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# ALTERATIONS OF BRAIN CATECHOLAMINES AND EXERCISE PERFORMANCE IN (+/-) NT3-KNOCK-OUT MICE

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## ABSTRACT

The influence of neurotrophin-3 (NT3) upon brain catecholamine concentrations and exercise performance were evaluated in mice carrying a targeted single copy inactivation of the NT3 gene (+/-). Concentrations of dopamine and norepinephrine were sampled from the hypothalamus, corpus striatum and olfactory bulb, and were found to be lower in +/- NT3 than +/+ wild type mice. Following exercise to exhaustion, the dopamine concentrations of +/- NT3 mice remained lower than their +/+ wild type littermate controls, however, the direction of the changes in dopamine (increases within hypothalamus and decreases within corpus striatum and olfactory bulb) were very similar between the +/+ and +/- NT3 mice. In contrast to dopamine, the profiles of changes in norepinephrine concentrations following exercise diverged with analyses of these data showing a significant genotype x exercise interaction. The times required for +/- NT3 mice to achieve exhaustion during exercise were significantly lower compared with +/+ NT3 mice. These data provide evidence supporting the theory suggesting central nervous system catecholaminergic involvement in exercise fatigue that can be studied within the +/- NT3 mouse model.

## Introduction

Due to the general consensus that exercise is associated with significant physiological, psychological and social benefits, the number of studies examining the effect of short and long term exercise on brain neurotransmission has appeared to increase substantially (2, 7, 8, 11, 12, 18, 23, 30, 31, 32,33, 34, 35, 36, 46). Transgenic animals have served as valuable models for the study of physiological processes and analysis of perturbations in these processes, which result from targeted gene mutations (1, 10, 13, 39, 45). Mice with a targeted deletion for one of the copies of the neurotrophin-3 (NT3) gene show a number of structural and functional deficits in their cardiac system resulting from a diminished sympathetic innervation (45). Since NT3 represents a member of the neurotrophin family which is required for growth, differentiation and survival of neurons (29), this reduction in sympathetic innervation of +/- NT3 mice is not unexpected. Moreover, significant decreases in sensory neurons within the peripheral nervous system are observed in these +/- NT3 mice (14, 15,16). While +/- NT3 mice do not appear to show any salient differences to their wild type littermates and are capable of reproducing (14), some abnormal postures and movements have been

reported in these mice (27). It has been shown recently that heterozygous mutant NT-3 (+/-) knockout mice expressed a different cardiac sympathetic response during exhaustive endurance exercise compared to wild type mice (24). To the best of our knowledge, there exists no report observe changes in central nervous system catecholamine concentrations within +/- NT3 mice. Nor are there any data on some of the behavioral consequences resulting from this single copy deletion of the NT3 gene. Due to the insufficient amount of information available in this research area, we were particularly interested in examining basal and post-exercise catecholamine concentrations within selected brain sites of +/+ and +/- NT3 mice. The hypothalamus and corpus striatum were selected for assay due to their importance in generalized metabolic functions and control of locomotion, respectively (3, 5, 9, 17,19, 20, 28, 31, 32, 40, 41). In addition, catecholamine concentrations were determined within the olfactory bulbs since this area may serve as a control site, which would not ordinarily be considered to be affected by exercise. The two basic questions which served as the bases for this report are: 1) What is the effect of the +/- NT3 condition upon brain catecholamine concentrations? and 2) Does this condition alter exercise performance?

## Materials and Methods

*Animals*

Mice were housed individually in plastic cages. These animals were fed and watered ad libitum and maintained on a 12:12 hour light/dark cycle (lights on at 0600 h). Approval from the Animal Care and Use Committees at Northeastern Ohio Universities in accordance with NIH Guidelines for the Care and Use of Laboratory Animals was granted for all experimental procedures. The NT-3 mutant strain used was developed on a 129 background by insertion of a partially deleted NT-3 coding exon for the NT3 gene (14, 45). NT-3 +/- mice were mated to produce offspring and mice were genotyped as +/+ or +/- by PCR amplification and 2% agarose gel electrophoresis of tail DNA. Oligonucleotide primers used for genotyping mice were oIMR130, oIMR131, and oIMR132. Two separate reactions were run for each sample using oIMR130 in combination with oIMR131 and oIMR132 which amplified products from the wild type and targeted alleles, respectively (Jackson Laboratory IMR protocol) (45).

