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RESEARCH ARTICLE

AMPK interacts with DSCAM and plays an important role in Netrin-1 induced neurite outgrowth

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ABSTRACT

Down syndrome cell adhesion molecule (DSCAM) acts as a netrin-1 receptor and mediates attractive response of axons to netrin-1 in neural development. However, the signaling mechanisms of netrin-DSCAM remain unclear. Here we report that AMP-activated protein kinase (AMPK) interacts with DSCAM through its γ subunit, but does not interact with DCC (deleted in colorectal cancer), another major receptor for netrin-1. Netrin-treatment of cultured cortical neurons leads to increased phosphorylation of AMPK. Both AMPK mutant with dominant-negative effect and AMPK inhibitor can significantly suppress netrin-1 induced neurite outgrowth. Together, these findings demonstrate that AMPK interacts with DSCAM and plays an important role in netrin-1 induced neurite outgrowth. Our study uncovers a previously unknown component, AMPK, in netrin-DSCAM signaling pathway.

KEYWORDS AMP-activated protein kinase (AMPK), neurite outgrowth, Down syndrome cell adhesion molecule (DSCAM), netrin

INTRODUCTION

AMP-activated protein kinase (AMPK) is a key metabolic sensor of energy balance at the cell and organism levels (Hardie et al., 1999). It is a heterotrimeric kinase composed of catalytic α, regulatory β, and AMP/ATP binding γ subunits (Hardie et al., 2003). The activation of AMPK requires phosphorylation of Thr^{172} in the activation loop of the catalytic α subunit. Several upstream activators of AMPK have been identified, including tumor suppressor LKB1 (Hawley et al., 2003; Hong et al., 2003), calcium/calmodulin-dependent protein kinase kinase-β (CaMKKβ) (Hawley et al., 2005; Woods et al., 2005).

As a key energy sensor, AMPK regulates the energy homeostasis at the cellular level (Carling, 2004; Kahn et al., 2005; Hardie, 2007). In addition to its metabolic function, AMPK also plays an important role in neuronal development by modulating neurite outgrowth (Dasgupta and Milbrandt, 2007; Wang et al., 2010). A recent study in knockout mice suggests that deletion of AMPKα subunit does not cause major defects or changes in gross morphology of the nervous system, although it is not clear whether netrin signaling is compromised in these mice (Williams et al., 2011). There is no clear evidence showing whether the phenotype of AMPKα subunit deficient mice is dependent on specific genetic background or whether redundant kinases exist in those mice to compensate for the loss of AMPKα subunit (Williams et al., 2011). Consistent with this idea, the same group shows that AMPK overactivation suppresses axon outgrowth through mTOR pathway (Williams et al., 2011), indicating that appropriate level of AMPK is critical for neuronal development, consistent with a recent study (Amato et al., 2011). Therefore, the roles of AMPK in neuronal development may be complex, and more studies are required to elucidate the underlying mechanisms.

Netrins are a family of evolutionarily conserved axon guidance cues that promote neurite outgrowth and guide growth cone navigation (Tessier-Lavigne et al., 1988; Hedgecock et al., 1990; Kennedy et al., 1994; Kolodziej et al., 1996; Mitchell et al., 1996). In *C. elegans*, the receptors for netrin have been identified as UNC40 and UNC5 (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Chan et al., 1996).

In mammals, the homologues of UNC40 are DCC and neogenin (Fazeli et al., 1997; Keino-Masu et al., 1996), and the orthologue of UNC5 is UNC5A-D (Ackerman et al., 1997; Leonardo et al., 1997).

The human DSCAM (Down Syndrome Cell Adhesion Molecule) gene was originally cloned from the human chromosome band 21q22.2-22.3, a critical region associated with neurological phenotypes of Down syndrome (Yamakawa et al., 1998). The *Dscam* gene encodes a type I transmembrane protein of the immunoglobulin (Ig) superfamily. DSCAM protein contains ten Ig domains, six fibronectin type III (FN) repeats, one transmembrane, and one intracellular domain (Yamakawa et al., 1998; Agarwala et al., 2000). The *Drosophila Dscam* gene possesses extraordinary molecular diversity. It can generate 38,016 distinct isoforms through alternative splicing (Schmucker et al., 2000; Wojtowicz et al., 2004). *Drosophila Dscam* gene plays an important role in dendritic self-avoidance and tilling (Hughes et al., 2007; Matthews et al., 2007; Soba et al., 2007). However, vertebrate *Dscam* gene does not undergo extensive alternative splicing (Agarwala et al., 2000). In the retina, *Dscam* is critical for soma spacing, dendrite arborization and synaptic formation (Fuerst et al., 2008, 2009).

