# Effects of annexins II and V on survival of neurons and astrocytes *in vitro*[1]

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**KEY WORDS** annexin II; annexin V; neurons; astrocytes; cell culture; lactate dehydrogenases; hydrogen peroxide; hypoxia

## ABSTRACT

**AIM:** To study the effects of annexins II and V on the survival and neurite outgrowth of primary cultured neurons and the survival of astrocytes after peroxide and hypoxia insults *in vitro*. **METHODS:** Annexins II and V proteins and/or corresponding antibodies were added to the medium of primary neocortical cultures. $H_2O_2$ and $NaN_3$ were used to induce neuron injury, respectively. Lactate dehydrogenase (LDH) release was measured. **RESULTS:** Addition of annexin II or V into the culture medium did not affect the normal survival and neurite outgrowth of cortical neurons. However, when an antibody against annexin II or V was added to the culture, the survival and neurite outgrowth of these neurons markedly declined. Further, addition of the two annexins into cortical cultures after peroxide and hypoxia insults markedly reduced the LDH release and cell death. **CONCLUSION:** Annexins II and V are essential for the survival and neurite outgrowth of developing cortical neurons, the survival of glial cells, and protect neurons and glial cells against peroxide and hypoxia injuries.

## INTRODUCTION

Annexins are a family of structurally and functionally related proteins that exhibit $Ca^{2+}$-dependent binding to phospholipids[1-4]. Previous studies suggest that these proteins play a role in the development of the central nervous system (CNS)[5]. In some pathological conditions such as traumatic hemorrhage, embolism, and thrombotic infarction[6] or some diseases of the CNS such as encephalomyelitis and Alzheimer's disease[7], annexin expressions are upregulated. During development, annexins are involved in establishing the midline structures of the CNS, directing the growth and decussation of sensory fibers, and promoting neuronal survival through regulating certain neural processing pathways such as signal transduction[8]. Annexins are also suggested to mediate the anti-inflammatory effect of endogenous glucocorticoids after various insults[9-12]. However, a complete understanding of the role of annexins in these pathological diseases is still lacking.

In a previous study, we demonstrated that both annexin II and V mRNAs and proteins increased in the spinal cord after either a spinal cord transection[13] or

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contusion[14]. In the present study, we investigated whether addition of annexin II or V proteins would enhance the survival and neurite outgrowth of cultured embryonic cortical neurons *in vitro*. In addition, we tested whether the beneficial effect of an annexin could be blocked by an antibody against this protein. Finally, we examined the protective effect of an annexin on cultured neurons and glia after peroxide and hypoxia insults.

## MATERIALS AND METHODS

**Neocortical primary culture** Neocortex was isolated from SD rat embryos at embryonic d 18 (E18). The tissue was rinsed in Hanks' buffered saline solution (HBSS), cut into small pieces, digested with trypsin, dissociated with a fire polished glass pipette and centrifuged to separate undissociated tissue. Cells were resuspended and plated onto poly-*D*-lysine-coated 6-mm glass cover slides placed within 35-mm culture dishes. For observing the survival and neurite outgrowth of cultured neural cells, 25 000 cells were seeded onto each cover-slide. Cells were grown in serum-free Neurobasal medium supplemented with 2 % $B_{27}$ and glutamine 0.05 mmol/L.

**Neural survival and neurite outgrowth assays *in vitro*** On d 2 one of the following proteins or antibodies was added to each culture: 1) annexin II protein (100 μg/L; Biodesign International, USA), 2) annexin V protein (100 μg/L; BD Biosciences, USA), 3) annexin II antibody (10 mg/L; Santa Cruz Biotechnology, USA), 4) annexin V antibody (10 mg/L; Santa Cruz Biotechnology, USA), 5) heat-inactivated annexin II antibody (10 mg/L; heated at 100 ºC for 10 min), 6) heat-inactivated annexin V antibody (10 mg/L; heated at 100 ºC for 10 min), and 7) no protein or antibody as control. On d 7 cultures were fixed in 4 % paraformaldehyde and processed for β-tubulin III (Sigma) and glial fibillary acidic protein (GFAP, Sigma) immunofluorescence double staining to identify neurons and astrocytes *in vitro* respectively. The nuclei of all cells were stained with a Hoechst 33342 nuclear dye. Briefly, the cultures were rinsed in phosphate-buffered saline (PBS) and incubated with the GFAP primary antibody (1:200) plus 1 % bovine serum albumin (BSA) in PBS overnight at 4 ºC. On d 2 cultures were washed in PBS, incubated with fluorescein (FITC)-conjugated goat anti-rabbit IgG secondary antibody (Jackson Immuno Research Lab, Inc, West Grove, PA) for 1 h at 37 ºC. The cultures were then washed three times with PBS and incubated with the β-tubulin III primary antibody (1:400) overnight at 4 ºC, and then washed with PBS and incubated with rhodamine (RHO)-conjugated secondary antibodies for 1 h at 37 ºC (Jackson ImmunoResearch Lab). The cultures were finally coverslipped with Gelmount (Blomeda, USA) supplemented with the Hoechst 33342 nuclear dye (0.02 mmol/L, Sigma). All control cultures were incubated in PBS with the omission of primary antibodies.

