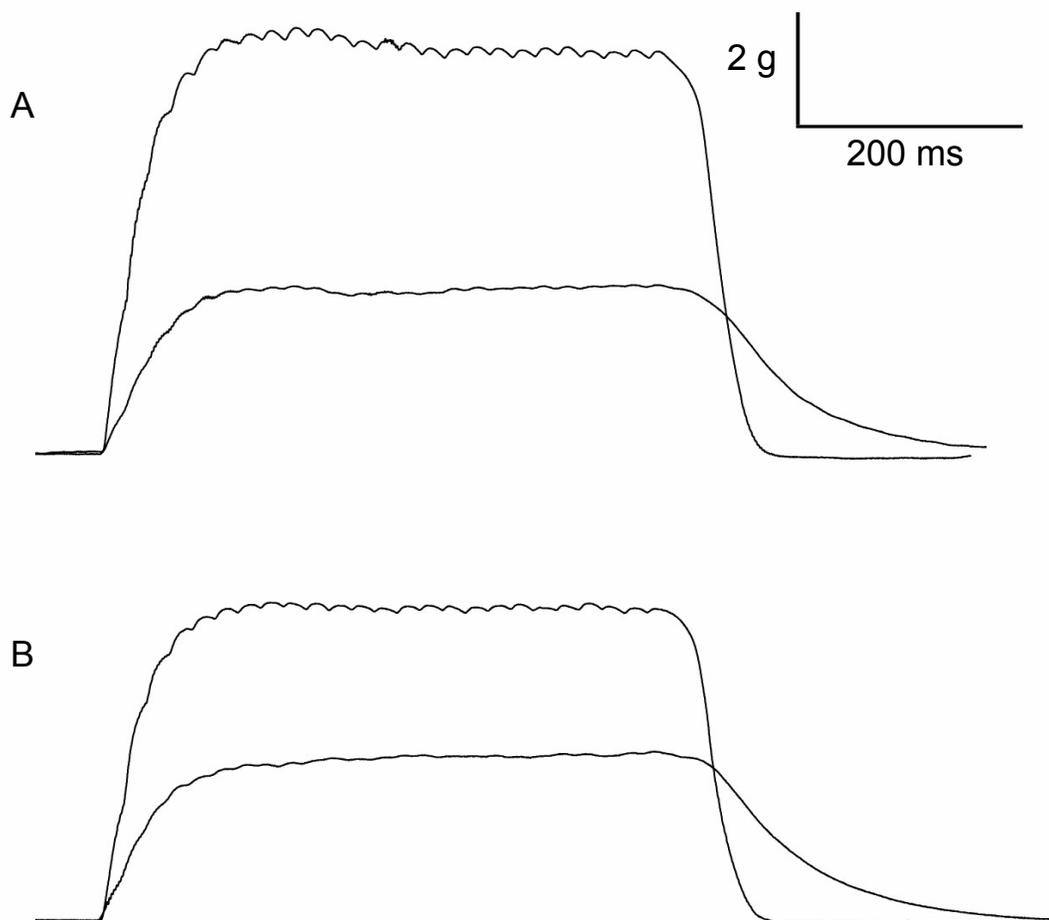


Figure 4: Comparison of fatigue indexes of genioglossus muscles in postnatal day 14 rats between artificially reared (A) and dam reared (B) groups. In both groups, the upper trace represents the first 500 ms 50 hz train while the bottom trace represents the last such train following a 2 minute period of 1 train/second stimulation. Mean fatigue index in the artificially reared group is 0.39 while the mean dam reared fatigue index is 0.50. These values represent a significant difference.

Postnatal day 42 rat genioglossus muscle

By 42 days postnatal, all the measured contractile characteristics were statistically similar except fatigue index. Fatigue index in artificially reared rats was 0.77 while dam reared fatigue index was 0.68 ($p=0.014$). See table 6 for a summary of all the contractile characteristics.

Table 6: Summary of genioglossus contractile properties in artificially reared (AR) and dam reared (DR) rats at postnatal day 42. Significant results are indicated by (*). Significance is where $p<0.05$. Results are listed as means +/- SD. N=10 in both the DR and AR groups unless otherwise indicated. † indicates a group size of 7.

Parameter	AR	DR
preload (g)	2.29 +/- 0.83	2.32 +/- 1.05
twitch force (g)	4.66 +/- 0.858	5.06 +/- 0.666
contraction time (ms)	13.8 +/- 0.129	13.3 +/- 0.183
half decay time (ms)	11.5 +/- 0.153	10.5 +/- 0.173
fusion frequency (hz)	170 +/- 11.5†	172 +/- 14.8
max tetanic force (g)	16.02 +/- 3.13	17.68 +/- 2.84
frequency at maximum tension (hz)	136 +/- 26.3	126 +/- 25.9
fatigue index	0.77 +/- 0.064*	0.68 +/- 0.082*

Figures 5-7 are representative of the genioglossus contractile properties seen in postnatal day 42 dam reared and artificially reared rat. Figure 5 is the typical whole genioglossus muscle twitch in response to a single stimulus pulse. Figure 6 shows genioglossus response to 200 ms pulses at varying frequencies and figure 7 shows fatigue indexes in artificially and dam reared rats.

