Journal of Neuroscience Research 79:114–118 (2005)

Selective Regulation of 14-3-3η in Primary Culture of Cerebral Cortical Neurons and Astrocytes During Development

Xiao Qian Chen,[1] Shuang Liu,[1,2] Lu Ye Qin,[1] Chen Ran Wang,[1] Yin-Wan Wendy Fung,[1,2] and Albert Cheung-Hoi Yu[1,2]*

[1]Neuroscience Research Institute, Peking University, Key Laboratory of Neuroscience (Peking University), Ministry of Education, Department of Neurobiology, Peking University Health Science Center, Beijing, China
[2]Hong Kong DNA Chips Limited, Hong Kong SAR, China

The 14-3-3 proteins exist predominantly in the brain and may play regulatory roles in cellular processes of growth, differentiation, survival, and apoptosis. The biological functions, however, of the various 14-3-3 isoforms (β, ϵ, η, γ, and ζ) in the brain remain unclear. We have reported previously upregulation of 14-3-3γ in ischemic astrocytes. In the present study, we report selective regulation of 14-3-3η in cultured cerebral cortical neurons and astrocytes during in vitro development. In cultured neurons, gene expression levels of 14-3-3η increase with culture age (0–10 days). Brain-derived neurotrophic factor and neurotrophin-3 upregulate 14-3-3η gene expression. In cultured astrocytes, 14-3-3η is downregulated with culture age (1–5 weeks). The gene expression level of 14-3-3η is not affected by scratch injury in astrocytes or by ischemia in neurons. These data suggest a possible role of 14-3-3η in growth and differentiation of neurons and astrocytes, indicating an intricate mechanism governing coordinated and well-controlled developmental events in the brain to ensure normal neural functions.

Key words: 14-3-3; 14-3-3 eta; neuron; astrocytes; gene expression; RT-PCR

The 14-3-3 proteins are multifunctional and exist predominantly in the brain (Berg et al., 2003). Associated with more than 100 other proteins, including protein kinases (e.g., Raf, MEKK, and cdc25) and transcription factors (e.g., FKHRL) (van Hemert et al., 2001), the binding of 14-3-3 regulates activities of other proteins (Kimura et al., 2001; Gannon-Murakami and Murakami, 2002; Saito et al., 2004), facilitates protein–protein interaction, and relocalizes other proteins (Benton and St. Johnston, 2003; Margolis et al., 2003; Tsuruta et al., 2004). The 14-3-3 proteins may be involved in regulating cell division, differentiation, survival, and apoptosis (Chen and Yu, 2002; Margolis et al., 2003; Saito et al., 2004).

The mammalian brain expresses five (β, ϵ, η, γ, and ζ) of seven 14-3-3 isoforms (β, ϵ, η, γ, ζ, σ, and τ). Two isoforms considered brain and neuronal specific are 14-3-3η and γ, respectively, and they share similar mRNA distribution pattern in the brain (Watanabe et al., 1993). We have reported recently that 14-3-3γ is expressed and upregulated by ischemia in astrocytes (Chen et al., 2003). Because the biological function of 14-3-3η in the brain remains obscure, we use our experience in studying 14-3-3γ to investigate the putative function of 14-3-3η in the brain. We present evidence that 14-3-3η is the major isoform specifically upregulated in cerebral cortical neurons during in vitro development and by neurotrophic factors, indicating a role of 14-3-3η in neuronal growth and differentiation.

MATERIALS AND METHODS

Primary Cultures of Cerebral Cortical Neurons and Astrocytes

Cultures were prepared from cerebral cortices of ICR mouse embryos of 16-day-old embryos (E16) as described previously for neurons (Li et al., 2002) and astrocytes (Yu et al., 2003). All cultures were incubated in a Napco CO_2 incubator (Precision Scientific, Inc.) at 37°C with 95% air/5% CO_2 (vol/vol) and 95% humidity.

Drug Treatment

Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) (all from Invitrogen, Canada) prepared in phophate-buffered saline (PBS) were added to 5-day-old neuronal cultures in a final concentration of 25 ng/ml. All treated cells were harvested 48 hr after treatment.

Contract grant sponsor: Natural Science Foundation of China; Contract grant number: 30270426; Contract grant number: 30470543; Contract grant sponsor: Beijing National Science Foundation; Contract grant number: 703026.