For counting the number of β-tubulin III-positive neurons and GFAP-positive astrocytes, 5-8 visual fields from each coverslip (containing approximately 600-1000 cells) were randomly selected. The percent of β-tubulin III-positive neurons or GFAP-positive astrocytes was calculated according to the following formula using the total counted number of blank control group as 100 %.

The percent of survival neurons=(the number of β-tubulin III-positive cells in each group)/the number of β-tubulin III-positive cells in the blank control group×100 %.

The percent of survival astrocytes=the number of GFAP-positive cells in each group/the number of GFAP-positive cells in the blank control group×100 %;

To measure neurite outgrowth from neurons, an unbiased counting frame containing 20×20 grids was superimposed on image of neurons and neurites under microscope. We randomly selected 5-8 neurons from each culture and counted the number of intersections of each neurite with grid lines, thereby allowing quantification of average neurite length per neuron using the following formula: $L$=JI/2×$d$×$J$, in which $L$ is neurite length in mm, $d$ is the vertical distance between two grid lines, and $J$ is the number of intersections between grid lines and neurites[15].

**LDH release from cortical cultures after peroxide and hypoxia insults** E18 neocortex was isolated from SD rat embryos. The tissue digestion and cell dissociation procedures followed the methods described above. Dissociated cells were seeded onto 96-well cell culture plates at a density of $1\times10^9$ cells/L and grown in serum-free Neurobasal medium supplemented with 2 % $B_{27}$ and glutamine 0.05 mmol/L. On d 5 two concentrations of $H_2O_2$ (low: $H_2O_2$ 25 μmol/L; high: $H_2O_2$ 50 μmol/L) and sodium azide (sodium azide 3 mmol/L) were added to the medium to produce peroxide and hypoxia insults, respectively. In the injury groups, only the insulting factors were added to the culture medium whereas in the treatment groups, annexin II and V proteins (500 mg/L) were added individually into the cul-

ture medium at the same time with the insulting factors. In addition, there was a positive control with the addition of 0.1 % Triton X-100 into the culture medium and a blank control with the addition of neither insulting factors nor annexins. Each group had six duplicate wells. The cultures were maintained for 3 additional days after various treatments before the culture medium of each well was removed for LDH release assay using a LDH-cytotoxicity assay kit (Biovision Inc, USA) according to the manufacturer's protocol. The relative absorbance of all samples was measured at 490 nm with an ELX 800UV microtiter plate reader (Bio-Tek Instruments, Winooski, VT). The measurement was repeated three times at a 5-s interval and the numbers of each group were calculated with the following formula:

Cytotoxicity=($A_{\text{test sample}}$-$A_{\text{blank control}}$)/( $A_{\text{positive control}}$-$A_{\text{blank control}}$)×100 %

The cells left in culture wells were used for the following experiments:

(1) 0.4 % trypan blue staining to quantify cell death in each group;

(2) β-tubulin III-immunofluorescence labeling to quantify the number of survival neurons in each group using the same method described above.

**Statistics** The percent of survival neurons/astrocytes, the lengths of all neurites in each neuron, and LDH release were expressed as mean±SD and the average values among different groups were statistically compared with *t*-test using the Microsoft Excel software.

## RESULTS

**Annexin II or V alone had no effect on neuron culture** The primary cultures of neurons and astrocytes taken from the E18 neocortex expressed annexins II and V (Fig 1, 2). In cultures containing annexin II and V proteins, neurons formed clusters with neurites radiating out in fascicles. No apparent morphological difference was found between the annexin V-treated and control groups (Fig 3A, B). Double immunofluorescence assay showed that annexin II and V protein did not induce marked morphological changes in cultured neocortical neurons and astrocytes *vs* control (Fig 4A, B, C, D). The percent of survival neurons and average length of their neurites were close to those of the blank control group ($P$>0.05, Fig 5, Fig 6).

**Effect of annexin II or V antibody on neuron culture** Under light microscope after addition of annexin

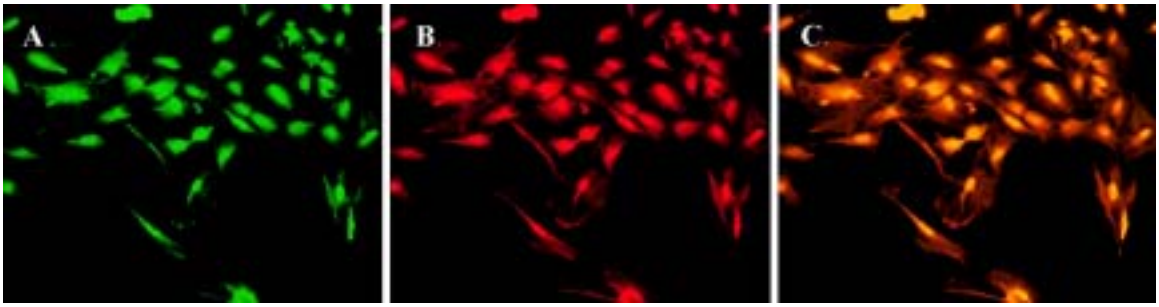


**Fig 1. Annexin II expression in astrocytes derived from the brain of E18 rats. Annexin II (A; green) immunoreactivity was found in GFAP-positive astrocytes (B; red) which could be seen in the merged image (Yellow). ×400.**

