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Cyclin-dependent kinase 5 influences Rohon-Beard neuron survival in zebrafish

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Abstract

Cyclin-dependent kinase 5 (cdk5), a member of the cyclindependent kinase family, is expressed predominantly in postmitotic cell populations. Unlike the other cdks, cdk5 is abundant and most active in differentiated neurons. Here, we describe the function of a cdk5 ortholog in zebrafish. Cdk5 catalytic activity is meager but present in early stages of development. However, at 24 h post-fertilization (hpf), the activity is remarkably higher and continues to be high through 48 and 72 hpf. Knocking down cdk5 by micro-injection of a specific siRNA resulted in decreased cdk5 protein level accompanied by reduced kinase activity. In the cdk5 siRNAinjected embryos, the number of primary sensory RohonBeard (RB) neurons was significantly reduced and there were more apoptotic cells in the brain. These phenotypes were rescued by co-injection of cdk5 mRNA. Within the first two days of development, RB neurons undergo apoptosis in zebrafish. To examine whether cdk5 has a role in RB neuron survival, cdk5 mRNA was injected into the oneto two-cell embryos. In these embryos, RB neuron apoptosis was inhibited compared with the uninjected control embryos. These results suggest that in zebrafish, cdk5 influences RB neuron survival and potentially regulates early neuronal development.

Keywords

apoptosis; cyclin-dependent kinase 5; RohonBeard neuron; siRNA; zebrafish

Cyclin-dependent kinase 5 (cdk5) was initially identified as a member of the cyclin-dependent kinase family (Meyerson *et al.* 1992). Although cdk5 is found in mitotic cells (Dhavan and Tsai 2001), it is not involved in the cell cycle. In contrast to other cdks, cdk5 activity is stimulated not by an associated cyclin but by neuron-specific activators, p35 and p39. The major activator, p35, is expressed predominantly in postmitotic neurons of the central nervous system, and correlates well with cdk5 kinase activity (Lew *et al.* 1994; Tsai *et al.* 1994; Zheng *et al.* 1998).

Cdk5 knockout mice exhibit defects in organization of the cortex and cerebellum and are embryonically lethal (Ohshima *et al.* 1996). Moreover, mice lacking p35 reveal cortical lamination defects (Chae *et al.* 1997). Overexpression of cdk5 and p35 promotes neurite

outgrowth of cultured cortical neurons, whereas a dominant-negative mutant of cdk5 inhibits neurite outgrowth (Nikolic *et al.* 1996). In *Xenopus*, cdk5 has two p35 activators and plays a role in eye, muscle and neuronal development (Gervasi and Szaro 1995; Philpott *et al.* 1997, 1999). Cdk5 is known to phosphorylate a large number of proteins, including the neurofilaments and tau (Pant *et al.* 1997; Sharma *et al.* 1999; Li *et al.* 2000; Grant *et al.* 2001; Bu *et al.* 2002; Ackerley *et al.* 2003; Shea *et al.* 2004).

Cdk5 kinase activity has been implicated in neuronal survival and death. Amyloid- β peptide and ischemic insults (Patrick *et al.* 1999; Wang *et al.* 2003) induce the conversion of p35 to its truncated form, p25, which causes apoptosis in neurons. In contrast, the association of p35 and cdk5 is required for neuronal survival. Cdk5-mediated kinase crosstalk regulates neuronal survival (Harada *et al.* 2001; Li *et al.* 2002; Wang *et al.* 2006).

During vertebrate nervous system development, apoptosis eliminates unnecessary cell types (Oppenheim 1991). One such cell population is Rohon-Beard spinal sensory neurons (RBs). These are the early born primary sensory neurons of the central nervous system. The reason for the death of RBs during development is unknown. In amphibians, RB neurons die gradually and their death is coincident with the development of dorsal root ganglion (DRG) (Hughes 1957). Zebrafish RBs are the equivalent of the amphibian RBs, having large cell bodies with huge nuclei and granular cytoplasm (Metcalf *et al.* 1990). As in amphibians, zebrafish RBs die within the first two days of development (Williams *et al.* 2000; Cole and Ross 2001).

