

# Overexpression of hIGF-1 exclusively in skeletal muscle increases the number of dihydropyridine receptors in adult transgenic mice

M. Renganathan<sup>b</sup>, M.L. Messi<sup>a</sup>, R. Schwartz<sup>c</sup>, O. Delbono<sup>a,b,\*</sup>

<sup>a</sup>Department of Physiology and Pharmacology, Bowman Gray School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA

<sup>b</sup>Department of Internal Medicine, Gerontology, Bowman Gray School of Medicine, Wake Forest University, Medical Center Boulevard, Winston-Salem, NC 27157, USA

<sup>c</sup>Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, USA

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**Abstract** The number of dihydropyridine receptors (DHPR) and sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  release channels (RyR1) and their interaction determine the efficacy of the sarcolemmal excitation-SR  $\text{Ca}^{2+}$  release-contraction coupling (ECC). Both receptors play a central role in ECC as demonstrated in various animal species and muscle subtypes. In the present work we studied the effect of transgenic overexpression of human insulin-like growth factor 1 (hIGF-1) on the levels of these two  $\text{Ca}^{2+}$  channels in extensor digitorum longus (EDL) (fast-twitch), soleus (slow-twitch) and pool of fast- and slow-twitch muscles from adult C57BL/6 mice. Muscles from hIGF-1 transgenic mice showed a significant increase in IGF-1 concentration (20–30-fold) and in the number of DHPR (52% increase) whereas no significant change in RyR1 binding sites was detected. The differential effect on DHPR and RyR1 resulted in a 30% increase in DHPR/RyR1 ratio. Fast- and slow-twitch muscles showed 50 and 70% increase in the number of DHPR and 30 and 80% increase in DHPR/RyR1, respectively. These results support the concept that the increased autocrine/paracrine secretion of hIGF-1 exerts potent stimulatory effects on DHPR  $\alpha 1$  subunit expression in adult skeletal muscle.

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**Key words:** Skeletal muscle; Excitation-contraction coupling; Insulin-like growth factor 1; Aging; Dihydropyridine receptor; Ryanodine receptor

## 1. Introduction

Intracellular  $\text{Ca}^{2+}$  release in response to the dihydropyridine receptor (DHPR)  $\alpha 1$  subunit activation is a key process that modulates skeletal muscle contraction. Studies in various animal species and muscle subtypes demonstrated that the absolute number of DHPR and sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  release channels (RyR1) and their interaction determine the properties of excitation-contraction coupling [1,2]. Therefore, the modulation of DHPR and RyR1 expression and function may have significant impact on muscle contraction force and kinetics.

Insulin-like growth factor 1 (IGF-1) induces skeletal muscle proliferation, differentiation and expression of multiple genes [3]. We have demonstrated that in addition to effects on muscle development, IGF-1 facilitates skeletal muscle DHPR activity via tyrosine kinase-PKC-dependent phosphorylation [4]. Our laboratory has also communicated that IGF-1-dependent DHPR modulation is impaired in aging skeletal

muscles [5] which may explain, at least partially, the decline in muscle force with aging [6]. Despite the established fast modulation of voltage-sensitive  $\text{Ca}^{2+}$  channels by IGF-1, long-term effects of sustained high levels of the trophic factor in skeletal muscle on DHPR and RyR1 are unknown.

In the present study we explored the effects of exclusive overexpression of IGF-1 on skeletal muscle DHPR and RYR1 in transgenic S1S2 mice [7]. In this animal model the role of auto/paracrine secretion of IGF-1 in muscle proteins can be investigated separately from the endocrine influence. S1S2 mice exhibit pronounced muscle hypertrophy without increases in overall body weight or circulating IGF-1 concentration [7]. Because specific muscle force declines with age in several animal species and in humans [8–10] mechanisms of muscle weakness not associated with muscle mass have been explored. Among these mechanisms, age-related alterations in muscle DHPR-RyR1 coupling have been demonstrated in humans and rodents [11] [12].

We hypothesize that age-dependent DHPR-RyR1 uncoupling and the associated decline in muscle strength can be prevented or delayed by IGF-1-mediated increase in DHPR expression. Therefore, in this study we investigated whether high autocrine/paracrine IGF-1 secretion is associated with higher levels of DHPR  $\alpha 1$  subunit and RyR1 in adult fast- and slow-twitch muscles and muscles consisting of fast and slow fibers from S1S2 transgenic and wild-type FVB mice.