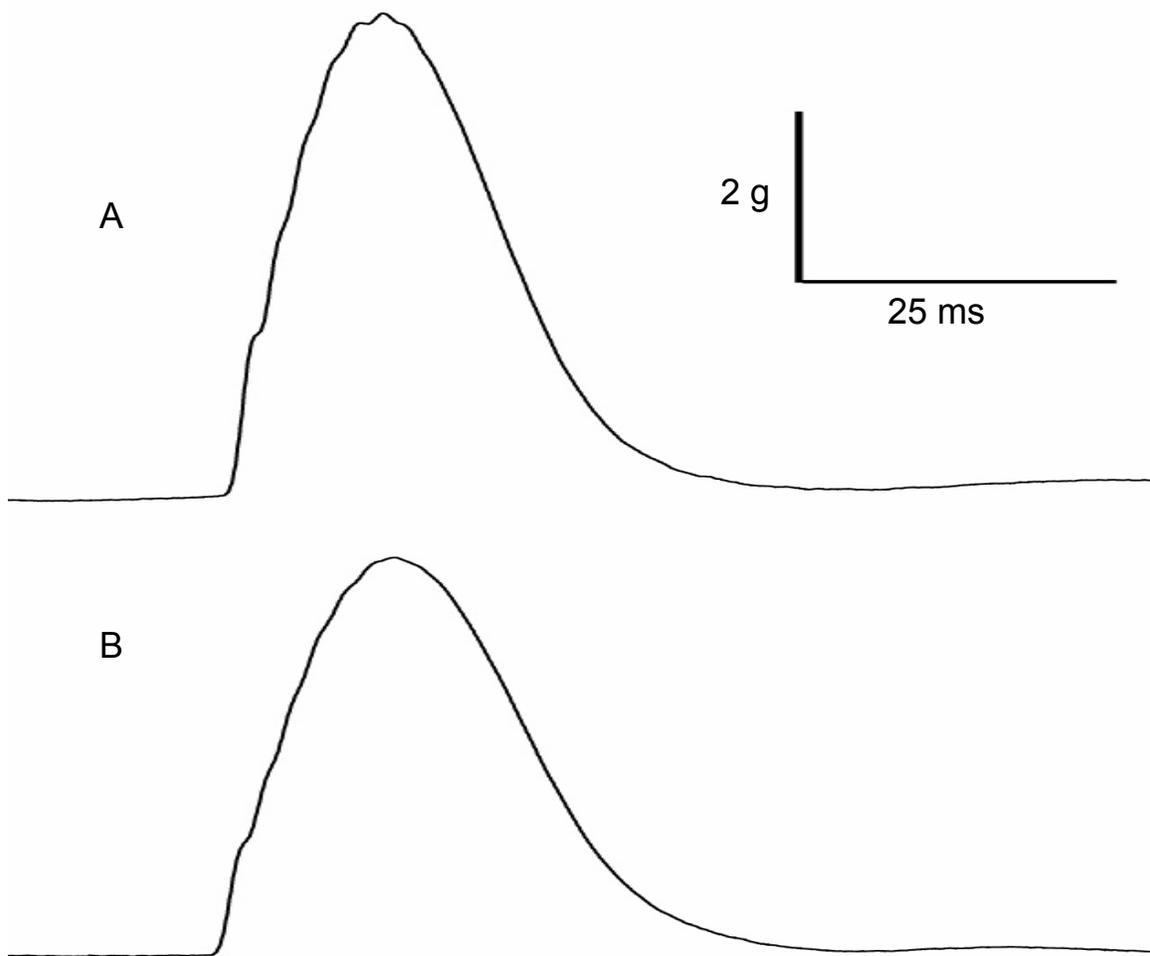


Figure 5: Comparison of single whole muscle genioglossus twitch in a dam reared rat (A) and an artificially reared rat (B). Mean dam reared force is 5.06 grams and mean artificial reared force is 4.66 grams. No significant differences in contraction time, half decay time or tension were observed.

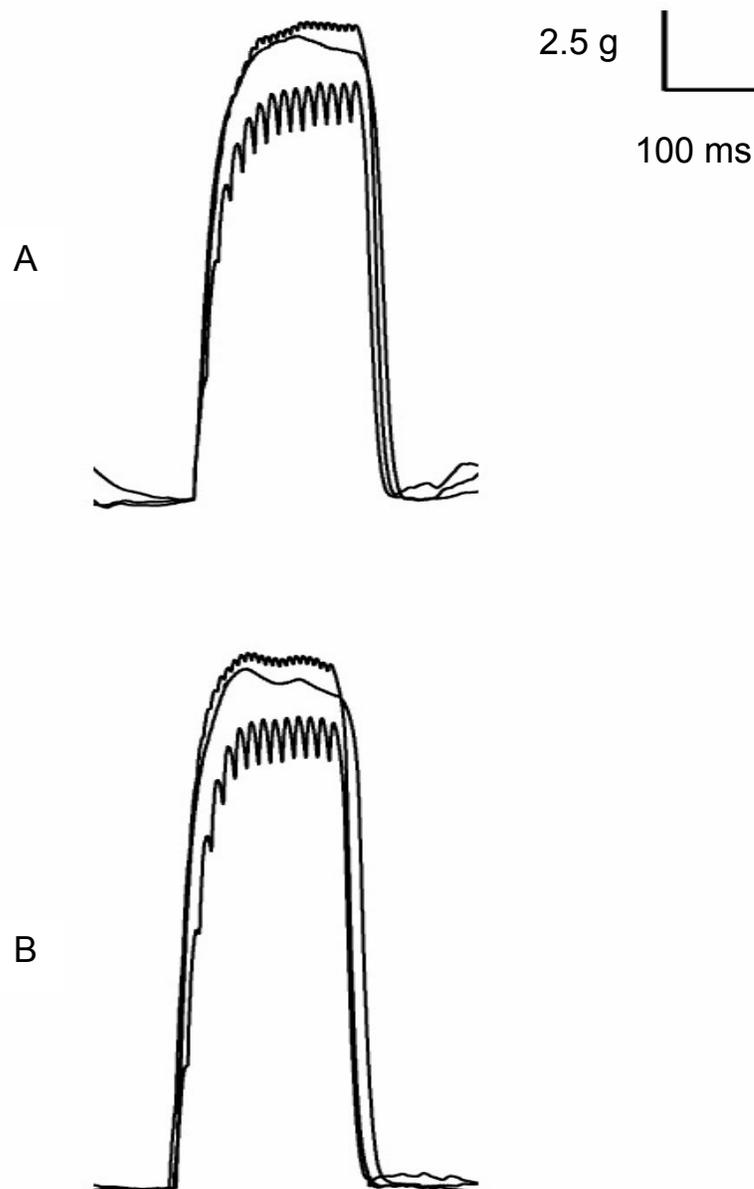


Figure 6: Comparison between force at frequency milestones in artificially reared (A) and dam reared (B) rat genioglossus muscle at postnatal day 42. All trains are 200 ms in length. In the artificially reared rat the frequencies shown, from least to most force, are: 70 Hz, 170 Hz (fusion) and 140 Hz (maximum tension). In the dam reared rat the frequencies shown, from least to most force, are 70 Hz, 170 Hz (fusion), and 130 Hz (maximum force). The frequencies for fusion and maximum tension reported here represent the means for their groups (dam reared and artificial reared).

