

1 **SKELETAL MUSCLE GLUCOSE UPTAKE DURING TREADMILL EXERCISE IN**  
2 **NEURONAL NITRIC OXIDE SYNTHASE  $\mu$  KNOCKOUT MICE**

3

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22 RUNNING TITLE: Glucose uptake during exercise in nNOS $\mu$  KO mice

23

24 **ABSTRACT**

25 Nitric oxide influences intramuscular signaling that affects skeletal muscle glucose uptake during  
26 exercise. The role of the main NO-producing enzyme isoform activated during skeletal muscle  
27 contraction, neuronal nitric oxide synthase mu (nNOS $\mu$ ), in modulating glucose uptake has not  
28 been investigated in a physiological exercise model. In this study, conscious and unrestrained  
29 chronically catheterized nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  mice either remained at rest or ran on a  
30 treadmill at 17 m/min for 30 min. Both groups of mice demonstrated similar exercise capacity  
31 during a maximal exercise test to exhaustion (17.7 $\pm$ 0.6 vs 15.9 $\pm$ 0.9 min for nNOS $\mu^{+/+}$  and  
32 nNOS $\mu^{-/-}$  respectively,  $P > 0.05$ ). Resting and exercise blood glucose levels were comparable  
33 between genotypes. Very low levels of NOS activity were detected in skeletal muscle from  
34 nNOS $\mu^{-/-}$  mice and exercise increased NOS activity only in nNOS $\mu^{+/+}$  mice (4.4 $\pm$ 0.3 to 5.2 $\pm$ 0.4  
35 pmol/mg/min,  $P < 0.05$ ). Exercise significantly increased glucose uptake in gastrocnemius  
36 muscle (5 to 7-fold) and surprisingly, more so in nNOS $\mu^{-/-}$  than nNOS $\mu^{+/+}$  mice ( $P < 0.05$ ). This  
37 is in parallel with a greater increase in AMPK phosphorylation during exercise in nNOS $\mu^{-/-}$  mice.  
38 In conclusion, nNOS $\mu$  is not essential for skeletal muscle glucose uptake during exercise and the  
39 higher skeletal muscle glucose uptake during exercise in nNOS $\mu^{-/-}$  mice may be due to  
40 compensatory increases in AMPK activation.

41

42 **Keywords:** glucose transport, nitric oxide, AMPK

## 43 INTRODUCTION

44 Skeletal muscle glucose uptake during exercise is an important physiological process for  
45 blood glucose and cellular energy homeostasis. It is regulated by intramuscular signaling that  
46 modulates membrane permeability to glucose (42). Nitric oxide (NO) is a signaling mediator that  
47 can alter membrane permeability to glucose via modulation of GLUT4 translocation (7, 43). The  
48 production of NO increases with skeletal muscle contraction/ exercise (4, 27, 45) and a series of  
49 studies using NOS inhibitors show that NO mediates skeletal muscle glucose uptake during  
50 contraction/ exercise (3, 5, 23, 35, 43, 45). In contrast, some studies found that NO does not play  
51 a role in muscle glucose uptake during contraction (7, 12, 21, 46). Methodology differences are  
52 believed to contribute to some of the conflicting results (32).

53 In skeletal muscle, NO from contraction may be derived from several NOS isoforms  
54 including endothelial NOS (eNOS) and neuronal NOS (nNOS), which are constitutively  
55 expressed in skeletal muscle of rodents (24, 25). Inducible NOS (iNOS) is expressed under  
56 inflammatory or disease states (1, 9) and therefore is not likely to be involved in acute  
57 contraction-mediated events of animals/ healthy subjects. The most commonly used NOS  
58 inhibitors in studies investigating the role of NO in contraction-stimulated glucose uptake, N-G-  
59 Monomethyl-L-arginine (L-NMMA) and N-G-Nitro-L-Arginine Methyl Ester (L-NAME), are  
60 non-specific competitive inhibitors that inhibit all of the NOS isoforms (54). Therefore, these  
61 NOS inhibitors cannot isolate the role of different NOS isoforms in skeletal muscle glucose  
62 uptake during contraction/ exercise. As such, genetically modified rodent models are imperative  
63 in this regard.

64           Skeletal muscle glucose uptake during treadmill exercise has previously been determined  
65 in eNOS<sup>-/-</sup> mice which were found to have higher glucose uptake compared with wild type  
66 controls (28). This was postulated to be due to the exercise-induced hypoxia in contracting  
67 muscle which, in turn, may have stimulated a greater muscle glucose uptake (28) since hypoxia  
68 is a potent stimulator of skeletal muscle glucose uptake (6). In addition, NO production during ex  
69 vivo contraction was not different between eNOS<sup>+/+</sup> and eNOS<sup>-/-</sup> muscles (13) suggesting that  
70 eNOS may not be directly involved in NO-mediated intramuscular signaling. Given that nNOS $\mu$   
71 is the major NOS isoform activated during contraction (27), it was surprising to find that nNOS $\mu$   
72 knockout muscles did not have attenuated muscle glucose uptake during ex vivo contraction  
73 (16). Nevertheless, NOS inhibition of isolated nNOS $\mu$  knockout (nNOS $\mu$ <sup>-/-</sup>) and wild type  
74 (nNOS $\mu$ <sup>+/+</sup>) muscles still attenuated the increase in muscle glucose uptake (16) suggesting that  
75 NO was still playing a role in muscle glucose uptake during contraction. It should be considered  
76 that ex vivo contraction lacks the complex integrated interactions underlying in vivo exercise  
77 conditions such as neural input, blood flow and hormonal changes. Highly relevant to this  
78 context is that nNOS has been shown to mediate arterial relaxation in contracting skeletal muscle  
79 (27). Thus, in vivo studies are essential to define the role of nNOS $\mu$  in muscle glucose uptake  
80 during exercise.

81           In this study, nNOS $\mu$ <sup>+/+</sup> and nNOS $\mu$ <sup>-/-</sup> mice were used to investigate the effect of nNOS $\mu$   
82 on skeletal muscle glucose uptake in conscious and unrestrained chronically catheterized mice  
83 running on a treadmill. This allows examination of the role of nNOS $\mu$  in skeletal muscle glucose  
84 uptake in a physiological unstressed condition with intact hemodynamic and intramuscular  
85 signaling responses. We hypothesized that the increase in muscle glucose uptake during

86 treadmill running would be attenuated in nNOS $\mu^{-/-}$  mice because nNOS $\mu$  is the major NOS  
87 isoform activated during contraction (27).