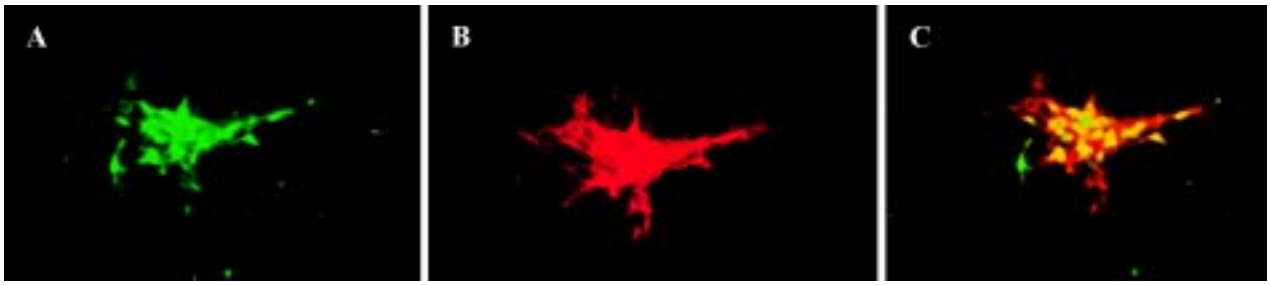


**Fig 2. Annexin V expression in neurons derived from the brain of E18 rats. Annexin V (A; green) immunoreactivity was found in β-tubulin-III positive neurons (B; red) which could be seen in the merged image (Yellow). ×400.**

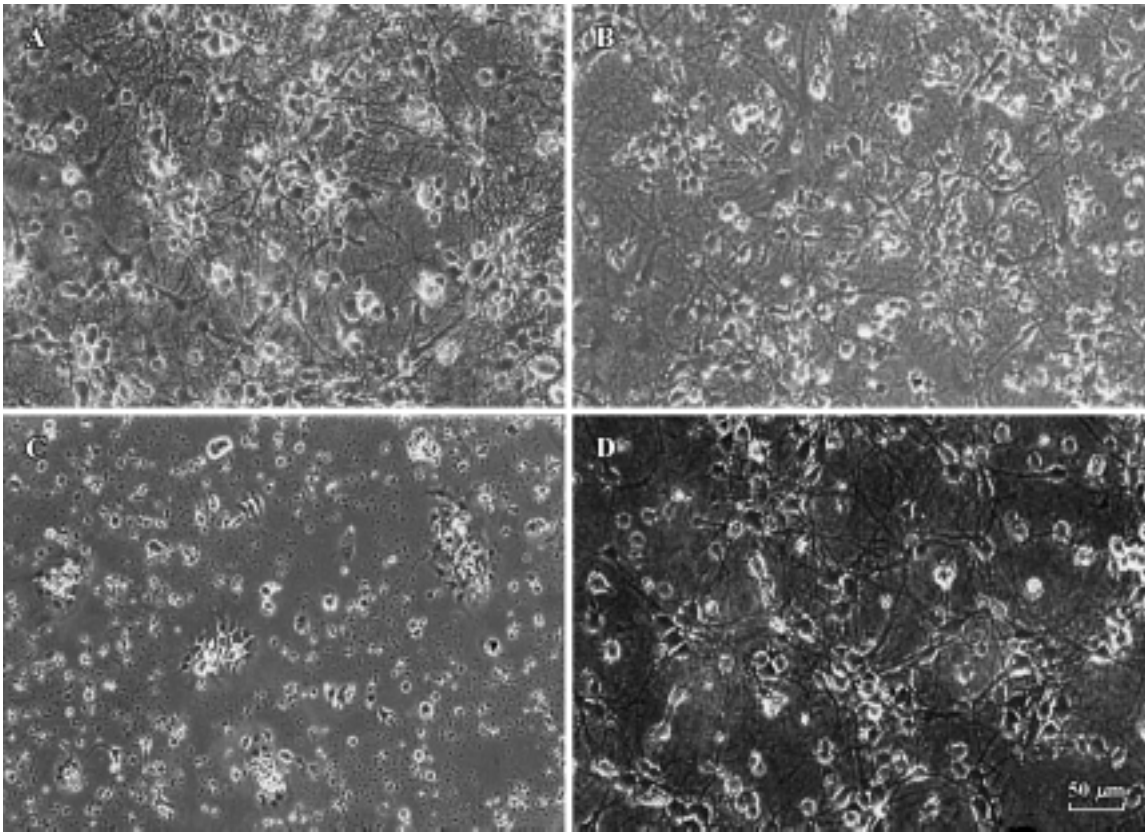


**Fig 3. Effects of annexin V protein and antibody on the survival and neurite outgrowth of cultured cortical neurons isolated from the E 18 SD rat. (A) Control; (B) At d 7 post-annexin V protein treatment; (C) At d 7 post-annexin V antibody treatment; (D) Inactivated annexin V antibody treatment. ×240.**

