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GDF-15 is a novel member of the transforming growth factor (TGF)-β superfamily that has critical roles in the central and peripheral nervous systems. We previously reported that GDF-15 increased delayed rectifier outward K+ currents and Kv2.1 α subunit expression through the TGF-β receptor (TβR) II to activate Src kinase and Akt/mammalian target of rapamycin (mTOR) signaling in rat cerebellar granule neurons (CGNs). Here, we found that treatment of CGNs with GDF-15 for 24 h increased intracellular Ca2+ concentration [Ca2+]i in response to membrane depolarization, as determined by Ca2+ imaging. Whole cell current recordings indicated that GDF-15 increased the inward Ca2+ current (ICa) without altering steady-state activation of Ca2+ channels. Treatment with nifedipine, an inhibitor of L-type Ca2+ channels, abrogated GDF-15-induced increases in [Ca2+]i and ICa. The GDF-15-induced increase in ICa was mediated via upregulation of the Cav1.3 α subunit, which was attenuated by inhibiting Akt/mammalian target of rapamycin and extracellular signal-regulated kinase pathways and by pharmacological inhibition of Src-mediated TβRII phosphorylation. Given that Cav1.3 is not only a channel for Ca2+ influx but also a transcriptional regulator, our data confirm that GDF-15 induces protein expression via TβRII and activation of a non-Smad pathway, and provide novel insight into the mechanism of GDF-15 function in neurons.
GDF-15 enhances intracellular Ca\(^{2+}\) by increasing Cav1.3 expression in rat cerebellar granule neurons

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Running Title: GDF-15 enhances intracellular Ca\(^{2+}\)

Summary statement
We report that GDF-15 increased [Ca\(^{2+}\)]\(i\) and Cav1.3 expression through T\(\beta\)RII to activate Akt/mTOR and ERK, which confirm our previous report that GDF-15 induces protein expression via T\(\beta\)RII–dependent non-Smad pathway, and provide novel insight into GDF-15 function in neurons.

# Two authors are equal contribution to this work

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**Abbreviations used**
GDF-15, growth/differentiation factor 15; TGFβ, transforming growth factor β; TßR, TGF-β receptor; CGNs, rat cerebellar granule neurons; ERKs, extracellular signal regulated kinases; mTOR, mammalian target of rapamycin; DMEM, Dulbecco’s modified Eagle medium; FCS, fetal calf serum; DIC, days in culture; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TTX, tetrodotoxin; 4-AP, 4-aminopyridine; SDS, sodium dodecyl sulfate; BSS, balanced salt solution; qPCR, quantitative real-time PCR; CT, cycle threshold; Fluc, firefly luciferase; Rluc, Renilla luciferase.

**ABSTRACT**
GDF-15 is a novel member of the transforming growth factor (TGF)-β superfamily that has critical roles in the central and peripheral nervous systems. We previously reported that GDF-15 increased delayed rectifier outward K⁺ currents and Kv2.1 α subunit expression through the TGF-β receptor (TßR) II to activate Src kinase and Akt/mammalian target of rapamycin (mTOR) signaling in rat cerebellar granule neurons (CGNs). Here, we found that treatment of CGNs with GDF-15 for 24 h increased intracellular Ca²⁺ concentration [Ca²⁺]ᵢ in response to membrane depolarization, as determined by Ca²⁺ imaging. Whole cell current recordings indicated that GDF-15 increased the inward Ca²⁺ current (I_Ca) without altering steady-state activation of Ca²⁺ channels. Treatment with nifedipine, an inhibitor of L-type Ca²⁺ channels, abrogated GDF-15-induced increases in [Ca²⁺]ᵢ and I_Ca. The GDF-15-induced increase in I_Ca was mediated via upregulation of the Cav1.3 α subunit, which was attenuated by inhibiting Akt/mammalian target of rapamycin and extracellular signal-regulated kinase pathways and by pharmacological inhibition of Src-mediated TßRII phosphorylation. Given that Cav1.3 is not only a channel for Ca²⁺ influx but also a transcriptional regulator, our data confirm that GDF-15 induces protein expression via TßRII and activation of a non-Smad pathway, and provide novel insight into the mechanism of GDF-15 function in neurons.