88

## 89 **MATERIALS AND METHODS**

### 90 *Animals*

91 All procedures were approved by The Alfred Medical Research and Education Precinct  
92 (AMREP) Animal Ethics Committee, and conformed to the Australian Code of Practice for the  
93 Care and Use of Animals for Scientific Purposes (2004, 7<sup>th</sup> Edition). nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$   
94 littermates were generated by mating C57Bl/6 nNOS $\mu^{+/+}$  mice originally obtained from Jackson  
95 Laboratories (Bar Harbor, ME). Genotyping was performed using tail samples obtained at day 21  
96 of age by a commercial vendor (Transnetyx Inc., Cordova, TN). Mice were housed in standard  
97 cages and maintained under constant temperature of  $21 \pm 1^\circ\text{C}$  with 12-hour light/ dark cycle in  
98 the AMREP Animal Facility. Animals had access to standard rodent chow and water ad libitum.  
99 Both male and female mice were used for experiments at 16 weeks of age.

100

### 101 *Exercise stress test*

102 Mice were subjected to an incremental exercise stress test as previously described (29) to  
103 determine their maximum exercise capacity. Briefly, two days following a familiarization test  
104 (10 m/min for 10 min) mice commenced running at a speed of 10 m/min on a 0% incline  
105 treadmill. Running speed was increased by 4 m/min every 3 min until mice were exhausted,  
106 which was defined as the point whereby mice continuously remained at the back of the treadmill

107 for more than five seconds despite tail prodding. Treadmill electrical stimulation was not used in  
108 any of the tests.

109

### 110 *Surgery and experimental procedures*

111           Surgery procedures were performed as previously described (2) except that only jugular  
112 vein cannulation was performed due to an observed intolerance of nNOS $\mu^{-/-}$  mice to chronic  
113 carotid cannulation. Briefly, mice were anaesthetized with 5% isoflurane in oxygen and  
114 maintained with 2% isoflurane in oxygen throughout the cannulation procedure. Carprofen was  
115 given subcutaneously for pain relief prior to the skin incision. The right jugular vein was  
116 cannulated with a silastic catheter. The free end of the catheter was tunneled under the skin to the  
117 back of the neck where it was exteriorized. The catheter was kept patent with saline containing  
118 200 U/ml of heparin and 5 mg/ml of ampicillin, and sealed with stainless steel plugs. Mice were  
119 housed individually after surgery and body weight was monitored. Mice were used for  
120 experiments at least three days post-surgery when they had fully recovered as indicated by  
121 normal activity, healthy appearance and weight regained after surgery.

122           On the day of the experiment, the exteriorized jugular catheter was connected, via a  
123 stainless steel connector, with Micro-Renathane tubing approximately one hour prior to the  
124 experiment. Mice were then placed in a single lane treadmill to acclimate to the environment.  
125 During the experiment, mice remained sedentary or began a single bout of exercise ( $t = 0$  min).  
126 Exercise started at 15 m/min (0% incline) for three min and then increased to 17 m/min  
127 throughout the rest of the experiment until  $t = 30$  min (28, 46). Sedentary mice were allowed to  
128 move freely on the stationary treadmill for 30 min. In all mice, a bolus of 13  $\mu$ Ci of [1,2- $^3$ H]2-

129 deoxy-glucose ( $[^3\text{H}]2\text{-DG}$ ) was injected into the jugular vein at  $t = 5$  min for evaluation of tissue-  
130 specific glucose uptake. At the end of the experiment, mice were anaesthetized with a jugular  
131 vein injection of sodium pentobarbital (3 mg). A tail blood sample was immediately obtained for  
132 determination of blood glucose levels. The gastrocnemius and superficial vastus lateralis muscles  
133 from each limb and the brain were rapidly excised, frozen with liquid nitrogen-cooled tongs and  
134 stored at  $-80^\circ\text{C}$ . A blood sample was collected via cardiac puncture after exercise and used for  
135 plasma insulin and lactate determination.

136

### 137 *Muscle glucose uptake determination*

138 The determination of muscle glucose uptake was performed as previously described (8).  
139 Muscle sample and brain tissue (~30 mg) were homogenized with 1.5 ml of MilliQ water.  
140 Phosphorylated  $[^3\text{H}]2\text{-DG}$  ( $[^3\text{H}]2\text{-DG-6-P}$ ) was extracted from an aliquot of centrifuged  
141 homogenates (6000 rpm for 10 min at  $4^\circ\text{C}$ ) using an anion exchange resin column (AG1-X8,  
142 Bio-Rad). Radioactivity of the samples was determined using a  $\beta$ -counter (Tri-Carb 2800TR;  
143 Perkin Elmer, Chicago, IL, USA). Glucose uptake for each muscle was expressed as an index of  
144  $[^3\text{H}]2\text{-DG-6-P}$  accumulation in the muscle normalized to  $[^3\text{H}]2\text{-DG-6-P}$  in the brain of that  
145 mouse, as done previously (8, 11). Brain glucose uptake was used as a control for the integrated  
146 plasma  $[^3\text{H}]2\text{-DG}$  concentration differences over the duration of the experiments (8) as glucose  
147 uptake into the brain except the hypothalamus occurs via passive diffusion that follows glucose  
148 concentration gradient between the blood and brain tissue (31). In addition, intracellular glucose  
149 phosphorylation under normoglycaemic condition and hexokinase II have no impact on brain

150 glucose uptake (14, 40). Importantly, [<sup>3</sup>H]2-DG-6-P in the brain was not different between  
151 genotypes.

152

### 153 *Blood and plasma biochemistry*

154 Plasma insulin concentrations were determined using an enzyme-linked immunosorbent  
155 assay (Mercodia, AB, Uppsala, Sweden) as per manufacturer's instructions. Plasma lactate  
156 concentrations were analyzed with the enzymatic method of Lowry and Passonneau (30). Blood  
157 glucose levels were determined directly from the tail blood using an ACCU-CHEK Advantage  
158 monitor (Roche Diagnostics, Indianapolis, Indiana, US).