## 2. Materials and methods

### 2.1. S1S2 hIGF-1 transgenic and FVB non-transgenic mice

S1S2 transgenic mice overexpressing human IGF-1 (hIGF-1) exclusively in skeletal muscle and control wild-type FVB mice (12 months old) were used. Animals were housed in a pathogen-free area at Bowman Gray School of Medicine (BGSM) and fed ad libitum. S1S2 mice were screened for the presence of hIGF-1 genomic DNA to identify transgenic from wild-type mice (see below). Animal procedures followed an approved protocol by the Animal Care and Use Committee of BGSM.

### 2.2. Screening for human IGF-1 (hIGF-1) genomic DNA

Mouse tail segments were excised and digested overnight at 55°C in digestion buffer containing 1 M Tris-NaOH, pH 8.0, 5 M NaCl, 0.5 M ethylenediamine-tetraacetic acid, 20% sodium dodecyl sulfate and 20 mg/ml proteinase K. From this mixture, DNA was extracted by phenol:chloroform:isoamyl alcohol (25:24:1). Human IGF-1 gene was screened from this DNA by polymerase chain reaction (PCR) with specific 25-base primers: hIGF-1 5': ATT TAA gTg Ctg CTT Ttg TgA TTT C and hIGF-1 3' TTC CTA CAT CCT gTA gTT CTT gTT T. Amplified DNA fragments were analyzed for the presence of a clear 450-bp fragment specific for hIGF-1 on 2.5% agarose gels.

### 2.3. Determination of IGF-1 concentration by radioimmunoassay

Frozen quadriceps muscles were powdered under liquid  $\text{N}_2$  and

\*Corresponding author. Fax: (1) (910) 716-7359.

E-mail: odelbono@bgsm.edu

then homogenized (1:5 w/v) in 1 M acetic acid using a Polytron tissue homogenizer. Homogenate was incubated for 2 h to separate the debris from the supernatant. Homogenate equivalent to 100 µg protein was added to the reaction mixture containing 50 mM phosphate, pH 7.5, 50 mM NaCl, 10 mM EDTA, 3 mM sodium azide and 0.25% BSA. IGF-1 antiserum was a kind gift from NIDDK. The concentration of antiserum required for the maximum detection of IGF-1 was determined from IGF-1-antiserum titration. IGF-1 antiserum was specifically withheld in some reaction tubes to determine non-specific binding of radiolabeled IGF-1. Thr<sup>69</sup>IGF-1 (Bachem, Inc., Torrance, CA) was radiolabeled with <sup>125</sup>I using the lactoperoxidase, glucose oxidase procedure [13]. The optimal counts needed for maximal IGF-1 detection were determined by titrating radiolabeled IGF-1 against known IGF-1 concentration. To initiate the reaction 50 000 counts per tube were added and incubated overnight at room temperature. IGF-1-antiserum complex was precipitated by protein A for 30 min and the precipitate was pelleted down by centrifuging at 3000×g for 15 min. Radioactive IGF-1 in the pellet was estimated in a γ-counter. Data are expressed per mg of protein in relation to IGF-1 standards. The minimum and maximum detectable doses were 15 and 1000 pg IGF-1.

#### 2.4. Muscle homogenate and radioligand binding to DHPR and RyR1

Extensor digitorum longus (EDL), soleus and whole leg muscles were studied. Muscles were homogenized as described [5]. Protein concentration was determined by Coomassie protein assay with bovine serum albumin as the protein calibration standard.

DHPR and RyR1 concentrations were determined using the radioligands [<sup>3</sup>H]PN200-110 and [<sup>3</sup>H]ryanodine, respectively. Homogenate (1–2 mg/ml protein) were incubated either with 0.05–5 nM [<sup>3</sup>H]PN200-110 for 1 h at 23°C in 50 mM Tris-HCl, pH 7.5, 10 µM Ca<sup>2+</sup>, 1 mM diisopropyl fluorophosphate (DIFP) and 5 µM leupeptin or 0.5–50 nM [<sup>3</sup>H]ryanodine for 24–48 h at 10°C in 20 mM PIPES-NaOH, pH 7.0, 1.0 M NaCl, 100 µM Ca<sup>2+</sup>, 5 mM AMP, 1 mM DIFP, and 5 µM leupeptin. Membrane bound [<sup>3</sup>H]PN200-110 and [<sup>3</sup>H]ryanodine were determined by filtration through Whatman GF/B filters using a Millipore unit (XX2702550, Millipore Corporation, Bedford, MA). Filters were rinsed three times with 5 ml of ice cold 200 mM choline chloride, 20 mM Tris-HCl, pH 7.5. Non-specific [<sup>3</sup>H]PN200-110 and [<sup>3</sup>H]ryanodine binding was assessed in the presence of 10 µM unlabeled nifedipine (Sigma Chemical Company, St. Louis, MO) or PN200-110 (Sandoz Pharmaceutical, East Hanover, NJ) and 10 µM unlabeled ryanodine (Calbiochem, San Diego, CA) respectively. Radioligand concentrations used resulted in occupancy of >95% of the high-affinity binding sites [1].