*Correspondence to: Albert C.H. Yu, PhD, Professor, Neuroscience Research Institute, Peking University, 38 Xue Yuan Road, Beijing 100083, China. E-mail: achy@bjmu.edu.cn

Received 8 July 2004; Revised 23 August 2004; Accepted 26 August 2004

Published online 22 November 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.20323

In Vitro Ischemia

An anaerobic chamber-induced ischemia model was used as described previously (Yu and Lau, 2000; Yu et al., 2001, 2003). Briefly, glucose- and serum-free medium (referred to as ischemia media thereafter) was equilibrated for 4 hr in an anaerobic chamber (Forma Scientific, Inc.) saturated with N_2:CO_2:H_2 (85:5:10). The oxygen concentration in the glucose-free medium was less than 0.1 parts per million (ppm) as measured by a dissolved oxygen meter (Hanna Instruments, Italy). Cultured neurons (5 days old) were washed three times with the ischemia media, after which the cells were covered with 0.78 ml (for 35-mm culture dish) of ischemia medium. All cultures were wrapped with parafilm and incubated at 37°C inside the anaerobic chamber. Cultures with normal culture medium and incubated in a normal CO_2 incubator were used as controls.

Scratch-Wound Injury

Scratch-wound injury was introduced to cultured astrocytes with a yellow plastic pipet tip as described previously (Wu and Yu, 2000; Lau and Yu, 2001). Immediately after injury, the culture was given fresh medium and incubated in a Napco CO_2 incubator at 37°C with 95% air/5% CO_2 (vol/vol) and 95% humidity for various durations.

RT-PCR

Total RNA from cultured cells was extracted with TRIzol reagent according to the manufacturer's instructions (Invitrogen). Gene expression levels of 14-3-3 isoforms were determined by reverse transcription-polymerase chain reaction (RT-PCR) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (Yu et al., 2003). Reverse transcription was carried out using 2 μg total RNA, 20 μM dNTP (Invitrogen), 1 μM random hexamers (Invitrogen), and 100 U reverse transcriptase (Invitrogen) in a total volume of 20 μl. PCR (with 29 cycles of 94°C, 45 sec; 68°C, 45 sec; 72°C, 45 sec; and a final extension at 72°C for 10 min) was carried out using 1 μl cDNA in a total volume of 10 μl. Forward and reverse primers for amplifying the 14-3-3 isoforms were based on GenBank sequences of murine 14-3-3 isoforms and are listed respectively: for β, 5′-ctcttcctggcgtgtcatct and 5′-actttgctttctgcctgggt; for ε, 5′-ccccattcgtttaggtcttg and 5′-ggtccacagcgtcaggttat; for η, 5′-atgggcatttgctggactg and 5′-aaggaatgagttgtcgctgtg; for γ, 5′-gttggtctggctcttcatcat and 5′-aggtgcagagtagacttgggtg; and for ζ, 5′-tgctggtgatgacaagaaagg and 5′-gaggcagacaaaggttggaag. Murine GAPDH primers were 5′-tgatgacatcaagaaggtggtgaag and 5′-tccttggaggccatgtaggccat. DNA sequencing was carried out to identify the PCR amplification products.

RESULTS

Gene Expression of 14-3-3η in Cultured Neurons

Cerebral cortical neurons exhibited a prominent in vitro developmental course when grown in primary culture. At initial seeding (0 hr), neurons in culture seemed phase bright, and began to send out processes 12 hr after seeding (Fig. 1A; 0 and 0.5 day). The number and length of processes steadily increased with culture age within 4 days. From Day 4 to 7, neuronal processes formed networks, and neurons were considered mature in vitro (Fig. 1A; 4 and 7 days). From Day 7 to 10, mature cultured neurons migrated to form colonies and thereafter (Fig. 1A), cultured neurons began to show signs of degeneration (data not shown).

Gene expression of 14-3-3 isoforms was examined in cultured neurons by RT-PCR. Isoforms 14-3-3 β, ε, η, γ, and ζ, but not σ or τ, were PCR amplifiable from primary culture of neurons (Fig. 1B). During in vitro neuronal development, gene expression levels of 14-3-3η exhibited an increasing trend from Day 0 to 10 (Fig. 1B), whereas expression levels of GAPDH remained constant. Expression levels of 14-3-3 β, ε, γ, and ζ did not alter when compared to that of GAPDH (Fig. 1B).

