Research report
Seasonal plasticity of neuromuscular junctions in adult male Siberian hamsters (*Phodopus sungorus*)

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Accepted 1 December 1998

Abstract

Transfer of adult Siberian hamsters, *Phodopus sungorus*, from long day (16 h light and 8 h dark; 16L:8D) to short day (8L:16D) photoperiods induces an involution of the gonads and a cessation of reproductive behavior 8–10 weeks later. The motoneurons of the spinal nucleus of the bulbocavernosus and their target muscles, the bulbocavernosus and the levator ani, are sexually dimorphic and are necessary for successful reproduction by male mammals. We demonstrate that after transfer of adult male Siberian hamsters to short photoperiods, the bulbocavernosus motoneurons, their target muscles and neuromuscular junctions are all significantly smaller than those of males that remain under long day conditions. Photoperiod also affected the number of active zones within each neuromuscular junction, an apparent remodeling of these synapses. Thus, this neuromuscular system of adult Siberian hamsters demonstrates considerable seasonal plasticity in response to changes in photoperiod.

Keywords: Siberian hamster; Photoperiod; Spinal nucleus of the bulbocavernosus; Neuromuscular junctions; Sexual dimorphism

1. Introduction

Like many temperate zone rodent species, the Siberian hamster (*Phodopus sungorus*), usually breeds only during the spring and summer months [12]. This naturally occurring seasonal variation in behavior is mediated in part by changes in melatonin secretion from the pineal gland in response to changes in photoperiod [2,25]. Melatonin is released during the dark, so as days get shorter, the nightly duration of melatonin secretion increases. Long durations of melatonin exposure (> 10 h/day) initiate the phenotypic changes associated with the transition to the winter, non-breeding condition [9]. These include a gradual change in pelage color from dark to light, decreased body weight, and gonadal regression. In males, gonadal regression is mediated by decreases in gonadotropin (luteinizing hormone, LH; and follicle stimulating hormone, FSH) and androgen production [15] and results in a cessation of spermatogenesis. Short-day conditions also result in behavioral changes such as a reduction in copulation (as shown in Syrian hamsters [17]). Changes in nervous system structure that mediate specific seasonal behavioral changes have not yet been documented in adult *P. sungorus*.

The motoneurons of the spinal nucleus of the bulbocavernosus (SNB) and their target muscles, the bulbocavernosus (BC) and the levator ani (LA) comprise a neuromuscular system active during copulation. In rats and mice this system is highly sensitive to steroid hormones. Differences in plasma androgen concentrations between male and female rats during development result in sexual dimorphism: the BC/LA muscle complex is much larger and there are more and larger SNB motoneurons in adult males compared to females [6]. This sensitivity to androgens persists into adulthood; castration results in a shrinking of the BC and LA muscles [23,21] and SNB motoneuron somata [5], whereas androgen replacement ameliorates this shrinkage. Likewise, the dendritic trees of SNB motoneurons shrink after castration in rats, and androgen treatment can reverse this effect [16].

So far, the neuromuscular junctions (NMJs) of the SNB system have demonstrated only limited plasticity. When castration and androgen replacement were used to manipulate the size of adult BC muscle fibers in male mice, the NMJs shrank and expanded in conjunction with fiber size as shown by Balice-Gordon, et al. [1]. However, the change in the size of the NMJs was a result of each nerve...
terminal branch and its underlying post-synaptic receptor region becoming smaller and re-enlarging in concert with fiber size, with no change in the number of post-synaptic receptor clusters (active zones) or terminal branches [1].

In male Siberian hamsters gestated and raised under short photoperiods the development of the SNB system is delayed compared with those raised in long days. Along with smaller SNB motoneurons and BC/LA muscles, the NMJs were also significantly smaller in juvenile hamsters raised under short photoperiods compared to hamsters raised in long days [11]. Additionally, the number of active zones per NMJ was smaller in short-day raised animals suggesting that NMJs may be more plastic in *P. sungorus* than in mice. Alternatively, the different outcomes in mice and Siberian hamster may reflect differences in plasticity of adult vs. juvenile animals.

