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Differential Regulation of Smac/DIABLO and Hsp-70 during Brain Maturation

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Abstract

The heat shock protein (Hsp) system is a cell defense mechanism constitutively expressed at the basal state and essential for cell survival in response to damaging stimuli. Apoptosis is a physiological cell death program that preserves tissue homeostasis. We investigated the intrinsic pathway of apoptosis at various stages of brain maturation in CD-1 mice, triggered by two mitochondrial proapoptotic proteins, cytochrome *c* and Smac/DIABLO, and the pathway's regulation by Hsp-70. Smac/DIABLO and Hsp-70 proteins were upregulated 2-fold and 1.5–3-fold, respectively, after birth. In contrast, in the presence of cytochrome *c*/2'-deoxyadenosine 5'-triphosphate (dATP), caspase activity in mouse brain cell-free extracts increased 90-fold and 61-fold, at fetal and neonatal stages, whereas no activation was detected 15 days postnatally or at any subsequent times. These results indicate that the activation pattern of the intrinsic pathway of apoptosis undergoes a marked shift during postnatal maturation.

Keywords

Aging; Apoptosis; Brain maturation; Caspase activation; Cytochrome *c*-mediated apoptosis; Development; Hsp-70 regulation; Intrinsic pathway; Neuronal cell death; Smac/DIABLO regulation

Introduction

During the development of the nervous system, a large number of neurons are eliminated through naturally occurring neuronal death, and many morphological and biochemical properties of such dying neurons resemble apoptosis (Kuan et al. 2000). Apoptosis is tightly regulated through multiple independent signaling pathways that are initiated either from

triggering events within the cell—the ‘intrinsic pathway’—or at the cell surface—the ‘extrinsic pathway’ (Jin and El-Deiry 2005). However, other organelles have been shown to be involved, as well (Ferri and Kroemer 2001).

Mitochondria are considered to be key organelles of apoptotic signaling pathways, mediating apoptosis through the release of apoptogenic proteins (van Loo et al. 2002). However, based on the results of knock-out studies in mice, not all released mitochondrial factors play apoptogenic roles (Ekert and Vaux 2005). Proapoptotic proteins may be released by pore formation or outer membrane permeabilization (van Loo et al. 2002). Cytochrome *c* (cyt *c*), one of the best characterized proteins, plays a major role in apoptosis as a cellular activator of caspase-3 (Liu et al. 1996). In the presence of adenosine 5'-triphosphate (ATP) or 2'-deoxyadenosine 5'-triphosphate (dATP), cyt *c* and the Apoptosis protease activating factor-1 (Apaf-1) (Li et al. 1997) assemble into a very large caspase-activating complex containing multiple copies of Apaf-1 and caspase-9 molecules, named the ‘apoptosome’ (Zou et al. 1999; Saleh et al. 1999), that activates procaspase-9. Caspase-9 then activates effector caspases such as caspase-3 and caspase-7, and the cell undergoes apoptosis (Li et al. 1997).

Smac/DIABLO (the second mitochondrial activator of caspases) (Du et al. 2000; Verhagen et al. 2000) is the second mitochondrial protein (cyt *c* being the first) that activates apoptosis. Smac/DIABLO functions at the levels of both the Apaf-1-caspase-9 apoptosome and effector caspases (Srinivasula et al. 2000). Its N-terminus was shown to be essential for its interaction with the baculo-virus IAP repeat (BIR3) of X-linked IAP (XIAP) and to promote cyt *c*-dependent caspase activation (Srinivasula et al. 2000). However, the N-terminus is less critical for Smac/DIABLO's ability to interact with BIR1/BIR2 of XIAP and promote the activity of the effector caspases (Srinivasula et al. 2000). It has been proposed that Smac/DIABLO potentiates caspase-9 activity by disrupting the interaction of the linker peptide of caspase-9 with the BIR3 domain (Srinivasula et al. 2001). A recent report suggested that Smac/DIABLO potentiates apoptosis by simultaneously antagonizing caspase-IAP interactions and repressing IAP ubiquitin ligase activities (Creagh et al. 2004). Binding to the BIR3 domain by two conserved peptides, one from Smac/DIABLO and the other one from caspase-9, has opposing effects on caspase activity and apoptosis (Srinivasula et al. 2001; Shiozaki and Shi 2004).