The mechanism of how 14-3-3 is regulated is currently unknown. If 14-3-3η is associated with development of neurons in culture, neurotrophic factors may be involved in regulating 14-3-3η expression. We investigated the effects of NGF, BDNF, and NT-3 on gene expression of various 14-3-3 isoforms, because these three neurotrophic factors are the most extensively studied and play critical roles in neuronal growth and differentiation (Ma et al., 2002; Burkhalter et al., 2003; Itami et al., 2003). In the presence of NGF, gene expression levels of 14-3-3 β, η, γ, and ζ did not alter evidently as compared to that in the controls (Fig. 1C). BDNF and NT-3, however, exhibited an upregulatory effect on expression levels of 14-3-3η (Fig. 1C), whereas expression levels of other 14-3-3 isoforms were not affected (Fig. 1C).

Gene Expression of 14-3-3η in Cultured Astrocytes

Similar to cultured neurons, astrocytes also exhibited a prominent in vitro developmental course when grown in culture (Fig. 2A). At 3 days after initial seeding, dissociated cerebral cortical astrocytes started to proliferate rapidly, and the culture became confluent in 2 weeks (Fig. 2A; 14 days). Cultured astrocytes are considered mature in vitro at the fourth week (Yu and Hertz, 1982; Yu et al., 1986). Gene expression levels of 14-3-3 β, ε, η, γ and ζ, in astrocytes cultured for 1, 2, 3, 4, and 5 weeks were revealed by RT-PCR, using GAPDH as normalizing controls (Fig. 2B). In contrast to the increasing trend of 14-3-3η as observed in cultured neurons, gene expression levels of 14-3-3η in cultured astrocytes exhibited a downward trend with culture age (2, 3, 4, and 5 weeks; Fig. 2B). Gene expression levels of 14-3-3β, ε, γ, and ζ in cultured astrocytes did not differ with culture age (Fig. 2B).

Gene Expression of 14-3-3η in Injured Neurons and Astrocytes

Expression levels of 14-3-3η in astrocytes and neurons matured in culture were compared. RT-PCR revealed that 14-3-3η was expressed in cultured astrocytes and neurons, but not in COS7 cells (Fig. 3A), supporting the brain-specific nature of the 14-3-3η isoform. To examine whether 14-3-3η is involved in protecting neurons and astrocytes against injury, astrocytes and neurons were subjected to scratch-wound injury and ischemia, respectively. In astrocytes in-

jured by scratch-wound, gene expression levels of 14-3-3η at various points after scratch injury did not vary evidently, when compared to levels of GAPDH (Fig. 3B). Similarly, in cultured neurons treated with ischemia of various duration, gene expression levels of 14-3-3η did not show an obvious difference when compared to levels of GAPDH (Fig. 3C).

DISCUSSION

Neuronal growth and differentiation are complicated processes regulated by various genes (Barak et al., 2003;