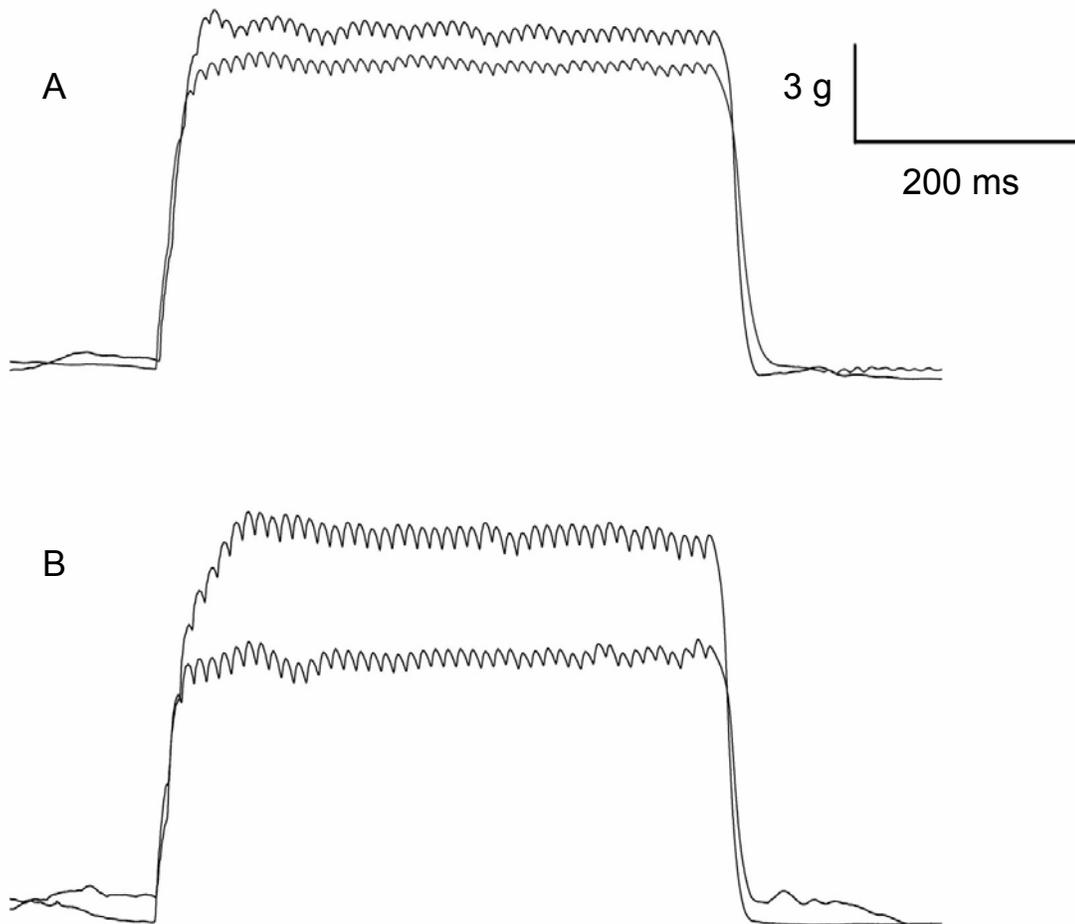


Figure 7: Comparison of fatigue indexes of genioglossus muscles in postnatal day 42 rats between artificially reared (A) and dam reared (B) groups. In both groups, the upper trace represents the first 500 ms 50 Hz train while the bottom trace represents the last such train following a 2 minute period of 1 train/second stimulation. Mean fatigue index in the artificially reared group is 0.77 while the mean dam reared fatigue index is 0.68. These values represent a significant difference.

Anatomy

Postnatal day 14

Anatomical data on 14 day old rats is the result of SDS-PAGE gel electrophoresis on protein of a total of 8 animals, 4 AR and 4 DR. Each of the eight animals had a portion of the genioglossus and biceps brachii removed and stored for this part of the study. Table 7 shows a comparison between artificially reared and dam reared MHC composition in 14 day old rat genioglossus. None of the values were within the statistically significant range of $p < 0.05$.

Table 7: Mean MHC phenotypes in artificial reared (AR) and dam reared (DR) postnatal day 14 rat genioglossus muscle. Each mean +/- SD represents a percentage of the total MHC complement in the AR and DR groups. N=4 per group. No significant differences were seen in this group.

IIa	AR	15.0 +/- 3.2
	DR	14.3 +/- 3.2
IIx	AR	20.1 +/- 7.1
	DR	24.0 +/- 9.4
neonate	AR	35.6 +/- 2.3
	DR	37.9 +/- 3.8
IIb	AR	31.5 +/- 6.4
	DR	23.9 +/- 4.0
I	AR	not detected
	DR	not detected

The bar graph in figure 8 illustrates the mean MHC distribution in genioglossus of all eight rats. Figure 9 is a representative SDS-PAGE of an artificially reared and dam reared genioglossus.