V antibody, numerous degenerated cells and fragmented neurites were observed (Fig 3C). Inactivated antibody of annexin V had no such effects (Fig 3D). Double immunofluorescence assay indicated that annexin II and V antibody substantially reduced the survival of neurons and astrocytes and reduced neurite outgrowth, respectively (Fig 4E, F). Heat-inactivated annexin II and V antibodies did not have a detrimental effect on survival and neurite outgrowth of cortical neurons or astrocytes (Fig 4G, H). In contrast, when annexin antibodies were added to the culture medium, the numbers of neurons and astrocytes markedly declined, as compared with the other treatment groups. For example, the number of survival neurons after annexin II and V antibody-treatment was only 16.8 % and 15.7 % of the blank control, respectively ($P<0.01$) and the number of survival astrocytes was 60.6 % and 69.0 % of the blank control group, respectively ($P<0.01$, Fig 5). Furthermore, the average length of neurites in both the annexin II and V antibody-treated groups was significantly reduced as compared with the control group ($P<0.01$, Fig 4, 6). Such a reduction was not observed when these antibodies were inactivated before being added into the cultures ($P>0.05$ *vs* control and annexin protein-added groups, Fig 5).

**Annexin II and V protected neurons against peroxide or hypoxia injury** After either a peroxide ($H_2O_2$ 25 μmol/L and $H_2O_2$ 50 μmol/L) or hypoxia (sodium azide 3 mmol/L) injury, the average value of LDH was similar to that of the positive control group treated with 0.1 % Triton-X100 ($P>0.05$, Fig 7). When annexins II and V were added to the cultures immediately after these insults, the average value of LDH greatly declined as compared with control groups ($P<0.01$). No statistical difference was found between the two annexin-treated groups ($P>0.05$, Fig 7). After peroxide and hypoxia insults, a substantial reduction of β tubulin III-positive neurons in cortical culture was found. Annexin II and V treatments successfully rescued β-tubulin III-positive neurons from insult (Fig 8).