159

### 160 *Immunoblotting*

161 Immunoblotting was performed using ground frozen gastrocnemius muscle homogenized  
162 with 200 times volume of solubilizing buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 10%  
163 glycerol, 10 mM EGTA, 0.1 M DTT and 0.01% bromophenol blue) as described previously (15,  
164 38). Five µg of total protein from whole homogenates were separated on SDS-PAGE gels (Bio-  
165 Rad Laboratories, Hercules, CA), which was then wet transferred onto polyvinylidene fluoride  
166 (PVDF) membranes. Following membrane blocking with 5% skim milk in TBS solution, they  
167 were probed with the following primary antibodies overnight: phospho-AMPKα Thr<sup>172</sup> (1:1000),  
168 phospho-TBC1D1 Ser<sup>660</sup> (1:1000), AMPKα (1:1000), TBC1D1 (1:500), α-tubulin (1:1000) (Cell  
169 Signaling Technology, Danvers, MA, USA); nNOS (1:10,000), eNOS (1:10,000), iNOS (1:2000)  
170 (BD Biosciences, San Jose, California, USA); GLUT4 (1:8000) (Thermo Scientific, Rockford,  
171 IL, USA), and actin (1:40,000) (Sigma Aldrich, St Louis, MO, USA). Chemiluminescent signal



172 was developed with ECL substrate (SuperSignal West Femto, Pierce, MA, USA) and it was  
173 captured with a charge-coupled device (CCD) camera using Quantity One software (Bio-Rad).  
174 Pre-stained molecular weight markers were immediately imaged under white light source  
175 without changing the membrane position. To quantify both phosphorylated and total protein  
176 abundance, phosphorylation-specific primary antibody signal was first determined and then  
177 stripped (62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.8%  $\beta$ -mercaptoethanol), re-blocked and re-  
178 probed with primary antibody against the total protein. Loading control proteins were always  
179 probed on non-stripped membranes and actin was used for all proteins except GLUT4. Actin and  
180 GLUT4 have similar molecular weights and it was not possible to probe both of these proteins  
181 without undertaking the stripping process, therefore  $\alpha$ -tubulin was used as a loading control for  
182 GLUT4 abundance.

183

#### 184 *NOS activity assay*

185 NOS activity was determined as described previously (29) using radiolabeled L-  
186 [ $^{14}$ C]arginine. NOS activity was expressed as picomoles of L- [ $^{14}$ C]citrulline formed per min, per  
187 mg of protein. It was determined based on the difference between samples incubated with and  
188 without L-NAME.

189

#### 190 *Statistical analysis*

191 All data are expressed as means  $\pm$  SEM. Statistical analysis was performed using SPSS  
192 statistical package using one factor ANOVA (genotype) or two-factor ANOVA (genotype and  
193 exercise). If there was a significant interaction, specific differences between mean values were

194 identified using Fisher's least significance test. The significance level was set at  $P < 0.05$ . No  
195 sex-specific differences were observed in muscle glucose uptake during exercise (male vs  
196 female: nNOS $\mu^{+/+}$ :  $1.72 \pm 0.23$  vs  $1.50 \pm 0.14$ ,  $p > 0.05$ ; nNOS $\mu^{-/-}$ :  $1.72 \pm 0.10$  vs  $2.10 \pm 0.17$ ,  $p >$   
197  $0.05$ ) and therefore, data from male and female mice were pooled and analyzed together.

198

## 199 **RESULTS**

### 200 *Body weight and exercise capacity of nNOS $\mu^{+/+}$ and nNOS $\mu^{-/-}$ mice*

201 At 16 weeks of age, the body weight of nNOS $\mu^{-/-}$  mice was significantly ( $P < 0.05$ ) lower  
202 than that of nNOS $\mu^{+/+}$  littermates (Table 1). The ratio of male to female mice was not  
203 significantly different in either genotype (Table 1). The maximum running speed achieved  
204 during the exercise stress test was similar between genotypes (Table 1). Similarly, the maximum  
205 running times were not different between these mice although nNOS $\mu^{-/-}$  mice tended ( $P = 0.10$ )  
206 to run for a shorter time than nNOS $\mu^{+/+}$  littermates (Table 1).

207

### 208 *Blood glucose level*

209 At the end of the experiment, blood glucose concentration from the sedentary mice was  
210 not significantly different between genotypes ( $7.9 \pm 0.5$  mmol/l vs  $7.3 \pm 0.8$  mmol/l for  
211 nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  respectively,  $P > 0.05$ ). Exercise had no effect on the blood glucose  
212 concentration compared with the sedentary state and remained similar between genotypes ( $8.7 \pm$   
213  $1.0$  mmol/l vs  $7.1 \pm 0.3$  mmol/l for nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  respectively,  $P > 0.05$ ).

214

215 *Skeletal muscle glucose uptake*

216           Gastrocnemius muscle glucose uptake at rest (sedentary state) was not different between  
217 genotypes (Fig. 1A). Exercise significantly increased glucose uptake in gastrocnemius muscle (5  
218 to ~7-fold) and the exercise-induced glucose uptake (fold-increase) was significantly higher in  
219 nNOS $\mu^{-/-}$  compared with nNOS $\mu^{+/+}$  mice ( $P < 0.05$ ) (Fig 1B). A similar muscle glucose uptake  
220 pattern was observed in the superficial vastus lateralis (SVL) muscle (Fig 1C & 1D).

221

222 *Plasma insulin and lactate levels*

223           At the end of the exercise, plasma insulin was not different between genotypes ( $1.00 \pm$   
224  $0.16$  vs  $0.89 \pm 0.17$   $\mu\text{g/l}$  for nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  respectively,  $P > 0.05$ ). Plasma lactate was  
225 significantly elevated following exercise compared with the sedentary state (main effect,  $P <$   
226  $0.05$ ), and the increases following exercise were similar across genotypes ( $6.0 \pm 0.5$  vs  $5.4 \pm 0.7$   
227  $\text{mmol/l}$  for nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  respectively,  $P > 0.05$ ).

228

229 *Protein expression and phosphorylation*

230           The expression of actin and  $\alpha$ -tubulin proteins was not different between genotypes and  
231 they were used as loading controls. Total AMPK $\alpha$  expression in gastrocnemius muscle was not  
232 different between genotypes (Fig 2A and 2B). For sedentary muscles, AMPK $\alpha$  Thr<sup>172</sup>  
233 phosphorylation relative to AMPK $\alpha$  abundance was also not different between genotypes.  
234 Exercise significantly increased skeletal muscle AMPK $\alpha$  Thr<sup>172</sup> phosphorylation of both nNOS $\mu^{-/-}$   
235  $^{-/-}$  and nNOS $\mu^{+/+}$  mice compared with their respective sedentary group (Fig 2C). The increase in

236 AMPK $\alpha$  Thr<sup>172</sup> phosphorylation was significantly greater in nNOS $\mu^{-/-}$  mice compared with  
237 nNOS $\mu^{+/+}$  mice (Fig 2C). Expression of TBC1D1 in gastrocnemius muscle was also similar  
238 between genotypes (Fig 3A and 3B) and there was no difference in sedentary TBC1D1 Ser<sup>660</sup>  
239 phosphorylation relative to TBC1D1 abundance between genotypes (Fig 3C). Exercise increased  
240 TBC1D1 Ser<sup>660</sup> phosphorylation (Fig 3C; main effect,  $P < 0.05$ ). There was no iNOS detected in  
241 either nNOS $\mu^{+/+}$  or nNOS $\mu^{-/-}$  skeletal muscle. Endothelial NOS (eNOS) (Fig 4A) and GLUT4  
242 (Fig 4B) protein expressions were not different between genotypes.