The role of cdk5 in early primary neurogenesis is not known. Here, we investigate the role of cdk5 in RB primary neuron development in zebrafish. We have used injection of cdk5 siRNA, designed against the cdk5 homolog in zebrafish, to knockdown cdk5 expression in the embryos. In the siRNA-mediated cdk5 knockdown embryos, the number of RB neurons was reduced compared with the control siRNA injected embryos. These embryos also exhibited an enhanced level of apoptosis in the brain. Significantly, the defects could be rescued by co-injection of cdk5 mRNA. Furthermore, overexpression of cdk5 mRNA inhibits the normal progression of RB neuron death during development, suggesting that cdk5 functions as an anti-apoptotic molecule in zebrafish RB neurons. These results suggest that cdk5 plays a pivotal role in neuronal cell survival during zebrafish development.

Experimental procedures

Animals

Zebrafish (*Danio rerio*) were kept and raised essentially according to standard procedures (NIH facility). Fertilization was achieved by natural spawning, and embryos were raised at 28.5 C and staged according to Kimmel *et al.* (1990).

Materials

Mouse monoclonal anti-acetylated α -tubulin antibody was purchased from Sigma (St Louis, MO, USA). Rabbit polyclonal antibodies against mammalian cdk5 (C-8) and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Western blotting

Extracts were prepared from developmentally-staged embryos or embryos micro-injected with siRNAs. Total protein (50 µg) was separated by 10–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted to polyvinylidene difluoride (PVDF) membrane. Cdk5 protein was detected using an antibody (C-8) against mammalian cdk5. The immunoblots were developed for signal visualization by enhanced chemiluminescence (ECL) (Amersham, Chicago, IL, USA).

Cdk5 kinase activity assay

Wild-type zebrafish embryos were allowed to develop for 72 h postfertilization (hpf). Embryos were collected at various time points and were homogenized in 120 µL lysis buffer (10 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, protease and phosphatase inhibitors). Homogenates were then sonicated and centrifuged for 5 min at 14 000 *g*. Immunoprecipitations of extracts containing 200 µg of total protein were performed by adding the cdk5 antibody (C-8) and incubating overnight at 4 °C with constant rotation. Then, 30 µL of a 50% slurry of Protein A-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were added and incubated under the same conditions for an additional 4 h. Immunocomplexes were centrifuged for 2 min at 14 000 *g*, then washed twice in lysis buffer and twice in kinase assay buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerol phosphate, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol, 10 mM magnesium chloride). Kinase activity assays were performed as described earlier (Li *et al.* 2000).

SiRNA micro-injection

To explore cdk5 function during early development, we cloned the zebrafish cdk5 ortholog. Zebrafish cdk5 has a predicted 293 aminoacid residue open reading frame (ORF; GenBank no. AF203736). We designed siRNAs to specifically silence cdk5 expression. Control non-silencing and cdk5 siRNAs (silencing) were designed as follows. Control siRNA (non-silencing) sense and antisense sequences were: 5'-r(UUUUCCGAACGUGUCACGU)d(TT)-3' and 5'-r(ACGUGACACGUUCGGAGAA)d(TT)-3', respectively. Cdk5 siRNA (silencing) sense and antisense sequences were: 5'-r(AAGCCGUACCCGAUGUAUC)d(TT)-3' and 5'-r(GAUACAUCGGGUACGGCUU)d(TT)-3', respectively. The sense and antisense strands were annealed to create the double-stranded siRNA at a 20 µM concentration. Cdk5 siRNA is designed against the cDNA nucleotide sequence spanning 859 to 877. Control siRNA and cdk5 siRNA were dissolved in Danieau's buffer (Nasevicius and Ekker 2000) before injection. siRNAs were injected into each egg at the one to two-cell stage (2 nL of a 10 µM stock solution per embryo). Rescue experiments were performed by co-injecting the siRNA mixed with 200 pg of *in vitro*-transcribed mRNA, obtained from the zebrafish cdk5 cDNA cloned into the *HindIII/XbaI* sites of the PCS2 vector using the SP6 RNA polymerase (Ambion Inc., Austin, TX, USA). Overexpression of cdk5 mRNA was achieved by injecting 5 pg mRNA/embryo at the one to two-cell stage.

Immunohistochemistry

For whole-mount immunohistochemistry, embryos were fixed in 4% paraformaldehyde overnight, blocked in 5% sheep serum and 2 mg/ mL bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and incubated overnight (4 C) in the monoclonal antibody against acetylated α -tubulin (1 : 2000). Embryos were then incubated in horseradish peroxidase (HRP)-conjugated anti-mouse antibody at 4 C overnight. Color development for positive signals was performed using the Vectastatin ABC kit (Vector Laboratories, Burlingame, CA, USA).

