Interfering with Apoptosis: Ca^{2+}-Binding Protein ALG-2 and Alzheimer's Disease Gene *ALG-3*

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Two apoptosis-linked genes, named *ALG-2* and *ALG-3*, were identified by means of a functional selection strategy. *ALG-2* codes for a Ca^{2+}-binding protein required for T cell receptor–, Fas-, and glucocorticoid-induced cell death. *ALG-3*, a partial complementary DNA that is homologous to the familial Alzheimer's disease gene *STM2*, rescues a T cell hybridoma from T cell receptor– and Fas-induced apoptosis. These findings suggest that *ALG-2* may mediate Ca^{2+}-regulated signals along the death pathway and that cell death may play a role in Alzheimer's disease.

The normal development of multicellular organisms is dependent on the removal of "unwanted" cells by a genetically controlled process termed programmed cell death (PCD) that is typically mediated by apoptosis (*1*). Disregulation of this process contributes to the pathogenesis of several diseases, including neurodegenerative disorders, cancer, immunodeficiency, and autoimmune diseases (*2*). Although the intracellular events that induce PCD are beginning to be defined, much remains to be understood. We have designed a method to select genes involved in apoptosis, using as a model PCD induced in a mouse T cell hybridoma (3DO) by T cell receptor (TCR) cross-linking (*3*). The selection system, which we named "death trap," is based on the assumption that a transfected complementary DNA (cDNA) library, constructed in the mammalian expression vector pLTP, should protect some recipient cells from death (*4*). Such inhibition may depend on inactivation of apoptotic genes by either antisense RNA or dominant negative mutants or on overexpression of proteins with anti-apoptotic activity.

Using this system, we isolated six cDNA clones, designated apoptosis-linked genes (*ALG-1* to -6), that were able to inhibit TCR-induced cell death in a transient transfection assay (*5*). Here we describe two of them: *ALG-2*, a Ca^{2+}-binding protein, and *ALG-3*, the mouse homolog of a human gene linked to Alzheimer's disease. *ALG-2* consisted of a 435–base pair (bp) cDNA insert that identified a single 1.3-kb transcript in 3DO cells and in all adult mouse tissues analyzed; the thymus and liver showed the most expression, whereas the testis and skeletal muscles showed the least (Fig. 1A). *ALG-3* consisted of an 850-bp fragment that hybridized to a transcript of about 2.4 kb that was present in the liver and, in lesser amounts, in all other adult mouse tissues analyzed (Fig. 1B), the thymus included (*5*). The *ALG-3* probe also detected a major ~1.3-kb mRNA in the liver and a transcript of ~7 kb in the heart and skeletal muscles (Fig. 1B). The 2.4-kb mRNA is also expressed in 3DO cells and, like *ALG-2* (Fig. 1A), is not regulated by TCR triggering.

DNA sequence analysis of the *ALG-2* insert and of five clones isolated from a 3DO cDNA library, together with Northern (RNA) blot analysis with single-strand probes (*5*), revealed that the transfected *ALG-2* is transcribed in the antisense orientation from the expression vector pLTP. The full-length *ALG-2* cDNA is identical to a partial cDNA previously identified (*6*) and has an open reading frame predicted to encode a protein of 191 amino acids, containing two canonical Ca^{2+}-binding EF hand structures (*7*) (Fig. 2A). A rabbit antiserum raised against an ALG-2–histidine tag fusion protein specifically recognized a polypeptide of the expected molecular mass (21.9 kD) in 3DO (Fig. 2B). The *ALG-3* cDNA instead is expressed from the vector as a truncated sense transcript coding for a putative polypeptide that is 98% identical to the 103 COOH-terminal amino acids of *STM2*, the chromosome 1 familial Alzheimer's disease gene (*8*) (Fig. 2C). Initial characterization of a full-length clone obtained from a mouse liver cDNA library indicates that the 2.4-kb mRNA encodes a protein that is highly homologous to *STM2* (*5*). Thus, *ALG-3* is the mouse homolog of *STM2*.