Recent studies identified that DSCAM is another receptor of netrin-1 in vertebrates in addition to UNC5 and DCC (Ly et al., 2008; Liu et al., 2009). DSCAM interacts with netrin-1 and plays an important role in netrin-induced neurite outgrowth and commissural axon projection (Ly et al., 2008; Liu et al., 2009). However, the downstream signaling pathways mediating netrin-DSCAM signaling are unclear.

In this study, we demonstrate that AMPK interacts with DSCAM through its γ subunit, whereas neither α nor γ subunits of AMPK interacts with DCC. Netrin-1 treatment increases AMPK phosphorylation in mouse cortical neurons. Inhibition of AMPK activity significantly decreases axon outgrowth induced by netrin-1. Together, these results indicate that AMPK interacts with DSCAM and plays an important role in netrin-1 induced neurite outgrowth, suggesting that AMPK is a downstream signaling molecule in the netrin-DSCAM pathway.

RESULTS

Identification of AMPK as a DSCAM-interacting protein

The yeast two-hybrid system was used to screen proteins interacting with the intracellular domain of the human DSCAM protein (DSAM-ICD), and the LexA yeast two-hybrid system was used as described (Wu and Maniatis, 1993; Zervos et al., 1993). A number of protein-coding cDNA clones were identified and they are described elsewhere. Here, we focused on a group of the independent cDNA clones that encode the non-catalytic 1 subunit of AMPK, AMPKγ1. Re-transformation of AMPKγ1 cDNA confirmed that interaction between DSCAM-ICD and AMPKγ1 was specific and dependent on the expression of DSCAM-ICD.

AMPK interacts with DSCAM through γ subunit

To examine the potential interaction between DSCAM and AMPK, we cotransfected HEK293 cells using plasmid expressing Flag-tagged full-length human DSCAM (DSCAM-Flag) and Myc-tagged AMPKγ (AMPKγ-Myc). As shown in Fig. 1, DSCAM-Flag was co-immunoprecipitated with AMPKγ-Myc. We also observed that the interaction between DSCAM and AMPKγ was not affected by treatment with netrin-1 (Fig. 1).

A previous study proposed that DSCAM may collaborate with DCC to mediate axonal response to netrin-1 (Ly et al., 2008). We then tested whether DSCAM interacts with endogenous AMPK, and whether DCC is also associated with AMPK. We expressed Myc-tagged full-length human DSCAM (DSCAM-Myc) or Myc-tagged full-length human DCC (DCC-Myc) in HEK293 cells. DSCAM was co-immunoprecipitated with the endogenous AMPKγ subunit (Fig. 2A) but not the α subunit (Fig. 2B). However, DCC did not interact with either α or γ subunit of AMPK (Fig. 2A and 2B). Together, these results indicate that AMPK interacts with DSCAM through its γ subunit but not with DCC.

Netrin treatment increases AMPK phosphorylation in cortical neurons

DSCAM has been identified as a netrin receptor that mediates attractive response in neurons (Ly et al., 2008; Liu et al., 2009). The finding of interaction between DSCAM and AMPK γ subunit prompted us to test the role of AMPK in netrin-DSCAM signaling. We asked whether netrin-1 treatment altered the activity of AMPK in cultured mouse cortical neurons. We treated mouse E15 cortical neurons with

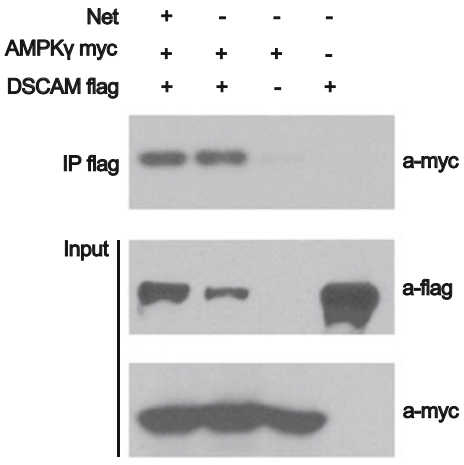

Figure 1. DSCAM interacts with AMPKγ subunit. HEK293 cells were co-transfected with flag-tagged DSCAM or control vector together with myc-tagged AMPKγ subunit or control vector as indicated. Following treatment with netrin-1 (Net) or control, cells were harvested for immunoprecipitation (IP) using anti-flag and Western blotting with anti-myc antibodies.