## DISCUSSION

Previous studies have shown that expression of

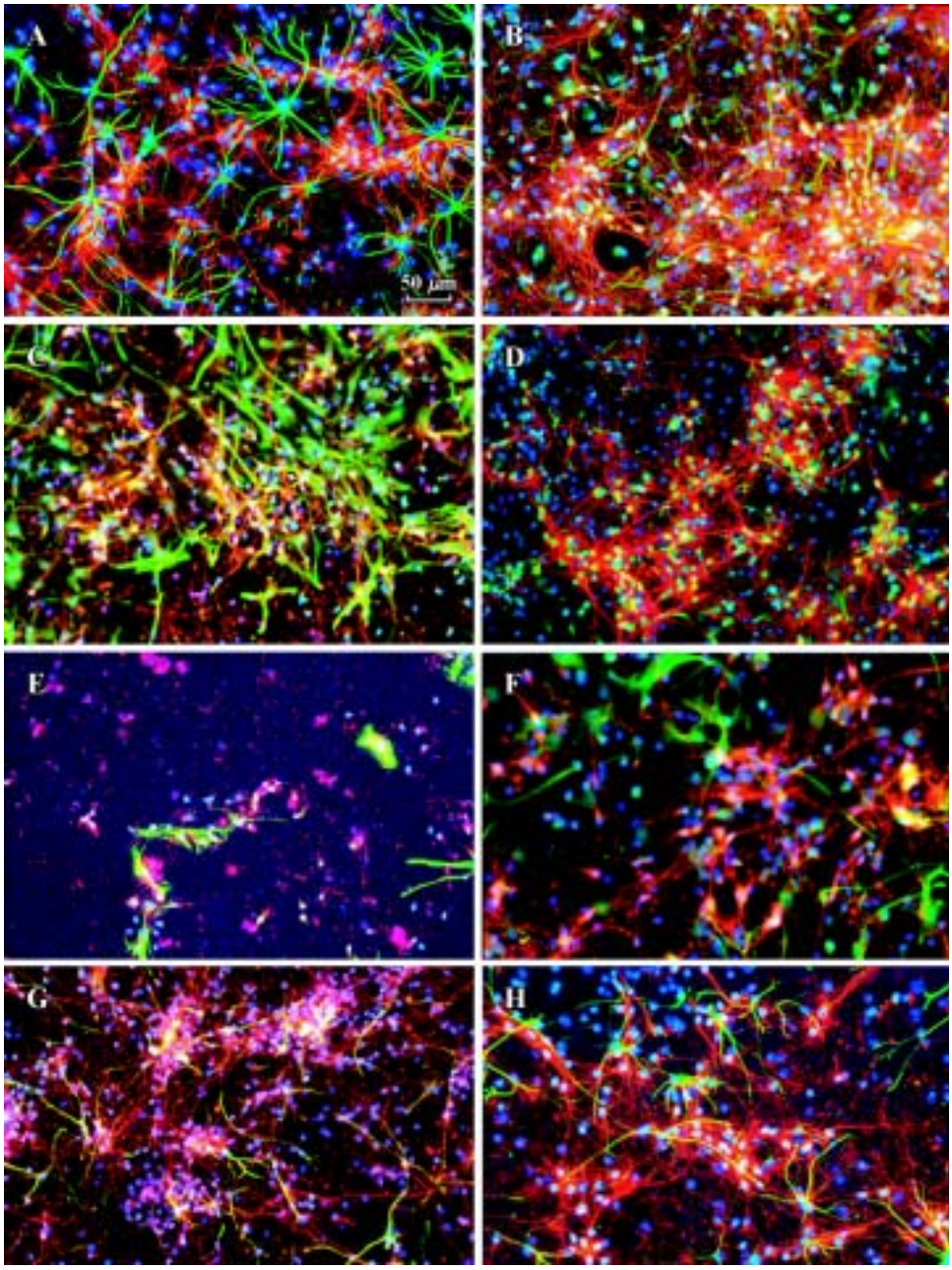


**Fig 4. Effects of annexin II and V proteins and antibodies on cultured neocortical neurons and astrocytes isolated from the E18 SD rat at d 7. Sections were double-stained with GFAP (green) and β-tubulin-III (red). All cell nuclei were stained with Hoechst 33342 nuclear dye (blue). (A,B) Control; (C,D) Annexin II and V protein treatment; (E, F) Annexin II and V antibodies treatment ; (G, H) Heat-inactivated annexin II and V antibodies treatment. ×200.**

annexins increased in the CNS during the course of embryo's development. This expression is transient and gradually declined during early postnatal developmental stages[8]. In the neocortex of the embryonic rat brain, annexin V mRNA was expressed in non-neuronal cells, such as astrocytes, microglia and fibroblasts, but not neurons [16]. However, our study demonstrated that both neurons and glial cells expressed annexins II and V in the neocortical culture.

In some pathological conditions, upregulation of annexins was seen[6,7]. In these instances, annexin expressions were mainly detected in reactive astrocytes and macrophages surrounding the injury or disease regions[6,7]. In a previous study, we demonstrated the presence of annexin II- and V-positive cells surrounding a complete spinal cord transection in adult rats[14]. These cells were identified as neurons and glial cells such as astrocytes, oligodendrocytes and microglia.