It has been suggested that Smac/DIABLO and cyt *c* pathways may act in a concerted fashion (Srinivasula et al. 2000); however, the link between these pathways during brain maturation is still undefined. Moreover, oxidative stress levels increase with aging (Shigenaga et al. 1994) and heat-shock or stress proteins, such as Hsp-70, are activated following damaging stimuli in order to maintain cellular function and survival (Beere 2004). Although most oxidative insults can be overcome by the cell's natural defenses, sustained perturbation of this balance may result in cell death (Beere 2004; Skulachev 2001). Since the regulation of some proteins during brain maturation is still unclear, we monitored by Western blotting the protein expression of Smac/DIABLO and Hsp-70, and correlated these results with caspase activation at the same developmental stages.

During the course of brain maturation, multiple effects seem to abrogate the response to cyt *c*/dATP seen in the fetus and neonate. Besides downregulation of Apaf-1, and/or procaspases-9 and -3, we show that Hsp-70 upregulation may prevent apoptotic signal transduction. In addition, the upregulation noted for Smac/DIABLO during brain maturation may not be a critical mechanistic factor in the loss of caspase activation.

Materials and Methods

Materials

All chemicals were of analytical grade and were purchased from Sigma-Aldrich, unless otherwise stated.

Animals

Healthy mice CD-1 strain was from Charles River Laboratories and paired at the Vivarium of the Buck Institute for Age Research (Novato, CA). Experimental animals were handled according to the Guide for the Care and Use of Laboratory Animals from the Institute of Laboratory Animal Research. All procedures fulfill the Animal Welfare Act (AWA), thus in agreement with the Public Health Service (PHS) Policy on Human Care and Use of Laboratory Animals as well as the Institutional Animal Care and Use Committee (IACUC) policy. The ages of the animals used were fetal (14–16 days) and postnatal (0–1 day, 15–30 days, 3–4 and 8–12 months), respectively.

Preparation of Brain Cell-free Extracts

The extracts were prepared from mice at five stages of maturation as previously described (Stoka et al. 2006).

Protein Concentration Determination

Protein amount was determined using a Coomassie Plus Protein Assay Reagent Kit (Pierce #23236) as recommended by the suppliers.

Kinetics of Activation

Caspase activation was assessed as previously described (Stoka et al. 2005). Briefly, 50 μ g of cell-free extracts of mouse brain at different stages of maturation were activated with 10 μ M horse heart cyt *c* (Sigma, #C7752) and 1 mM dATP (Invitrogen, #10216-018) at 37°C for 60 min. Control samples in the absence of cyt *c* and dATP were run under the same conditions. About 20 μ l samples were loaded in a 96-well microplate and the reaction started by addition of 80 μ l of substrate Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) (Ana-spec, #25273) dissolved in caspase buffer (100 μ M final concentration). Caspase buffer consisted of 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 1 mM EDTA, 100 mM sodium chloride, 10% sucrose (w/v), 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (w/v), and 10 mM dithiothreitol. The residual activity was continuously monitored for 30 min at 37°C in a thermostated Molecular Devices SpectraMax Gemini spectrofluorimeter, at excitation and emission wavelengths of 405 and 510 nm, respectively. The steady-state rates of substrate hydrolysis were obtained from the linear parts of the curves. The results were expressed in μ M/min as standardized using a calibration curve based on known concentrations of the 7-amino-4-(trifluoromethyl)coumarin (AFC) fluorogen (Anaspec, #23480).

Western Blotting and Image Analysis

The NuPAGE Bis-Tris system, which includes electrophoresis and electroblotting apparatus, gels, running buffers, sample preparation buffers, transfer buffers and molecular weight protein standards, Mark 12 and Multi-mark colored standard, were purchased from Invitrogen.