The present study focuses on the effect of photoperiod on SNB motoneurons, the BC/LA muscles and the NMJs of adult male Siberian hamsters. It has been shown in the seasonally breeding white-footed mouse (*Peromyscus leucopus*), that changes in photoperiod induce changes in the SNB system of adult males [8], but no other animals have yet been reported to display seasonal changes in the adult SNB. Furthermore, NMJ morphology was not addressed in the studies of *Peromyscus*. *P. sungorus* are more amenable to experimental manipulations than *Peromyscus*, because we have found them to be more fecund in the laboratory and more tolerant of surgical anesthesia.

2. Materials and methods

Eighteen male Siberian hamsters were gestated and raised under short-day (LD) photoperiods (16L:8D; 16 h light, 8 h dark; lights on 0200 h) until 3 to 4 months of age. At this time, the animals were divided into two groups, siblings being randomly distributed between groups. Ten animals were moved into short day (SD) photoperiods (8L:16D; lights on 1000 h; SD males), while eight were maintained under the long photoperiod condition (LD males). All males were housed either singly or with male siblings. In each condition, animals were housed at 22°C with continuous access to food and water.

After 10–11 weeks all animals from both long and short photoperiods were weighed and their BC muscles injected with 4.5 μl of 2% cholea-toxin conjugated horseradish peroxidase (CT-HRP; a retrograde neuronal tracer; List Laboratories, Campbell, CA) under ketamine cocktail anesthesia (21 mg ketamine, 2.4 mg xylazine, 0.3 mg acepromazine/ml at 3.3 μl/gm body weight i.p.). Two days later, the animals were administered a lethal i.p. injection of 39 mg pentobarbital sodium and perfused with phosphate buffered saline (PBS: pH 7.2) followed by 10% phosphate buffered formalin. Spinal cords, BC/LA muscles, and testes were harvested, and post-fixed in buffered formalin for about an hour. Testes and BC/LA muscles were trimmed of fat and weighed. Spinal cords were transferred to 20% sucrose solution at 4°C overnight, then frozen sectioned transversely at 50 μm and mounted on gelatin-coated slides. The tissue sections were then reacted with the chromagen diaminobenzidine (DAB, 0.05%; with 0.08% NiCl) to stain CT-HRP labeled cells according to the method of Watson and Burrows [24]. The slides were rinsed, dehydrated through a graded ethanol series, cleared in hemo-D and coverslipped using Permout.

The size of the retrogradely labeled SNB motoneurons was determined using a Macintosh computer connected to a digital video camera and microscope. First, a digital image of the stained motoneuron was obtained, by focusing on the soma at its greatest extent. Then, using the software program NIH Image, the border of the stained soma was traced using the mouse and the 2-dimensional area was calculated for each motoneuronal soma. At least 12 somata from each animal were measured by an observer blind to the group identity of the subjects.

After weighing the BC/LA muscles complex, SNB axons and neuromuscular junctions (NMJs) were stained using immunocytochemistry. Briefly, whole muscles were blocked in PBS with 0.3% Triton-X-100 (PBS-TX) with 10% normal horse serum for 2 h to overnight. Then muscles were incubated in mouse monoclonal antibodies to synaptophysin (Sigma, St. Louis, MO) to label synaptic zones, and neurofilament (2H3, 165 kDa; Developmental Studies Hybridoma Bank, Iowa City, IA) to label axons, simultaneously diluted in PBS at 1:400 and 1:200, respectively, with 1% normal goat serum Sigma, St. Louis, MO) and 0.05% sodium azide overnight at 4°C. After rinsing at least five times over 1.5 h in PBS-TX, muscles were incubated in biotinylated goat anti-mouse IgG (Vector Labs, Burlingame, CA; 1:200 in PBS-TX) overnight, with 1% normal goat serum Sigma, St. Louis, MO) overnight, then rinsed, dehydrated through a graded ethanol series, cleared in xylene and mounted on gelatin-coated slides. The tissue sections were then reacted with the method of Watson and Burrows 24 . The slides were then rinsed at 8°C overnight, then rinsed at 8°C overnight, then dehydrated through a graded ethanol series, cleared in xylene and mounted on gelatin-coated slides. The tissue sections were then reacted with the method of Watson and Burrows 24.