243

#### 244 *Expression of nNOS $\mu$ , nNOS splice variants and NOS activity*

245 Neuronal NOS $\mu$  and nNOS $\beta$  (which are splice variants of nNOS) were detected in  
246 gastrocnemius muscles of nNOS $\mu^{+/+}$  but not nNOS $\mu^{-/-}$  mice (results not shown), as we have  
247 previously reported in EDL muscles (16). Exercise caused a significant increase in NOS activity  
248 in gastrocnemius muscles from nNOS $\mu^{+/+}$  mice ( $P < 0.05$ ). Low levels of NOS activity were  
249 detected in gastrocnemius muscle from nNOS $\mu^{-/-}$  mice (Fig 5) which is in accordance with  
250 previous data from EDL muscles (16) and brain tissues of nNOS $\mu^{-/-}$  mice (18).

251

## 252 **DISCUSSION**

253 In this study we observed that skeletal muscle glucose uptake increased to a significantly  
254 greater extent during 30-min of moderate intensity treadmill running in nNOS $\mu^{-/-}$  mice than  
255 nNOS $\mu^{+/+}$  littermates. The higher muscle glucose uptake in nNOS $\mu^{-/-}$  mice was observed together  
256 with a greater increase in skeletal muscle AMPK phosphorylation during exercise in nNOS $\mu^{-/-}$   
257 mice.

258           Given that NO is involved in GLUT4 translocation and nNOS $\mu$  is the main NOS isoform  
259 that produces NO during contraction in skeletal muscle (27), it is surprising and interesting to  
260 find that glucose uptake during physiological in vivo exercise was enhanced instead of  
261 attenuated in mice genetically lacking nNOS $\mu$ . Nevertheless, it was recently reported that  
262 although NO is involved in mediating skeletal muscle glucose uptake during ex vivo contraction,  
263 nNOS $\mu$  is not essential in this process (16). Skeletal muscle glucose uptake during ex vivo  
264 contraction was normal in mice with or without nNOS $\mu$  however glucose uptake was attenuated  
265 by NOS inhibition (L-NMMA) in both groups. The reduction in glucose uptake during  
266 contraction with L-NMMA was reversed by L-arginine indicating a critical role of NO in  
267 mediating glucose uptake in skeletal muscle during ex vivo contraction (16). Under  
268 physiological in vivo exercise conditions, various factors beyond the signaling events within the  
269 muscle including endocrine, vascular, neural and internal milieu inputs that work in an integrated  
270 fashion could affect skeletal muscle glucose uptake.

271           Neuronal NOS $\mu^{-/-}$  mice used in this study were generally comparable with their nNOS $\mu^{+/+}$   
272 littermates in a number of phenotypic features that may directly or indirectly influence muscle  
273 glucose uptake. The blood glucose level at rest (sedentary) and during exercise was similar in  
274 both genotypes implying that the higher glucose uptake in nNOS $\mu^{-/-}$  mice was not due to higher  
275 blood glucose levels (17). Similarly, plasma insulin levels after exercise were not different  
276 between genotypes suggesting that the observed higher glucose uptake in nNOS $\mu^{-/-}$  mice was not  
277 due to a potential additive effect of insulin on contraction-stimulated glucose uptake (57).

278           Exercise stimulated a greater muscle AMPK phosphorylation in nNOS $\mu^{-/-}$  mice compared  
279 with nNOS $\mu^{+/+}$  littermates. AMPK is a metabolic fuel sensor that can be activated following  
280 metabolic stress/ perturbations in which the degradation of ATP and the consequent

281 accumulation of ADP and AMP increase the ADP/ATP and AMP/ ATP ratio which leads to an  
282 increase in phosphorylation of AMPK (51). The higher AMPK phosphorylation in nNOS $\mu^{-/-}$   
283 mice suggests that they may have endured a higher metabolic stress. However, both groups of  
284 mice had similar maximum exercise capacity (maximal running speed and time) which suggests  
285 that the metabolic stress levels may have been similar. Although not statistically significant, it is  
286 possible that the 10% longer running time in the control mice compared with the nNOS $\mu^{-/-}$  mice  
287 could be important during high intensity exercise. We unfortunately did not measure oxygen  
288 uptake or carbohydrate oxidation during this study. Alternatively, AMPK can also be activated  
289 under hypoxic conditions (10, 56). nNOS has been shown to be involved in mediating arteriolar  
290 relaxation in contracting muscles (27, 50). Therefore, it is plausible that nNOS $\mu^{-/-}$  mice might  
291 have attenuated blood flow during exercise causing some degree of muscle hypoxia and a higher  
292 intramuscular metabolic stress (48) leading to a subsequent increase in phosphorylation of  
293 AMPK. It is unfortunate that we were unable to measure blood flow in these mice during  
294 exercise due to intolerance of the nNOS $\mu^{-/-}$  mice to chronic carotid artery catheterisation.  
295 However, eNOS $^{-/-}$  mice with lower exercise-induced increases in blood flow to the contracting  
296 muscle and a likely greater hypoxic state in the muscles have no greater increase in AMPK  
297 phosphorylation during exercise (28). Indeed, we have shown previously that there is little effect  
298 of hypoxia on glucose uptake during exercise in humans (56). Therefore, hypoxia-induced  
299 increases in AMPK phosphorylation in nNOS $\mu^{-/-}$  mice during exercise appear to be an unlikely  
300 stimulus for the greater increase in AMPK phosphorylation during exercise and thus the reasons  
301 for this finding remain unclear.

302           Though the higher muscle glucose uptake in nNOS $\mu^{-/-}$  mice could be due to the increased  
303 AMPK phosphorylation, we have no direct evidence to prove a causal relationship between these

304 parameters in nNOS $\mu^{-/-}$  mice as we have not investigated glucose uptake during exercise in these  
305 mice while preventing the increase of AMPK activation. It may be worthwhile to compare  
306 skeletal muscle glucose uptake during ex vivo contraction in nNOS $\mu^{-/-}$  muscles that are crossed  
307 with an AMPK dominant negative mouse strain.