To confirm the data obtained in the transient transfection assay and to better characterize the steps along the death pathway that are affected by these two cDNAs, we established stable transfected 3DO cell clones. To this end, we cotransfected a plasmid carrying the neomycin resistance gene (pcDNA3) with either the *ALG-2* or *ALG-3* expression vectors into 3DO cells to generate G418-resistant clonal populations. Cell clones transfected with the empty plasmid or with pLTP expressing two cDNAs, called *A25* and *B15* (*9*), which scored ambiguously in the transient transfection experiment, were also produced. Thirty-six 3DO clones, expressing amounts of surface TCR comparable to those expressed by the 3DO hybridoma cells (*10*), were analyzed for susceptibility to TCR-induced death. Unlike clones pc.1 to -.4, A25.1 to -.7, and B15.1 to -.6, transfected with the empty pLTP or with this same vector expressing the *A25* and *B15* cDNAs, 11 of the 16 *ALG-2*–transfected clones and all 4 *ALG-3*–transfected clones were resistant to TCR-induced cell death (Fig. 3A).

Because of its antisense orientation in the pLTP expression vector, the *ALG-2* cDNA is predicted to produce, upon transfection, an antisense RNA that should reduce the steady-state amount of the corresponding protein required for TCR-induced cell death. To verify this hypothesis, we analyzed the amount of ALG-2 protein present in 13 of the *ALG-2*–transfected clones by protein immunoblot analysis (clones ALG-2.12, -.15, and -.16 were not analyzed). 3DO clones ALG-2.1, -.2, -.3, -.6, -.7, -.10, -.11, -.13, and -.14, which were resistant to TCR-induced death, expressed reduced amounts of ALG-2 protein (representative clones ALG-2.6, -.7, and -.11, which were further analyzed, are shown in Fig. 3B). The clones ALG-2.4, -.5, -.8, and -.9, which were susceptible to receptor-trig-

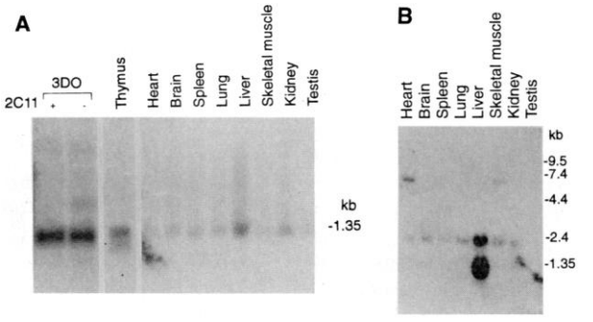

Fig. 1. (**A**) Northern blot analysis of *ALG-2* mRNA expression in 3DO cells untreated or stimulated with immobilized antibody to CD3, and in adult mouse tissues. Poly(A)$^+$ mRNA was prepared from untreated 3DO cells (−) or from cells treated with 2C11 for 1 to 5 hours (+). Thymic mRNA was isolated from adult BALB/c mice. Each lane was loaded with 2 μg of mRNA. (**B**) *ALG-3* transcript in different adult mouse tissues. The blot containing mRNA from the other adult mouse tissues was purchased from Clontech. The filters were hybridized with the *ALG-2* and *ALG-3* inserts and with an actin probe as an internal standard control to allow normalization to the amount of mRNA loaded in each lane (*5*).

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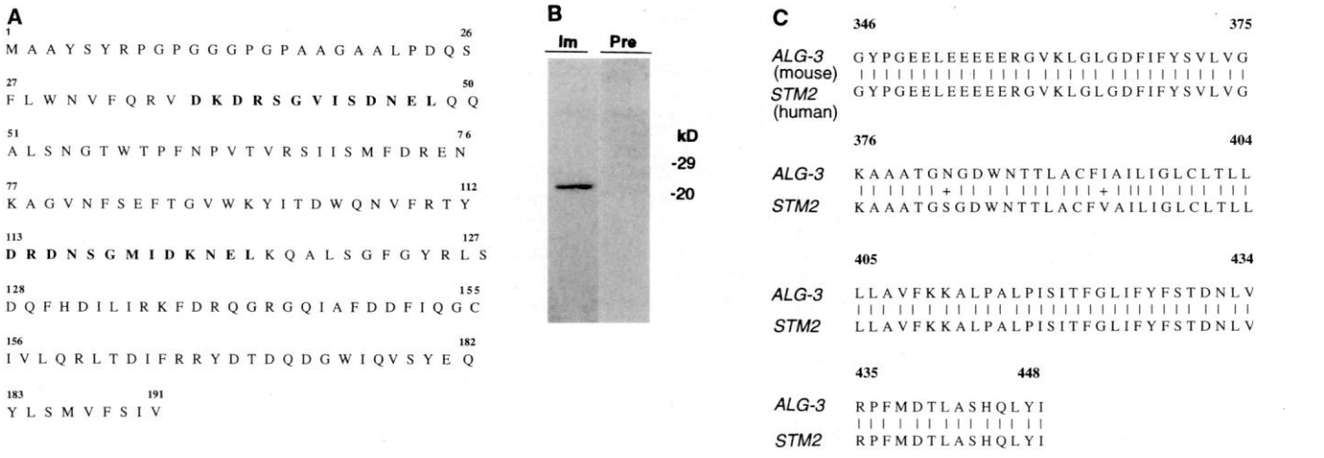