#### 2.5. Data analysis

Linear regression and non-linear least squares analysis were used to calculate non-specific and total binding of the radioligands to the receptors. Specific binding of [<sup>3</sup>H]PN200-110 and [<sup>3</sup>H]ryanodine at each concentration was calculated by subtracting the non-specific binding from the total binding obtained from the above analysis. The following equation

$$y = (xa)((x + b) + (xc)) \quad (1)$$

where  $a$  = receptor number ( $B_{\max}$ );  $b = K_D$ , dissociation constant;  $c$  = the non-specific binding or the low affinity site, was used to fit the binding isotherm. Data were also given in a graphical representation of the Scatchard plot. There was no significant difference in  $B_{\max}$  values obtained from the curve fit or Scatchard plot [12]. All values were analyzed for statistical significance using unpaired Student's  $t$ -test.

### 3. Results

Two groups of five 12-month-old S1S2 transgenic and FVB non-transgenic mice were used in this study. The presence of hIGF-1 gene was detected by PCR analysis of genomic DNA. PCR was conducted using specific primers for hIGF-1 [7] (see Section 2). PCR fragments from the five transgenic and non-transgenic mice were analyzed on 2.5% agarose gel. Transgenic mice showed a single DNA band of 450 bp indicating the presence of hIGF-1 in the genomic DNA (Fig. 1).

The extent of hIGF-1 gene expression in S1S2 transgenic skeletal muscles was determined by measuring IGF-1 concentration in quadriceps muscles (Table 1). The mean IGF-1 concentration in non-transgenic and transgenic mice was  $825 \pm 268$  and  $21\,870 \pm 5982$  pg/mg total muscle protein, respectively. The difference between both groups was statistically significant ( $P < 0.001$ ). Skeletal muscles from transgenic mice exhibited a 20–30-fold higher concentration of IGF-1 than non-transgenic mice. This increase is similar to that reported for S1S2 hIGF-1 transgenic mice [7].

The concentration of DHPR and RyR1 and their dissociation constant for high-affinity radioligands were determined in a pool of muscles consisting of both fast- and slow-twitch muscle fibers. DHPR and RyR1 were quantitated in mouse skeletal muscle homogenates with the use of [<sup>3</sup>H]PN200-110 and [<sup>3</sup>H]ryanodine. Fig. 2 shows the [<sup>3</sup>H]PN200-110 and [<sup>3</sup>H]ryanodine binding (mean  $\pm$  S.D.) analysis of five transgenic and five non-transgenic mice. The insets for both figures are the Scatchard analyses of ligand binding to the DHPR and RyR1, respectively.  $B_{\max}$  and  $K_D$  values were determined by Scatchard analysis or by fitting Eq. 1 to binding data.

Individual non-transgenic and transgenic [<sup>3</sup>H]PN200-110 and [<sup>3</sup>H]ryanodine  $B_{\max}$ ,  $K_D$ , and DHPR/RyR1 ratio values were calculated from Scatchard analyses of the binding assay and are given in Table 1.  $B_{\max}$  and  $K_D$  values for transgenic muscles were (mean  $\pm$  S.D.)  $4.03 \pm 0.27$  pmol/mg protein and  $1.4 \pm 1.03$  nM, respectively and for non-transgenic muscles were  $2.53 \pm 0.62$  pmol/mg protein and  $1.9 \pm 0.79$  nM, respectively. These results demonstrate that exclusive overexpression