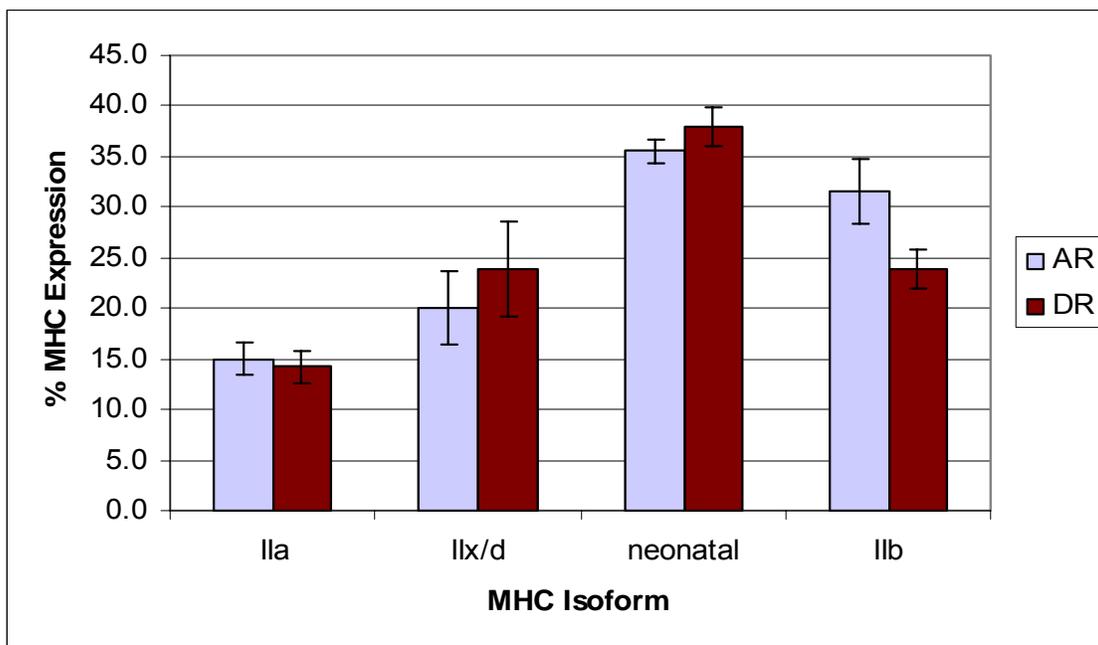


Figure 8: Comparison of relative mean percentages of MHC, +/- SE, in postnatal day 14 artificially reared (AR) and dam reared (DR) rat genioglossus muscle. No significant MHC expression differences were found between the AR and DR groups. N=4 per group.

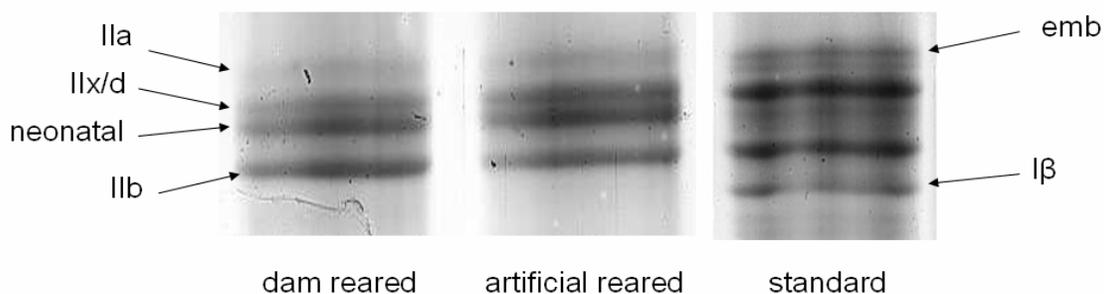


Figure 9: Representative SDS-PAGE image of dam reared and artificially reared 14 day old genioglossus muscle.

Biceps brachii was also evaluated to test for systemic effects of artificial rearing. No significant differences were found in the biceps among the two groups at 14 days of age. See table 8 for details.

Table 8: Mean MHC phenotypes in artificial reared (AR) and dam reared (DR) postnatal day 14 rat biceps brachii muscle. Each mean +/- SD represents a percentage of the total MHC complement in the artificially reared and dam reared groups. N=4 per group. No significant differences were seen in this group.

IIa	AR	8.9 +/- 3.2
	DR	9.5 +/- 6.2
IIx/d	AR	14.5 +/- 2.4
	DR	26.3 +/- 9.9
neonate	AR	30.6 +/- 1.5
	DR	23.7 +/- 7.5
IIb	AR	36.6 +/- 3.0
	DR	31.2 +/- 20
I	AR	9.4 +/- 2.0
	DR	8.6 +/- 6.8

Postnatal day 42

Once again eight animals were evaluated to determine MHC distribution in 42 day old animals; 4 dam reared and 4 artificially reared rats. While the genioglossus was the focus of this examination, biceps brachii was also evaluated to determine if any systemic effects, due to artificial rearing, occurred. At postnatal day 42 MHC IIa expression in genioglossus was significantly greater in dam reared rats than in artificially reared rats ($p=0.024$). Neonatal MHC was no longer detected in either dam reared or artificial reared rat genioglossus. See

table 9 for phenotype comparison and figure 10 for MHC distribution in genioglossus. Figure 11 is a representative SDS-PAGE of an artificially reared and dam reared genioglossus.

Table 9: Mean MHC phenotypes in artificial reared (AR) and dam reared (DR) postnatal day 42 rat genioglossus muscle. Each mean +/- SD represents a percentage of the total MHC complement in the artificially reared and dam reared groups. N=4 per group. Significant results are indicated by (*).