308 TBC1D1 has been implicated in the regulation of muscle glucose uptake during  
309 contraction/ exercise in which glucose uptake is decreased in muscle overexpressing TBC1D1  
310 mutated on several predicted AMPK phosphorylation sites (53). TBC1D1 Ser<sup>660</sup> phosphorylation  
311 is one of the downstream effectors of AMPK (53) that is stimulated during contraction in mice  
312 (53) and exercise in humans (22). The increase in TBC1D1 Ser<sup>660</sup> phosphorylation with exercise  
313 in nNOS $\mu^{-/-}$  mice suggests that an AMPK-TBC1D1 mechanism may potentially be involved in  
314 stimulating the higher glucose uptake in these mice which, however, remained to be investigated.  
315 AMPK can also phosphorylate other downstream mediators such as AS160 to stimulate muscle  
316 glucose uptake (26) although there is evidence that AMPK-mediated AS160 phosphorylation  
317 does not have a role in muscle glucose uptake during contraction (52).

318 A caveat to the interpretation of the data using genetically-modified mice needs to be  
319 considered. The loss of a protein of interest during development that spans the entire lifespan  
320 could possibly induce secondary adaptations including compensatory overexpression of closely  
321 related proteins (33). These changes could mask the effects elicited by the loss of the protein of  
322 interest. In this study, no compensatory increase in iNOS, eNOS, nNOS splice variants, or  
323 GLUT4, all of which could directly or indirectly affect muscle glucose uptake, were detected in  
324 nNOS $\mu^{-/-}$  mice. Likewise, there was no difference in total AMPK or TBC1D1 expression  
325 between genotypes. These data suggest that nNOS $\mu$ , similar to ex vivo contraction (16), may not  
326 play a role in muscle glucose uptake during in vivo exercise because total loss of nNOS $\mu$  did not

327 attenuate glucose uptake nor elicit a compensatory response in the proteins examined. It should  
328 be considered, however, that there may have been compensatory increases in the other potential  
329 proteins that may regulate skeletal muscle glucose uptake including  $\text{Ca}^{2+}$ / calmodulin-dependent  
330 protein kinase (CaMKII) (58), protein kinase C (20), and Rac1/PAK1 (49).

331 In addition, an exacerbated ROS accumulation during exercise in  $\text{nNOS}\mu^{-/-}$  mice may  
332 have contributed to the higher muscle glucose uptake. Muscle contraction/ exercise increases  
333 ROS production in the heart and skeletal muscles (41, 47), and ROS increases muscle glucose  
334 uptake during ex vivo contraction (36, 47). Following acute exercise, there is significantly higher  
335 accumulation of ROS in the myocytes from mice lacking nNOS compared with controls (44). If  
336 a similar effect is conferred by nNOS in skeletal muscle during exercise as in the myocytes, it is  
337 plausible that muscle glucose uptake in  $\text{nNOS}\mu^{-/-}$  mice could be increased as a result of ROS-  
338 induced glucose uptake. Nevertheless, some studies have shown that ROS has no stimulatory  
339 effect on muscle glucose uptake during in vivo conditions in rats (34) and humans (37).

340 The relative roles of  $\text{nNOS}\mu$  could also be affected by the exercise intensity. Given that it  
341 has been shown that nNOS is expressed at higher levels in fast-twitch muscles than slow-twitch  
342 muscles (24, 36) it would be expected that nNOS would have a greater contribution to glucose  
343 uptake during exercise in fast-twitch muscles and/ or at higher exercise intensities. In fact, we  
344 have shown that NOS inhibition significantly attenuates the increase in glucose uptake during ex  
345 vivo contraction in EDL (mainly fast-twitch) but not in soleus muscles (mainly slow-twitch)  
346 (36). However, the fiber type effects on muscle glucose uptake during in vivo exercise are  
347 unclear. It is possible that there was no effect of a lack of  $\text{nNOS}\mu$  on glucose uptake during  
348 exercise because the intensity of exercise was insufficient to substantially activate  $\text{nNOS}\mu$ .  
349 However, the observed increase in NOS activity during exercise suggests that  $\text{nNOS}\mu$  was



350 indeed activated. Further studies should examine the effects of nNOS $\mu$  on glucose uptake during  
351 exercise at different intensities.

352 In this study, we observed very low levels of NOS activity in nNOS $\mu^{-/-}$  mice while eNOS  
353 abundance was not different between the genotypes. Together with the previous finding that  
354 NOS activity is normal or increased in eNOS $^{+/-}$  and eNOS $^{-/-}$  mice, respectively (28), these data  
355 indicate that nNOS $\mu$  is the predominant NOS isoform responsible for NOS activity in skeletal  
356 muscle. This finding is in agreement with a study showing that nNOS is the predominant NOS  
357 isoform that activates NO downstream signaling via cGMP during ex vivo contraction (27).  
358 Interestingly, eNOS abundance in skeletal muscle was not different between nNOS $\mu^{-/-}$  and their  
359 wild type littermate control mice in this study, as opposed to our previous study that found a  
360 compensatory increase of eNOS expression in nNOS $\mu^{-/-}$  muscles (55). However, in that study the  
361 control mice were C57Bl/6 mice rather than littermate controls (55). Others have also found no  
362 compensation of eNOS expression in myocytes and uterus of mice lacking nNOS when  
363 comparing to their wild type littermates (19, 39). This highlights the importance of using  
364 littermate controls as a proper experimental control.

365 In summary, nNOS $\mu$  is not essential for skeletal muscle glucose uptake during in vivo  
366 exercise. The greater muscle glucose uptake observed in nNOS $\mu^{-/-}$  mice than nNOS $\mu^{+/+}$  mice  
367 during moderate intensity treadmill exercise may be due to the observed greater increase in  
368 AMPK activation during exercise.

369

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374

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378

## 379 **AUTHOR CONTRIBUTIONS**

380 Y.H.H., C.Y., A.C.B., R.S.L.Y., and G.K.M. contributed to the conception and design of the  
381 research; Y.H.H., C.Y., and R.S.L.Y., performed the experiments; Y.H.H. and A.C.B. analyzed  
382 the data; Y.H.H., C.Y., A.C.B., R.S.L.Y., and G.K.M. interpreted the results of the experiments;  
383 Y.H.H. and A.C.B. prepared the figures and drafted the manuscript; Y.H.H., C.Y., A.C.B.,  
384 R.S.L.Y., and G.K.M. edited, revised and approved the final version of the manuscript.