Fifty micrograms of S16 extracts of tissue or HEK 293T cells overexpressing Apaf-1, caspase-9 or caspase-3 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). About 4–12% Bis-Tris pre-casted gels were run under reducing conditions using MOPS running buffer at 200 V constant over 50 min and transferred to a PVDF

membrane (Schleider and Schuell) at 30 V constant for 60 min. Equal protein loading was confirmed by reversible stain of the membrane with Ponceau S stain solution (Sigma, #P7170). For Western blotting, all incubations were carried out on a rocking platform. The membrane was blocked with 5% skim milk in TBS buffer containing 0.2% Tween (TBST) for 60 min at room temperature and gentle shaking. Three washing steps of 10 min each were carried out after blocking and incubations with antibodies. All antibodies were diluted in blocking buffer and used at the appropriate concentration determined by titration. Each membrane was incubated overnight at 4°C with the appropriate primary antibody Smac/DIABLO (QED Biosciences, #1409) and Hsp-70 (Stressgen Bioreagents, #SPA-812). The polyclonal antibodies were raised in rabbit against epitopes of human molecules. Both polyclonal antibodies cross-reacted with mouse tissue. The positive controls were recombinant human Smac/DIABLO (R&D systems, #789-SM) and recombinant human Hsp-70 (Stressgen Bioreagents, #NSP-555), respectively. Each membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology) for 30 min at room temperature and the protein/antibody complexes were detected using enhanced chemiluminescence reagents (ECL) (Amersham Biosciences). The membranes were exposed to an X-ray film (Kodak) which was developed on a Hope film developer system.

Image analysis was carried out using the public domain NIH Image J software developed at the U.S. National Institutes of Health and available at <http://rsb.info.nih.gov/ni-image/>. Protein bands intensities were expressed in relative units. The data were reported as mean $\pm$ SD from three independent measurements of a representative experiment.

The statistical analysis of the data was carried out using the one-way analysis of variance (ANOVA) and a *P*-value below 0.05 was considered statistically significant.

Results

Smac/DIABLO Protein Level during Brain Maturation

Smac/DIABLO is a mitochondrial protein that upon stimulation of the intrinsic pathway is released together with cytochrome *c* and promotes apoptosis by binding to and antagonizing IAPs (Du et al. 2000; Verhagen et al. 2000). The expression levels of Smac/DIABLO protein were monitored with an antibody raised against the epitope corresponding to amino acids 225–239 mapping within the C-terminus of human Smac/DIABLO. No significant changes in Smac/DIABLO expression levels were seen between embryonic and neonatal stages (Fig. 1A, B; lanes 1–2). However, after birth, the protein was found almost 2-fold upregulated, a value that remained constant through the subsequent maturation stages (Fig. 1A, B; lanes 3–5).

Activation of the Intrinsic Pathway of Apoptosis during Brain Maturation

Previous studies have shown that mitochondria-targeted stimuli induce activation of the initiator procaspase-9, and that the signal amplification of this cascade proceeds through the activation of effector caspases (Li et al. 1997). In a cell-free system, the apoptotic pathway was induced by addition of 10 μ M cytochrome *c*/1 mM dATP, and caspase activation was monitored kinetically following DEVDase activities (Li et al. 1997). In mouse brain extracts, the cytochrome *c*/dATP pathway of apoptosis was activated 90-fold at the fetal stage and 61-fold in the neonatal stage compared to control samples. In contrast, no activation was measured 15 days after birth or at later stages (Fig. 2). These results confirm that the intrinsic pathway of apoptosis is tightly controlled, with a major regulation step (shut-off of the pathway's direct activation by cytochrome *c*/dATP) within the first 15 days postnatally.

Hsp-70 Protein Level during Brain Maturation

HSPs are part of the cellular machinery that protects the cells from the deleterious effects of induced heat shock, oxidative stress, or other insults that result in protein misfolding. Many HSP-70s act as molecular chaperones (Kiang and Tsokos 1998), and Hsp-70 was reported to be a negative regulator of apoptosis by a mechanism that prevents the recruitment of procaspase-9 to the apoptosome (Beere et al. 2000; Saleh et al. 2000). Here, the expression levels of Hsp-70 were monitored with an antibody raised against the inducible form of human Hsp-70 that cross-reacted with mouse Hsp-70. During brain maturation, Hsp-70 was found to be upregulated 1.5–3-fold after birth (Fig. 3A, B; lanes 3–5). Specifically at these stages of Hsp-70 upregulation, no caspase activation was measured (Fig. 2), whereas prior to Hsp-70 upregulation, caspase activation occurred with cyt *c* and dATP. These findings suggest that Hsp-70 may play an important role as a regulator of the intrinsic pathway of apoptosis—specifically, after birth, by preventing further caspase cascade signal amplification due to interference with caspase-9 activation. However, these results are correlative, do not prove that Hsp-70 plays a role in this apoptotic shift, and do not exclude the possibility that other factors may also play a role in the described shift of the activation profile of the intrinsic pathway of apoptosis.