*Tissue preparation*

At euthanasia, the hypothalamus, corpus striatum and olfactory bulb were removed from each animal and prepared for assay of catecholamine concentrations. For dissection of the hypothalamus, two lateral cuts were made in the hypothalamic sulci, an anterior cut at the border of the optic chiasm and a posterior cut at the level of the mammillary bodies to enable removal of the hypothalamic block. For removal of the corpus striatum, the brain was bisected and the ventricles pried open revealing the corpus striatum. The corpus striatum was removed from within the perimeter of the corpus callosum of both hemispheres. To remove the olfactory bulbs, the olfactory tracts were severed and the olfactory bulbs were pried off the cribriform plate. The tissues were weighed and placed in vials containing 500 µl cold (4˚C) 0.1 N $HClO_4$. All samples were sonicated, centrifuged, and an aliquot was removed from the supernatant for assay of dopamine (DA) and norepinephrine (NE). Catecholamine concentrations were then expressed as picograms of DA or NE per milligram of tissue weight.

*Catecholamine assay*

Measurements of DA and NE were performed using HPLC-EC (ESA Inc.). A 100 X 4.6 mm, 3 um C-18 reverse phase co-

lumn (Biophase ODS, BAS Inc.) with an isogradient mobile phase (5.75 gm citric acid, 4.1 gm sodium acetate, 0.5 gm sodium hydroxide, 20 mg sodium octyl sulfate, 35 mg EDTA and 7% methanol in 1.0 liter of deionized water at a pH of 4.5) was used in this assay. The mobile phase was strained through a milipore filter (0.45 um) and degassed prior to use. The DA and NE standards were diluted in 0.1 N HCl04 and doses of 12.5, 25, 50, 100, 200, and 400 pg/20 ul were used to construct a standard curve. Tissue supernatant samples in 20 ul were injected into the HPLC-EC. DA and NE levels from tissue samples were determined by comparisons with peak heights and retention times of standards. The sensitivity of this assay was determined by the reliable appearance of chromatographic peaks over baseline with noise established at < 12.5 pg/20 ul.

*Exercise protocol*

Twenty six +/- NT-3 and 12 wild type littermate control (+/+) adult mice were randomly assigned into the following four groups: 1) +/-"sedentary" controls (SED) that were euthanized without treadmill exercise (n=15), 2) +/- exhaustively exercised (EX) and euthanized immediately after cessation of the exercise bout (n=11), 3) +/+ "sedentary" controls that were euthanized without treadmill exercise (n=7) and 4) +/+ exhaustively exercised euthanized immediately after cessation of the exercise bout (n=5). This proportion of cell sizes was a function of availability of animals. All animals were acclimated to the treadmill. The non-exercised animals were placed in the treadmill for the same amount of time as the exercised groups. Exercise capacity of the mice was evaluated with the graded run-to-exhaustion test. Animals were exercised using a motor-driven treadmill that had an adjustable belt speed. Treadmill speed was calibrated at the day when exercise groups performed the progressive run-to-exhaustion test. The protocol for the functional test involved a series of increasing speeds. Treadmill speed was initiated at 6 m/min, 4% inclination for the first 10 minutes of protocol, increased to 12m/min during the second 10 minutes of the protocol, and subsequently increased to 18 m/min during the third ten minutes of the protocol. This speed was maintained until exhaustion. The animals were continually monitored during the exercise test. No electric shock aversion was used. Exhaustion was defined as the point when a mouse, running on a motor driven treadmill at 18 m/min at 4% inclination, was unable to keep pace with the treadmill. The time (min) to exhaustion was recorded for each mouse and the mice were euthanized immediately by rapid decapitation.

*Statistical analysis*

An independent groups 2 X 2 two-way analysis of variance (ANOVA) with the factors being genotype (+/+ wild type mice vs. NT-3 +/- mice strains) and treatment (SED vs. EX) was used to analyze potential differences in tissue concentrations of DA and NE for the three brain sites sampled. If a significant interaction was observed, a Simple Effects Analysis with a Bonferroni adjustment was used to compare specific contrasts. The differences in running times between +/- and +/+ mice were examined using an independent groups t-test. Significance was set a priori at $p < 0.05$. All values are presented as means (M) ± standard error of the mean (SEM).

## Results

For the **hypothalamus** data, no difference between wild type +/+ mice and NT-3 +/- mice strains was evident for NE (see Fig. 1). For hypothalamus DA, NT-3 mice ($M = 932.4 \pm 35.4$) had lower values ($P = .001$) than the wild mice ($M = 1146.3 \pm 66.2$) (see Fig. 2).