IIa	AR	17.4 +/- 3.7*
	DR	24.2 +/- 2.7*
IIx	AR	45.7 +/- 8.6
	DR	37.6 +/- 2.1
neonate	AR	not detected
	DR	not detected
IIb	AR	36.9 +/- 9.8
	DR	38.2 +/- 2.2
I	AR	not detected
	DR	not detected

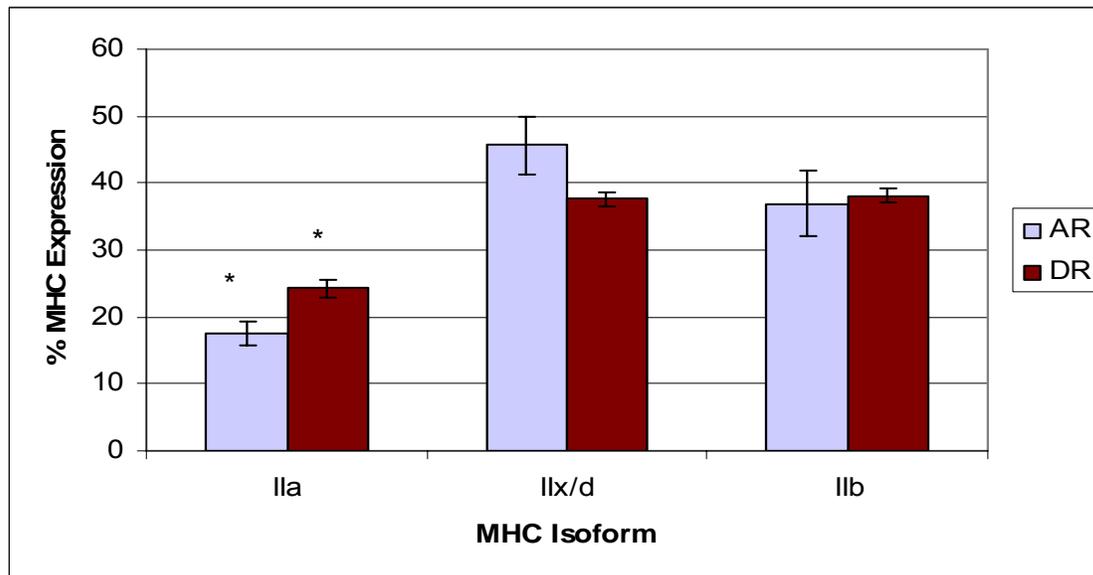


Figure 10: Comparison of relative mean percentages of MHC, \pm SE, in postnatal day 42 AR and DR rat genioglossus muscle. Significant results are indicated by (*). N=4 per group.

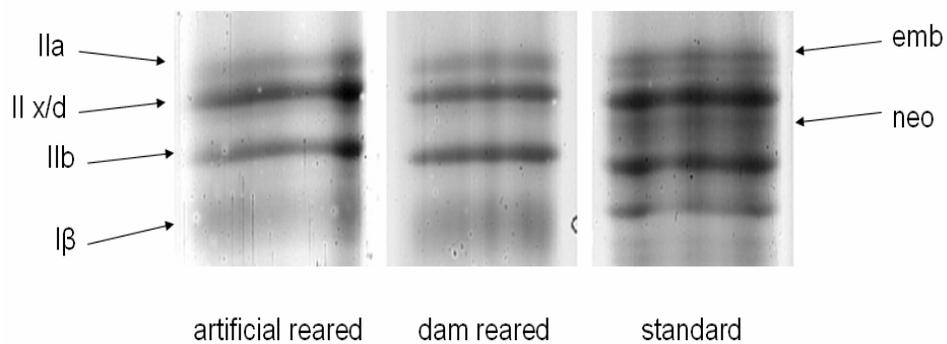


Figure 11: Representative SDS-PAGE image of dam reared and artificially reared 42 day old genioglossus muscle.

As with the postnatal day 14 rats, muscle samples from the biceps brachii were taken from this group of animals as well. As table 10 below indicates, no significant differences were found between biceps brachii phenotypes of artificial reared and dam reared rats.

Table 10: Mean MHC phenotypes in artificially reared (AR) and dam reared (DR) postnatal day 42 rat biceps brachii muscle. Each mean +/- SD represents a percentage of the total MHC complement in the artificially reared and dam reared groups. N=4 per group. No significant differences were seen in this group.

IIa	AR	8.2 +/- 3.0
	DR	8.2 +/- 3.4
IIx/d	AR	22.5 +/- 3.3
	DR	25.7 +/- 5.1
neonate	AR	not detected
	DR	not detected
IIb	AR	57.6 +/- 4.5
	DR	53.9 +/- 5.8
I	AR	11.8 +/- 5.7
	DR	10.5 +/- 6.7

Discussion

The major findings of this study in regard to contractile properties were: 1) the genioglossus muscle of postnatal day 14 rats subjected to artificial rearing from postnatal day 3 to 14 was more easily fatigued compared to their dam reared counterparts, 2) the genioglossus muscle of postnatal day 14 rats subjected to artificial rearing from postnatal day 3 to 14 produces less maximum force when stimulated at high frequency for 200 ms compared to their dam reared counterparts, 3) the genioglossus muscle of postnatal day 42 rats subjected to artificial rearing from postnatal day 3 to 14 was more resistant to fatigue compared to their dam reared counterparts.

The major findings of this study in regard to anatomical evaluation of myosin heavy chain (MHC) phenotype were: 1) the genioglossus muscle of postnatal day 42 dam reared rats had a higher percentage of MHCIIa than their artificially reared counterparts, 2) differences in MHC content in a muscle used as a control, the biceps brachii, were not observed between dam reared and artificial reared groups at postnatal days 14 or 42.