Discussion

In the last few years, several studies have contributed to clarifying the mechanism of action of the proapoptotic molecule Smac/DIABLO at the functional (Srinivasula et al. 2000, 2001; Creagh et al. 2004) and structural (Shiozaki and Shi 2004) levels, respectively. However, the regulation of this protein during the course of maturation remains undefined.

The results presented by Western blotting confirmed that Smac/DIABLO was upregulated ~2-fold after birth (Fig. 1A, B; lanes 3–5), thus favoring apoptosis. Similarly, Smac/DIABLO expression was found to be increased in the cytosol in experimental models of Alzheimer's disease (Yin et al. 2002; Yao et al. 2005), stroke (Hu et al. 2004) and transient focal (Shibata et al. 2002; Saito et al. 2003, 2004; Siegelin et al. 2005a, b) and global (Sugawara et al. 2002; Tanaka et al. 2004) ischemia. On the other hand, Smac/DIABLO expression levels were unaffected in lysates of rat brains subjected to moderate traumatic brain injury, when compared to their controls (Lotocki et al. 2003).

Endogenously released Smac, however, has been shown to be insufficient to trigger apoptosis (Hunter et al. 2003; Bartling et al. 2004), and the requirement for both Smac/DIABLO and cyt *c* to induce apoptosis has been confirmed by microinjection in sympathetic neurons (Deshmukh et al. 2002) and prostate cancer cells (Carson et al. 2002).

The unexpected upregulation of Smac/DIABLO protein during the course of maturation might favor apoptosis. Therefore, we evaluated whether cyt *c* would exert the same effect on the system at various ages. To do this, we evaluated the cyt *c*/dATP caspase cascade activation in a cell-free system (Li et al. 1997) of CD-1 mouse brain at various developmental stages (Fig. 2). A significant finding in the presence of cyt *c*/dATP is that mouse brain extracts were highly activated at the fetal stage and, to a slightly lesser extent, at the neonatal stage. In contrast, no DEV-Dase activation occurred at 15 days after birth, or at any subsequent time (Fig. 2). This finding is compatible with previous work showing that, in rat brain, a similar loss of activation occurred at approximately two weeks postnatally (Ota et al. 2002).

Since Smac/DIABLO and cyt *c* are both proapoptotic proteins, a high apoptotic activity during brain development might be expected in order to ensure the appropriate synaptic connections (Kuan et al. 2000). However, it is less clear why Smac/DIABLO upregulation (Fig. 1A; lanes 3–5) occurs after birth, in a setting in which DEV-Dase activation by cyt *c*/dATP is abolished

(Fig. 2). Unlike Smac/DIABLO, cyt *c* is downregulated during brain aging (Takai et al. 1995).

The upregulation noted for Smac/DIABLO with development and maturation may not be a critical mechanistic factor in the loss of caspase activation. In addition to Smac/DIABLO's effect of preventing the inhibitory function of IAP (Du et al. 2000; Verhagen et al. 2000), we found, based on mixing experiments, that during brain maturation, cytosolic caspase inhibitors do not play an active role in the loss of caspase activation by cyt *c*/dATP (Stoka et al. 2005). Omi/HtrA2 is another mitochondrial protein released into the cytosol during apoptosis that binds and inhibits IAPs. This protein has a trypsin-like domain and induces caspase-independent cell death (Verhagen et al. 2002). Additional caspase-independent death effectors include apoptosis-inducing factor (AIF) and endonuclease G (Arnoult et al. 2003). Interestingly, in a model of cerebral hypoxia-ischemia, despite the constant levels of AIF (although with greater nuclear translocation), caspase-dependent and caspase-independent mechanisms were activated to a much greater extent in the immature brains compared to the juvenile and adult stages (Zhu et al. 2005).