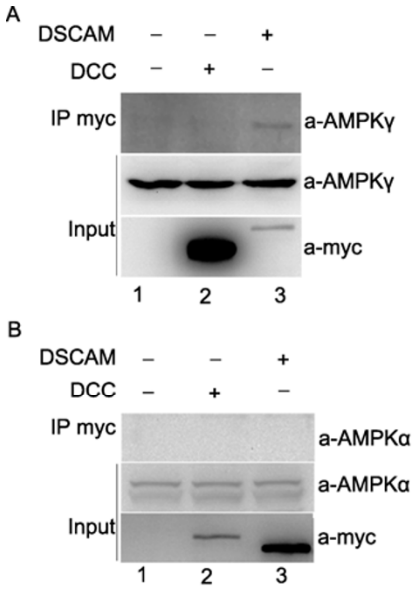

Figure 2. DSCAM interacts with endogenous AMPKγ subunit while DCC does not interact with either AMPKα or AMPKγ subunit. Protein lysates of HEK293 cells transfected with DSCAM-myc or DCC-myc were subjected for immunoprecipitation (IP) using anti-myc antibody. (A) AMPKγ subunit associated with DSCAM or DCC was detected by anti-AMPKγ antibody. (B) AMPKα subunit interacted with DSCAM or DCC was detected by anti-AMPKα antibody.

netrin-1, and AMPK activation was examined using phospho-AMPK-specific antibody. As shown in Fig. 3, AMPK Thr^{172} phosphorylation was increased 15-30 minutes after the treatment by netrin-1 (Fig. 3). This indicates that netrin treatment activates AMPK phosphorylation in neurons.

AMPK plays an important role in netrin-induced neurite outgrowth in cortical neurons

Since netrin-1 can induce neurite outgrowth (Liu et al., 2004; Ly et al., 2008; Liu et al., 2009), we next investigated the effect of AMPK on neurite outgrowth induced by netrin-1. Because there are multiple subunits of AMPK, it is difficult to use siRNA approach to knock down all subunits of AMPK, and we employed a mutant AMPK acting in a dominant negative manner to inhibit AMPK activity (Inoki et al., 2003). Vector control or dominant-negative mutant AMPK (dnAMPK) was expressed in mouse E15 cortical neurons, and then netrin-1 was added to treat the neurons for 20 h. The YFP plasmid was used to co-transfect with control or dnAMPK plasmid to mark the transfected neurons. The longest neurites of individual neurons were quantified. In control plasmid transfected cell, netrin-1 significantly induced neurite outgrowth in cortical neurons. However, expression of dnAMPK inhibited netrin-induced axon outgrowth without affecting the basal level of axon outgrowth (Fig. 4). To further confirm the role of AMPK in netrin-1 mediated neurite outgrowth, we treated cortical neurons with either vehicle control (DMSO) or 5 μmol/L Compound C (CC), a specific AMPK inhibitor.

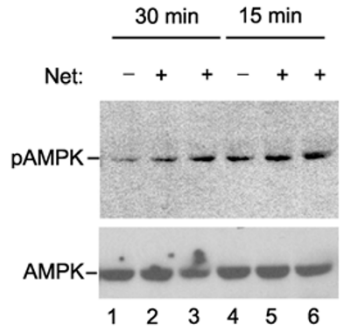

Figure 3. Netrin-1 induces the phosphorylation of AMPK in embryonic cortical neurons. Protein lysates of embryonic mouse cortical neurons (E15) stimulated with netrin-1 for 15 min or 30 min were examined by Western blotting using specific anti-AMPK and anti-pAMPK antibodies.