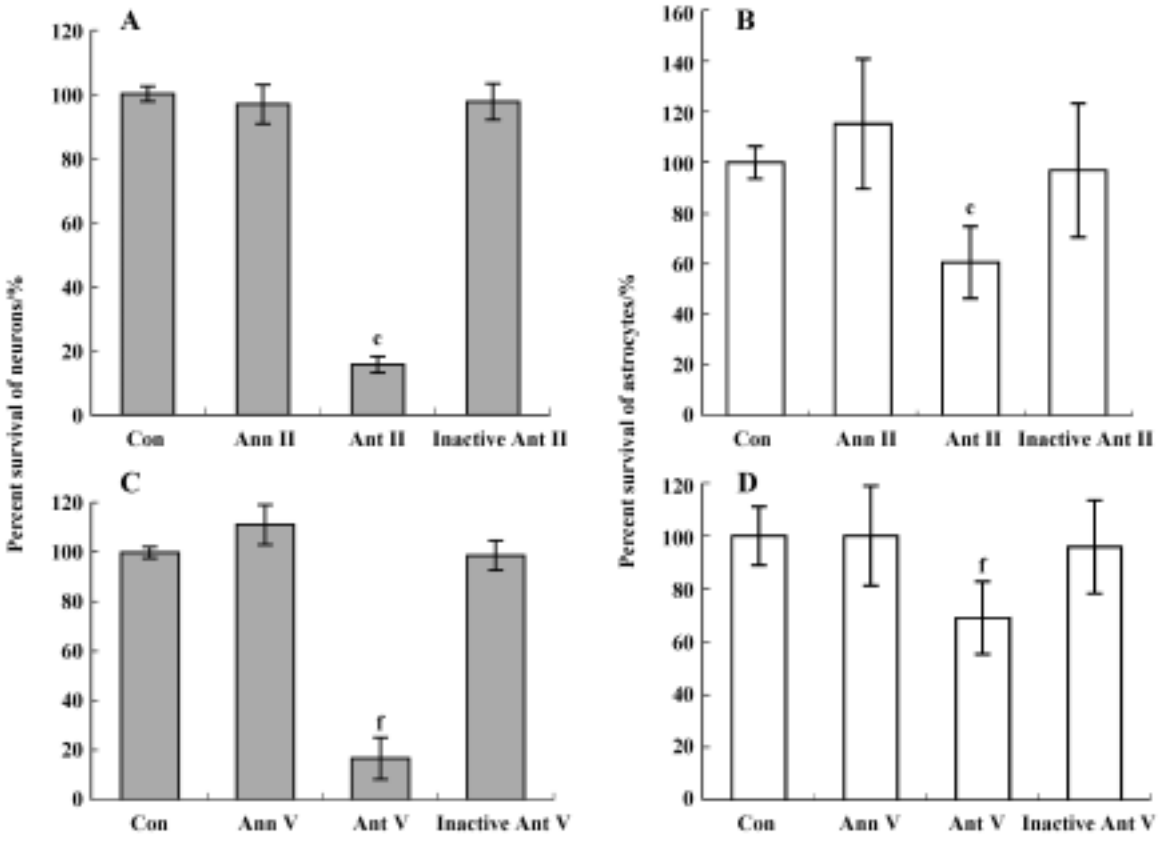


**Fig 5. Effects of annexin II (A,B) or annexin V (C, D) protein and antibody on the survival of neurons (A,C) and astrocytes (B, D). *n*=18. Mean±SD. $^{c}P<0.01$ *vs* control for Ann II. $^{f}P<0.01$ *vs* control for Ann V. Con: control; Ann II: annexin II; Ant II: annexin II antibody.**

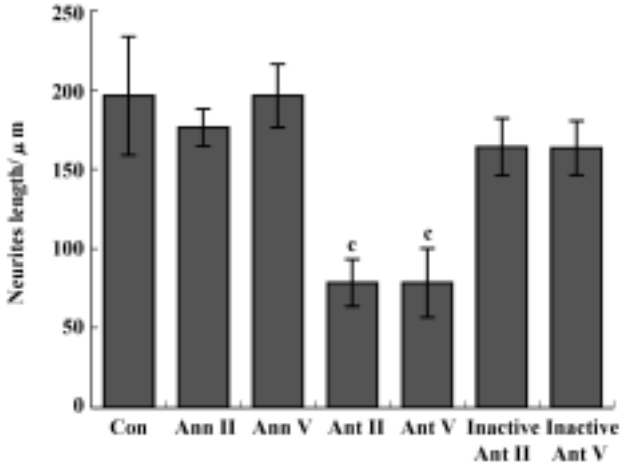


**Fig 6. Effects of annexin II and V proteins and antibodies on neurite lengths of cultured neurons. *n*=18. Mean±SD. $^{c}P<0.01$ *vs* control. Con: control; Ann II: annexin II; Ann V: annexin V; Ant II: annexin II antibody; Ant V: annexin V antibody.**

The expression peaked at 1 and 2 weeks after the injury respectively for annexins II and V[14].

The functional efficacy of increased expression of annexins following CNS injury is still unclear. Annexins may be involved in the process of cell death, alternatively, they may play a role in cell survival and regeneration. In the present study, we demonstrated that endogenous annexins were essential for neuronal survival. This is confirmed by the fact that blocking the endogenous annexins with specific-annexin antibodies resulted in a reduction of neuronal survival and neurite outgrowth. These results suggest that annexins have neurotrophic effects and that they are essential for the survival and neurite outgrowth of neurons at least at special stages of embryonic development. Whether annexins have a direct protective effect on neurons or an indirect effect through glial cells remains to be elucidated. Since annexins are reported to be secreted by glial cells[17], the presence of an indirect neuroprotective effect of annexins on neurons through annexin-producing glial cells is possible. The observation that heat-inactivated annexin antibodies did not have a blocking effect further confirms the neuroprotective

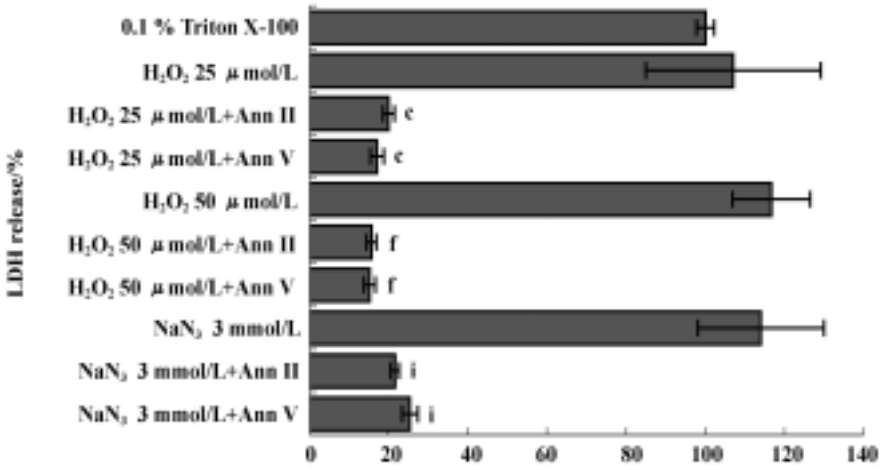


**Fig 7. Effects of annexin II and V proteins on LDH-release after peroxide or hypoxia insults. The mean value of LDH-release of the positive control group was considered as 100 %. *n*=18. Mean±SD. $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* $H_2O_2$ 50 μmol/L. $^{i}P<0.01$ *vs* $NaN_3$ 3 mmol/L.**