Fig. 2. (**A**) Inferred polypeptide sequence of the ALG-2 protein. The two Ca^{2+}-binding consensus sequences are in bold characters (*27*). The *ALG-2* insert has been used as a probe to isolate the full-length cDNA from a 3DO library constructed in the Uni-ZAP phage (Stratagene). The *ALG-2* cDNA insert overlapped the 5′ untranslated region and the 5′ 320 coding nucleotides of the full-length cDNA. (**B**) Protein immunoblot analysis of ALG-2 protein in 3DO cells. A rabbit polyclonal antiserum recognizing ALG-2 was prepared by Spring Valley Laboratories with the use of a fusion protein containing the full ALG-2 amino acid sequence fused to a histidine tag (Novagen). After SDS-PAGE separation, proteins were transferred to nitrocellulose membranes and probed with either preimmune serum (Pre) or antiserum (Im) to ALG-2. Immunoblots were developed with the ECL System (Amersham). (**C**) Comparison of the predicted amino acid sequence encoded by the *ALG-3* cDNA with the 103 COOH-terminal amino acids predicted for the *STM2* gene product (*27*). Identical residues are indicated by vertical lines and similar residues by plus signs; numbering is from the *STM2* sequence.

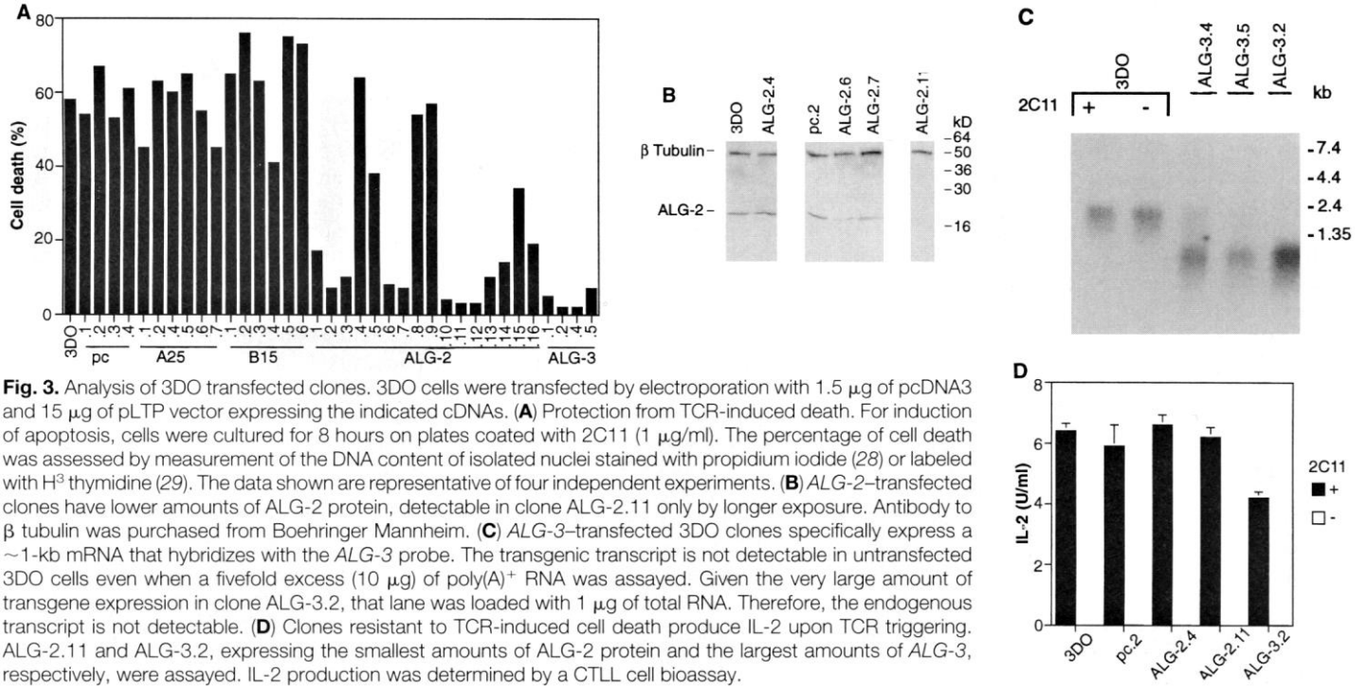
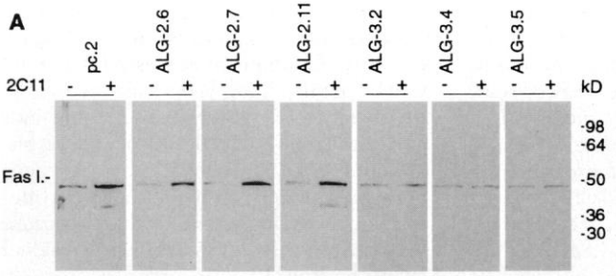