Cell death assay

TUNEL (terminal transferase-mediated dUTP nick end labeling) was used to assay for apoptosis in the 24 hpf embryos. Embryos were fixed, as for *in situ* hybridization, and stored in methanol. After re-hydration, embryos were permeabilized by treatment with proteinase K (10 μ g/mL), re-fixed in buffered 4% paraformaldehyde and washed in PBST (PBS with 0.05% Tween 20). Embryos were first placed in terminal transferase buffer (Roche Molecular Biochemicals, Indianapolis, IN, USA), then incubated on ice for 1 h with terminal transferase and alkaline phosphatase-labeled ddUTP (Roche) followed by a 1 h incubation at 37 C. Embryos were extensively washed in PBST and the signal was detected with the ABC kit (Vector Laboratories).

Results

Cdk5 protein expression and kinase activity are developmentally regulated

Previously, we have cloned the zebrafish cdk5 ortholog (GenBank no. AF203736). To examine the developmental expression pattern of the cdk5 protein, we used an antibody against the carboxyl terminus of human cdk5 (C-8) for immunoblot analysis (Fig. 1a). A single band of the predicted molecular mass, 33 kDa, was detected, which approximates the size of zebrafish cdk5. The cdk5 protein expression was low but detectable during 1.5–12 h of development. The expression of cdk5 at these early stages of cleavage suggests the presence and translation of maternal cdk5 mRNA. The protein level was moderately higher at 24 h, increased significantly at 48 hpf and reached a robust level of expression at 72 hpf. A densitometric analysis of cdk5 protein level is presented in Fig. 1(b), representing one of three separate experiments. To determine whether the change in the cdk5 level correlated with its catalytic activity, we performed an *in vitro* kinase activity assay, after immunoprecipitating 200 μ g total protein extracted from various embryonic stages, using the anti-cdk5 antibody. Histone H1 was used as the substrate. Between 1.5 and 12 h of development cdk5 kinase activity was barely detectable, but it increased dramatically (almost 20-fold) in embryos at 24–72 hpf (Fig. 1c, upper panel). The histone H1 substrate level used for each reaction is shown after staining the gel with Coomassie blue (Fig. 1c, bottom panel). Quantitation of cdk5 activity in three separate experiments is graphically presented in Fig. 1(d). Whole-mount immunohistochemistry showed ubiquitous expression of cdk5 in the zebrafish embryos (data not shown).

Cdk5 siRNA knocks down cdk5 protein in zebrafish embryos

Expression of cdk5 in the early embryonic stages of zebrafish suggests an early developmental role. To explore this possibility, we carried out loss-of-function experiments by injecting siRNA, designed specifically against the zebrafish cdk5, into the one to two-cell embryos (3 nL of a 20 μ M stock solution per embryo). At 24 hpf there were no gross morphological defects in these embryos when compared with the control (data not shown). To determine whether cdk5 had been reduced in the cdk5 siRNA-injected embryos, total protein extracts were prepared from injected embryos at 24 hpf, and 20 μ g total proteins were used for immunoblot analysis using an antibody against cdk5. In the control siRNA-injected embryos, there was an approximately 33 kDa polypeptide corresponding to the size of cdk5 protein (Fig. 2, lane 1; upper panel). The results demonstrated a loss of the cdk5 protein in the cdk5 siRNA-injected embryos (Fig. 2, lane 2; upper panel). A duplicate immunoblot was probed with an antibody against actin (Fig. 2, lower panel), confirming an equal amount of total protein load in the two lanes.

SiRNA-mediated cdk5 knockdown embryos have fewer primary sensory spinal Rohon-Beard (RB) neurons

In order to determine the effect of siRNA-mediated cdk5 knockdown on neuronal development, we examined the RB neurons. RB neurons are the primary sensory neurons to develop along the neural axis and may be the first to respond to siRNA-mediated cdk5 knockdown. At 24 hpf, in the control siRNA-injected embryos, the RB neurons appeared to be distributed normally (Figs 3a and d). However, in the cdk5 siRNA-injected embryos, there was a drastic reduction in the number of RB neurons along the body axis (Figs 3b and e). In the rescue experiments in which the cdk5 siRNA was co-injected with cdk5 mRNA, the number of RB neurons was restored to the control level (Figs 3c and f), as observed in the control siRNA-injected embryos (Fig. 3a). These results suggest that cdk5 in zebrafish may have an early developmental role in neurogenesis.