Countering the proapoptotic effects of cyt *c* and Smac/DIABLO, heat shock proteins (Hsp) have been shown to be involved in the intrinsic pathway of apoptosis as negative regulators. Specifically, the activation of effector caspases was prevented by interaction of Hsp-27 with procaspase-9 (Garrido et al. 1999; Pandey et al. 2000), whereas Hsp-70 prevented the recruitment of procaspase-9 to the apoptosome (Beere et al. 2000; Saleh et al. 2000). A 1.5–3-fold upregulation of Hsp-70 during brain maturation may therefore be an additional regulatory feature affecting the intrinsic pathway of apoptosis (Fig. 3). Interference with apoptosome formation may potentially be of major importance because it would prevent caspase-9 activation and further signaling cascade amplification. Basal synthesis of Hsp-70 mRNA was found to be upregulated in young and old rat kidneys compared with their adult controls (Maiello et al. 1998). In rat alveolar macrophages, the effect of aging on Hsp-70 expression varied depending on the physiological status of the cell (Moore et al. 1998).

However, other studies have demonstrated a decrease of Hsp-70 with age (Gutsmann-Conrad et al. 1998, 1999; McArdle et al. 2004; Tandara et al. 2006). Muscles of young mice adapt rapidly following exercise by an increase in the production of Hsps, whereas muscles of old mice show a severely diminished response; therefore, a diminished production of Hsp-70 in muscles of old mammals may have a major effect on age-related functional deficits (McArdle et al. 2004). A recent study of human dermal fibroblasts showed a decreased resistance to ischemic and oxidative stress due to a decrease in Hsp-70 expression (Tandara et al. 2006).

The decline in Hsp-70 expression during cellular senescence in vitro and in cells derived from old human subjects was correlated with a decrease in the levels of heat shock transcription factor HSF1 (Gutsmann-Conrad et al. 1998). A second mechanism may also be at work: besides the effect of HSF1 to mediate the heat-induced transcription of Hsp-70, splenocytes from old female rats displayed lower levels of heat shock element binding than younger rats despite similar levels of HSF1 protein itself (Gutsmann-Conrad et al. 1999).

Earlier studies suggested that there is a link between Smac/DIABLO and cyt *c* pathways and that these may act in a concerted fashion (Srinivasula et al. 2000). However, the analysis of the current data does not fit into a single model. Thus, depending on the system and/or triggering stimuli, various models have been suggested: (a) cyt *c* and Smac/DIABLO are released from mitochondria in a more or less synchronized event (Rehm et al. 2003; Zhou et al. 2005), (b) cyt *c* and Smac/DIABLO are differentially regulated (Kandasamy et al. 2003), (c) Smac/DIABLO release is a caspase-catalyzed event that occurs downstream of cyt *c* release (Okada et al. 2002), (d) Smac/DIABLO may mediate a pathway of apoptosis that is cyt *c*/Apaf-1/

caspase-9 pathway independent (Chauhan et al. 2001; Dirsch et al. 2003), (e) in *cyt c* deficient cells (*cyt c*^{-/-}) Smac/DIABLO was not released from mitochondria (Hansen et al. 2006), and (f) a Smac/DIABLO-independent pathway was demonstrated in Smac-deficient mouse and its derivative cells (Smac^{-/-}) (Okada et al. 2002).

If Smac/DIABLO is released from mitochondria prior to or concomitantly with *cyt c*, it should block IAP function, thus further enhancing caspase-9 activation and downstream signaling (Srinivasula et al. 2000; Zhou et al. 2005). On the other hand, if *cyt c* is released from mitochondria prior to the release of Smac/DIABLO, this would promote apoptosome formation and caspase activation (Adrain et al. 2001). It is evident that there is a delicate balance between all of these factors, and depending on the magnitudes of their effects, the overall result may vary during the course of brain maturation. In our system, a major change occurred within the first 15 days after birth, as revealed by changes in caspase activation (Fig. 2) and the expression pattern of the proteins tested (Fig. 1, Fig 3). In addition, during brain development and maturation of CD-1 mice there is downregulation of the protein expression levels of Apaf-1 and caspase-3 and also some degradation of caspase-9 (Table 1). Our studies indicated that a decrease in the expression levels of key components of the pathways were responsible for the lack of caspase activation measured during brain maturation of CD-1 mice, which could indeed have translated into an overall reduction in apoptosome formation. In our studies the components of the pathway were found to be functional, and they responded in a manner commensurate with their expression levels, which were downregulated, in some cases, during maturation. Moreover, in order to confirm that caspase-3, a key enzyme in this pathways, was fully functional, we induced its activation in a direct fashion using granzyme B as activator (Stoka et al. 2006). Our studies indicate that there is a correlation between kinetic (Fig. 2) and protein expression data (Table 1, Fig. 1, Fig 3). In addition, Yakovlev et al. demonstrated that mRNA and protein expression for both Apaf-1 and caspase-3 were markedly decreased in rat cortex during brain development. Profiles of both Apaf-1 and caspase-3 gene expression were comparable with the developmental profile of *cyt c*-mediated caspase-3 activation in rat brain (Yakovlev et al. 2001).