Fig. 3. Analysis of 3DO transfected clones. 3DO cells were transfected by electroporation with 1.5 μg of pcDNA3 and 15 μg of pLTP vector expressing the indicated cDNAs. (**A**) Protection from TCR-induced death. For induction of apoptosis, cells were cultured for 8 hours on plates coated with 2C11 (1 μg/ml). The percentage of cell death was assessed by measurement of the DNA content of isolated nuclei stained with propidium iodide (*28*) or labeled with H^3 thymidine (*29*). The data shown are representative of four independent experiments. (**B**) *ALG-2*–transfected clones have lower amounts of ALG-2 protein, detectable in clone ALG-2.11 only by longer exposure. Antibody to β tubulin was purchased from Boehringer Mannheim. (**C**) *ALG-3*–transfected 3DO clones specifically express a ~1-kb mRNA that hybridizes with the *ALG-3* probe. The transgenic transcript is not detectable in untransfected 3DO cells even when a fivefold excess (10 μg) of poly(A)$^+$ RNA was assayed. Given the very large amount of transgene expression in clone ALG-3.2, that lane was loaded with 1 μg of total RNA. Therefore, the endogenous transcript is not detectable. (**D**) Clones resistant to TCR-induced cell death produce IL-2 upon TCR triggering. ALG-2.11 and ALG-3.2, expressing the smallest amounts of ALG-2 protein and the largest amounts of *ALG-3*, respectively, were assayed. IL-2 production was determined by a CTLL cell bioassay.

gered death, expressed amounts of ALG-2 protein equivalent to those present in the mock transfectants and 3DO cells (only clones ALG-2.4 and pc.2, which were used for further analysis, are shown in Fig. 3B). This may be the consequence of either poor expression of the *ALG-2* transgene or integration of pcDNA3 alone. An antibody specific for β tubulin was used to allow normalization to the amount of protein loaded on each lane (Fig. 3B). Thus, reduction of the amount of ALG-2 protein protects 3DO cells from receptor-induced cell death.

The *ALG-3* cDNA was in sense orientation in the expression vector pLTP. Therefore, *ALG-3*–transfected 3DO clones, protected from TCR-induced PCD, should express a truncated *ALG-3* transcript. Northern blot analysis with the *ALG-3* cDNA probe, which detected the endogenous 2.4-kb mRNA in all tested populations, hybridizes to a ~1-kb transgenic transcript expressed in *ALG-3*–transfected clones and not present in 3DO cells (only ALG-3.2, -.4, and -.5 clones, used for subsequent studies, are shown in Fig. 3C). Thus, expression of the truncated *ALG-3* transcript correlates with resistance of 3DO cells to receptor-induced cell death.