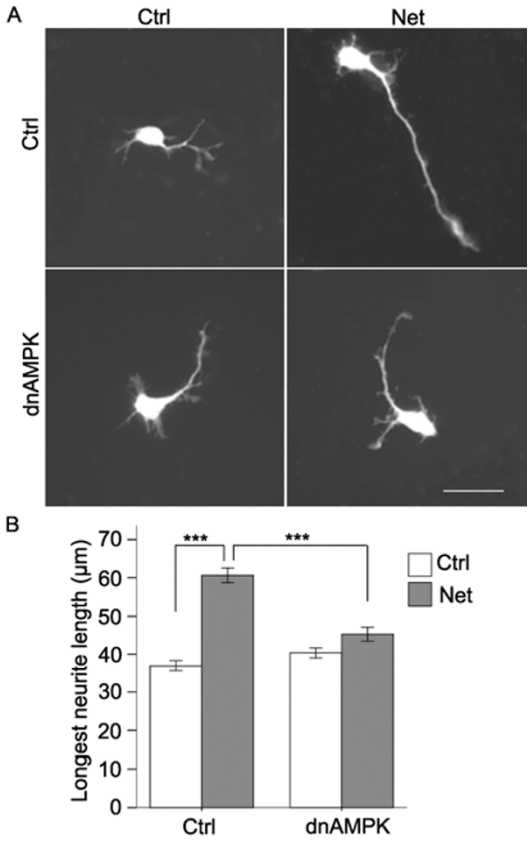

Figure 4. Expression of a dominant-negative mutant AMPK (dnAMPK) inhibits netrin-1 induced neurite outgrowth. (A) Cortical neurons from E15 mouse embryos were co-transfected with YFP and dnAMPK or control vectors. Following stimulation with netrin-1 (Net) or control (Ctrl) preparation for 20 h, fluorescent microscopic images were taken. The neurites of YFP-positive neurons were quantified. (B) Quantification of the length of the longest neurite from individual neurons. The data were presented as the mean ± SEM (Two way ANOVA, ***P < 0.001). Scale bar, 25 μm.

Netrin-induced neurite outgrowth was blocked in the presence of 5 μmol/L CC (Fig. 5), further supporting the importance of AMPK activity in netrin-stimulated neurite outgrowth. Collectively, these findings indicate that AMPK is required in axon outgrowth induced by netrin-1.

DISCUSSION

Our results provide evidences that AMPK plays an important role in netrin-DSCAM signaling pathway in neuronal development. We demonstrate that AMPK interacts with DSCAM through its γ subunit, and netrin-1 activates AMPK phosphorylation in cortical neurons. Moreover, DCC does not associate with either γ or α subunit of AMPK, suggesting that the involvement of AMPK in netrin signaling pathway is dependent on DSCAM instead of DCC. Finally, inhibiting AMPK activity blocks netrin-1-induced neurite outgrowth without affecting the basal level in cortical neurons, which further confirms the role of AMPK in netrin-1 signaling pathway. Previous studies indicated that knocking down DSCAM blocked netrin-1 induced neurite outgrowth (Liu et al., 2009), which showed a similar phonotype to inhibiting AMPK activity.

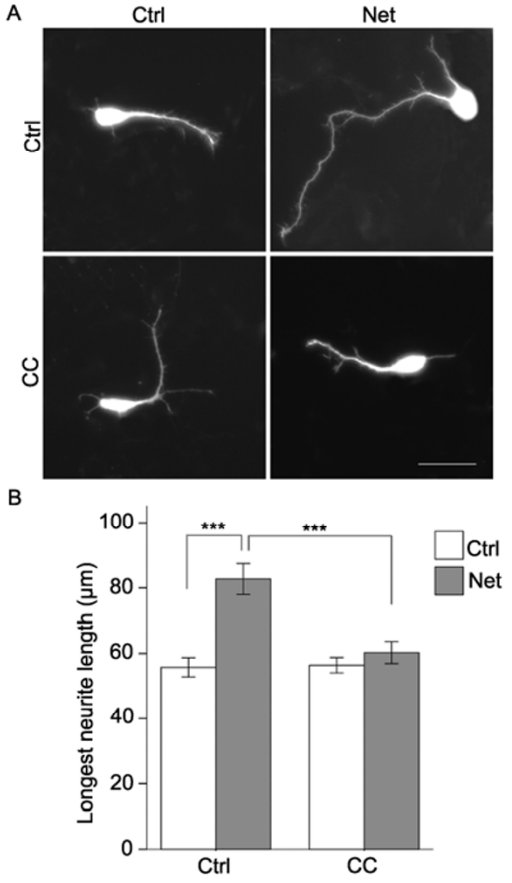

Figure 5. An AMPK inhibitor, compound C (CC), inhibits netrin-1 induced neurite outgrowth. (A) Cortical neurons from E15 mouse embryos were transfected with YFP and then treated with CC or DMSO, incubated for 20 h with netrin-1 or control preparation. Fluorescent microscopic images were taken after fixation of neurons. (B) Quantification of the length of the longest neurite from individual neurons. Same as in Fig. 4B.