Cdk5 knockdown increases apoptosis in the nervous system

In mammalian cells, cdk5 has been implicated in regulating cell survival (Neystat *et al.* 2001; Li *et al.* 2002; Zhu *et al.* 2002). Whole-mount immunohistochemical analyses demonstrated a ubiquitous expression of cdk5 (data not shown). We hypothesized that the reduction in the number of RB neurons could have resulted from the enhanced apoptosis of the cells in cdk5 siRNA-injected embryos, the number being restored to normal when cdk5 mRNA was co-injected. In order to test this possibility, we performed TUNEL assay in 24 hpf embryos to identify cells undergoing apoptosis. The results demonstrated a number of apoptotic cells in the brain and eyes of control siRNA-injected embryos (Fig. 4a; $n = 23$). However, in the brain and eyes of cdk5 siRNA-injected embryos, the number of apoptotic cells was much higher (Fig. 4b). The number of apoptotic cells shown in Fig. 4(b) is representative of 17 out of 25 embryos injected with the cdk5 siRNA. The number of apoptotic cells in the brain was significantly reduced in the embryos co-injected with cdk5 siRNA and cdk5 mRNA (Fig. 4c; 25 out of 25 injected), suggesting the specificity of the effect of cdk5 on apoptosis. The relative numbers of apoptotic cells in these three groups of embryos obtained from three separate experiments are presented graphically in Fig. 4(d).

Furthermore, in the control siRNA-injected embryos ($n = 25$), TUNEL assay demonstrated a number of apoptotic signals in the dorsal axial region wherein the RB neurons reside (Fig. 4e). In contrast, apoptotic signals were higher in the cdk5 siRNA-injected embryos (Fig. 4f; 18 out of 25 injected) compared with the control siRNA-injected embryos. In a rescue experiment, in which cdk5 siRNA and cdk5 mRNA were co-injected, 25 of the 25 injected embryos demonstrated barely detectable apoptotic signals in the spinal region harboring the RB neurons (Fig. 4g; $n = 25$), suggesting that cdk5 may prevent apoptosis in these embryos.

Cdk5 overexpression protects Rohon-Beard neurons from undergoing apoptosis during development

In zebrafish, the RB neurons are primary sensory neurons positioned in the dorsal neural tube (Grunwald *et al.* 1988; Kimmel *et al.* 1990; Metcalfe *et al.* 1990). Apoptotic depletion of RB neurons occurs through the second day of development (Williams *et al.* 2000). In the uninjected embryos (24 hpf), TUNEL assay demonstrated a number of apoptotic signals in the spinal region wherein RB neurons reside (Figs 5a and c). However, in embryos injected with cdk5 mRNA (5 pg/embryo), TUNEL-positive apoptotic signal was significantly reduced (Figs 5b and d). Immunostaining with acetylated α -tubulin revealed the presence of approximately similar numbers of RB neurons in both uninjected and cdk5 mRNA-injected embryos (Figs 5e and f). The numbers of TUNEL-positive RB neurons in the uninjected control and cdk5 mRNA-injected embryos, derived from three separate experiments, are presented statistically in Fig. 5(g). These results suggest that RB neuron death during the progression of normal development is prevented by ectopic expression of cdk5, otherwise indicating that cdk5 has an anti-apoptotic role in the RB neurons. To determine whether cdk5 activity changed in the cdk5 mRNA-injected embryos, a cdk5 activity assay was performed using immunoprecipitated cdk5 from the embryos to phosphorylate histone H1. Embryos injected with cdk5 mRNA showed slightly up-regulated cdk5 activity compared with control uninjected embryos (Fig. 5h). Densitometric analysis of phosphorylated histone H1 from three separate experiments showed a significant increase in cdk5 activity in the cdk5 mRNA-injected embryos compared with the uninjected embryos (Fig. 5i). The observed up-regulation of activity could have resulted from the presence of multiple cdk5 activators, such as p35 and p39 in zebrafish, that exist as unbound subunits of the holoenzyme. Nonetheless, these results suggest that the up-regulated cdk5 activity may have contributed toward cell survival.

Discussion

In this study, we have used the zebrafish cdk5 ortholog (GenBank no. AF203736) to study the role of cdk5 in apoptosis of the nervous system. Compared with other vertebrates, particularly the closely related *Xenopus* (Szaro *et al.* 1989), cdk5 protein is expressed very early during cleavage, before the mid blastula transition. Moreover, cdk5 kinase activity, although low, was measurable during the first 12 h of development. The presence of cdk5 in zebrafish embryos much earlier than muscle or neuronal differentiation implies a function devoted to early developmental processes, apart from a possible role in neurogenesis and its related events. These results are in contrast with the expression pattern of cdk5 in *Xenopus*, which first appears after the mid blastula transition (Gervasi and Szaro 1995).