Contractile properties of genioglossus muscle

Comparison of 42 day old rat genioglossus with existing literature

In this part of the discussion we compare the dam reared postnatal day 42 rats used in this study to adult rats used in other studies since no other studies using young/adolescent rat genioglossus muscle were found in the literature. A review of the literature indicates that genioglossus contraction time and half decay time of the 42 day old dam reared rats used in this study were similar to the times reported by Sutlive et al. (2000) in adult rats. Sutlive et al., using normal rats at approximately 90 days of age, reported a mean genioglossus contraction time of 14.22 +/- 0.56 ms and a half decay time of 11.68 +/- 1.05 ms, compared to mean times of 13.3 +/- 1.8 ms and 10.5 +/- 1.7 ms in the present study. Although the means from the current study are close to the means from the Sutlive et al. study, they are not within the range reported by Sutlive et al. These minor differences may easily be explained by examining differences in MHC phenotype as well as age. While neither study reported finding any MHC I, both found MHC IIa, MHC IIx and MHC IIb. The dam reared rats in this study had muscle that consisted of 38.2% MHC IIb, 37.6% MHC IIx and 24% MHC IIa compared to 28% MHC IIb, 27% MHC IIx and 45% MHC IIa in the Sutlive et al. study. MHC IIb is the isoform that corresponds to the fastest fiber type (IIB), so naturally one would expect muscle with primarily this type of MHC isoform to have the shortest contraction time with all other factors being equal (Pette and Staron, 2001). Furthermore, in the present study, 42 day old dam reared rats

exhibited higher fusion frequencies than the adult rats in the Sutlive study. This is not surprising given that the 42 day old genioglossus has shorter contraction and half decay times (McComas, 1996). In addition to the predictable differences in contraction time based on MHC phenotype, it has also been observed that age related differences in contraction time exist. Elber and McComas (1987) found that contraction time and half decay time in rat plantaris decreased from postnatal day 18 to postnatal day 46 and then increased to 30% more than day 18 levels by day 224. The authors attribute these differences to differences in sarco-tubular calcium kinetics rather than myosin ATPase activity.

As far as twitch force and maximum tetanic tension are concerned, significant differences apparently do exist between the 42 day old dam reared rats used in this study and the adult rats used in the study by Sultive et al. (2000). Sutlive et al. reported a single whole muscle twitch force of 7.02 ± 1.44 grams in adult rats compared to 5.06 ± 0.67 grams found presently. Additionally, Sutlive et al. reported a maximum tension of 37.22 ± 7.4 grams at around 104 Hz, while 42 day old dam reared rats in this study produced a maximum force of only 17.7 ± 2.84 grams at 126 Hz. These differences are not surprising. González et al. (2000) examined force production of single fibers in extensor digitorum longus in 2-6 month old mice, 12-14 month old mice and 20-24 month old mice, and found that single fiber twitch force and tetanic force continued to increase into the 14th month. Another study by Martin et al. (2000) presented similar findings in humans; the authors found that strength increased

every decade up to the 4th decade of life, even when differences in body mass were discounted. Obviously age was an important factor here as well.

Fatigue index of the 42 day old dam reared rats used in this study was comparable to the FI found in adult rats in the Sutlive et al. (2000) study. Sutlive et al. reported a fatigue index of 0.72 while the current study reported a fatigue index of 0.68. This disparity was, once again, consistent with observed differences in MHC composition. Sutlive et al reported finding 45% MHCIIa and 27% MHCIIx versus only 24% MHCIIa and 37.6% MHCIIx in this study. The majority of our 42 day old genioglossus muscle was composed of MHCIIb (38.1%), which has a low resistance to fatigue compared to MHCIIa (Polla, 2005). In addition to differences in MHC composition, slight age related differences in the muscles' ability to handle biochemical products produced during a fatigue protocol may exist (McComas, 1996).

Mean fusion frequency of the genioglossus muscle in 42 day old dam reared rats used in this study was approximately 170 Hz, while Sutlive et al. (2000) reported a genioglossus muscle mean fusion frequency of 105 Hz in adult rats. Kinirons et al. (2003) reported a fusion frequency of 130 Hz in retractor musculature of 42 day old dam reared rats, and Gilliam and Goldberg (1995) reported a fusion frequency of around 90 Hz for the adult rat tongue's retractor musculature. In both the genioglossus muscle and the retractor musculature fusion frequency was much higher in 42 day old rats than in the older rats. Perhaps this difference may be explained by looking at contraction and half

decay times. In both the genioglossus muscle and the retractor musculature the contraction time and the half decay time in the younger animals were less than the times in the older animals. Although the differences in contraction and half decay times were minor when one examines an individual twitch, the effect on fusion frequency is significant.

Comparison of contractile properties between artificial reared and dam reared groups

At postnatal day 14 significant differences in maximal tetanic tension and fatigue index between dam reared groups and artificial reared groups were observed. Dam reared rats produced a higher maximum tetanic force and were more resistant to fatigue. Other disuse models have presented similar findings. Elder and McComas (1987) found that soleus and plantaris single twitch force and maximum tetanic tension decreased in neonatal rats that underwent hindlimb suspension, and Fitts et al. (2001) report a decrease in maximum lower extremity strength in rats in as few as 6 days of space flight. Where fatigue is concerned, Kinirons et al. (2003), using a model very similar to the one used here, found that artificially reared rat styloglossus fatigued significantly more than their 14 day old dam reared counterparts. Differences in twitch and maximal tetanic tension are usually well explained by the decreases in muscle mass and fiber size observed in animals under the influence of altered activity (Fitts et al., 2001, Asmussen & Soukup, 1991, Elder & McComas, 1987, D'Antona et al., 2003). Observed differences in fatigue are not as easily explained. Differences in fatigue may be

multi-factorial as alterations at the level of the cortex down to the muscle fiber have been indicated in fatigue (Berne and Levy, 1998).