Table 1

Mouse	[IGF-1]	Pool					EDL			Soleus				
		DHPR		RyR1		Ratio	DHPR		RyR1	Ratio	DHPR		RyR1	Ratio
		$B_{\max}$	$K_D$	$B_{\max}$	$K_D$		$B_{\max}$	$B_{\max}$			$B_{\max}$	$B_{\max}$		
Non-transgenic														
W	–	2.43	1.25	2.49	7.7	0.98	5.98	6.1	0.99	2.23	5.85	0.38		
ASE	1185	2.96	2.5	2.83	11.4	1.05	5.40	6.1	0.88	2.08	5.80	0.36		
BQ	725	2.95	1.69	2.89	9.6	1.02	9.45	8.5	1.12	3.03	7.76	0.39		
BC	550	1.48	1.17	1.55	16.9	0.95	8.13	6.8	1.19	5.02	6.40	0.78		
BX	840	2.81	2.97	3.14	8.0	0.89	6.13	6.1	1.01	2.82	6.33	0.45		
Transgenic														
AO	–	3.80	1.04	2.8	7.3	1.36	9.8	7.8	1.26	5.20	6.78	0.77		
AG	17290	4.42	0.40	3.2	5.9	1.37	8.2	6.1	1.34	5.80	6.50	0.89		
AP	20165	4.06	0.85	3.31	3.3	1.24	13.7	9.7	1.42	5.63	8.92	0.63		
AQ	19380	3.76	2.08	3.32	6.9	1.16	11.0	7.9	1.39	3.98	4.96	0.80		
BB	30660	4.09	0.68	3.2	7.6	1.28	10.1	9.9	1.01	5.26	5.68	0.93		

IGF-1 concentration is expressed in pg/mg protein.  $B_{\max}$  and  $K_D$  are expressed in pmol/mg protein and nM, respectively.

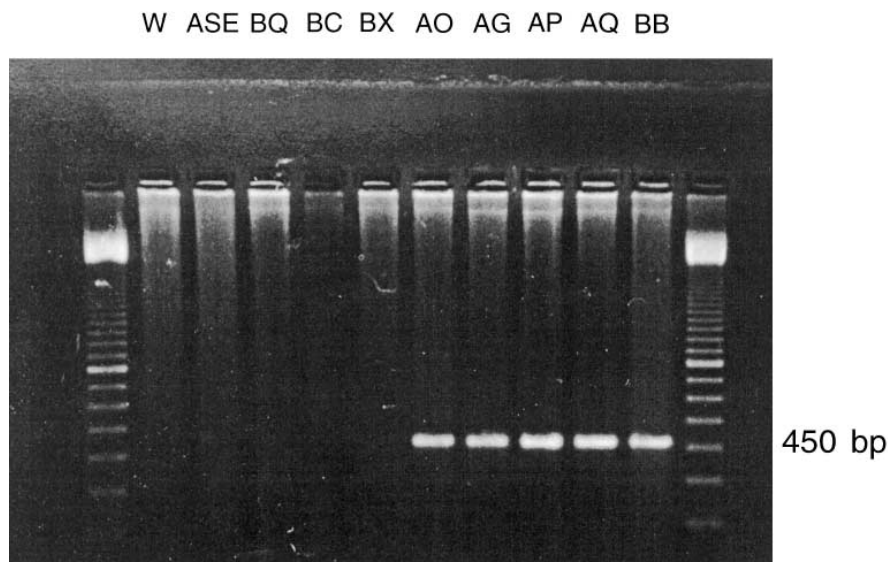


Fig. 1. Screening for hIGF-1 genomic DNA in mouse skeletal muscle on 2.5% agarose gel. Lanes 1 and 12 depict DNA markers of 200–1000 bp. Lanes 2–6 are non-transgenic mice labeled W, ASE, BQ, BC and BX. Lanes 7–11 are transgenic mice AO, AG, AP, AQ and BB. hIGF-1 band is located at 450 bp.