As an approach to investigating the role of cdk5 in development, we injected siRNA into the one-to-two-cell stage to knockdown cdk5 expression. Immunodetection techniques confirmed that cdk5 siRNA was functionally effective in knocking down the cdk5 protein at least by 24 hpf. Nevertheless, in spite of the reduction in cdk5 no delays in development, or gross morphological abnormalities, were observed. It is possible, however, that the low level of maternal cdk5 protein expression may have been sufficient to sustain these developmental events. As cdk5 has been reported to affect neurite outgrowth and axonogenesis in both mammalian and *Drosophila* development (Tsai *et al.* 1993; Nikolic *et al.* 1996; Ohshima *et al.* 1996; Connell-Crowley *et al.* 2000; Smith 2003), we explored the possibility that a more subtle phenotypic effect could be demonstrated in cdk5 siRNA-injected embryos. Accordingly, we studied the effect of cdk5 on the formation of primary sensory RB neurons and apoptosis in 24 hpf embryos. In all cases, we could detect unequivocal defects in RB neuron number. Furthermore, the specificity of the effects could be shown by restoring the normal phenotype after co-injection of cdk5 mRNA with cdk5 siRNA.

The difference in number of RB neurons in the cdk5 siRNA-injected embryos suggests either an effect on early neuronal differentiation or increased apoptosis, or both. During vertebrate nervous system development, as many as half of the developing neurons die (Clarke *et al.* 1984; Cowan *et al.* 1984; Oppenheim 1991). Moreover, an antiapoptotic role for cdk5 has been reported in mammalian systems (Neystat *et al.* 2001; Li *et al.* 2002; Zhu *et al.* 2002), although its precise role in apoptosis is still uncertain. On the other hand, cdk5 has been implicated in the induction of apoptosis (Henchcliffe and Burke 1997; Shirvan *et al.* 1998; Morris *et al.* 2001; Neystat *et al.* 2001; Zhang *et al.* 2004; Zheng *et al.* 2005), while others have reported a reduction in cdk5/p35 in apoptotic cells (Kerokoski *et al.* 2001). Inhibition of de-regulated cdk5 activity has been shown to promote cell survival of neurons challenged by β -amyloid peptide (Alvarez *et al.* 1999; Lee *et al.* 2000). However, cdk5-deficient cultured cortical neurons exhibit increased sensitivity to apoptotic stimuli (Li *et al.* 2002). In the present study, we show that zebrafish embryos injected with cdk5 siRNA show enhanced apoptosis in the brain, eye, and RB neurons along the body axis.

Zebrafish RBs die within the first two days of development (Williams *et al.* 2000; Cole and Ross 2001). The DNA of RBs is fragmented before neuronal degeneration (Reyes *et al.* 2004) and this can be detected using the TUNEL assay. In our studies, in the 24 hpf embryos, TUNEL-positive cells in an otherwise normal-looking RB population were consistent with this report. It is also of interest to note that a drastic reduction in TUNEL-positive RB neurons in the cdk5 mRNA-injected embryos occurred coincidentally with an up-regulation in cdk5 catalytic activity. These results further support the observation that cdk5 may have a role in RB neuron survival. A tight regulation of cdk5 expression during RB neuron development, and their (RBs) disappearance in two days, must occur for the normal development of zebrafish embryos. In this context, other signaling cascades, along with cdk5, could have a major role in the proper development of the spinal sensory nervous system.

In conclusion, we show that a significant reduction of cdk5 protein in zebrafish embryos can lead to disrupted primary sensory RB neuron formation, partly due to increased apoptosis in the absence of overt phenotypic abnormalities. We believe that, with the proper application

of cdk5 knockdown experiments (siRNA or morpholinos), the zebrafish system lends itself to an analysis of the role of cdk5 in the initial stages of neurogenesis.