The interaction of wild type and NT-3 mice with condition (see Fig. 1) was significant ($P$ = .048) for NE in the hypothalamus. Post hoc testing of this interaction, using Analysis of Simple Effects and Bonferroni comparisons, did not yield any comparisons sufficiently strong to be predictable. The strongest comparison ($P$ = .076) suggested the wild mice had higher NE values in the hypothalamus in the sedentary condition than when exercised.

A condition main effect occurred for DA ($P \leq .0005$) in the hypothalamus. Exercise values were higher than sedentary (see Fig. 2) for both mice strains.

Catecholamine/metabolic concentrations in the **corpus striatum** yielded a main effect for mice type for DA ($P$ = .002) with NT-3 mice evidencing lower DA ($M$ = 14163.1 $\pm$ 650.7) than wild mice ($Ms$ = 17263.2 $\pm$ 678.3) (see Fig. 3). A condition main effect for DA ($P$ = .005) was found, with sedentary values (M = 16274.0 $\pm$ 685.7) higher than exercise ($M$ = 13481.3 $\pm$ 731.5).

The interaction effect for NE was significant ($P$ = .015) in the corpus striatum. Simple Effects Analysis with Bonferroni comparisons indicated (see Fig. 4) that sedentary NT-3 mice had higher NE than the wild mice ($P$ = .024). Further, this difference between mice strains reversed under the exercise condition, with wild mice NE higher (P = .008) than NT-3 mice. Within the wild mice only, sedentary NE was lower ($P$ = .001) than exercise (see Fig. 4). There was no condition effect ($P$ = .323) for NE found for NT-3 mice, with no differences between sedentary values compared to exercise.

**Olfactory bulb** data indicated that the wild mice type was different from NT-3 mice for DA (P $\leq$ .0005) and that sedentary DA was higher than exercise values ($P$ = .001). A significant mice type by condition interaction effect for DA ($P$ = .042),

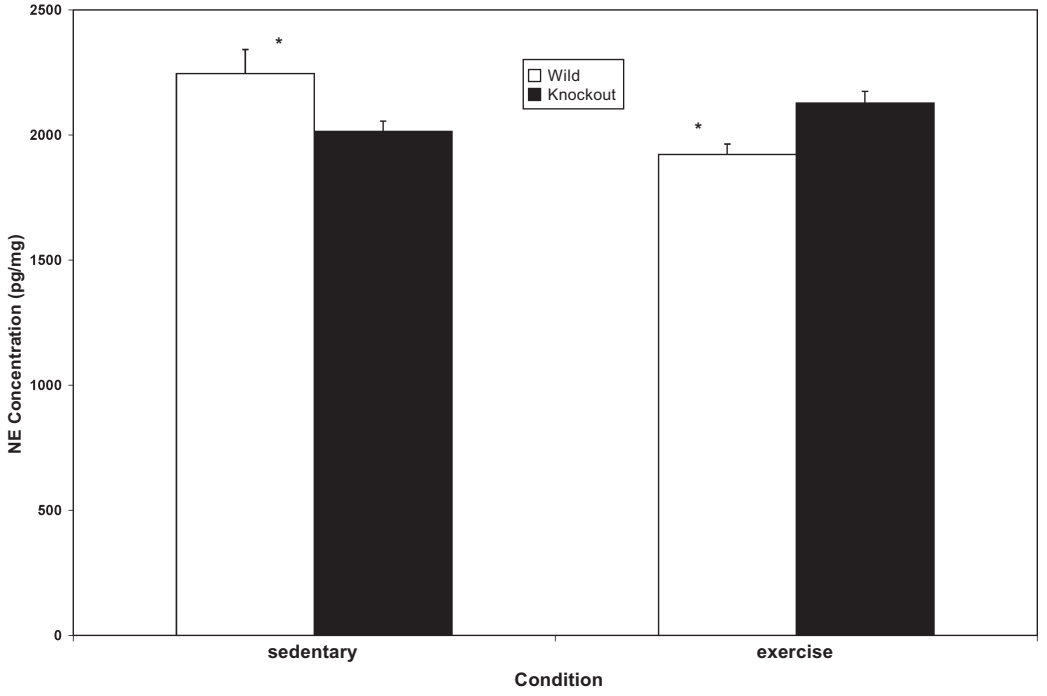


Fig. 1. *Hypothalamus norepinephrine (NE) concentrations for NT3 and wild mice under each condition (sedentary and after exercise). Values are means $\pm$ SEM.*
* $p$ = .076