The transfected *ALG-2* and *ALG-3* constructs could either specifically inter-



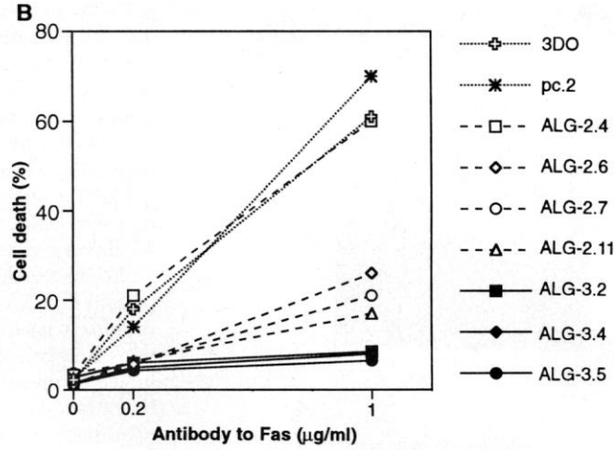


Fig. 4. (**A**) Fas ligand up-regulation upon TCR triggering. Protein immunoblot analysis was performed as described in Fig. 2. Fas ligand (Fas l.) induction was measured on total cell lysates prepared from untreated cells or from cells stimulated for 4 hours with 2C11, with the use of an antibody to Fas ligand (Santa Cruz). The nature of the cross-reactive smaller protein is unknown. (**B**) Susceptibility to Fas-mediated PCD. To induce cell death, 3DO cells were incubated at room temperature for 15 min with the indicated concentrations of the antibody to Fas Jo2 (Pharmingen), then washed and plated on wells coated with an antibody to hamster immunoglobulin G (5 μg/ml) (Pharmingen).

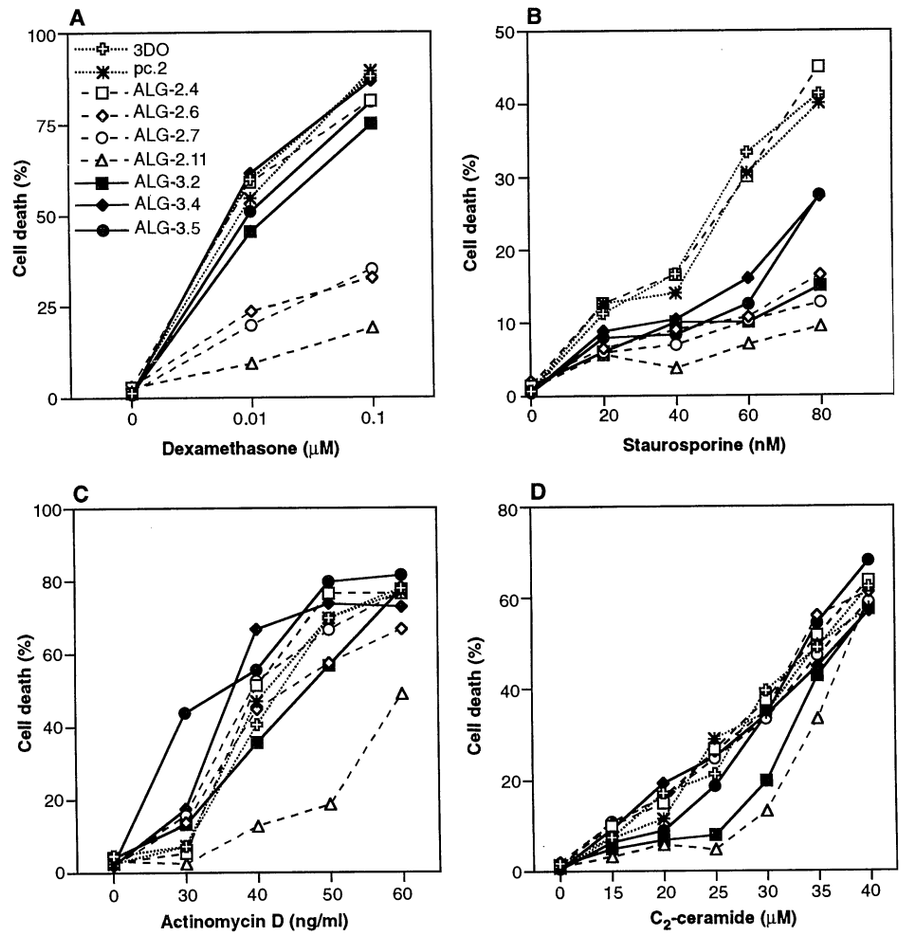


Fig. 5. Analysis of apoptosis induced by other stimuli. Cells were cultured with the indicated concentrations of (**A**) dexamethasone (Ascot Pharmaceutical) for 18 hours, (**B**) staurosporine (Sigma) (9 hours), (**C**) actinomycin D (U.S.B.) for 20 hours, or (**D**) the cell-permeable synthetic ceramide C_2-ceramide (BIOMOL Research Labs) for 14 hours. Cell death was measured as indicated in Fig. 3.

fere with PCD or compromise early events in TCR signaling, events that lead to activation followed by death. In order to differentiate between these two possibilities, we measured interleukin-2 (IL-2) amounts in the culture supernatant at 8 hours after TCR triggering. All the clones analyzed produced equivalent amounts of IL-2 (Fig. 3D). Thus, signal transduction upon TCR stimulation is not generally affected in the clones transfected with *ALG-2* and *ALG-3*.