At postnatal day 42 the only significant difference between dam reared and artificial reared rats in terms of contractile properties was fatigue index. Artificially reared rats were more fatigue resistant than their dam reared counterparts at this age. This may seem surprising but it is consistent with unpublished results from this laboratory comparing dam and artificially reared rat retractor contractile properties at postnatal day 42 following artificial rearing from postnatal day 3 to 21. Additionally, Kinirons et al (2003) reported a similar, albeit non-significant, finding at postnatal day 42 in retractor musculature subjected to just 10 days of artificial rearing. While Kinirons found artificially reared rats expressed more of the fatigue resistant MHCIIa than dam reared rats and that dam reared rats express more of the non-fatigue resistant MHCIIx than artificial rats, this study did not. To the contrary, artificially reared rats in this study expressed less of the fatigue resistant MHCIIa than the dam reared rats at postnatal day 42 even though artificially reared rats were more fatigue resistant. One has to question why artificially reared rats are more fatigue resistant than dam reared rats at postnatal day 42. Phenotype is apparently not the whole answer given the contrary finding that dam reared rats express a greater percentage of MHCIIa than do artificially reared rats. Perhaps an important developmental period has been disrupted and thus altered neurological development of genioglossus motor control. This may be reasonable given that

this study seeks to disrupt the period where neurons in the hypoglossal nucleus that innervate the genioglossus undergo a period of dendritic simplification from birth to 13-15 days followed by a period of regrowth and proliferation lasting until about postnatal day 30 (Nunez et al., 1994). Also, observations from altered activity studies in rats and humans show that unloaded muscles display increased utilization of glycogen, which may help hold off fatigue (Fitts et al., 2001 and Grichko et al., 2000).

Comparison of contractile properties between age groups

As expected, differences between age groups were seen. Two of the more interesting differences were contraction time and half decay time. At postnatal day 14 mean contraction time was 29.2 ± 4 ms and half decay time was 24 ± 4.7 ms compared to a contraction time of 13.6 ± 1.6 ms and half decay time of 11 ± 1.7 ms in 42 day old rats. As previously discussed this is an expected change; Elder and McComas (1987) measured contractile properties of rat plantaris and soleus at several different ages and found that both the relatively slow soleus and the fast plantaris tended to display decreasing contraction and half decay times from birth to day 46 followed by increasing times through day 224. A few possible mechanisms may have a role in initially increasing contraction and half decay time before they start to decline: 1) fiber type changes; fibers tend to transition from slow to fast (Pette and Staron, 2000), 2) maturation of the sarcotubular system leads to a faster rate of calcium release

(Elder and McComas, 1987), and 3) changes in myofilament geometry (Thompson and Brown, 1999).

Isometric twitch and maximal isometric tetanic tension also increased from day 14 to day 42 in this study. The mean twitch tension in the 14 day old rats was 3.3 grams while the 42 day old rats produced a mean twitch force of 4.9 grams. Maximal isometric tetanic tension increased from a mean of 7.2 grams at 14 days to a mean of 16.8 at 42 days; this is consistent with what is reported in the literature. Elder and McComas (1987) showed that isometric twitch and maximal tetanic tension more than doubled in plantaris and soleus from day 18 to day 46 in normal rats. Several factors could contribute to the differences in tension development between 14 day old rats and 42 day old rats. Some of these factors include metabolic, hormonal and nervous system differences and differences in muscle mass and fiber density (Van Praagh & Dore, 2002, Fellmann & Coudert, 1994, and Boisseau & Delamarche, 2000).

Lastly, fusion frequency in this study increased 98% from postnatal day 14 to 42, similar to the study by Kinirons et al. (2003), where fusion frequency increased by 92.7% in tongue retractor musculature during the same time period. The percent change is very similar between the two muscles and correspond well to changes in contraction and half decay times.

Genioglossus Myosin Heavy Chain Phenotypes

The role of MHC phenotypes in regard to their impact on contractile properties has already been discussed, so this section will focus on the changes that take place from birth to early adolescence in dam reared and artificially reared rats and discuss those changes in view of what is already known in the literature.

At postnatal day 14 the genioglossus of artificial and dam reared rats were very similar except that artificially reared rats were trending towards having significantly more MHCIIb than their dam reared counterparts ($p = 0.092$). This is noteworthy because Adams et al. (2000) reported subtle increases of MHCIIb in fast muscles following 16 days of space flight in neonatal rats, while other authors have only reported changes in slow muscle phenotype following spaceflight or other disuse scenarios (Elder & McComas, 1987, Huckstorf et al., 2000, and Asmussen & Soukup, 1991). The reason typically given for the disparity between slow and fast muscles is that slow muscles require weight bearing during the neonatal period to make the conversion from developmental isoforms to slow isoforms, while fast muscles are dependent on an intact thyroid state (Baldwin and Haddad, 2001). As anticipated, rats in both the dam reared and artificial reared groups expressed more MHCneonatal than any other isoform, this isoform transitions completely to fast isoforms before day 42.

By day 42, MHC phenotype showed a significant difference in mean MHCIIa between the dam reared and artificial reared groups. MHCIIa made up

24.2% of the total MHC complement in dam reared rats versus 17.4% of the MHC complement in artificial reared rats. A corresponding, but non-significant shift was noted in MHCIIx complement. This finding is consistent with a finding by Jänkälä et al. (1997) that fast fiber types transition to faster fiber types when influenced by altered activity.

As with contractile properties, an attempt to compare the results presented here with published results follows. LaFramboise et al. (1992) and Brozanski et al. (1993) both published results that are similar to the findings presented here in adult and/or adolescent rats. LaFramboise et al., studying only adult rats, and Brozanski et al., studying rats from postnatal day 4 to adult, found that rat genioglossus, after postnatal day 30, expressed primarily MHCIIb followed by MHCIIx and MHCIIa. In addition, Brozanski et al., and the present study, found the following order of expression in postnatal day 14 rats (from most to least): MHCneo, MHCIIx, MHCIIb, MHCIIa. However, LaFramboise et al. and Brozanski et al. found that adult genioglossus also expressed a small amount MHCI, which was not found in this study.