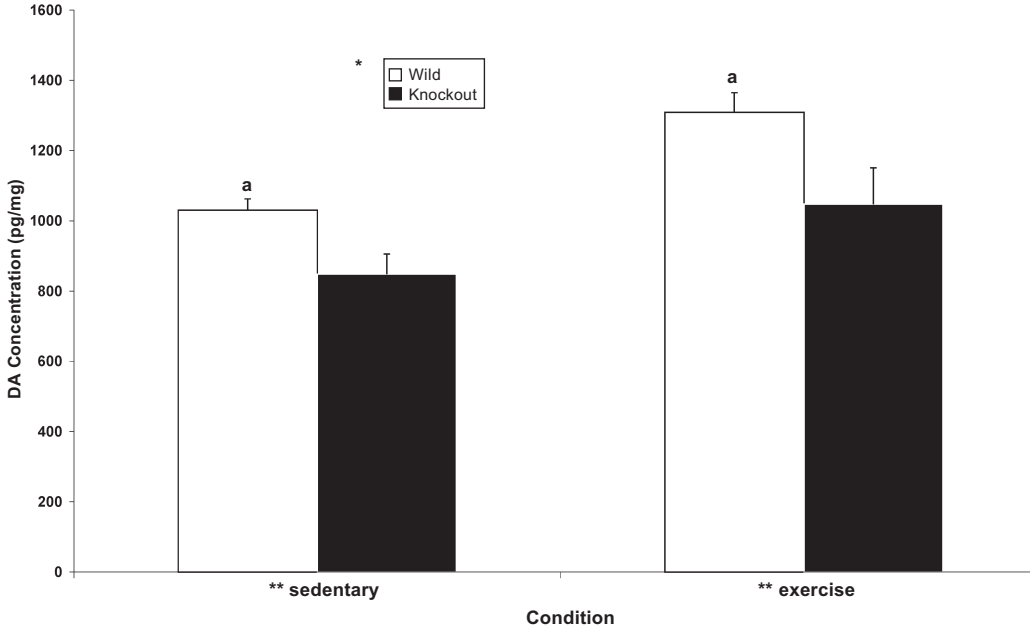


Fig. 2. *Hypothalamus dopamine (DA) concentrations for NT3 and wild mice under each condition (sedentary and after exercise). Values are means ± SEM.*
* $p = .001$; ** $^{a}p \leq .0005$

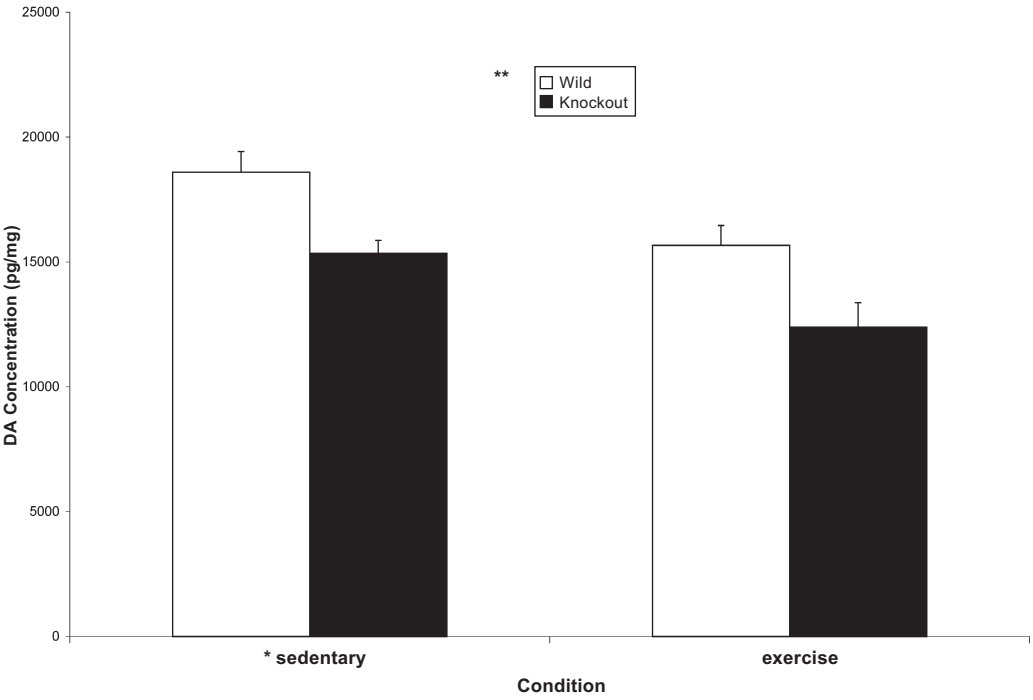


Fig. 3. *Corpus Striatum dopamine (DA) concentrations for NT3 and wild mice under each condition (sedentary and after exercise). Values are means ± SEM.*
* $p = .005$; ** $p = .002$