**Keywords:** GDF-15, TßRII, Akt/mTOR, ERK, [Ca²⁺]ᵢ, Cav1.3

**INTRODUCTIONS**
Growth/differentiation factor (GDF)-15, also known as macrophage inhibitory cytokine-1, is a distant member of the transforming growth factor (TGF)-β superfamily [1]. GDF-15 plays key roles in prenatal development and the regulation of cellular responses to stress signals and inflammation as well as in tissue repair after acute injury [2]. Recent studies have also shown that GDF-15 expression is upregulated during myocardial injury, ischemia, and remodeling, suggesting that it may act as a cytokine that protects the heart from ischemia/reperfusion injury [3, 4]. The mechanism of action of GDF-15 is not fully understood, although it was shown to block norepinephrine-induced myocardial hypertrophy by inhibiting the phosphorylation of epidermal growth factor receptor and the downstream kinases AKT and extracellular signal-regulated kinase (ERK)1/2 [5].
GDF-15 is widely expressed in the brain, specifically in the cortex, striatum, and thalamus [6] and acts as a potential neurotrophic factor for midbrain dopaminergic neurons in vivo, promoting the survival of damaged mesencephalic dopaminergic neurons following cortical lesioning [6, 7]. GDF-15 is upregulated in a central nervous system (CNS) model of ischemia induced by middle cerebral artery occlusion [8], and GDF-15 knockout mice exhibit progressive postnatal loss of spinal, facial, and utrigrinal motoneurons and sensory neurons in dorsal root ganglia [9]. A recent study suggested that GDF-15 is involved in neuronal synaptic development and integration and may promote axonal elongation [10]. These data indicate that GDF-15 has critical roles in CNS development, although its mechanisms of action are poorly understood.

Cerebellar granule neurons (CGNs) are glutamatergic cells that differentiate postnatally into various types of neuron in the mammalian brain. Primary rat CGN cultures are used as a model for studying neuronal maturation, apoptosis, differentiation, and synaptic plasticity [11]. Growth and differentiation factors such as TGF-β1 and neuregulin can stimulate or inhibit CGN development and maturation via regulation of multiple signaling pathways [12, 13]. GDF-15 prevented the death of K+-deprived CGNs by activating Akt and inhibiting constitutively active ERK [14]. We recently demonstrated that GDF-15 increased delayed rectifier outward K+ currents (IK) and Kv2.1 α subunit expression by Src kinase activation via TβRII in non K+-deprived CGN cultures [15]. These data showed for the first time that the modulation of K+ channel expression and the downstream signaling pathways by GDF-15 is receptor-mediated, and demonstrated that CGNs are an effective cell model for investigating the mechanism of action of GDF-15.

Increases in intracellular Ca2+ concentration [Ca2+]i activate signaling pathways that induce the expression of genes essential for dendritic development, neuronal survival, and synaptic plasticity [16-18]. [Ca2+]i also regulates gene expression during CGN development [17, 19]. Whether GDF-15 modulates [Ca2+]i in CGNs and the mechanisms that are involved are unknown. In this study, we evaluated the effect of GDF-15 on [Ca2+]i by Ca2+ imaging while simultaneously recording inward Ca2+ current (Ica), since changes in [Ca2+]i in CGNs are associated with Ca2+ influx-dependent Ca2+ release [20, 21]. We also examined whether the same signaling pathways and receptors identified in our previous report are activated by GDF-15 under these conditions.

**EXPERIMENTAL PROCEDURES**

**Cell culture**-All experimental procedures were carried out in accordance with European guidelines for the care and use of laboratory animals (Council Directive 86/609/EEC). CGNs were derived from the cerebellum of 7-day-old Sprague-Dawley rat pups as previously described [22]. Briefly, isolated cells were plated in 35-mm Petri dishes coated with poly-l-lysine (1 μg/ml) at a density of 10^6 cells/ml and cultured at 37°C under 5% CO2 in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), insulin (5 μg/ml), KCl (25 mM), and 1% antibiotic-antimycotic solution. After 24 h of culture, cytosine β-D-arabinofuranoside (5 μM) was added to the culture medium to inhibit the proliferation of non-neuronal cells. Cells were used for experiments after 4–5 days in culture (DIC) unless otherwise indicated.