Fas ligand is induced after TCR stimulation in T cell hybridomas, and the engagement of Fas by Fas ligand activates the cell death program (*11*). Because all 3DO clones expressed equivalent amounts of Fas protein on the cell surface (*10*), we examined whether Fas ligand induction or Fas-mediated cell death (or both) were affected. Protein immunoblot analysis of whole-cell extracts revealed that Fas ligand up-regulation, which was detectable in the mock transfectant pc.2 and in all *ALG-2*–transfected clones, was completely blocked in the *ALG-3*–expressing cells (Fig. 4A). Stimulation of the Fas receptor with an antibody to Fas readily induced death of 3DO cells, of the mock transfectant pc.2 clone, and of the ALG-2.4 clone. In contrast, the clones resistant to TCR-mediated cell death were also protected from Fas-induced cytotoxicity (Fig. 4B). Thus, the ALG-2 protein functions downstream of Fas–Fas ligand interaction, and the *ALG-3* transgenic transcript interferes with the TCR death pathway by inhibiting Fas ligand induction and, consequently, Fas-mediated autocrine suicide; and by blocking the death signal transduced by the Fas molecule.

To investigate whether the effect of these two cDNAs is limited to TCR-induced PCD, we treated 3DO clones with other apoptosis-inducing agents. Dexamethasone, a synthetic corticosteroid that causes apoptosis in lymphoid cells (*12*), triggers a pathway that is independent of Fas–Fas ligand interaction and antagonizes TCR-induced death by inhibiting Fas ligand up-regulation (*13*). Ceramide has been implicated as a mediator of Fas-induced death and is a potent inducer of apoptosis (*14*). Staurosporine and actinomycin D induce apoptosis in many cell lines (*15*). Clones expressing reduced amounts of ALG-2 protein were less sensitive to steroid- and staurosporine-induced cell death

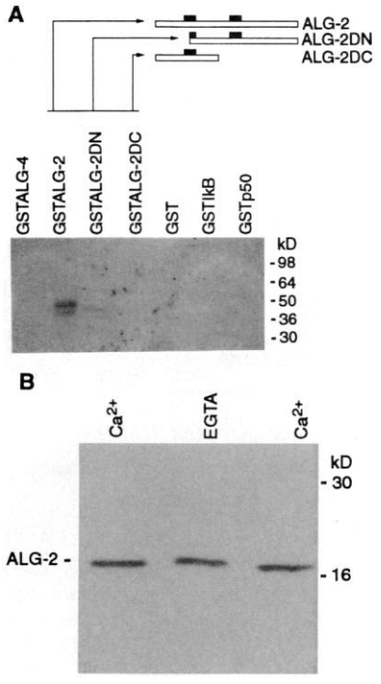


Fig. 6. Ca^{2+}-binding property of ALG-2. (**A**) $^{45}Ca^{2+}$ ligand-blotting analysis of ALG-2. Partial removal of the NH_2-terminal Ca^{2+}-binding domain (GSTALG-2DN) does not completely abrogate Ca^{2+} binding. Glutathione-S-transferase (GST) fusion proteins (2 μg) were separated by electrophoresis, blotted on nitrocellulose membrane, and assayed for Ca^{2+}-binding activity as previously described (*30*). The fusion proteins were produced with the use of constructs based on the inducible prokaryotic expression vector pGEX (Pharmacia). The GSTALG-2 and the two mutant proteins are schematically represented (open boxes) and the two Ca^{2+}-binding domains are indicated (black boxes). GSTALG-2 contains the almost complete ALG-2 amino acid sequence (Arg^7 to Val^{191}); GSTALG-2DN has the NH_2-terminal Ca^{2+}-binding domain partially deleted (Val^{42} to Val^{191}), and GSTALG-2DC has the COOH-terminal EF hand structure completely removed (Arg^7 to Phe^{95}). The other controls are GST, GSTALG-4 (*10*), GSTIkB, and GSTp50 (*31*) (Fig. 2A). After $^{45}Ca^{2+}$ binding, the membrane was stained with Ponceau Red to verify that all lanes were loaded with equal amounts of recombinant proteins. (**B**) Ca^{2+}-dependent gel mobility shift of ALG-2. Total 3DO cell lysates were incubated for 5 min with 2.5 mM $CaCl_2$ or 5 mM EGTA and then subjected to 15% SDS-PAGE, followed by protein immunoblot analysis with the antiserum to ALG-2. The small shift observed has been reproduced in six independent experiments.