Fig. 1. Short days affect the reproductive system of photoreponsive animals. Testes (left) and BC/LA muscles (right) are significantly smaller in adult Siberian hamsters responding to short photoperiods. Means ± standard errors of the means. n = 8 for each group. Each p < 0.0005, two-tailed t-test.
were then rinsed, trimmed into thin sections, and dried overnight on slides before dehydration, clearing and mounting as above. The area of the NMJs was determined by an observer blind to the experimental condition using a Sony digital camera to capture an image of a magnified NMJ, then the NIH Image software was used to calculate the area. The two dimensional area of 12 NMJs was measured to produce an average value for each animal. To determine the number of synaptophysin-immunopositive zones within each junction, the number of synaptophysin-immunopositive zones of six randomly selected NMJs were counted and averaged for each of four animals from each condition. Although sometimes the labeling of individual synaptic zones appeared to merge, it was usually possible to delineate individual junctions by adjusting the focus of the microscope. To determine the average size of the synaptophysin-immunopositive zones, the two-dimensional area was calculated and averaged for 10 well-delineated zones from each of four animals in each condition. Differences were assessed for statistical significance by two-tailed t-tests with \( n \) = number of animals.

### 3. Results

#### 3.1. Photoreponse of testes

As in other studies [12,22], the body weight of the LD males was heavier than that of SD males (41.9 ± 7.0 g vs. 34.9 ± 8.1 g; mean ± standard deviation; \( p < 0.05 \)). We used testes weights as an indication of photoresponsiveness. Testes of two SD males failed to respond to short photoperiods (i.e., they weighed more than 400 mg) and these animals were thus determined to be nonresponders [19], and were not used for any further analysis. The testes of responsive SD males were significantly lighter than those from LD males (Fig. 1, left; \( p < 0.0001 \)).
3.2. Photoresponse of BC/LA

The BC/LA muscle complex was also significantly lighter in males responding to short photoperiods compared to those of males maintained under long days (Fig. 1, right; \( p < 0.001 \)).

3.3. Photoresponse of SNB motoneurons

The average area of the somata of SNB motoneurons was smaller in males responding to short photoperiods than those of males maintained under long photoperiods (Fig. 2, left; \( p < 0.001 \)).

3.4. Photoresponse of NMJs

The average area of the NMJs of the hamsters responding to short photoperiods was also smaller than that of males maintained under long photoperiods (Fig. 2, right; \( p < 0.001 \); and Fig. 3). There were fewer synaptophysin-immunopositive synaptic zones per NMJ in the SD males than in the LD males (Fig. 4, left; \( p < 0.001 \)). In animals from both photoperiods there was a range in the size of the synaptic zones, however, the average size of individual synaptophysin-immunopositive zones was not significantly different between conditions (Fig. 4, right; \( p > 0.3 \)). NMJs from animals from both photoperiod conditions frequently had small regions in the center of the junctions where synaptic zones were absent (Fig. 3), a feature common among mammalian NMJs.

The neurofilament staining of axons did not reveal any detectable difference in the pattern of innervation of BC/LA muscle long- vs. short-day conditions. There was little if any multiple innervation in either group. Preterminal branching patterns of axons were identical in both groups.

4. Discussion

It has been shown previously that adult male hamsters undergo a seasonal regression of testes, and a drop in androgen production and plasma concentrations in response to changes in photoperiod [13]. We found that, as in Peromyscus [8], there are photoperiod-induced changes in sexually dimorphic SNB neuromuscular system of P. sungorus. Because the SNB system has now been found to vary with photoperiod conditions in each of the two seasonally breeding species examined, this may be a general feature of all seasonally breeding mammals. The unique aspect of the present report is evidence that photoperiod can also influence the organization of neuromuscular synapses.