**Patch-clamp recordings**-Whole-cell CGN currents were recorded with a conventional
patch-clamp technique using multiclamp 200B amplifier (Axon Instruments, Foster City, CA, USA) operated in voltage-clamp mode. Data acquisition and analysis were carried out using pClamp 8.01 (Axon Instruments) and/or Origin 8 (Microcal Software, Northampton, MA, USA) software. Prior to recording $I_{ca}$, the culture medium was replaced with a bath solution containing 147 mM tetraethylammonium chloride, 10 mM BaCl₂, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 2 mM MgCl₂, 1 μM tetrodotoxin (TTX), 2 mM 4-aminopyridine (4-AP), and 10 mM glucose. Soft glass recording pipettes were filled with an internal solution containing: 145 mM CsCl, 10 mM ethylene glycol tetraacetic acid, 10 mM HEPES (pH 7.3), 5 mM Na₂-ATP, and 0.5 mM Na₂-GTP. The pipette resistance was 4–6 MΩ after filling with internal solution. All recordings were carried out at room temperature. CGNs selected for electrophysiological recording exhibited the typical morphological characteristics of healthy cells, such as fusiform soma with two principal neurites of similar size. There was no difference in the mean capacitance of cells recorded in the control and GDF-15 treatment groups ($9.17 \pm 0.23$ and $9.36 \pm 0.21$ pF) [15].

Western blot analysis-Cells were lysed on ice for 30 min in lysis buffer containing 20 mM HEPES, 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 2 mM ethylenediaminetetraacetic acid, 100 μM Na₃VO₄, 50 mM NaF (pH 7.5), and 1% proteinase inhibitor cocktail. After centrifugation, the supernatant was mixed with 2× sodium dodecyl sulfate (SDS) loading buffer and boiled for 5 min. Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), which was blocked with 10% nonfat milk and incubated at 4°C overnight with mouse monoclonal antibody against Cav1.2 or 1.3 (1:1000; UC Davis, Davis, CA, USA) and mouse monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (1:10,000; KangChen Bio-Tech, Shanghai, China). After extensive washing in Tris-buffered saline with 0.1% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:10,000, KangChen Bio-Tech) for 2 h at room temperature. Protein bands were visualized by chemiluminescence using the SuperSignal West Pico trial kit (Pierce, Rockford, IL, USA) and detected using a ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). Quantity One v.4.6.2 software (Bio-Rad) was used for background subtraction and quantification of immunoblotting data.

Measurement of $[Ca^{2+}]_i$- $[Ca^{2+}]_i$ in single cells was detected based on Fura-2 fluorescence intensity as previously described [23]. Briefly, CGNs grown on coverslips were rinsed twice with balanced salt solution (BSS) containing 145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1 mM MgCl₂, 10 mM glucose, and 2 mM CaCl₂, and incubated at 37°C for 45 min in the presence of 5 μM Fura-2 AM with 0.1% dimethylsulfoxide in BSS. After two washes with BSS, cells were incubated for an additional 20 min in BSS prior to imaging. The coverslips were transferred to a chamber mounted on the stage of an inverted phase contrast microscope (Eclipse Ti; Nikon, Tokyo, Japan); fresh BSS was added to the chamber, and images were acquired at 4-s intervals for the duration of the experiment. Excitation wavelengths for Fura-2 were 340 and 380 nm, with emission at 505 nm. Baseline $[Ca^{2+}]_i$ was determined for 60 s immediately prior to the addition of high K⁺ solution (27 mM KCl). Fluorescence intensity was quantified using Metafluor software (Universal Imaging Corporation, Downingtown, PA, USA).
Transfection and dual luciferase reporter assays—Rat Cav1.3 promoter (-1400bp to +497 bp) synthesized from Magorbio company (Shanghai, China) were inserted into pGL3 luciferase reporter plasmid. Lentiviral vectors for coordinately expressing CACNA1D promoter following fluorescent protein and Renilla luciferase protein only were constructed, respectively. CGNs cells were co-transfected with Cav1.3 promoter and renilla reporter plasmids. Luciferase assays were performed at 7 DIC which is 3d after transfection using the Dual Luciferase Reporter Assay system (E1910, Promega, Madison, USA) according the manufacturer’s instructions. The results were expressed as a ratio of firefly luciferase (Fluc) activity to Renilla luciferase (Rluc) activity, and the Renilla-luciferase reporter gene (50 ng) was used as an internal control. For each sample, the relative luciferase activity was normalized to control group Fluc/Rluc ratio. All experiments were performed in triplicate.

qPCR—To measure the Cav1.2 and Cav1.3 mRNA levels, qPCR (quantitative real-time PCR) analysis was performed with the following sequences: Cav1.2 forward primer 5’- TCAAAAGCTACTGGACTGGAT-3’ and reverse primer 5’- CCATGGCCCTCG TCCTCAT-3’; Cav1.3 forward primer, 5’- CTTCCCTCTCATCATCTCTTC-3’ and reverse primer, 5’- TCATACTACCGCATTCC-3’. To control for sampling errors, qPCR for the housekeeping gene GAPDH was performed with the primer sequences forward, 5’- TGCTCCTCTGCTTGC-3’ and reverse, 5’- AGCCTTGACT GTGCC-3’. The reaction solution contained 1.0 μg of diluted reverse transcription PCR product, 0.2 μM of each paired primer and Power SYBR Green PCR master mix (Toyobo). The annealing temperature was set at 58°C and 40 amplification cycles were used. The absolute mRNA levels in each sample were calculated according to a standard curve determined using serial dilutions of known amounts of specific templates plotted against the corresponding cycle threshold (CT) values. The normalized ratio of the target gene over GAPDH in each sample was calculated. The specificity of the primers was verified by both gel electrophoresis and sequencing of the PCR products.