The previous study proposed that AMPK activity is not required for neuronal development based on the observation that no obvious defects in neurogenesis, neuronal migration, axon formation, or neuronal survival in *AMPKα1/2*-null animals as compared with those in the control mice (Williams et al., 2011). However, these findings do not rule out the possibility that *AMPKα*-like genes compensate for the AMPKα deficiency. Besides DSCAM receptor, DCC and neogenin also mediate netrin signaling. It has been reported that *DSCAM*-null embryonic mice show normal axon outgrowth in response to netrin-1. It should be noted that there was a moderate reduction in netrin-induced neurite outgrowth and a moderate increase in neogenin level in *DSCAM*-null mice [see Fig. 2 and Fig. 1 in reference (Palmesino et al., 2012)]. Thus, it is possible that in *DSCAM* mutant mice, the loss of DSCAM is compensated by other netrin receptors. Moreover, it is interesting to note that our work together with others show that the phenotypes of *DSCAM* mutant mice are highly dependent on their genetic backgrounds. For example, in C57BJ/6 and BALB/c congenic background, homozygous *Dscam*^{del17} mutant mice can survive to adulthood but exhibit severe hydrocephalus and decreased motor function (Xu et al., 2011). The same *Dscam*^{del17} mutant mice on C57BJ/6 background died soon after birth (Xu et al., 2011). Similarly, it is possible that in *AMPKα*-null mice, the phenotypes are dependent on specific genetic backgrounds, and AMPKα deficiency is compensated by *AMPKα*-like genes. Additionally, AMPK pathway may not be the only pathway downstream of netrin-DSCAM. Other downstream pathways may also have compensatory effects.

Although two reports suggested that AMPK activation by AICAR suppressed axon outgrowth in cortical neurons (Amato et al., 2011; Williams et al., 2011), our results show that netrin treatment not only activates AMPK but also stimulates neurite outgrowth. Our findings are consistent with previous observation that AMPK activation by resveratrol is required for neuronal development, and that AMPK inhibition suppresses resveratrol-induced neurite outgrowth (Dasgupta and Milbrandt, 2007). Together, these studies suggest that the roles of AMPK in neural development may be highly complicated and further investigation is necessary to elucidate the function of AMPK in neurons.

Our findings provide evidences that AMPK play important roles in netrin-induced neurite outgrowth. In peripheral tissues, the signaling pathways of AMPK are extensively studied (Hardie, 2008; Shackelford and Shaw, 2009). However, our knowledge is limited about the role of AMPK in the nervous system. It has been suggested that AMPK activation may inhibit mTOR pathway and suppress axogenesis (Williams et al., 2011). Therefore, it is possible that AMPK suppresses mTOR pathway both inside and outside the nervous system. The role of mTOR in neuronal regulation is complex. Some studies demonstrate that mTOR activation increases neurite outgrowth, particularly regenerative growth (Park et al., 2008;

Abe et al., 2010; Christie et al., 2010; Liu et al., 2010), while other findings suggest that inhibiting mTOR by its inhibitor Rapamycin promotes neurite outgrowth in primary neurons (Guo et al., 2011). Thus detailed studies are required to explore the exact role of mTOR in AMPK induced neurite outgrowth.

Our results together with the findings by others [(Dasgupta and Milbrandt, 2007) and references therein] led us to propose one possible working model for the role of AMPK in netrin induced axon outgrowth (Fig. 6): netrin stimulates AMPK activity through the interaction of DSCAM receptor with AMPK γ subunit. AMPK may exert its effect on actin cytoskeleton by inhibiting mTOR. However, because of the complex role of mTOR in neurite outgrowth, it is also likely that AMPK affects actin cytoskeleton through other pathways which needs evidence in nervous system. In conclusion, our study uncovers a previously unknown component in the netrin-DSCAM signal transduction pathway and provides insights into potential function of AMPK in the nervous system. Further investigation is necessary to demonstrate the physiological role of the netrin-DSCAM-AMPK pathway in neuron.