Due to the complexity of the brain, different regions (may) exhibit a different expression pattern and thus the 'whole' (may) behave differently than the sum of the parts. Using primary cultures of neurons and astrocytes we proved the preceding statement for the activation of the intrinsic pathway of apoptosis at various stages of brain maturation (Stoka et al. 2005). Therefore, a kinetic approach (Fig. 2) represents an invaluable tool to monitor the overall function of the system, complementing the expression levels monitored by Western blotting (Fig. 1, Fig 3). Moreover, the sensitivity of neurons and various brain regions to cerebral hypoxia-ischemia injury varies with the animal's age and level of brain development (Zhu et al. 2005).

Here, we show that during brain development and maturation of CD-1 mice, there is an up-regulation of Hsp-70 and Smac/DIABLO and a down-regulation of *cyt c*/dATP dependent caspase activation (Fig. 1–Fig 3). However, it cannot be canonically assumed that these are just correlative events because we recently showed that a decrease in apoptosis activation during aging is not simply tissue-specific, but rather displays a complex dependence on species and strains of animals (Stoka et al. 2006). Therefore, the genetic background of the animals would favor a pathway and thus cannot necessarily be extrapolated from studies carried out on other organisms and/or strains. In cell-free extracts of primary cultures of neurons and astrocytes at various stages of maturation, we evaluated the intrinsic pathway of apoptosis (Stoka et al. 2005). Interestingly, neuronal extracts were activated 65-fold after 3 days, 9-fold after 7 days, and were not activated after 10 days in culture, whereas astrocyte extracts were activated to a similar extent at all stages, up to 60 days in culture. The loss of *cyt c*/dATP dependent caspase activation was not associated with an up-regulation of endogenous

inhibitors. This finding was also confirmed in cell-free extracts from brain tissue (Stoka et al. 2006).

A working model that summarizes the present data is shown in Fig. 4. During mitochondrial-dependent apoptotic cell death, cyt *c* is released into the cytosol where, in the presence of dATP, there assembles a large caspase-activating complex involving Apaf-1 and procaspase-9 (pC9). Caspase 9 (C9) activates executioner caspases such as procaspase-3 (pC3), thus leading to apoptosis. Caspase-9 and -3 (C3) activities are regulated by IAPs (Shiozaki and Shi 2004). The inhibitory role of the latter is prevented by Smac/DIABLO, thus favoring apoptosis. In cell-free extracts either from neuronal primary cultures (Stoka et al. 2005) or brain tissue (Stoka et al. 2006), no significant differences in the endogenous levels of protein inhibitors could be detected. Specifically, kinetic experiments did not show inhibition, even increasing the ratio of non-activatable over the activatable cell-free extracts of brain or liver (Stoka et al. 2006). In those studies we concluded that protein inhibitors were not responsible for the lack of activation observed and we searched for alterations in the protein expression levels of other components of the pathway such as caspases -3, -9 and Apaf-1 (Stoka et al. 2006; Table 1), Smac/DIABLO and Hsp-70 (this work), all of which lend support to our working hypothesis.

During the course of brain maturation, multiple effects were found, and all seem to favor the observed maturity-associated abrogation of the response to cyt *c*/dATP seen in the fetus and neonate. Specifically, downregulation of the apoptotic protease activator factor-1 (Apaf-1) and/or procaspase-9 should decrease signal transduction. Hsp-70 upregulation (this work) should block recruitment of procaspase-9 to the apoptosome, thus suppressing caspase-9 activation. In addition, procaspase-3 downregulation should prevent caspase cascade amplification. Although the various factors were each altered by only a few fold, the overall effect was to abolish caspase activation completely (although how many other factors may also be involved in this overall functional effect is not yet known).