Data analysis—Multiple groups were compared by one-way analysis of variance and two-sample comparisons were performed using the Student’s t test. Data are presented as mean ± SEM, with n as the number of neurons recorded, imaging experiments, or replicates. Electrophysiological data were collected from at least four different batches of neurons prepared on different days to minimize bias resulting from culture conditions. P < 0.05 was considered as statistically significant.

Chemicals—Recombinant human GDF-15 was purchased from Pepro Tech (Rocky Hill, NJ, USA). TTX, 4-AP, rapamycin, SB431542, PP1, LY2109761 and poly-L-lysine were purchased from Sigma (St. Louis, MO, USA). U0126 purchased from Selleckchem (Houston, USA). FCS, DMEM, and antibiotic-antimycotic solution were purchased from Gibco Life Technologies (Grand Island, NY, USA).
RESULTS
GDF-15 enhances [Ca^{2+}]_i and inward I_{Ca} in CGNs without affecting steady-state channel activation

We previously demonstrated that GDF-15 increases I_K of CGNs in a time- and dose-dependent manner at different developmental stages, and that incubating CGNs starting from 5 DIC with 100 ng/ml GDF-15 for 24 h produced the most significant increase in I_K [15]. We therefore applied 100 ng/ml GDF-15 to CGNs after 5 DIC for 24 h and evaluated the effects of GDF-15 on [Ca^{2+}]_i by Ca^{2+} imaging using the Ca^{2+}-sensitive fluorescent dye Fura-2. Since GDF-15 did not affect basal [Ca^{2+}]_i, we used a high-K^+ solution (27 mM KCl) to depolarize neurons and activate voltage-gated Ca^{2+} channels (VGCCs), inducing a rapid increase in [Ca^{2+}]_i. In control neurons, depolarization with high K^+ caused acute elevation of [Ca^{2+}]_i, with an increase in the F340/F380 ratio from 0.69 ± 0.01 (n = 43) to a maximum of 1.71 ± 0.06 (n = 127). After treatment with GDF-15 for 24 h, the ratio increased to a maximum value of 2.24 ± 0.01 (n = 115) (Fig. 1A, B), which was about 30.9 % higher than in the control (Fig. 1C).

To assess the role of GDF-15 in the activation of VGCCs in CGNs, we recorded whole cell I_{Ca}, which was evoked by a 200-ms depolarization from a holding potential of −80 to 10 mV. GDF-15 application (100 ng/ml for 24 h) increased I_{Ca} amplitude by 44.57% (from 155.71 ± 10.23 to 225.11 ± 16.31 pA, n = 41 and 54, P < 0.05) (Fig. 2A, B). We then investigated whether the effects of GDF-15 on I_{Ca} amplitude were exerted via modulation of the voltage-gating properties of I_{Ca} channels. An I_{Ca} was evoked by a 20-ms depolarizing pulse from a holding potential of −80 mV to between −80 and 40 mV in 10-mV steps at 10-s intervals (Fig. 2C). The current-voltage (I-V) curves of control and GDF-15-treated CGNs showed that I_{Ca} increased from a negative potential of −40 mV to a maximum value of 10 mV (Fig. 2D), indicating that GDF-15 did not affect Ca^{2+} channel activity. Steady-state I_{Ca} activation was determined by calculating conductance and normalizing this value to the command voltage. Data were fitted using the Boltzmann function. The steady-state I_{Ca} activation curves of CGNs with or without GDF-15 treatment showed half-activation potentials of 4.57 ± 1.36 and 3.24 ± 1.14 mV, respectively (n = 15 and 18, respectively; P > 0.05) (Fig. 2E). These results indicate that GDF-15-induced increases in I_{Ca} amplitude were not due to changes in the voltage-gating properties of I_{Ca} channels.