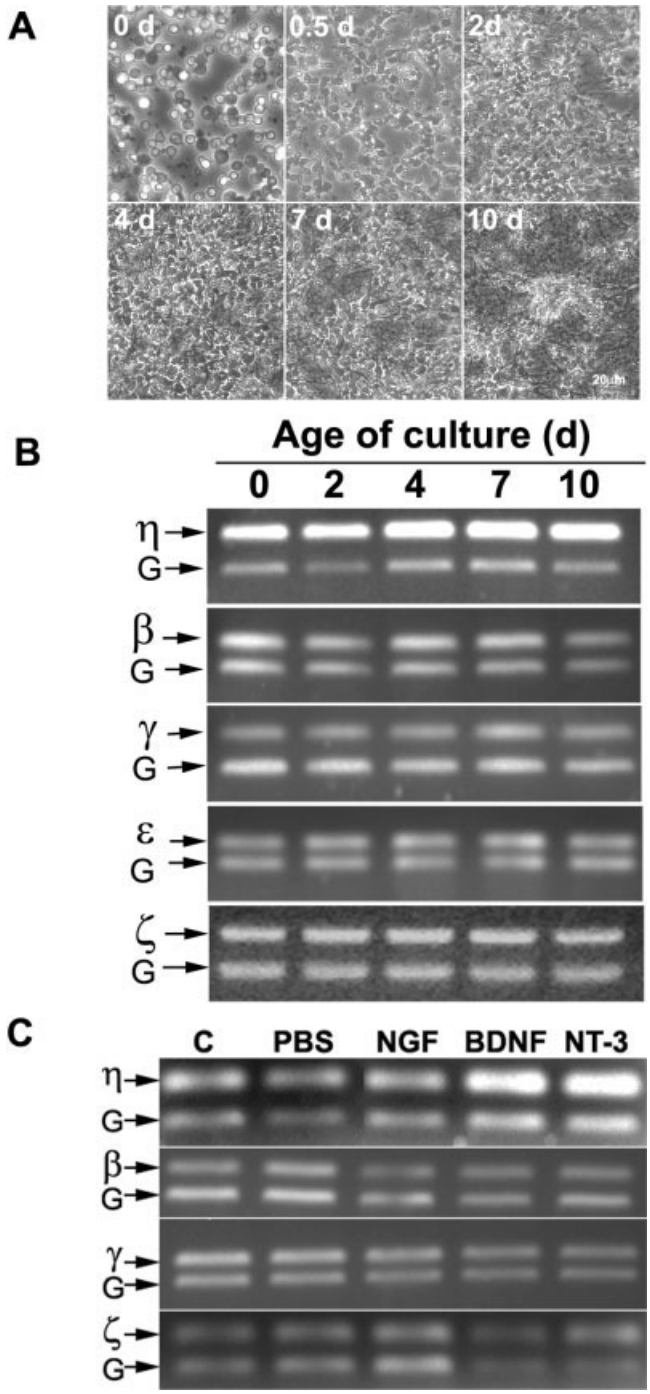

Fig. 1. Expression of 14-3-3η and in vitro development of cortical neurons in primary cultures. **A:** Cerebral cortical neurons from E16 mice exhibited prominent features of neuronal growth and differentiation in primary cultures. Phase contrast micrographs showed neurons at various developmental ages (d, day). **B:** Gene expression of 14-3-3 isoforms in cultured neurons as revealed by RT-PCR. Representative RT-PCR results showed an increasing trend of gene expression of 14-3-3η, but not β, ϵ, γ and ζ, with the age of cultured neurons. GAPDH (denoted as G) was amplified simultaneously as internal control. **C:** Neuronal cultures (5 days old) were treated with 35 ng/ml of NGF, BDNF, and NT-3 for 48 hr. RT-PCR revealed that 14-3-3η gene expression was increased in the presence of BDNF and NT-3, which did not seem to affect the expression levels of other 14-3-3 isoforms. NGF did not vary the expression levels of any 14-3-3 isoforms.