MATERIALS AND METHODS

Antibodies and plasmids

Antibodies were obtained from commercial sources: Myc (cwbiotech), Flag (Sigma-Aldrich), AMPKα1 (Proteintech Group, Cell signaling), phospho-AMPKα (Thr172) (Cell signaling), AMPKγ1 (Proteintech Group). Plasmids expressing the full-length DSCAM with a Flag tag at the C terminus and DCC with a Myc tag were as described previously (Liu et al., 2009). Full-length DSCAM tagged with a Myc plus a His at the C terminus was subcloned into pcDNA 3.1. Myc tagged AMPKγ1 was prepared by inserting the open reading frame of human AMPKγ1 cDNA in a CS2 vector (Li et al., 1999). The dominant-negative mutant AMPK plasmid was a generous gift from Dr. K Guan (Inoki et al., 2003).

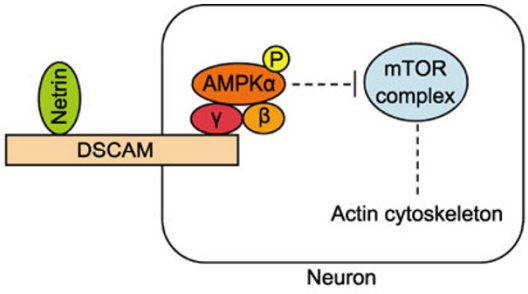

Figure 6. A model for the role of AMPK in netrin-induced neurite outgrowth. Netrin binds to the extracellular domain of DSCAM whose intracellular domain interacts with AMPK γ subunit. Netrin stimulation leads to the activation of AMPK by phosphorylation. Previous studies show that AMPK activation suppresses mTOR pathway and regulates neurite outgrowth in cortical neurons (Guo et al., 2011; Williams et al., 2011). AMPK may affect actin cytoskeleton through inhibition of mTOR pathway and thereby modulate neurite outgrowth.

Yeast two-hybrid screening to identify DSCAM-interacting proteins

The intracellular domain of the human DSCAM protein (DSAM-ICD) was used as a bait to search for proteins interacting with DSCAM. The LexA yeast two-hybrid system was used as described (Wu and Maniatis, 1993; Zervos et al., 1993).

Cell culture and transfection

Cell culture was maintained at 37°C with 5% CO_2. HEK293 cells were cultured in DMEM (GIBCO) with 10% fetal bovine serum (Thermo Scientific) and penicillin-streptomycin (GIBCO). HEK293 cells were cultured to 80% confluence for transfection using different plasmids with PEI (Polyplus).

Immunoprecipitation and immunoblotting

To test protein-protein interactions, cells were harvested 48 h after transfection and then lysed in cell lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 150 mmol/L NaCl, 0.5% Nonidet P-40, 1 mmol/L NaF, 1 mmol/L PMSF, proteinase inhibitors (Roche)]. The supernatant of the lysates was collected, incubated with corresponding antibodies at 4°C for 1 h, and then incubated with protein A/G-agrarose beads (Roche) at 4°C for another 3 h. Then the beads were washed with cell lysis buffer for 3 times. Co-immuoprecipitated proteins were detected by Western blot analysis using specific antibodies.

Primary cortical neuron culture and netrin treatment

Cortical neurons were dissected from E15 mouse embryos, and plated on 35 mm dishes coated with poly-L-lysine (50 μg/mL, Sigma-Aldrich) and laminin (5 μg/mL, Invitrogen). Neurons were cultured for 24 h in DMEM with 10% FBS. Then cells were starved for 12 h in DMEM with 0.1% bovine serum albumin, and then treated with purified netrin-1 (R & D Systems, Minneapolis) at 15 nmol/L concentration for 15 min and 30 min.

Neurite outgrowth assay

Neurite outgrowth assay was performed using the same protocol as described in the previous study (Liu et al., 2009). Fluorescent images were obtained under a Nikon Eclipse Ti Microscope.

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AUTHOR CONTRIBUTIONS

JYW, LZ and HY designed the study; KZ, XC and JYW performed the experiments and analyzed the data; HY, LZ, JL and JYW supervised the experiments and discussed and analyzed the data; KZ, HY, LZ

and JYW wrote the paper.

ABBREVIATIONS

AMPK, AMP-activated protein kinase; CaMKKβ, calcium/calmodulin-dependent protein kinase β; DCC, deleted in colorectal cancer; DSCAM, Down syndrome cell adhesion molecule; FN, fibronectin

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