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557  
558

559 **FIGURE LEGENDS**

560 Figure 1: Gastrocnemius muscle glucose uptake normalized to brain glucose uptake of that same  
561 animal (ratio) (A), and relative to sedentary state (fold change) (B), superficial vastus lateralis  
562 (SVL) muscle glucose uptake normalized to brain glucose uptake of that same animal (ratio) (C),  
563 and relative to sedentary state (fold change) (D). Data are means  $\pm$  SEM, n = 11 & 3 for  
564 sedentary nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  respectively, and 10 & 6 for exercise nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$   
565 respectively. \* P < 0.05 vs sedentary of the same genotype, # P < 0.05 vs exercise nNOS $\mu^{+/+}$ .

566  
567 Figure 2: Representative blots for AMPK, AMPK $\alpha$  Thr<sup>172</sup> phosphorylation and actin (A),  
568 gastrocnemius muscle AMPK $\alpha$  abundance in sedentary muscles (B), and gastrocnemius muscle  
569 AMPK $\alpha$  Thr<sup>172</sup> phosphorylation relative to AMPK $\alpha$  abundance (C). Data are means  $\pm$  SEM, n =  
570 9 & 4 for sedentary nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  respectively, and 9 & 5 for exercise nNOS $\mu^{+/+}$  and  
571 nNOS $\mu^{-/-}$  respectively. \* P < 0.05 vs sedentary of the same genotype; # P < 0.05 vs exercise  
572 nNOS $\mu^{+/+}$ .

573  
574 Figure 3: Representative blots for TBC1D1, TBC1D1 Ser<sup>660</sup> phosphorylation and actin (A),  
575 gastrocnemius muscle TBC1D1 abundance in sedentary muscles (B), gastrocnemius muscle  
576 TBC1D1 Ser<sup>660</sup> phosphorylation relative to TBC1D1 abundance (C). Data are means  $\pm$  SEM, n =  
577 9 & 4 for sedentary nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  respectively, and 9 & 5 for exercise nNOS $\mu^{+/+}$  and  
578 nNOS $\mu^{-/-}$  respectively. † P < 0.05 main effect for exercise.

579

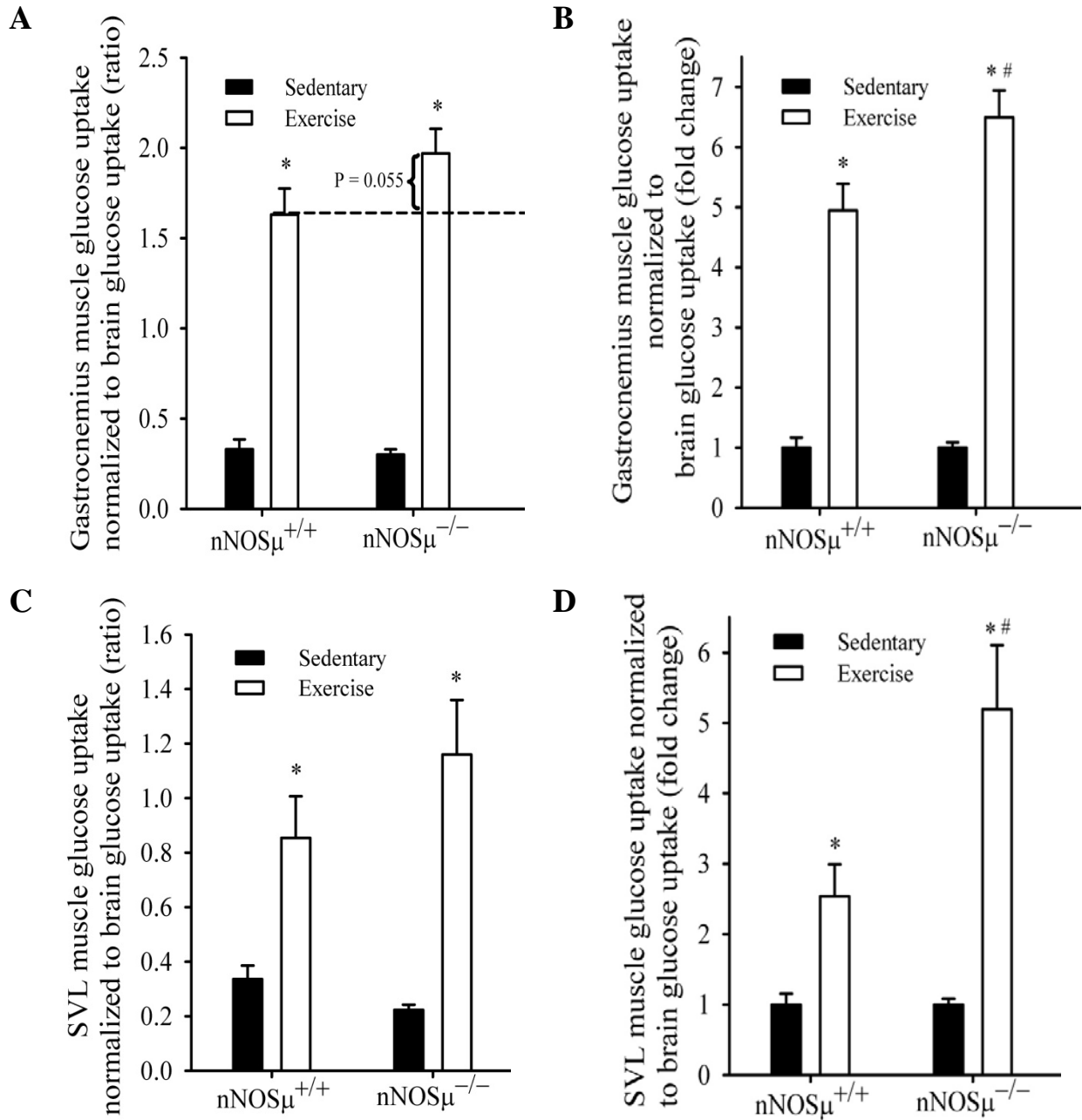


580 Figure 4: Gastrocnemius muscles eNOS (A) and GLUT4 (B) protein expressions in sedentary  
581 state relative to actin and tubulin abundance respectively. Data are means  $\pm$  SEM; n = 9 for  
582 nNOS $\mu^{+/+}$  and 4 for nNOS $\mu^{-/-}$ . For GLUT4 protein expression, bands at 45 and 40 kDa  
583 represented glycosylated and de-glycosylated GLUT4 respectively. Both bands were used for  
584 data analysis.