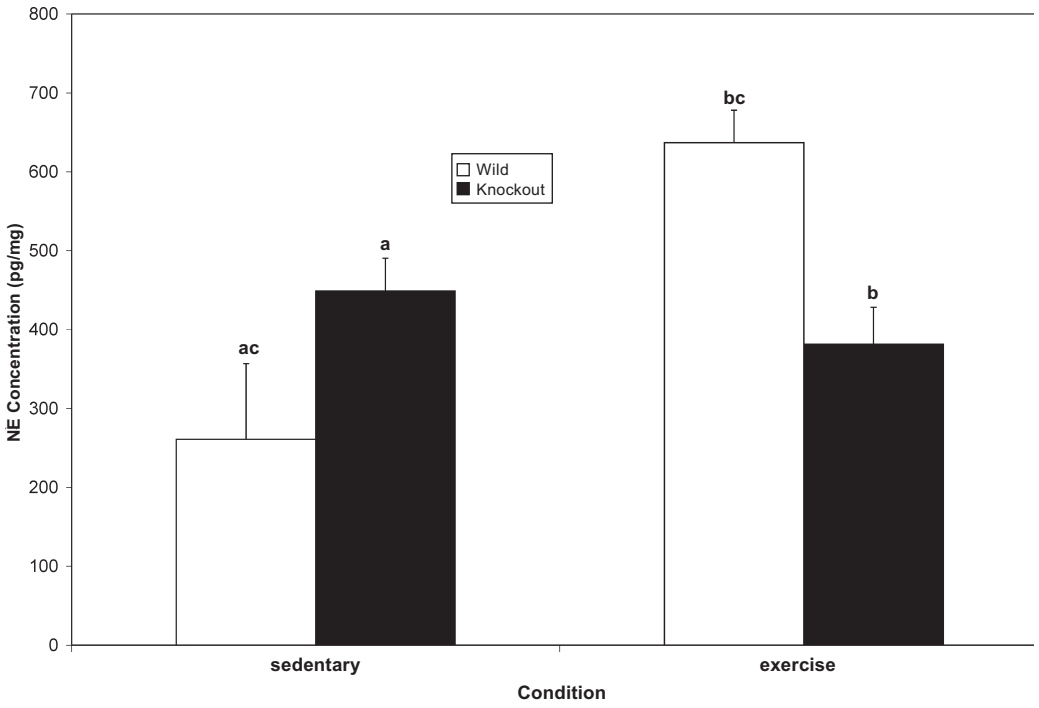


Fig. 4. *Corpus Striatum norepinephrine (NE) concentrations for NT3 and wild mice under each condition (sedentary and after exercise). Values are means ± SEM.*
*p* ≤ .05

when subjected to Simple Effect (Bonferroni) Analysis, indicated that the wild mice had higher concentrations of DA in the olfactory bulb tissue at the sedentary condition than after exercise ($P = .002$) and that while the wild mice demonstrated higher DA than the NT-3 mice under both conditions (see Fig. 5), the strongest effect was evident for the sedentary condition ($P \leq .0005$) and not the exercise condition ($P = .055$).

Wild mice (sedentary plus exercising $M = 216.2 \pm 11.5$) had higher NE values ($P = .002$) than NT-3 mice (sedentary plus exercising $M = 174.7 \pm 6.0$) over both conditions (main effect, see Fig. 6). Neither the condition effect nor the interaction of mice type and condition evidenced significance in olfactory bulb tissue DA ($P > .05$).

**Performance on the run-to-exhaustion test** is shown in Figure 7. Differences in running times during exercise between the two mice strains (NT-3 and wild) were analyzed using an independent groups t-test. Wild type mice (+/+) evidenced substantially greater ($P = .001$) running times ($M = 156.4$ min $\pm$ 29.2) than the NT-3 knockout mice ($M = 67.5$ min $\pm$ 3.5). Since Levene's Test for Equality of Variances was significant ($P \leq .0005$), an independent t-test for equal variances not assumed was conducted. The difference between the two mice strains remained significant ($P = .038$).