of IGF-1 in skeletal muscle is associated with a 52% increase in the number of DHPR  $\alpha 1$  subunit ( $P=0.001$ ). Scatchard analysis of ryanodine binding assays gave a  $B_{\max}$  of  $3.15 \pm 0.20$  pmol/mg protein and a  $K_D$  of  $12.18 \pm 8.69$  for transgenic muscles and  $B_{\max}$  of  $2.58 \pm 0.62$  pmol/mg protein and  $K_D$  of  $10.78 \pm 3.76$  nM for non-transgenic muscles. The difference in [ $^3\text{H}$ ]ryanodine  $B_{\max}$  between transgenic and non-transgenic muscles is not statistically significant ( $P=0.086$ ), which suggests that the ryanodine receptor gene expression is not susceptible to IGF-1 modulation. The DHPR/RyR1 ratio, which is an indication of the proportion of receptors coupled [2] [1], has been included in Table 1. The PN200-110/ryanodine (DHPR/RyR1)  $B_{\max}$  ratio of  $0.98 \pm 0.07$  (mean  $\pm$  S.D., 10 determinations from five different preparations) for non-transgenic FVB mice muscles is similar to that reported for matured rabbit muscles [1], fast-twitch rat EDL muscle [2] and 14-month-old rat EDL and mixed-fiber type muscles [12]. The ratio in transgenic skeletal muscles was  $1.28 \pm 0.09$ . The significant increase in PN200-110/ryanodine binding ratio ( $P<0.001$ ) in transgenic muscles is caused by the increase in the number of DHPR. It has been shown that only a fraction of the RyR1 is coupled to DHPR in fast-twitch rodent muscles [1,2]. Results from the present study support the concept that the fraction of RyR1 coupled to DHPR increases in transgenic mice. The increase in the maximum [ $^3\text{H}$ ]PN200-110 binding capacity cannot be attributed to a decrease in the dissociation constant of [ $^3\text{H}$ ]PN200-110 (Table 1).

### 3.1. DHPR and RyR1 in EDL and soleus muscles

To determine whether the increase in the number of DHPR in the pools of transgenic muscles was due to an increase in the number of DHPR in fast- and/or slow-twitch muscles, binding studies were performed in EDL or soleus muscles (Table 1). The  $B_{\max}$  values of DHPR and RyR1 in EDL and soleus muscles were determined by a single saturating concentration of [ $^3\text{H}$ ]PN200-110 and [ $^3\text{H}$ ]ryanodine. The DHPR  $B_{\max}$  values for transgenic and non-transgenic muscles were  $10.57 \pm 2.15$  and  $7.02 \pm 1.71$  pmol/mg protein, respec-

tively, and for RyR1 were  $8.28 \pm 1.56$  and  $6.70 \pm 1.03$  pmol/mg protein, respectively. Transgenic EDL muscles exhibited a statistically significant increase in DHPR ( $P=0.026$ ); how-

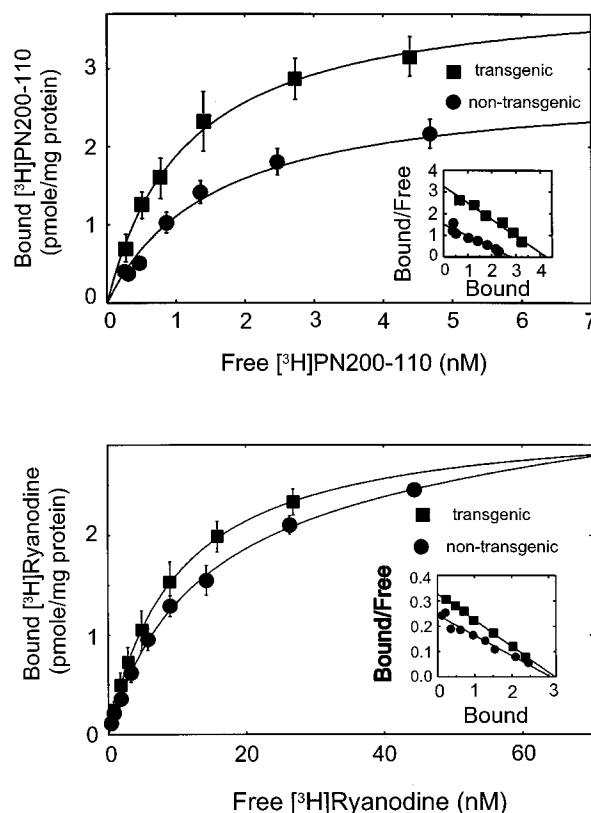


Fig. 2. [ $^3\text{H}$ ]PN200-110 (top) and [ $^3\text{H}$ ]ryanodine (bottom) binding to skeletal muscle homogenates in 12-month-old non-transgenic and transgenic mice.  $B_{\max}$  and  $K_D$  values were obtained from Scatchard analysis and fitting of Eq. 1 to binding data (inset). The plots correspond to representative experiments of the total number of studies (10 determinations from five samples) included in Table 1.

ever, no significant differences in RyR1 concentration were found ( $P=0.095$ ). The  $B_{\max}$  values for DHPR and RyR1 in non-transgenic EDL and soleus (see below) muscles are equivalent to those reported in rat EDL muscle [12,14], when expressed per gram muscle. In our hands, 1 g of EDL or soleus muscles gave  $100 \pm 30$  mg protein ( $n=10$ ). The DHPR/RyR1  $B_{\max}$  ratio for non-transgenic skeletal muscle was  $1.038 \pm 0.12$ . This value is similar to  $0.95 \pm 0.14$  reported for rat EDL muscles [12]. In transgenic skeletal muscle DHPR/RyR1 ratio increased significantly to  $1.28 \pm 0.16$  ( $P=0.027$ ) due to the increase in the amount of DHPR.