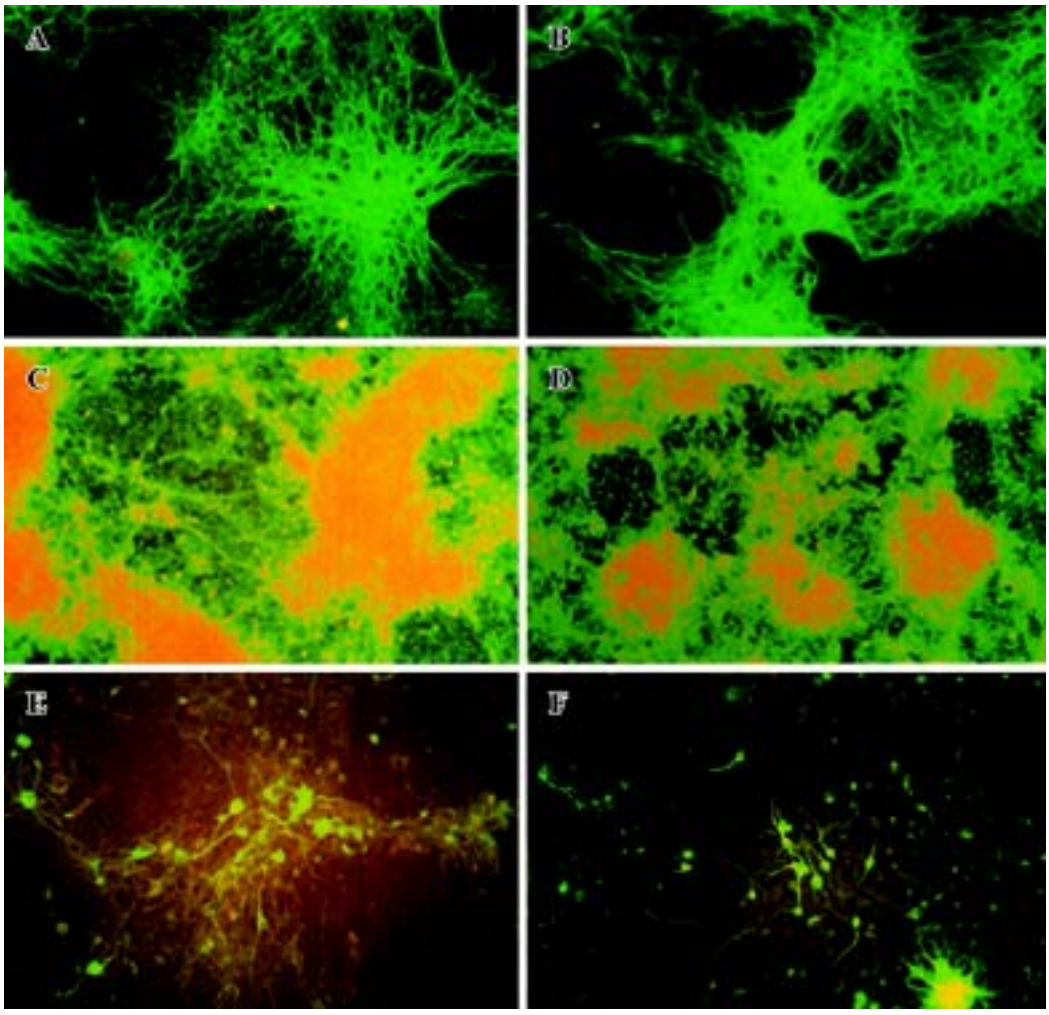


**Fig 8. Effects of annexin II proteins on neural survival and neurite outgrowth after peroxide or hypoxia injuries. (A, B) Control; (C) Sodium azide 3 mmol/L; (D) $H_2O_2$ 25 μmol/L; (E ) $H_2O_2$ +Annexins II treatment; (F) $NaN_3$+Annexin V treatment. ×240.**

function of annexins as well as their bioactivities.

Cytotoxic cell death is classically evaluated by quantification of plasma membrane damage. A standard method to measure the degree of cytotoxicity is to measure the level of LDH released from damaged cells[18]. Although the cultures prepared in the present study contained both neurons and glial cells, the use of serum-free neurobasal medium had selectively increased neuronal population. Moreover, the presence of glial cells in cultures provided an optimal milieu for the better growth of cortical neurons.

The peroxide and hypoxia cell injury was induced by $H_2O_2$ and sodium azide, respectively[19]. The present study measured cell cytotoxicity based on the release of LDH from damaged cells. A decrease in LDH release was found only in groups that received annexin treatments confirming that both annexins are neuroprotective. The β-tubulin III immunofluorescence labeling further confirmed the detrimental effects of the peroxide and hypoxia insults on neuronal survival and the rescuing effects of annexins. The notion that annexins are neuroprotective is further supported by the evidence that blocking endogenous annexins induces cell death.

We previously demonstrated that both annexin II and V mRNAs and proteins increased in the spinal cord after injury[13,14]. The injury-induced upregulation of annexins II and V may be a recapitulation of what happened during development. Since annexins provide guidance for growing neurites and promote neuronal survival during development, increases of annexins after injury may play a similar protective/growth role in the adult CNS.