L-type Ca^{2+} channels and Cav1.3 expression are mediated GDF-15-induced [Ca^{2+}]_i and I_{Ca} amplitude

The I-V curves suggested that the Ca^{2+} channels were L-type channels found in neurons [24]. To determine whether L-type Ca^{2+} channels are indeed responsible for the GDF-15-induced increases in Ca^{2+} influx and I_{Ca} amplitude, we treated CGNs with the selective blocker nifedipine. Pre-incubation of CGNs with nifedipine (10 μM) [25] abrogated the increase in [Ca^{2+}]_i, evoked by high K^+ and inhibited the GDF-15-induced increase in [Ca^{2+}]_i (Fig. 3A, B). In the presence of nifedipine, the increase in the F340/F380 ratio evoked by high K^+ solution without and with GDF-15 was reduced from 1.71 ± 0.06 (n = 127) to 1.15 ± 0.10 (n=58) and from 2.24 ± 0.01 (n = 115) to 1.23 ± 0.07 (n=45) respectively (Fig. 3C). Consistent with these findings,
nifedipine application alone reduced the amplitude of $I_{Ca}$ evoked by a 200-ms depolarization from −80 to 10 mV by 29.95 ± 5.9% (n = 24 and 7), and abolished the GDF-15-induced increase in $I_{Ca}$ amplitude (Fig. 4A, B), suggesting that L-type of Ca$^{2+}$ channels mediate the GDF-15-induced increase in $I_{Ca}$ amplitude and [Ca$^{2+}$].

We investigated whether the GDF-15-mediated increase in $I_{Ca}$ is due to an upregulation of channel expression. A previous study showed that Cav1.2 and 1.3 are the major $\alpha$-subunits of L-type of Ca$^{2+}$ channels [26]; we therefore assessed the expression of these two proteins in GDF-15-treated cells. Specific primers to amplify Cav1.2 and Cav 1.3 were used to measure mRNA expression levels by qPCR after incubation with and without GDF-15. The result revealed that there was significant increase in the mRNA levels both of the Cav1.2 and Cav 1.3 $\alpha$-subunit (Fig. 5A). However, western blotting indicated that only Cav1.3 but not Cav1.2 protein level was increased in CGNs by 53.34 ± 8.46% (n = 4; P < 0.05) following a 24h incubation with GDF-15 (100 ng/ml) at 5 DIC (Fig. 5B). Moreover, the effect of GDF-15 on Cav1.3 expression was gradually increased with incubation time (Fig. 5C). These result suggested that an upregulation in Cav1.3 protein expression induced by GDF-15 is responsible for the observed increased in $I_{Ca}$ and [Ca$^{2+}$]. We thus examined the effect of GDF-15 on the Cav1.3 gene promoter using luciferase reporter assays. Administration of GDF-15 increased luciferase expression driven by the rat Cav1.3 promoter by 51.4 ± 17.6% (n=3; Fig. 5D).

**Effect of GDF-15 on Cav1.3 expression requires Akt/mammalian target of rapamycin (mTOR) and mitogen-associated protein kinase (MAPK)/ERK activation via TβRII**

Our previous study showed that Akt/mTOR signaling and TβRII activity are required for the GDF-15-induced upregulation of $I_{K}$ and Kv2.1 $\alpha$ subunit expression [15]. We therefore investigated whether these are involved in the observed effect of GDF-15 on Cav1.3 expression. Blocking Akt/mTOR activity with 20µm LY294002 or 50 nM rapamycin [27] reduced the GDF-15-induced increase in Cav1.3 protein expression from 53.4% ± 6.7% to 11.6% ± 4.7% and 0.04% ± 4.2%, respectively (n = 3; P < 0.05) (Fig. 6A). Unexpectedly, inhibition of MAPK kinase with 1µm U0126 [28] also suppressed the increase in Cav1.3 expression induced by GDF-15 from 54.8% ± 4.7% to 18.4% ± 4.3% (n = 3, P < 0.05) (Fig. 6B). These data indicate that both the Akt/mTOR and MAPK/ERK pathways are required for the upregulation of Cav1.3 expression induced by GDF-15.