## Discussion

The present results show some intriguing changes in sedentary (basal) and post-exercise brain catecholamines between +/+ and +/- NT3 mice. Overall, the basal concentrations measured from sedentary mice for DA are consistently decreased in +/- NT3 mice within each of the three brain areas sampled. The basal concentrations

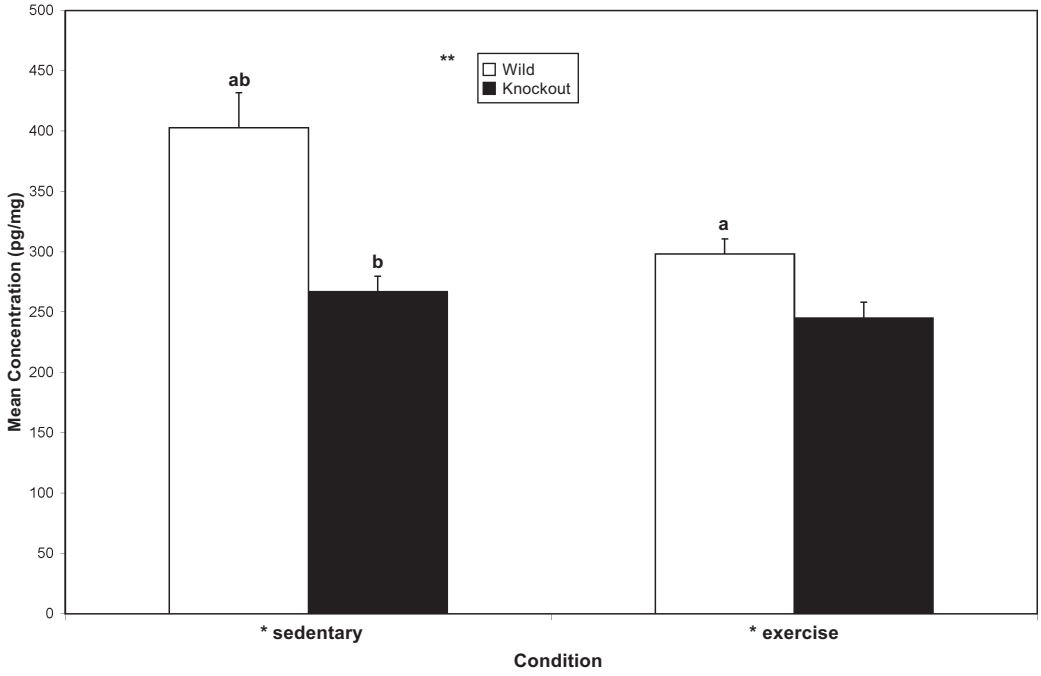


Fig. 5. *Olfactory Bulb dopamine (DA) concentrations for NT3 and wild mice under each condition (sedentary and after exercise). Values are means ± SEM.*
* $p = .001$; ** $p \leq .0005$; for like letters, $p \leq .05$