It has been proposed that each member of the annexin family of proteins has its unique structure, property and function[3]. Annexin II may be involved in the regulation of $Ca^{2+}$-dependent exocytosis and cell-cell adhesion mechanism[5,17], while annexin V may have the ability to act as a voltage-gated cation-selective channel[5]. However, studies have also shown that many annexin members have overlapping functions such as anti-inflammatory and anticoagulant properties[3]. Our study shows that both annexins II and V have an effect on the survival of developing neurons and glial cells *in vitro* and protect them from hypoxia- and peroxide-induced injuries.

Although the present study demonstrated protective effects of annexins II and V on the survival and neurite outgrowth of normal and injured neurons *in vitro*, their effects on the protection and functional recovery in injured animal models *in vivo* remain to be investigated. In further studies, we will test whether annexins II and V have a neuroprotective effect in a spinal cord contusion injury and, if so, what the underlying mechanism is in order to develop new repair strategies for the treatment of CNS injuries including those of the spinal cord.

## REFERENCES


1 Modi WS, Seuanez HN, Jaye M, Haigler HJ, Kaplan R, O'Brien SJ. The human endonexin II (ENX2) gene is located at 4q28 — q32. Cytogenet Cell Genet 1989; 52: 167-9.

2 Tait JF, Frankenberry DA, Shiang R, Murray JC, Adler DA, Disteche CM. Chromosomal localization of the human gene for annexin V (placental anticoagulant protein I) to 4q26 — q28. Cytogenet Cell Genet 1991; 57: 187-92.

3 Raynal P, Pollard HB. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochim Biophys Acta 1994; 1197: 63-93.

4 Spreca A, Rambotti MG, Giambanco I, Pula G, Bianchi R, Ceccarelli P. Immunocytochemical localization of annexin V (CaBP33), a $Ca^{2+}$-dependent phospholipid and membrane-binding protein, in the rat nervous system and skeletal muscles and in the porcine heart. J Cell Physiol 1992; 152: 587-98.

5 Naciff JM, Kaetzel MA, Behbehani MM, Dedman JR. Differential expression of annexins I-VI in the rat dorsal root ganglia and spinal cord. J Comp Neurol 1996; 368: 356-70.

6 Elderfield AJ, Bolton C, Flower RJ. Lipocortins 1 (annexin I) immunoreactivity in the spinal cord of Lewis rats with acute experimental allergic encephalomyelitis. J Neurol Sci 1993; 119: 146-153.

7 Johnson MD, Kamso-Pratt JM, Whetsell WO Jr, Pepinsky RB. Lipocortin-1 immunoreactivity in the normal human central nervous system and lesions with astrocytosis. Am J Clin Pathol 1989; 92: 424-9.

8 Hamre KM, Chepenik KP, Goldowitz D. The annexins: specific markers of midline structures and sensory neurons in the developing murine central nervous system. J Comp Neurol 1995; 352: 421-35.

9 Adcock IM, Ito K. Molecular mechanisms of corticosteroid actions. Monaldi Arch Chest Dis 2000; 55: 256-66.

10 Barnes PJ. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci 1998; 94: 557-72.

11 Lundgren JD, Hirata F, Marom Z, Logun C, Steel L, Kaliner M. Dexamethasone inhibits respiratory glycoconjugate secretion from feline airways *in vitro* by the induction of lipocortion (lipomodulin) synthesis. Am Rev Respir Dis 1988;137: 353-7.

12 Morris HG. Mechanisms of glucocorticoid action in pulmonary disease. Chest. 1985; 88 (2 Suppl): 133S-141S.

13 Zhang KH, Xiao HS, Lu PH, Shi J, Li GD, Wang YT, *et al*. Study on the spinal cord injury-induced differential gene expression. Proceedings of Human Genome Meeting; 2002;

Shanghai, China. p 168.
14 Liu NK, Xu XM. Increased expression of annexins I and II following spinal cord injury in adult rats. Program No 393-13. 2002 Abstract Viewer/Itinerary Planner. Washington (DC): Society for Neuroscience; 2002. CD-ROM.
15 Novitskaya V, Grigorian M, Kriajevska M, Tarabykina S, Bronstein I, Berezin V. Oligomeric forms of the metastasis-realated Mts1 (S100A4) protein stimulate neuronal differentiation in cultures of rat hippocampal neurons. J Biol Chem 2000; 275: 41278-86.
16 Takei N, Ohsawa K, Imai Y, Nakao H, Iwasaki A, Kohsaka S. Neurotrophic effects of annexin V on cultured neurons from embryonic rat brain. Neurosci Lett 1994;171: 59-62.
17 Siever DA, Erickson HP. Extracellular annexin II. Int J Biochem Cell Biol 1997; 29: 1219-23.
18 Mukhin AG, Ivanova SA, Allen JW, Faden AI. Mechanical injury to neuronal/glial cultures in microplates: Role of NMDA receptors and pH in secondary neuronal cell death. J Neurosci Res 1998; 51: 748-58.
19 Regan RF, Guo Y. Extracellular reduced glutathione increases neuronal vulnerability to combined chemical hypoxia and glucose deprivation. Brain Res 1999; 817: 145-50.

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