(Fig. 5, A and B). However, only clone ALG-2.11, expressing the lowest amounts of ALG-2 protein (Fig. 3B), was partially protected from actinomycin D and C_2-ceramide cytotoxicity (Fig. 5, C and D). The *ALG-3*–transfected clones, on the other hand, were sensitive to dexamethasone and actinomycin D (Fig. 5, A and C) and were partially protected from staurosporine-induced cell death (Fig. 5B). Like ALG-2.11, the ALG-3.2 clone, expressing the largest amounts of *ALG-3* transgene, showed some resistance to cytotoxicity at concentrations of C_2-ceramide between 20 and 30 μM (Fig. 5D). Thus, the ALG-2 protein is required for steps along the death pathway that are distinct from those affected by the *ALG-3* truncated transcript.

Because *ALG-2* is constitutively expressed in 3DO cells and is not regulated by receptor triggering, it is likely that the protein itself or some functionally interacting molecule must be modified after activation to carry out the apoptotic program. This hypothesis is reinforced by the evidence that fibroblasts overexpressing the full-length sense *ALG-2* cDNA undergo cell death after treatment with ionomycin and phorbol 12-myristate 13-acetate (*5*), two stimuli that partially mimic TCR engagement and cause PCD in T cells (*16*). This same treatment has no effect on fibroblasts transfected with an antisense *ALG-2* expression vector or with empty plasmid (*5*). Extracellular Ca^{2+} flux or Ca^{2+} release from intracellular storage compartments might mediate, at least in part, this functional activation. To test whether ALG-2 can bind Ca^{2+}, we performed a $^{45}Ca^{2+}$ ligand-blotting experiment with recombinant proteins. As shown in Fig. 6A, a GSTALG-2 fusion protein specifically binds $^{45}Ca^{2+}$ and both Ca^{2+}-binding EF hands are required for efficient binding. In addition, like other Ca^{2+}-binding proteins, ALG-2 migrates faster during SDS–polyacrylamide gel electrophoresis (PAGE) in the presence of calcium (Fig. 6B). Thus, by these two criteria, ALG-2 is identified as a Ca^{2+}-binding protein.

In this study, two cDNAs involved in PCD were described. One, *ALG-2*, codes for a Ca^{2+}-binding protein and is a component of both TCR- and glucocorticoid-triggered cell death. Apoptosis induced by other agents is also affected. The involvement of Ca^{2+} in apoptosis was first suggested by studies assessing the biochemical requirements for DNA cleavage (*17*). Subsequently, it has been shown that glucocorticoid and TCR triggering stimulate sustained calcium increases in thymocytes, and that cell death is prevented by blocking of the calcium increase (*18, 19*). A requirement for extracellular Ca^{2+} in TCR-induced Fas ligand up-regulation and for intracellular Ca^{2+} for Fas-mediated death has been recently demonstrated (*20*). Moreover, calmodulin (*19, 21*) and the calcium-dependent cysteine protease calpain (*22*) have been suggested to be involved in PCD. ALG-2 is the first Ca^{2+}-binding protein shown to be directly involved in PCD and might represent the prototype of a family of genes that mediate Ca^{2+}-regulated signals along the death pathway. Cysteine proteases of the ICE–CED-3 family have been suggested to be involved in Fas-mediated (*23*) but not glucocorticoid-induced (*24*) apoptosis. Whether the ALG-2 pathway is dependent on the activation of these enzymes has yet to be analyzed. However, because we analyzed cell clones expressing residual amounts of ALG-2, in order to precisely assess how general and physiological the role of this protein is, *ALG-2* knockout mice and cell lines should be generated. The other cDNA identified, *ALG-3*, is the mouse homolog of *STM2*, the familial Alzheimer's disease gene on chromosome 1. This finding raises the possibility that, as has also been suggested by other studies (*25*), cell death plays an important role in the pathophysiology of Alzheimer's disease and provides a clue to the physiological function of *STM2*. However, whether the truncated *ALG-3* RNA confers resistance to cell death by being translated into a polypeptide (*26*) and whether the endogenous ALG-3 protein is involved in the death process remain to be determined.