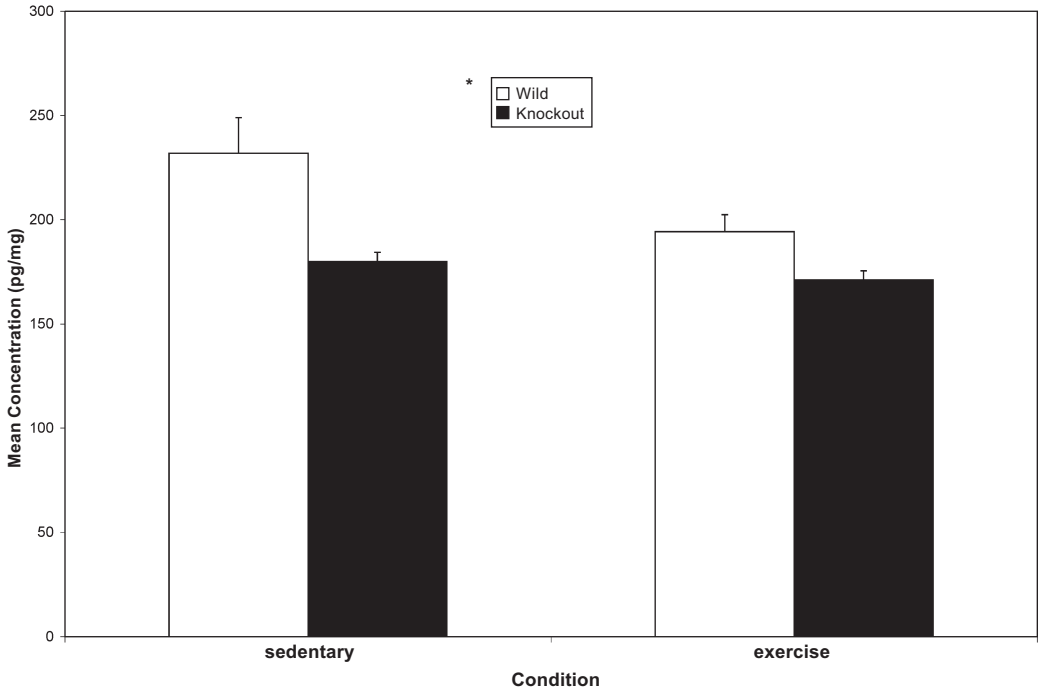


Fig. 6. *Olfactory Bulb norepinephrine (NE) concentrations for NT3 and wild mice under* each condition (sedentary and after exercise). Values are means ± SEM.
* $p = .002$

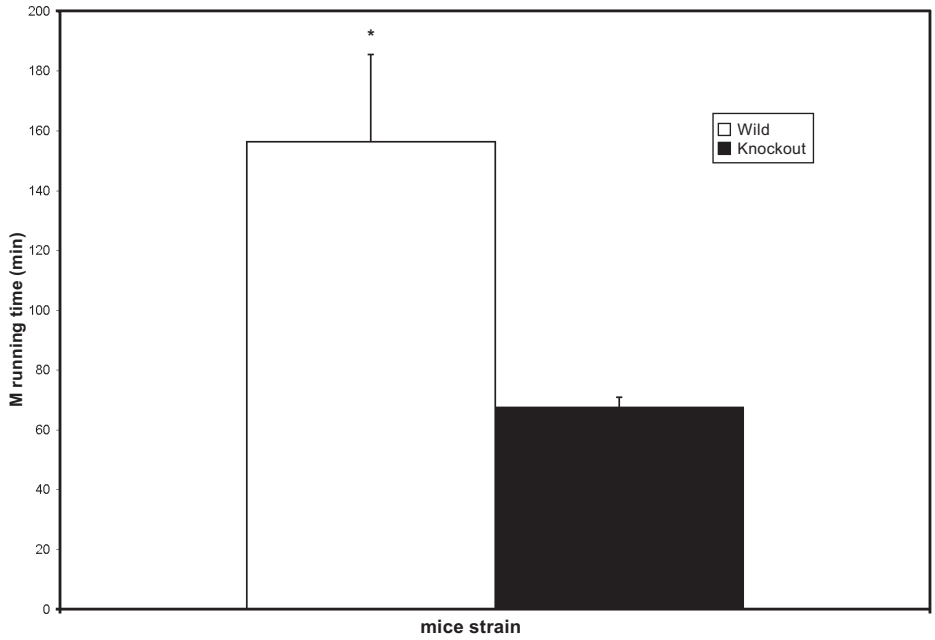


Fig. 7. *Treadmill running times to exhaustion (min) of NT3 and wild mice. Values are means ± SEM.*
* p = .001 (or .038 with equal variances not assumed)

measured from sedentary mice for NE are also decreased in +/- NT3 mice within hypothalamus and the olfactory bulb. These findings are similar to that observed for cardiac NE concentrations which are reduced in +/- NT3 mice (45) and can be related to the loss in sensory neurons reported in +/- NT3 mice (14). The present data now provide some evidence suggesting central nervous system effects upon brain catecholamines resulting from the targeted inactivation of a single copy of the NT3 gene. In general, reductions in basal catecholamine concentrations in +/- NT3 mice were more severe for DA (21% for hypothalamus, 16% for corpus striatum, 31% for olfactory bulb) versus NE (10% for hypothalamus, 22% for olfactory bulb); an exception is noted for NE in corpus striatum. These findings suggest that DA may be more adversely affected by the single copy deletion of NT3 in these mice.