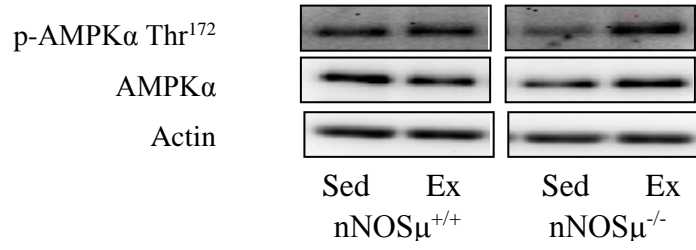
585

586 Figure 5: Gastrocnemius muscle NOS activity at rest (sedentary) and during exercise. Data are  
587 means  $\pm$  SEM; n = 7 & 3 for sedentary nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  respectively; and 7 & 5 for  
588 exercise nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  respectively. \* P < 0.05 vs sedentary of the same genotype; ‡ P  
589 < 0.05 vs nNOS $\mu^{+/+}$  of the same condition.

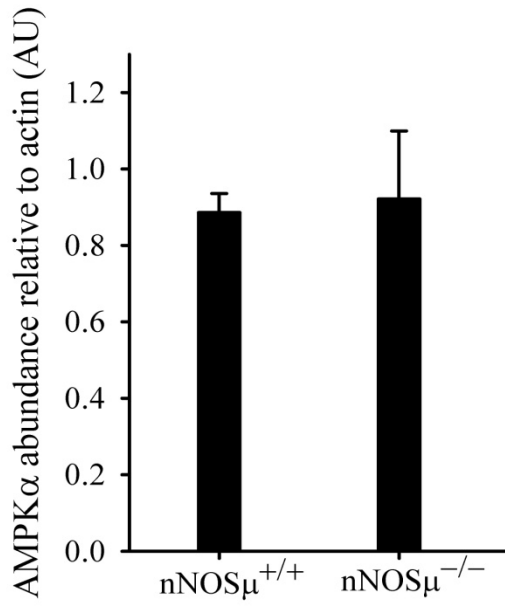
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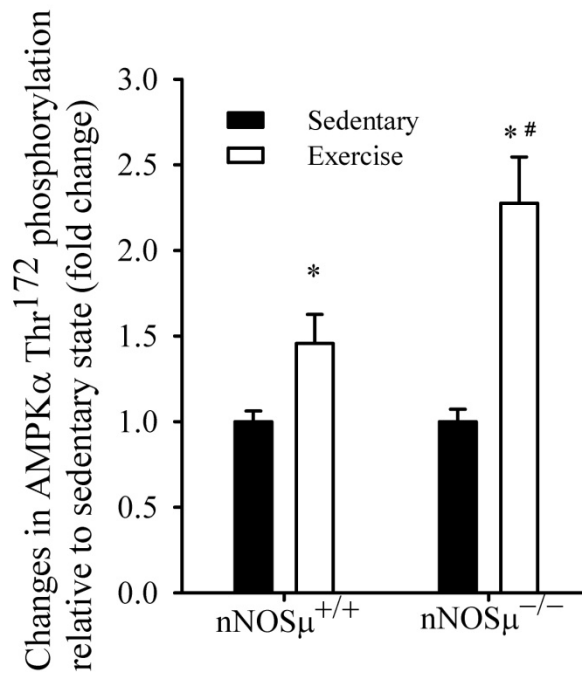
**A**