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27. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Newly Learned Auditory Responses Mediated by NMDA Receptors in the Owl Inferior Colliculus

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Alignment of auditory and visual receptive fields in the optic tectum of the barn owl (*Tyto alba*) is maintained through experience-dependent modification of auditory responses in the external nucleus of the inferior colliculus (ICX), which provides auditory input to the tectum. Newly learned tectal auditory responses, induced by altered visual experience, were found to be pharmacologically distinct from normal responses expressed at the same tectal sites. *N*-methyl-D-aspartate (NMDA) receptor antagonists administered systemically or applied locally in the ICX reduced learned responses more than normal responses. This differential blockade was not observed with non-NMDA or broad-spectrum antagonists. Thus, NMDA receptors preferentially mediate the expression of novel neuronal responses induced by experience during development.

Experience-dependent modification of neuronal responses tailors the function of neural circuits based on the sensory experience of the individual. Pharmacological studies of this process (*1*) have implicated the NMDA subtype of excitatory amino acid (EAA) receptor in the induction of experience-dependent synaptic modification. However, interpretation of these experiments is difficult because the specific effects of NMDA receptor blockade are usually confounded with nonspecific effects of blocking postsynaptic activity (*2*). We have used a different approach in a system in which normal and newly learned responses can be recorded simultaneously at single sites. Here we show that newly functional circuitry, once it has been induced by experience-dependent processes, is pharmacologically specialized: Transmission through this circuitry is preferentially mediated by NMDA receptors, relative to transmission through original circuitry (*3*).

Barn owls localize sounds using interaural timing difference (ITD) as a cue for sound source azimuth. In the ICX, where the owl's map of auditory space is synthesized, neurons are tuned to specific ITD values and are organized into a map of ITD, and hence of azimuthal space. The auditory space map is relayed topographically to the optic tectum, where it is aligned with the tectal map of visual space so that tectal neurons are tuned to the value of ITD produced by sounds at the locations of their visual receptive fields (VRFs) (*4*). This alignment is dynamically maintained by experience-dependent plasticity and can be altered systematically if owls are raised wearing prismatic spectacles that optically displace the visual field in azimuth (*5*, *6*). During prism-rearing, tectal neurons develop novel responses to sounds with ITDs that correspond to the location of their optically displaced VRFs (schematized in Fig. 1A). At many tectal sites, these novel responses, which are to ITDs that are systematically displaced from the normal ITD range (*6*), first appear while responses to ITDs in the normal range continue to be expressed, creating a "transition state" ITD tuning curve (*7*) (Fig. 1A, middle panel). Transition state tuning curves are often abnormally broad and sometimes double-peaked. They are defined here as those ITD tuning curves that include both responses to ITD values that are normally appropriate for that tectal site, termed "normal responses," and responses to ITD values corresponding to the prismatically displaced VRF, termed "learned responses" (*8*). Over subsequent weeks, normal responses are eliminated to produce a narrow ITD tuning curve centered on the learned ITD value (Fig. 1A, bottom panel).

The alteration of tectal ITD tuning can be accounted for by experience-dependent plasticity that occurs at the level of the ICX (*6*, *7*). In the study reported here, we compared the pharmacology of ICX circuits mediating normal and newly learned responses in prism-reared owls. We did this by applying EAA receptor antagonists either systemically or locally into the ICX while recording responses at tectal sites displaying transition state ITD tuning. We focused on NMDA and non-NMDA subtypes of EAA receptors, because auditory transmission in the ICX of normal owls is known to be mediated through these receptors (*9*). ITD tuning was monitored in the optic tectum rather than in the ICX, because tectal VRFs allow unambiguous determination of normal ITD tuning for any given site (*6*, *8*).

Systemic injection of the anesthetic and NMDA receptor antagonist ketamine HCl (*10*) at 10 to 15 mg per kilogram of body weight, a dose known to selectively antagonize NMDA receptors in the ICX (*11*),

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