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Abbreviations

cdk5	cyclin-dependent kinase 5
hpf	hours postfertilization
RB	Rohon-Beard
TUNEL	terminal transferase-mediated dUTP nick end labeling

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Fig. 1. Cdk5 protein and kinase activity levels vary during early development in zebrafish. (a) Immunoblot analysis using an antibody against human cdk5 shows the presence of cdk5 protein in various stages of development. (b) A densitometric analysis of the intensity of the signals in the immunoblot in (a) is plotted to demonstrate the developmental expression of the protein in different stages. (c) Kinase activity was measured in cdk5 immunoprecipitates of embryos at various developmental stages as indicated. Histone H1 was used as a substrate. The autoradiograph shows the phosphorylated histone H1 (upper panel). The amount of histone H1 substrate used for each reaction is presented in the bottom panel after staining the gel with Coomassie blue. (d) Densitometric analysis of the level of phosphorylated histone H1, derived from three separate experiments, is presented to demonstrate the variation of cdk5 activity at different stages of development.

Fig. 2.

Cdk5 siRNA injection strongly inhibits cdk5 protein translation in the embryos.

Immunoblots were obtained from extracts (100 µg total protein/lane) prepared from 24 h embryos after injecting the siRNAs. Immunoblots were probed with anti-cdk5 and anti-actin antibodies. Cdk5 protein levels in control siRNA-injected and cdk5 siRNA injected embryos are shown in the upper panel. The lower panel shows the immunoblot probed with an antibody against β -actin.

Fig. 3. Effect of *cdk5* on the development of Rohon-Beard (RB) neurons in zebrafish. Acetylated α -tubulin antibody was used to immunostain the 24 hpf embryos. Control siRNA, *cdk5* siRNA and *cdk5* siRNA plus *cdk5* mRNA (200 pg) were injected into the embryos at the one to two-cell stage following fertilization. Lateral views show the distribution of RB neurons across the spinal cord in the 24 hpf embryos injected with the control siRNA (a), *cdk5* siRNA (b) and *cdk5* siRNA with *cdk5* mRNA (c). YS indicates yolk sac and Y indicates yolk extension. Arrowheads indicate RB neurons. Scale bar = 40 μ m. Higher magnifications of (a) (b) and (c) are shown in (d) (e) and (f), respectively. Scale bar = 20 μ m. Arrowheads indicate the RB neurons.

Fig. 4.

Cdk5 knockdown enhances apoptosis in the nervous system. Control siRNA, cdk5 siRNA, and cdk5 siRNA with cdk5 mRNA (200 pg/ embryo), were injected into oneto two-cell stage embryos following fertilization. Apoptotic cells in the brain were visualized by TUNEL assay. Dorsal view of flat-mounted 24 hpf whole embryos (brain region) injected with control siRNA (a), cdk5 siRNA (b) and cdk5 siRNA plus cdk5 mRNA (c). Arrowheads indicate some of the dying cells in the brain and eyes. The numbers of apoptotic cells in the respective areas in the brain, as shown in the figures, are statistically presented (d). Dorsal view shows the apoptotic cells in the axial (spinal) region injected with control siRNA (e), cdk5 siRNA (f) and cdk5 siRNA plus cdk5 mRNA (g). Arrowheads indicate the apoptotic RB neurons.

Fig. 5. Cdk5 overexpression has an anti-apoptotic effect on the Rohon-Beard (RB) neurons of the spinal nervous system. Lateral views of the un.injected (a) and cdk5 mRNA-injected (5 pg/embryo) (b) embryos at 24 hpf show TUNEL-positive cells indicating apoptotic RB neurons. Scale bar = 60 μ m. Higher magnifications of the demarcated regions in (a) and (b) are presented in (c) and (d). Scale bar = 25 μ m. The whole embryos processed for TUNEL assays are focused for microscopy to visualize the RB neurons. Arrowheads indicate TUNELpositive RB neurons. Lateral views of the 24 hpf embryos immunostained with an antibody to acetylated α -tubulin reveal the RB neurons in the un.injected (e) and cdk5 mRNA-injected (f) embryos. Scale bar = 20 μ m. Arrowheads indicate the RB neurons. The relative numbers of apoptotic RB neurons in a specific area of the un.injected and cdk5 mRNA-injected embryos are graphically presented (g). Arrowheads indicate the RB neurons; Y indicates yolk extension. Uninjected and cdk5 mRNA-injected embryos at 24 hpf were analyzed to determine the cdk5 activity (h). A representative autoradiograph of three separate experiments shows phosphorylated histone H1 (upper panel) and Coomassie blue-stained histone H1 substrate (lower panel). Densitometric analysis of the level of phosphorylated histone H1 is presented to demonstrate the variation of cdk5 activity between the uninjected and cdk5 mRNA-injected embryos (i).