4.1. Androgen sensitivity of SNB system

The SNB system has been shown to be androgen sensitive in adult rats but androgen sensitivity by the SNB system of adult P. sungorus had not been demonstrated. Nevertheless, it was not surprising that the SNB motoneurons and BC/LA muscles were smaller in male P. sungorus that were responsive to short photoperiods. However, there are differences between castration/hormone replacement (the manipulations by which the SNB system of rats has been evaluated) and the conditions of our
experiment, where the only experimental manipulation between groups was the duration of a single daily environmental cue, photoperiod. In this experiment all animals remained gonadally intact. As has been shown previously [27], plasma androgen concentrations were likely reduced in SD animals compared to those of their LD counterparts. However, the drop in circulating androgens seen after castration is rapid and occurs within hours, whereas, the drop in circulating androgens in male hamsters after transfer to short photoperiods is gradual and occurs over several weeks (as has been demonstrated in Syrian hamsters [3]). There continue to be small amounts of androgens produced by the testes of animals housed under short photoperiods. Additionally, androgen sensitivity has been shown to be affected by photoperiod. The sensitivity of GnRH neurons to the feedback effects of androgens is augmented by short photoperiods [4]. Thus, unlike the case after castration, when levels of FSH and LH are elevated, the levels of FSH and LH are reduced in short-day animals [26].

4.2. Photoresponse of SNB system

Nevertheless, we saw the expected plasticity of the SNB neuromuscular system. The average area of the SNB motoneuronal somata and the weight of the BC/LA muscle complex were smaller in adult males transferred to short photoperiods than in those maintained under long photoperiod conditions. These findings are consistent with a study of the SNB in seasonally breeding *Peromyscus leucopus* [8].

4.3. Plasticity of neuromuscular junctions

However, in the present study, we also detected plasticity in the NMJs of BC/LA muscles from adult *Phodopus*. Similar to our findings in developing hamsters [11], the two dimensional area of the NMJs was smaller in males housed under short photoperiods than in males maintained under long day conditions. Within the NMJ, the average size of individual synaptophysin-immunopositive synaptic zones was similar for LD and SD males, but there were more such zones per NMJ in males from long days than in SD males. Thus, the number of synaptic zones within the adult NMJ is plastic, suggesting that these zones may be constructed and dismantled seasonally in concert with changes in muscle and motoneuron size. As each synaptic zone is normally innervated by a branch of the overlying motor axon, presumably LD and SD males also differ in the terminal branching pattern of SNB motoneuronal axons.

These results are in contrast to the effects of castration and androgen replacement in mice [1], where the overall size of the NMJ changes in concert with muscle fiber size, but the number of active zones remains constant. These differences between studies may be due to the different methods used to observe NMJs, or the different species used. Conversely, the difference may be due to the difference in hormonal manipulation: perhaps the plasticity seen in the present study is due to hormonal responses to photoperiod other than the loss of androgens mimicked through castration in mice.

Interestingly, it appears that in *P. sungorus* the size of individual synaptophysin-immunopositive synaptic zones is relatively constant throughout development; the size of the zones of the adult NMJs in this study was not significantly different from the zones of the juvenile (pre-pubertal) hamsters of an earlier study [11], even though the total area of the adult NMJs is much larger and there are many more such zones within each NMJ of LD adults compared to LD juveniles (40–45 days old).

4.4. Nonresponsiveness

Normally under short photoperiods, circulating concentrations of LH and FSH of adult male hamsters are reduced [20,27], and thus the testes are much smaller and circulating androgen concentrations are lower than those of their long-day counterparts [13]. However, in *P. sungorus* a sub-population has been identified whose gonads do not respond to short-photoperiods. The nonresponsiveness results from a failure to produce a long-duration melatonin pulse in short day lengths [19,11]. In our study, most males responded to short photoperiods as expected, however, 20% of the animals failed to respond as measured by testes weights. This is comparable to rates of non-responders in other studies [10,19].

4.5. Mammalian seasonal plasticity

Seasonal plasticity of neural areas involved in specific behaviors (song production, for example) has been known for some time in birds [18], but much less is known about seasonal neural plasticity of mammals. This study demonstrates in adult mammals that, in concert with the seasonal plasticity of reproductive behavior, there is a plasticity in the underlying neuromuscular anatomy. These results in *P. sungorus* are in agreement with those in *Peromyscus*, but we have additionally found differences in NMJ morphology in the Siberian hamster. There may be other examples of seasonal plasticity in adult mammalian neuronal systems in concert with seasonal changes in behavior. We propose that, although so far sparsely documented [7,14], instances of adult mammalian neuroplasticity in response to seasonally changing environmental conditions may be widespread.

Acknowledgements

The authors wish to thank Dr. Irving Zucker for advice. This work was supported by NSF grant IBN-9810342. Experiments were carried out using NIH guidelines for care of laboratory animals.
References