For DA, no real discriminative changes between +/+ and +/- NT3 mice were obtained as associated with the exercise treatment. That is, for each of the three brain areas sampled, similar patterns of responsiveness (increases in hypothalamus and decreases in corpus striatum and olfactory bulb) were seen within +/- and +/+ NT3 mice. In this way, the single copy deletion of NT3 appears to exert no influence upon DA responses, which are present following exercise to exhaustion. In contrast, for the NE concentration data, a marked divergence in responsiveness between +/+ and +/- NT3 mice following this exercise regimen was shown. This profile was particularly salient for the hypothalamus where the analyses revealed a statistically significant interaction between genotype and exercise. Even though to a lesser degree, such a divergence was also seen within the corpus striatum. The exact nature of the relationship between the significantly decreased exercise performance and changes in catecholamines following exercise for the +/- NT3 mice must be considered with caution since the +/- NT3 condition significantly impairs cardiac function (45), which

also has the potential of reducing exercise performance. Our recent study also showed that stress of exercise uncovers an effect of inactivation of one copy of the mouse NT3 gene on the left ventricle NE concentration (24). In response to exercise stress, wild type mice demonstrated higher cardiac sympathetic activity, which is in agreement with our previous data (25). NE concentrations significantly increased in the left ventricle of the exercised wild type mice, while mice with disruption of one NT3 gene (+/-) knockout failed to show comparable responses. It is this evidence that suggests that heterozygous mutant NT3 (+/-) knockout mice could not maintain increased cardiac sympathetic response of the left ventricle during exhaustive endurance exercise at the same level as wild type mice (24). Exercise performance could have been influenced in part by the different cardiac sympathetic response as well as by genetic differences.

While remaining cognizant of this caveat about cardiac response to exercise of NT3 mice, the exercise dependent changes in catecholamines which are present between +/+ and +/- NT3 mice suggest the potential for a central nervous system involvement. Similar to our findings, hypothalamic DA concentrations have been shown to be increased in rats following prolonged exercise (2, 4, 6, 22). An increase in brain dopaminergic activity during exercise has been implicated in delaying fatigue, with reliance on DA's ability to inhibit brain serotonin metabolism and by directly activating motor pathways (2, 6, 7). It is of interest to the present report that endurance performance is also decreased following partial destruction of dopaminergic neurons by 6-hydroxydopamine (22) or increased by dopaminergic activation by amphetamine (18). Dopamine (DA) within the nigrostriatal dopaminergic (NSDA) system is associated with locomotion, emotion, and learning (3, 17, 28, 31, 32, 40). It has recently been shown that treatment with methamphetamine, which is known to decrease vesicular transporter function in dopaminergic neurons and dopamine uptake (5, 41), significantly decreased running times to exhaustion (23). In this way, although the pattern of responsiveness for hypothalamic DA following exercise was similar between +/+ and +/- NT3 mice (as described above), the overall higher levels of DA within +/+ NT3 mice compared with their transgenic littermates may contribute to their higher exercise performance. Thus, decreased running time to exhaustion in +/- versus +/+ NT3 mice may provide additional evidence supporting the concept that brain DA levels represent an essential countermeasure, required to prevent increases in serotonin levels which in turn may cause so called "central" fatigue (2, 6, 12, 38).

An additional central nervous system factor of potential significance as revealed in the present experiment are the changes in NE concentrations that occur following exercise. Our data showing a decrease of the hypothalamic NE in wild type animals after an acute exhaustive exercise are consistent with the similar findings in other studies (21, 22, 26, 42, 43, 44, 47). For all three brain areas sampled in our study, a clear difference in the response pattern of NE was obtained between +/+ and +/- NT3 mice in response to exercise. Again, this contrasts markedly with the similarity in profiles obtained between +/+ and +/- NT3 mice for DA. While the +/+ NT3 mice are characterized by changes in NE concentrations when sampled after exercise versus their sedentary levels, relatively little change is present within +/- NT3 mice in general. This lack of a NE response to the exercise treatment may then indicate another deficit in central nervous functioning, which may contribute to their decreased exercise performance.

The recent changes in the interpretation of the existing central fatigue hypothesis proposed by Newsholme et al. (37, 38) causes a concern for documentation. Previously such theories have taken into account the primary involvement of the central serotonergic system in central fatigue. However, it has been recently suggested that mental and physical deficiency, as well as central fatigue, are not caused by metabolic and neuromuscular alterations, but rather by impairment of the *interaction* of central neurotransmitters (46). Our data provide evidence supporting such central nervous system catecholaminergic involvement in exercise fatigue and has an increasing potential to be studied within the +/- NT3 mouse model.

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