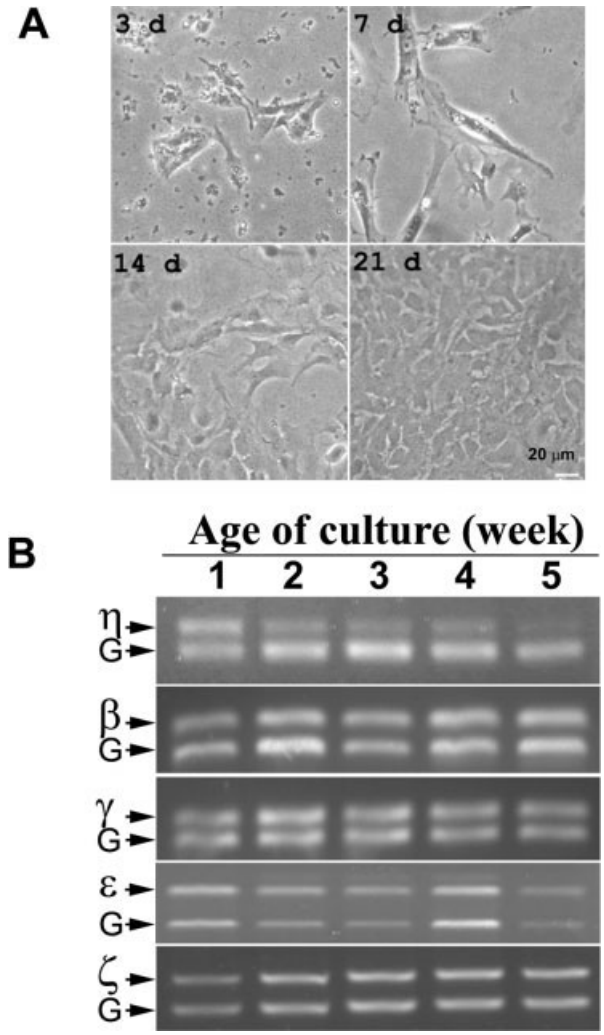

Fig. 2. Downregulation of 14-3-3η in cultured astrocyte during in vitro development. **A:** Cerebral cortical astrocytes dissociated from newborn mice underwent proliferation within the first 2 weeks and matured in culture on the fourth week. Phase contrast micrographs showed astrocytes at various culture ages (d, day). **B:** Gene expression of 14-3-3 isoforms in astrocytes of various culture ages. Representative RT-PCR results revealed that expression of 14-3-3η, but not β, ϵ, γ, and ζ, showed a downward trend with the culture ages. GAPDH (denoted as G) was amplified simultaneously as internal control.

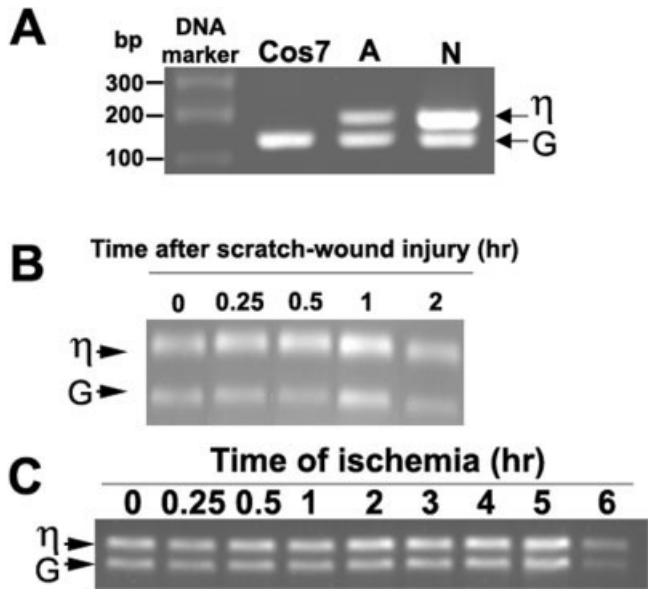

Fig. 3. Gene expression of 14-3-3η in cultured neurons and astrocytes, and treatment of scratch-wound injury and ischemia. **A:** Representative RT-PCR results indicated the gene expression levels of 14-3-3η in COS7 cells, astrocytes, and neurons in culture. **B:** Representative RT-PCR results showed constant gene expression levels of 14-3-3η in astrocytes at various time points after scratch-wound injury. **C:** Representative RT-PCR results showed constant gene expression levels of 14-3-3η in cultured neurons subjected to ischemia treatment for various time lengths.

Bulow and Hobert, 2004; Chang et al., 2004). Identifying differentially expressed genes in the brain is challenging because of the complexity of the brain. Furthermore, the use of cell lines such as PC12 may not reflect physiologic conditions. Neurons in primary cultures exhibit prominent neurite growth and functional maturation during in vitro development (Yu and Hertz, 1982; Yu et al., 1986; Li et al., 2002), mimicking conditions in vivo. Cultured cerebral cortical neurons are thus suitable for studying gene expression during neuronal growth and differentiation.

It is known that the brain contains 14-3-3 β, ϵ, η, γ, and ζ. We have shown that 14-3-3 β, ϵ, η, γ, and ζ, but not σ and τ, are expressed in neurons (Fig. 1B) and astrocytes (Fig. 2B), suggesting that astrocytes and neurons contribute to the pool of brain-specific 14-3-3 proteins. Being brain specific, the functional roles of various 14-3-3 isoforms are largely unclear. In this study, we demonstrated upregulation of 14-3-3η in neurons during in vitro development (Fig. 1B), corresponding to active axon and dendrite growth and ramification of neurons in culture (Fig. 1A). Because expression levels of 14-3-3η do not vary in cultured neurons subjected to ischemia, 14-3-3η may function specifically to regulate neuronal development and differentiation. Compared to neurons, 14-3-3η is downregulated during proliferation and maturation of astrocytes in culture (Fig. 2A,B). Freshly dissociated astrocytes, which may express glial fibrillary acidic protein (GFAP), from cerebral cortices of newborn mice are immature in culture. The higher expression level of 14-3-3η in astrocytes at earlier stage of in vitro development (Week 1; Fig. 2B) might associate with the primitive state of the dissociated astrocytes.