$B_{\max}$  values for DHPR in transgenic and non-transgenic soleus muscles were  $5.17 \pm 0.71$  and  $3.04 \pm 1.18$  pmol/mg protein, respectively. For RyR1 the values for both groups of muscles were  $6.57 \pm 1.50$  and  $6.43 \pm 0.79$  pmol/mg protein, respectively. The number of DHPR in transgenic soleus muscle was 70% higher than in non-transgenic ( $P=0.008$ ). However, no significant changes in the number of RyR1 were detected ( $P=0.858$ ). The DHPR/RyR1 ratio of  $0.47 \pm 0.18$  observed in non-transgenic soleus muscle is consistent with the value of  $0.44 \pm 0.12$  seen in 14-month-old rat soleus muscle fiber [12]. This ratio increased to  $0.80 \pm 0.12$  in transgenic soleus muscle. The increase in DHPR/RyR1 ratio is due to the increase in the number of DHPR. In summary, these results indicate that the increase in the number of DHPR and DHPR/RyR1 ratio observed in mixed-fiber type muscles is due to the increase in the number of DHPR in both fast- and slow-twitch muscles.

#### 4. Discussion

In the present work we report that transgenic overexpression of IGF-1 exclusively in skeletal muscle increases the number of DHPR  $\alpha 1$  subunit in adult mouse. The DHPR  $\alpha 1$  subunit functions both as a voltage sensor and as a permeation pathway for  $\text{Ca}^{2+}$  ions in skeletal muscles [15]. When muscles from S1S2 transgenic mice were compared with FVB non-transgenic mice, a significant increase in DHPR was evident. The levels of RyR1, which functions as the  $\text{Ca}^{2+}$  release channel in EC coupling [16], did not change significantly in transgenic compared to non-transgenic muscles. Because the DHPR/RyR1 ratio increased 30% in transgenic compared to non-transgenic muscle, we postulate that the IGF-1-mediated increase in DHPR may overcome reported defects in EC coupling with aging [5,11,17].

IGF-1 plays a crucial role in skeletal muscle growth and maintenance [3]. Systemic transgenic overexpression of IGF-1 has shown a modest improvement in muscle mass [18,19]. However, overexpression of IGF-1 targeted to striated muscles causes uniform muscle hypertrophy without increase in overall body weight or circulating IGF-1 concentration [19]. The mechanism of IGF-1 action in the signaling pathway has been extensively studied, however the mechanism of  $\text{Ca}^{2+}$  channel upregulation reported in this work is unknown. Because DHPR expression has been associated with skeletal muscle activity [14], it should be mentioned that both transgenic and non-transgenic mice were housed in similar cages and exhibited similar physical activity. IGF-1 is known to induce the transcription of the immediate-early genes *c-fos* and *c-jun*. Members of the Fos and Jun protein families dimerize to form the AP-1 complex which binds to the TPA responsive element (TRE) present in the promoter of responsive genes [20]. IGF-1 has been shown to stimulate *c-fos* and

*c-jun* transcription in PC12 cells [21]. Transfection of PC12 cells with *c-fos* and *c-jun* increased  $\omega$ -conotoxin-sensitive calcium current 2–4-fold in 2 days thereby suggesting that renewed induction of both *c-fos* and *c-jun* may be required for sustained enhancement of  $\text{Ca}^{2+}$  influx [22]. Kojima et al. have shown that IGF-1 stimulates  $\text{Ca}^{2+}$  influx by activating a  $\text{Ca}^{2+}$ -permeable cation channel via the IGF-1 receptor and  $\text{Ca}^{2+}$  influx may play a critical role in the mitogenic action of IGF-1 [23]. Although the mechanism by which sustained overexpression of IGF-1 induces upregulation of DHP-sensitive  $\text{Ca}^{2+}$  channels in skeletal muscle is unknown, based on observations in other cell types, it may occur via modulation of AP1 or CRE sites.

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