In conclusion, the resistance of the intrinsic pathway to cyt *c*/dATP activation during brain maturation does not appear to be associated with the alteration of a single factor. Instead, this switch in activation profile is associated with alterations in the expression of several different factors, some proapoptotic (such as Smac/DIABLO, Apaf-1, pro-caspase-9, and pro-caspase-3) and some anti-apoptotic (such as Hsp-70).

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Fig. 1.

Western blot analysis of Smac/DIABLO protein during brain maturation. **(A)** Representative Western blot of 50 μ g of cell-free extracts of mouse brain tissue at various stages of maturation: fetal (14–16 days) (lane 1) and postnatal (0–1 day; lane 2), 15–30 days (lane 3), 3–4 months (lane 4) and 8–12 months (lane 5). A positive control consisted of recombinant Smac/DIABLO protein, which was observed as a single band of 25 kDa (lane c). **(B)** Densitometric values for relative Smac/DIABLO protein from **(A)** were obtained as described in Materials and Methods section. Values are mean $\pm$ SD, representative of three independent experiments. A one-way ANOVA analysis gave a *P*-value 0.004

Fig. 2.

Activation of the intrinsic pathway of apoptosis during brain maturation. Fifty micrograms of cell-free extracts of mouse brain tissue at several stages of maturation—fetal (14–16 days) (lane 1) and postnatal (0–1 day; lane 2), 15–30 days (lane 3), 3–4 months (lane 4) and 8–12 months (lane 5)—were activated with cyt *c*/dATP. Control samples in the absence of cyt *c*/dATP were run under the same conditions. Caspase activation was measured fluorometrically as described in Materials and Methods section and expressed as rates of substrate (Ac-DEVD-AFC) hydrolysis (V). The data are presented as means $\pm$ SD, representative of three independent experiments

Fig. 3. Western blot analysis of Hsp-70 during brain maturation. **(A)** Representative Western blot of 50 μ g of cell-free extracts of mouse brain tissue at various stages of maturation: fetal (14–16 days) (lane 1) and postnatal (0–1 day; lane 2), 15–30 days (lane 3), 3–4 months (lane 4) and 8–12 months (lane 5). A positive control consisted of 50 μ g of recombinant Hsp-70 protein (lane c). **(B)** Densitometric values for relative Hsp-70 protein concentrations from **(A)** were obtained as described in Materials and Methods section. Values shown are means $\pm$ SD, and are representative of three independent experiments. A one-way ANOVA analysis gave a *P*-value below 0.001

Fig. 4.

Model of the intrinsic pathway of apoptosis during brain maturation. In the intrinsic pathway of apoptosis, *cyt c* is released to the cytosol, where in the presence of dATP it assembles with Apaf-1 and procaspase-9 (pC9). Caspase 9 (C9) activates procaspase-3 (pC3) thus leading to apoptosis. Caspase-9 and -3 (C3) activities are regulated by the inhibitor of apoptotic proteins (IAPs). Smac/DIABLO counteracts IAP's inhibitory role, thus favoring apoptosis. During brain maturation, the mechanism by which apoptosis activation by *cyt c*/dATP is decreased during maturity is linked to the alteration in the expression levels of Apaf-1, Hsp-70 and/or procaspase-9 and -3. Further details are provided in the main text

Table 1
Protein expression pattern of other regulators of the *intrinsic* pathway of apoptosis during brain maturation of CD-1 mice

	Fetus	Neonate	Young	Adult	Old	ANOVA
Apaf-1	245 ± 19	193 ± 9	105 ± 19	107 ± 14	83 ± 8	<i>P</i> < 0.0001
Caspase-9 p11	293 ± 14	319 ± 20	340 ± 32	439 ± 8	567 ± 81	<i>P</i> < 0.0001
Caspase-3	290 ± 41	254 ± 35	106 ± 10	108 ± 28	84 ± 14	<i>P</i> < 0.0001

The protein expression data was expressed in relative units and resulted from Western blotting experiment and quantification thus carried out as described in Stoka et al. 2006. The statistical significance of the data was evaluated using a one-way analysis of variance (ANOVA)