The upstream signaling for 14-3-3 inductions is unknown. We first showed that BDNF and NT-3 could specifically upregulate 14-3-3η (Fig. 1C), consistent with the roles of BDNF (Burkhalter et al., 2003; Itami et al., 2003) and NT-3 (Ma et al., 2002; Peng et al., 2003) in neuronal development. It is known that BDNF and NT-3 are upregulated during neuronal development (Lessmann et al., 2003; Sadakata et al., 2004). Increasing levels of 14-3-3η in developing neurons might result from increases in endogenous BDNF and NT-3. NGF, BDNF, and NT-3 exert their effects through their distinct receptors Trk A, B, and C, respectively (Huang and Reichardt, 2003). Trk B and C, but not Trk A, are highly expressed in the cerebral cortex (Patapoutian and Reichardt, 2001). Neurons dissociated from the cerebral cortex might also express Trk B and C only, and thus 14-3-3η is not expected to be induced by NGF, whose signaling is mediated by other 14-3-3 isoforms (Kimura et al., 2001; Gannon-Murakami and Murakami, 2002; Saito et al., 2004). Increasing levels of 14-3-3η might be required for modulating other intracellular proteins or signaling pathways in developing neurons. Many 14-3-3-binding proteins such as protein kinase C (Gannon-Murakami and Murakami, 2002), Raf (Hekman et al., 2004), and proline-rich Akt substrate (Saito et al., 2004) are induced or activated and may play important roles in neuronal development. The proper function of these proteins might require 14-3-3η binding.

In conclusion, data presented here suggest specific roles of 14-3-3η during growth and differentiation of neurons in culture. Selective regulation of 14-3-3η in cultured neurons and astrocytes may indicate an intricate mechanism governing coordinated and well-controlled developmental events in the brain to ensure normal neural functions.

REFERENCES

Barak O, Lazzaro MA, Lane WS, Speicher DW, Picketts DJ, Shiekhattar R. 2003. Isolation of human NURF: a regulator of Engrailed gene expression. EMBO J 22:6089–6100.

Benton R, St. Johnston D. 2003. *Drosophila* PAR-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells. Cell 115:691–704.

Berg D, Holzmann C, Riess O. 2003. 14-3-3 proteins in the nervous system. Nat Rev Neurosci 4:752–762.

Bulow HE, Hobert O. 2004. Differential sulfations and epimerization define heparan sulfate specificity in nervous system development. Neuron 41:723–736.

Burkhalter J, Fiumelli H, Allaman I, Chatton JY, Martin JL. 2003. Brain-derived neurotrophic factor stimulates energy metabolism in developing cortical neurons. J Neurosci 23:8212–8220.

Chang CW, Tsai CW, Wang HF, Tsai HC. 2004. Identification of a developmentally regulated striatum-enriched zinc-finger gene, Nolz-1, in the mammalian brain. Proc Natl Acad Sci USA 101:2613–2618.

Chen XQ, Yu ACH. 2002. The association of 14-3-3γ and actin played a role in cell division and apoptosis in astrocytes. Biochem Biophys Res Commun 296:657–663.

Chen XQ, Chen JG, Zhang Y, Hsiao WW, Yu ACH. 2003. 14-3-3gamma is upregulated by in vitro ischemia and binds to protein kinase Raf in primary cultures of astrocytes. Glia 42:315–324.

Gannon-Murakami L, Murakami K. 2002. Selective association of protein kinase C with 14-3-3 zeta in neuronally differentiated PC12 Cells. Stimulatory and inhibitory effect of 14-3-3 zeta in vivo. J Biol Chem 277:23116–23122.

Hekman M, Wiese S, Metz R, Albert S, Troppmair J, Nickel J, Sendtner M, Rapp UR. 2004. Dynamic changes in c-Raf phosphorylation and 14-3-3 protein binding in response to growth factor stimulation: differential roles of 14-3-3 protein binding sites. J Biol Chem 279:14074–14086.

Huang EJ, Reichardt LF. 2003. Trk receptors: roles in neuronal signal transduction. Annu Rev Biochem 72:609–642.