Since there are no specific inhibitors of TβRII, we used the TβRI inhibitors SB431542 and PP1 and the TβRI/TβRII inhibitor LY2109761 to determine whether the effect of GDF-15 on Cav1.3 expression involves TβRII. There was no change in GDF-15-induced Cav1.3 expression relative to the control upon treatment with 10 µM PP1 [15] (29.5% ± 3.91% without PP1vs. 34.11% ± 4.94% with PP1, n = 5, P > 0.05) (Fig. 7A) or 10 µM SB431542 [15] (29.5% ± 3.91% without SB431542 vs. 25.22% ± 3.41% with SB431542, n = 5, P >0.05) (Fig. 7B). In contrast, 5 µM LY2109761 [15] treatment reduced the GDF-15-induced upregulation of Cav1.3 expression from 29.5% ± 3.91% to 1.41% ± 4.99% (n = 5; P < 0.05) (Fig. 7C). These results indicate that the effects of GDF-15 on $I_{Ca}$ and [Ca$^{2+}$] are exerted via modulation of Cav1.3 expression, which involves the activation of Akt/mTOR and MAPK/ERK signaling downstream of TβRII. Further, we also examined whether TβRII and ERK signal pathway are involved in the observed effect of GDF-15 on Cav1.3 gene promoter using luciferase reporter assays. Similar, in the present SB431542, administration of GDF-15 significantly increased luciferase expression driven by the rat Cav1.3 promoter by 36.23 ± 11.82% relative to the control upon treatment with SB431542 alone.
LY2109761 and U0126 inhibited the GDF-15-induced upregulation of luciferase expression driven by the rat Cav1.3 promoter (n=3, Fig.7D).

**DISCUSSION**

GDF-15 plays various roles in neuroprotection, neural regeneration, and axonal elongation [6, 8, 9]. However, there is little known about the mechanism of action of GDF-15 and its downstream effectors. Our previous study suggested that GDF-15 activates TβRII and phosphoinositide 3-kinase (PI3K)/Akt/mTOR signaling to increase \(I_K\) amplitude and Kv2.1 expression in CGNs, which may have developmental significance [15]. Here, we found that GDF-15 also increased the expression of Cav1.3 and thereby modulated the inward \(I_{Ca}\) and \([Ca^{2+}]_i\), which involved activation of the same receptor and some of the same downstream signaling components as those previously reported by our group.

VGCCs are voltage sensors that convert membrane depolarization into intracellular \(Ca^{2+}\) signals. In neurons, VGCCs are L-, N-, P/Q-, R-, and T-type \(Ca^{2+}\) channels [16, 24]. L-type \(Ca^{2+}\) channels are widely distributed on the neuronal cell body throughout the mammalian CNS, including in CGNs [26, 29]. \(Ca^{2+}\) influx in response to membrane depolarization occurs via L-type \(Ca^{2+}\) channels and regulates intracellular \(Ca^{2+}\) homeostasis [18, 30]. Our results demonstrate that intracellular basal \(Ca^{2+}\) was not increased by GDF-15 treatment; however, \([Ca^{2+}]_i\) in response to membrane depolarization and nifedipine-sensitive \(I_{Ca}\) were upregulated, suggesting the involvement of L-type \(Ca^{2+}\) channels, although we cannot exclude the possibility that nifedipin-insensitive \(Ca^{2+}\) channels or N-, P/Q-, R-type \(Ca^{2+}\) channels were also modulated by GDF-15. A recent study of rat CGNs indicated that administration of pituitary adenylate cyclase-activating polypeptide (PACAP) induced a rapid rise in \([Ca^{2+}]_i\) and thereby stimulated \(Ca^{2+}\) influx through N- but not L-type \(Ca^{2+}\) channels. This difference may be explained by the fact that PACAP affects basal \([Ca^{2+}]_i\) but not the response to membrane depolarization. Moreover, PACAP modulated the channels through rapid phosphorylation of channel proteins rather than regulation of \(Ca^{2+}\) channel α subunit expression [31].

L-type channels consist of subtypes Cav1.1–1.4. Cav1.1 and 1.4 are mainly expressed in skeletal muscle and retinal cells [32, 33], while Cav1.2 and 1.3 are abundant in the brain [29, 34]. Both of the latter isoforms show broad expression patterns in many types of neuron [35, 36], where they regulate neuronal excitability, synaptic plasticity, and activity-dependent gene transcription [37-39]. Cav1.2 and 1.3 account for 89% and 11%, respectively, of L-type channel transcripts in mouse CGNs, and Cav1.2 comprises the pore-forming subunits of anomalous L-type channels in these cells [40]. However, our data showed that there was no difference in the expression of the two isoforms in CGNs, consistent with a previous study reporting that functionally distinct L-type \(Ca^{2+}\) channels coexist in rat CGNs [41]. Besides species differences, variations in protein stability likely underlie the higher abundance of the Cav1.3 α subunit than what is predicted from mRNA levels, leading to a higher number of functional Cav1.3 channels in the membrane.