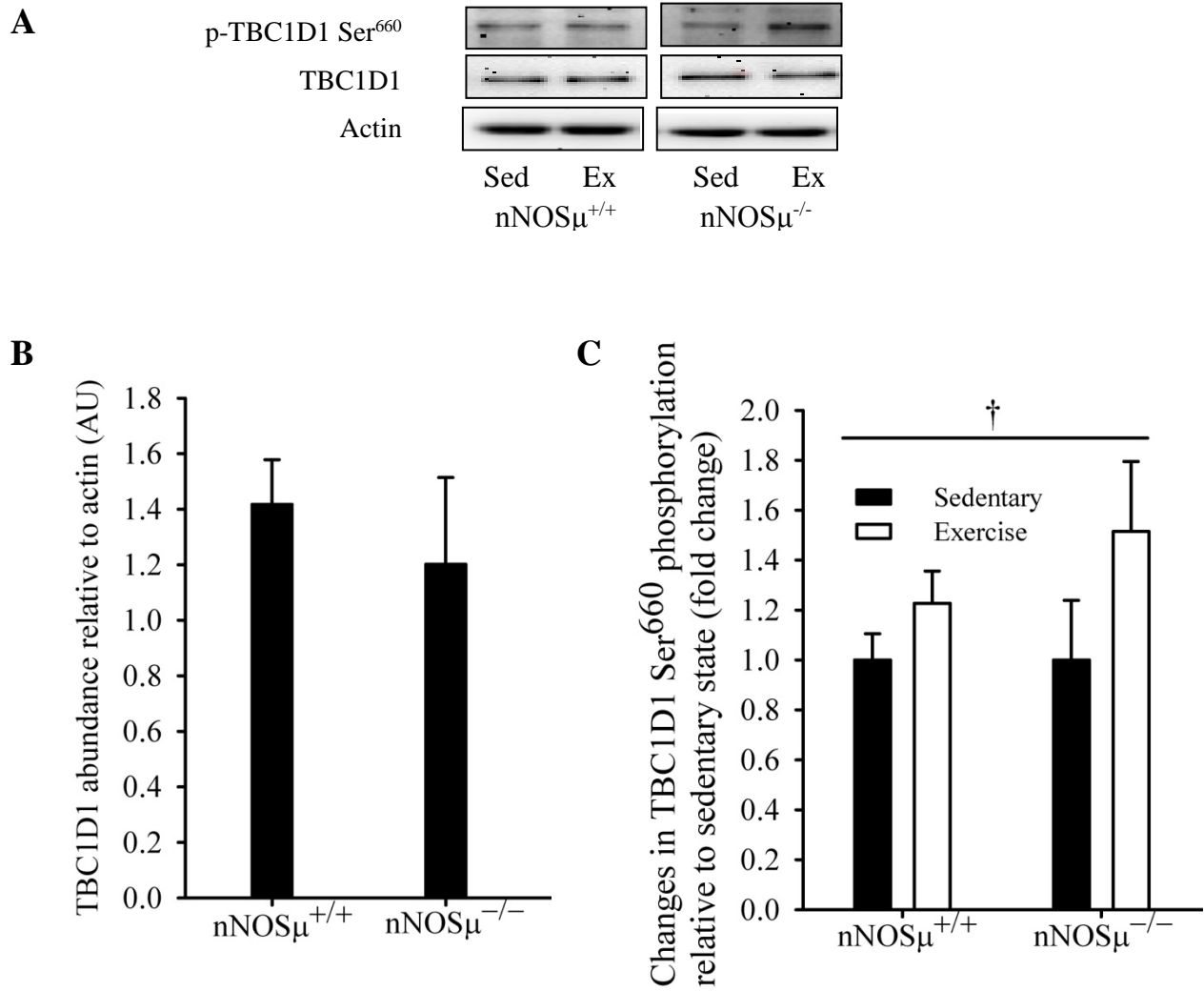


**B**

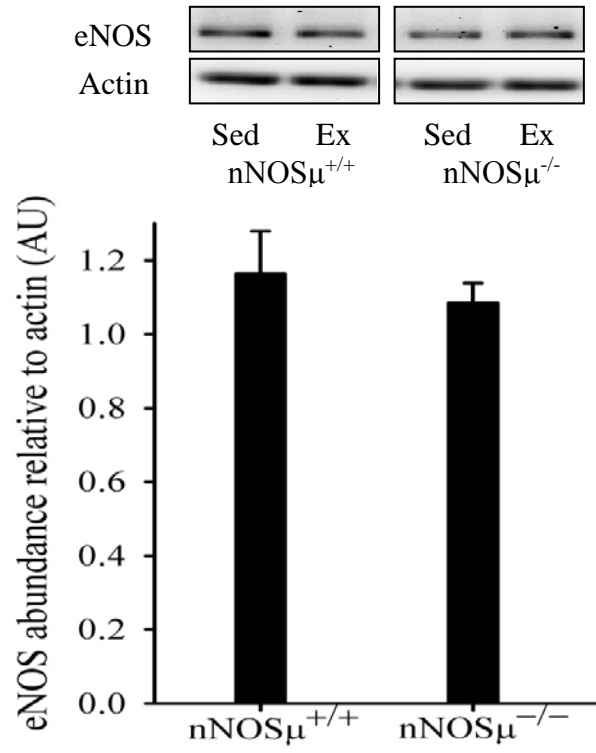


**C**

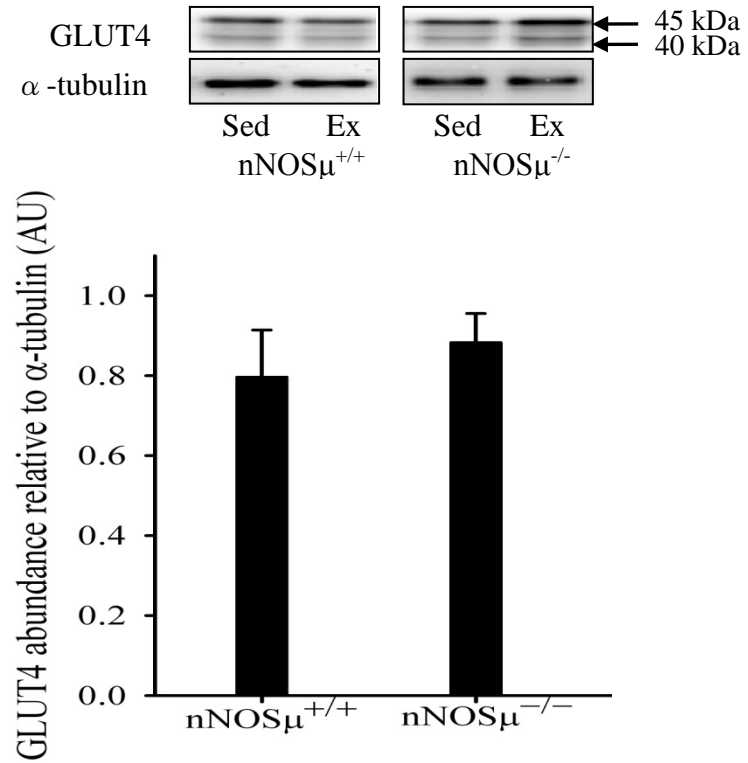


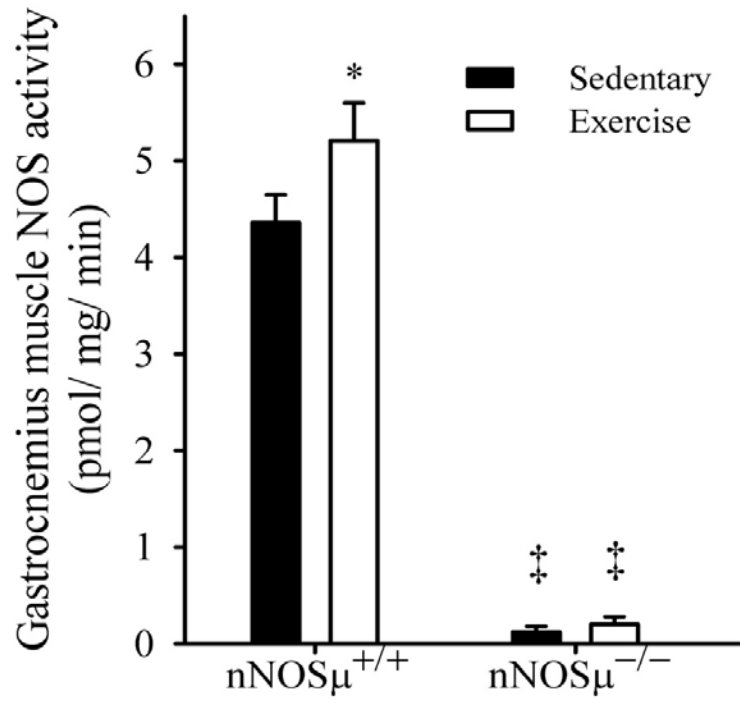


**A**



**B**





600 Table 1: Body weight and exercise capacity of nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  mice

	nNOS $\mu^{+/+}$	nNOS $\mu^{-/-}$
Male : female (number)	15 : 15	6 : 8
Body weight (g)	29.0 $\pm$ 0.8	23.6 $\pm$ 1.0 ‡
Max running speed (m/min)	31.5 $\pm$ 0.9	29.4 $\pm$ 1.2
Max running time (min)	17.7 $\pm$ 0.6	15.9 $\pm$ 0.9

601 Values are means  $\pm$  SEM, n = 30 and 14 for nNOS $\mu^{+/+}$  and nNOS $\mu^{-/-}$  respectively. ‡ P < 0.05 vs

602 nNOS $\mu^{+/+}$ .