Itami C, Kimura F, Kohno T, Matsuoka M, Ichikawa M, Tsumoto T, Nakamura S. 2003. Brain-derived neurotrophic factor-dependent unmasking of "silent" synapses in the developing mouse barrel cortex. Proc Natl Acad Sci USA 100:13069–13074.

Kimura MT, Irie S, Shoji-Hoshino S, Mukai J, Nadano D, Oshimura M, Sato TA. 2001. 14-3-3 is involved in p75 neurotrophin receptor-mediated signal transduction. J Biol Chem 276:17291–17300.

Lau LT, Yu ACH. 2001. Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. J Neurotrauma 18:351–359.

Lessmann V, Gottmann K, Malcangio M. 2003. Neurotrophin secretion: current facts and future prospects. Prog Neurobiol 69:341–374.

Li Q, Li Z, Sun CX, Yu ACH. 2002. Identification of transcripts expressed under functional differentiation in primary culture of cerebral cortical neurons. Neurochem Res 27:147–154.

Ma L, Harada T, Harada C, Romero M, Hebert JM, McConnell SK, Parada LF. 2002. Neurotrophin-3 is required for appropriate establishment of thalamocortical connections. Neuron 36:623–634.

Margolis SS, Walsh S, Weiser DC, Yoshida M, Shenolikar S, Kornbluth S. 2003. PP1 control of M phase entry exerted through 14-3-3-regulated Cdc25 dephosphorylation. EMBO J 22:5734–5745.

Patapoutian A, Reichardt LF. 2001. Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 11:272–280.

Peng HB, Yang JF, Dai Z, Lee CW, Hung HW, Feng ZH, Ko CP. 2003. Differential effects of neurotrophins and Schwann cell-derived signals on neuronal survival/growth and synaptogenesis. J Neurosci 23:5050–5060.

Sadakata T, Mizoguchi A, Sato Y, Katoh-Semba R, Fukuda M, Mikoshiba K, Furuichi T. 2004. The secretory granule associated protein CAPS2 regulates neurotrophin release and cell survival. J Neurosci 24:43–52.

Saito A, Narasimhan P, Hayashi T, Okuno S, Ferrand-Drake M, Chan PH. 2004. Neuroprotective role of a proline-rich Akt substrate in apoptotic neuronal cell death after stroke: relationships with nerve growth factor. J Neurosci 24:1584–1593.

Tsuruta F, Sunayama J, Mori Y, Hattori S, Shimizu S, Tsujimoto Y, Yoshioka K, Masuyama N, Gotoh Y. 2004. JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. EMBO J 23:1889–1899.

van Hemert MJ, Steensma HY, van Heusden GP. 2001. 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. Bioessays 23:936–946.

Watanabe M, Isobe T, Ichimura T, Kuwano R, Takahashi Y, Kondo H. 1993. Molecular cloning of rat cDNAs for beta and gamma subtypes of 14-3-3 protein and developmental changes in expression of their mRNAs in the nervous system. Brain Res Mol Brain Res 17:135–146.

Wu BY, Yu ACH. 2000. Quercetin inhibits c-fos, heat shock protein, and glial fibrillary acidic protein expression in injured astrocytes. J Neurosci Res 62:730–736.

Yu ACH, Chan PH, Fishman RA. 1986. Effects of arachidonic acid on glutamate and gamma-aminobutyric acid uptake in primary cultures of rat cerebral cortical astrocytes and neurons. J Neurochem 47:1181–1189.

Yu ACH, Hertz L. 1982. Uptake of glutamate, GABA, and glutamine into a predominantly GABA-ergic and a predominantly glutamatergic nerve cell population in culture. J Neurosci Res 7:23–35.

Yu ACH, Lau LT. 2000. Expression of interleukin-1 alpha, tumor necrosis factor alpha and interleukin-6 genes in astrocytes under ischemic injury. Neurochem Int 36:369–377.

Yu ACH, Wong HK, Yung HW, Lau LT. 2001. Ischemia-induced apoptosis in primary cultures of astrocytes. Glia 35:121–130.

Yu ACH, Yung HW, Hui MH, Lau LT, Chen XQ, Collins RA. 2003. Cyclohexamide and actinomycin D delay death and affect bcl-2, bax, and Ice gene expression in astrocytes under in vitro ischemia. J Neurosci Res 15:318–325.