Cav1.3 and 1.2 differ in terms of biophysical properties, distribution in the brain, and function [42, 43]. We observed that the expression of the two channel types is also differentially regulated, since GDF-15 upregulated the expression of Cav1.3 protein but not Cav1.2 protein, but both of Cav1.2 and 1.3 mRNA levels detected by
using quantitative real-time PCR were increased by GDF-15 for reasons that are as yet unclear. Cav1.2 and 1.3 are encoded by the *cacna1C* and *cacna1D* genes, respectively [44]. The regulatory properties of Cav1.2 and 1.3 channels differ according to interaction with different intracellular proteins [45, 46]. For instance, the association between Cav1.2 and PSD-95/Dlg/ZO1 domain proteins plays an important role in coupling L-type Ca\(^{2+}\) channel activity with the phosphorylation of nuclear cAMP response element-binding protein (CREB) [47], while interaction of Cav1.3 with Shank results in its targeting to phosphorylated (p)CREB at synapses [45, 48]. Structurally distinct forms of Cav1.3 have also been reported in which the C-terminal modulatory domain confers unique gating properties [49, 50]. Whether the differential regulation of Cav1.2 and Cav1.3 protein expression by GDF-15 is due to variation in protein structure, or a post-transcriptional mechanism remains an open question.

Our previous study found that Akt/mTOR and MAPK/ERK pathways were activated in CGNs by GDF-15 treatment, consistent with findings in non-neuronal cells[51, 52], although activation of ERK signaling was not required for the GDF-15-induced increases in Kv2.1 expression and \(I_k\) [15]. Moreover, the effect of GDF-15 on Kv2.1 expression may be exerted via TβRII-induced activation of Src [15]. Our present results suggest that the upregulation of Cav1.3 expression induced by GDF-15 is required for the activation of TβRII and PI3K/Akt/mTOR signaling pathways, conforming our previous finding of a non-Smad mechanism [15]. However, we observed that blocking ERK signaling did abolish the GDF-15-induced increase in Cav1.3 expression, suggesting that activation of the ERK pathway is required for this effect. A previous study showed that ERK activation regulates Kv4 K\(^+\) channel subunits at the transcriptional and posttranslational levels [53]. ERK can directly phosphorylate ion channel subunits and may alter the gating properties of K\(^+\) channels, as in the regulation of \(I_A\) by growth factors [54]. Since GDF-15 neither alters the gating properties of \(I_{Ca}\) nor has an immediate effect on Ca\(^{2+}\) amplitude (data not shown), alternately it increased the mRNA level of Cav1.3 and luciferase expression driven by Cav1.3 promoter, we believed that the activation of ERK signaling by GDF-15 regulates Cav1.3 expression at the transcriptional level.

Neuronal L-type Ca\(^{2+}\) was known to play a critical role in coupling neuronal activity to gene transcription. Ca\(^{2+}\) influx via postsynaptic L-type Ca\(^{2+}\) channels activates pCREB [47, 48] and nuclear factor activated T cells cytoplasmic 4 [55], which stimulate the transcription of target genes [48]. However, a recent study demonstrated that the C terminus of Cav1.3 translocates to the nucleus where it functions as a transcriptional regulator to modulate the transcription of Ca\(^{2+}\)-activated K\(^+\) channels in atrial myocytes [56], and studies in Cav1.3\(^{-/-}\) mice have implicated Cav1.3 channels in auditory brainstem physiology and development [57, 58]. Various pathologies have been linked to Cav1.3 channels; for instance, Cav1.3 channel deficiency reduces long-term fear memory, antidepressant-like behavior, and congenital deafness [59-61]. In conclusion, our findings provide important insight into the mechanisms underlying the various functions of GDF-15 in the brain.

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AUTHOR CONTRIBUTION
Jun-Mei Lu and Chang-Ying Wang performed experiments, analyzed data, interpreted results of experiments, prepared figures and drafted manuscript; Changlong Hu help to analyzed data and interpreted results of experiments; Yan-Jia Fang and Yan-Ai Mei is design of research, drafted manuscript and approved final version of manuscript.

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FIGURE LEGENDS

Figure 1. Effect of GDF-15 on [Ca2+]i induced by high K+ in rat CGNs. (A) Intracellular Ca2+ imaging of control and GDF-15-treated CGNs before (Base) and after (HK) depolarization by acute perfusion with 27 mM K+. Changes in Fura-2 fluorescence excitation ratios with increasing [Ca2+]i are depicted as a color gradient from purple to red. Scale bar, 50 μm. (B) Changes in [Ca2+]i upon application of a depolarizing stimulus, as measured by quantification of fluorescence excitation ratios. The arrow represents a 30-s perfusion with a depolarizing solution of 27 mM K+. (C) Statistical analysis of [Ca2+]i induced by high K+ in the presence or absence of GDF-15. Data represent the mean ± SEM. *, p < 0.05 for two groups connected with a straight line.