Artificial Rearing

To study the effects of the cannulation procedure itself, a previous study published by this lab cannulated a group of rats at postnatal day 4, and then immediately removed the cannula and returned the rats to the dam to be raised normally (Kinirons et al. 2003). No significant differences were found between

these so called sham operated rats and the dam reared rats in any anatomical or physiological parameter. Therefore, further evaluation of sham operated rats was decided against.

Complications

Approximately 53% of the rats that started the artificial rearing process survived to day 14. Many of the rats that did not survive the full artificial rearing term died within the first 24 hours following cannulation as a result of a misplaced cannula or trauma resulting from the cannulation procedure. The remainder of the rats that died typically died starting about day 10 from complications related to abdominal distension or bloat. The exact cause of the distension is debatable but the treatment is always reduced feeding volume until the distension resolves. Unresolved distension will eventually impede breathing and the animal will die. A similar condition, known as necrotizing enterocolitis, exists in preterm newborn human infants that require enteral feeding. As with the rats in our study these infants become bloated and usually lose weight as a result of a reduction in feeding volume. The exact cause of necrotizing enterocolitis is unknown (Noerr, 1993).

Body weights

Artificially reared rats at postnatal day 14 and 42 weighed significantly less than their dam reared counterparts. This is a common complication (Tonkiss et al., 1987, Kinirons et al, 2003) and in this study can be traced back to the desire

to prevent death from complications related to bloat. This is also a common finding among human neonates requiring enteral feedings (Noerr, 1993).

Systemic effects

Muscle biopsies from the longhead of the biceps brachii were taken and analyzed by SDS-PAGE gel electrophoresis in order to determine if any phenotype changes occurred as the result of artificial rearing. No changes were observed; this suggests that artificial rearing did not have a systemic impact on myosin heavy chain phenotype.

Summary

Artificial rearing has been used to study, among other things, the behavior of rats raised without access to a dam and/or litter mates, and the effects of specific nutrients and drugs on the developing brain and organs, but this is the first time artificial rearing has been used to compare MHC phenotype and contractile properties of the genioglossus muscle in dam and artificially reared rats. The results indicate that as few as 11 days of artificial rearing significantly alters the short and long term development of the genioglossus. In addition, this study confirms that artificial rearing is an appropriate altered activity model to study tongue dysfunction associated with preterm birth.

Conclusion

Human infants born before the completion of the 37th gestational week accounted for 12.1% of all live births in 2002, up 29% from 1981 (Martin et al., 2003). A large number of infants, especially those born before the 29th gestational week, experience lengthy neonatal intensive care unit (NICU) stays and frequently require nasogastric tube feeds for the first several weeks following birth (Medoff-Cooper et al., 2002 and Dodrill et al., 2004). Many of these infants go on to experience delays in other feeding behaviors and delayed acquisition of speech and language skills (Jennisch and Sedin, 1999). Successful treatment of this growing group of infants is in the public interest because, if survival rates and healthcare costs continue to rise as they have in recent decades, so too shall the financial burden of caring for these infants.

While it was never the intention of this study to present a cure for speech/language and feeding disorders associated with preterm birth, it is appropriate to encourage the therapies specifically aimed at increasing endurance and strength in the genioglossus muscle of neonates through methods such as sucking on nipples of different shapes and resistances. While this will not address all the deficits, it will address some of the deficits documented in the present study.

Future and Present Studies

This lab has emphasized two major areas of tongue research related to the ultimate goal of being able to provide treatment rationale for infants exhibiting oral feeding delays: 1) examination of tongue musculature through measurement of contractile properties and MHC phenotype in adult and developing rats, and 2) examination of motoneuron morphology and location within the hypoglossal nucleus in adult and developing rats.

So far this lab has taken a close look at adult retractor and protrusor whole muscle and motor unit contractile properties and myosin heavy chain phenotype. Additionally, postnatal development of protrusor and retractor musculature in terms of contractile properties and myosin heavy chain phenotype has now been studied in dam reared rats and rats subjected to artificial rearing for 10 to 11 days. Recently, we have successfully interrupted a critical period where rats begin to transition to solid foods (Maeda et al., 1987) by extending the period of artificial rearing to 18 days (postnatal day 3 to 21) and collected retractor contractile data at postnatal days 21 and 42 in this group of rats. In the future, we will examine the contractile properties and phenotype of the tongue's intrinsic muscles and extend the period of artificial rearing even more by delivering rats via cesarean section in an attempt to further disrupt the period in which rats acquire suckling skills.

Within the hypoglossal nucleus, the organization and morphological properties of motoneurons of adult and developing rats has been realized, and

we have begun to collect similar data in artificially reared rats to determine the possible influence of altered activity. As with the tongue musculature, we will continue to increase the length of the artificial rearing period.

In addition to these broad areas, we hope to increase understanding of the suckling deficits observed in preterm human infants and human infants necessarily deprived of suckling, through the use of imaging ultrasound and examination of the deficits already observed, and yet to be observed, in artificially reared rats. Through this critical examination is likely to come the first generation of specific therapies and treatments aimed specifically at treating neonatal patients.

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Vita

Wayne Allen Moore, Jr. was born a citizen of the United States of America on September 24, 1971 in Charlotte, North Carolina. He graduated from East Mecklenburg High School in Charlotte, North Carolina in 1990 and then attended Appalachian State University where he earned a Bachelor of Science degree in Exercise Science In 1995. Following his undergraduate work, Allen was accepted into Western Carolina University's Master of Physical Therapy program where he graduated in 1998. Following Graduation, Allen accepted a position as an acute care physical therapist at McLeod Regional Medical Center in Florence, South Carolina.

Following three years at McLeod Regional Medical Center, Allen was accepted into Virginia Commonwealth University's School of Medicine to pursue a doctoral degree. While at Virginia Commonwealth University Allen was trained in Dr. Stephen J. Goldberg's lab in the measurement and assessment of skeletal muscle contractile properties and the anatomy of the hypoglossal motor system. Allen will be presenting his work at the Society of Neuroscience's annual conference in November of 2005.