Figure 2. Effect of GDF-15 on inward ICa amplitude and steady-state Ca2+ channel activation. (A) Representative traces of control and GDF-15-treated CGNs. ICa was elicited by depolarization to 10 mV from a holding potential of −80 mV. (B) Statistical analysis of the effect of GDF-15 on ICa amplitude. Data represent mean ± SEM. *, p < 0.05 for two groups connected with a straight line. (C) Representative traces obtained with a steady-state voltage protocol of control and GDF-15-treated CGNs. ICa was elicited by 200-ms depolarizing pulses from a holding potential of −80 mV to between −60 and +40 mV in 10-mV steps at 10-s intervals. (D) Voltage-dependent activation curves of ICa. *, p < 0.05 for comparing with corresponding control. (E) Steady-state activation curves of ICa obtained by plotting normalized conductance as a function of command potential. Data points were fitted using the Boltzmann function. Data represent mean ± SEM.

Figure 3. Effect of nifedipine on the increase in [Ca2+]i elicited by high K+ in CGNs with or without GDF-15 treatment. (A) Ca2+ imaging before and after depolarization by application of a 27-mM K+ solution in GDF-15-treated CGNs in the presence or absence of nifedipine. Scale bar, 50 μm. (B) Changes in [Ca2+]i upon application of a depolarizing stimulus, as measured by quantification of fluorescence excitation ratios. Each arrow represents a 30-s perfusion with a depolarizing 27-mM K+ solution. (C) Statistical analysis of [Ca2+]i in control and GDF-15-treated CGNs in the presence or absence of nifedipine. Data represent mean ± SEM. *, p < 0.05 for two groups connected with a straight line.

Figure 4. Effect of nifedipine on ICa in control and GDF-15-treated CGNs. (A) Representative traces obtained with a steady-state voltage protocol of control and
GDF-15-treated CGNs in the presence or absence of nifedipine. (B) I-V curve of $I_{\text{Ca}}$. Data were obtained from six independent experiments and represent the mean ± SEM. *P < 0.05. (C) Representative traces of control and GDF-15-treated CGNs in the presence or absence of nifedipine. $I_{\text{Ca}}$ was elicited with depolarizing pulses to 10 mV from a holding potential of −80 mV. (D) Statistical analysis of the effect of nifedipine on $I_{\text{Ca}}$. Data represent mean ± SEM. *, p < 0.05 for two groups connected with a straight line.

**Figure 5.** Effect of GDF-15 on Cav1.2 and 1.3 expression in CGNs. (A) Statistical analyses of Cav1.2 and Cav1.3 mRNA levels detected using quantitative real-time PCR. CGNs was incubated with GDF-15 from 15 min to 36 h. (B) Western blot and statistical analyses of the effect of GDF-15 on Cav1.2 and Cav1.3 expression in CGNs. (C) Western blot and statistical analyses of Cav1.3 levels in CGNs after incubation with GDF-15 for 15 min to 36 h. (D) Statistical analyses of the effect of GDF-15 on Cav1.3 promoter expression in CGNs was determined by luciferase reporter assays. Promoter information is illustrated. Data represent mean ± SEM. *, p < 0.05 for two groups connected with a straight line.

**Figure 6.** Effect of Akt/mTOR and ERK pathway inhibition on the GDF-15-induced increase in Cav1.3 α subunit expression. (A, B) Western blot and statistical analyses of the effects of the Akt inhibitor LY294002 and mTOR inhibitor rapamycin (A) and the MEK inhibitor U0126 (B) on GDF-15-induced upregulation of Cav1.3 protein levels. Data represent mean ± SEM. *, p < 0.05 for two groups connected with a straight line.

**Figure 7.** Effects of TβRI and TβRI/TβRII inhibitors on the GDF-15-induced increase in Cav1.3 protein level and gene promoter expression. (A-C) Western blot and statistical analyses of the effects of TβRI inhibitors (SB431542 and PP1) (A,B) and TβRI/TβRII dual inhibitor (LY2109761) (C) on GDF-15-induced upregulation of Cav1.3 protein levels. (D) Statistical analyses of the effect of the effects of SB431542, LY2109761 and U0126 on GDF-15-induced upregulation Cav1.3 promoter expression in CGNs determined by luciferase reporter assays. Data represent mean ± SEM. *, p < 0.05 for two groups connected with a straight line.
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