The Rho family of small GTPases regulates numerous signaling pathways that control the organization of the cytoskeleton, transcription factor activity, and many aspects of the differentiation of skeletal myoblasts. Now demonstrate that the kinase Mirk (minibrain-related kinase)/dyrk1B is induced by members of the Rho family in myoblasts and that Mirk is active in skeletal muscle differentiation. Mirk is an arginine-directed serine/threonine kinase which is expressed at elevated levels in skeletal muscle compared with other normal tissues. A Mirk promoter construct was activated when C2C12 myoblasts were switched from growth to differentiation medium and was also activated by the Rho family members RhoA, Cdc42, and to a lesser degree Rac1, but not by MyoD or Myf5. Mirk protein levels increased following transient expression of constitutively active Cdc42-QL, RhoA-QL, or Rac1-QL in C2C12 cells. High concentrations of serum mitogens down-regulated Mirk through activation of the Ras-MEK-Erk pathway. As a result, Mirk transcription was induced by the MEK1 inhibitor PD98059 and by the switch from growth to differentiation medium. Mirk was induced with similar kinetics to another Rho-induced differentiation gene, myogenin. Mirk protein levels increased 10-fold within 24–48 h after primary cultured muscle cells; C2C12 mouse myoblasts or L6 rat myoblasts were induced to differentiate. Thus Mirk was induced following the commitment stage of myogenesis. Stable overexpression of Mirk enabled myoblasts to fuse more rapidly when placed in differentiation medium. The function of Mirk in muscle differentiation was established by depletion of endogenous Mirk by small interfering RNA, which prevented myoblast fusion into myotubes and inhibited induction of markers of differentiation, including myogenin, fast twitch troponin T, and muscle myosin heavy chain. Other members of the dyrk/minibrain/HIPK family of kinases in lower organisms have been shown to regulate the transition from growth to differentiation, and Mirk is now shown to participate in skeletal muscle development. The Rho family of evolutionarily conserved small GTPases regulates numerous signaling pathways that control the organization of the cytoskeleton and influence cell polarity, microtubule dynamics, membrane transport, and transcription factor activity (1). Rho family members have been implicated in many aspects of the differentiation of skeletal myoblasts. Rho-GTP activators have been observed to increase during myogenic differentiation, while inhibition of Rho blocks myogenesis (2, 3). Rho family proteins are required for the transcription of the myogenin gene and various other muscle specific genes during myogenesis including skeletal α-actin and β-myosin heavy chain (β-MHC) (4). Rho GT-Pase activity was shown to regulate an adipogenesis-myogenesis decision in vivo in response to IGF-1, using mice lacking the major Rho inhibitory protein p190-B RhoGAP (5). Murine embryonic fibroblasts lacking p190-B RhoGAP underwent the “default pathway” of myogenic differentiation in response to IGF-1 unless the Rho effector Rho-kinase was inhibited (5). Conversely, IGF-1, together with oncogenically activated RhoV14, stimulated myogenic differentiation in wild-type murine embryonic fibroblasts. In the current study, we have established that the Mirk (minibrain-related kinase) gene (6) is induced by certain Rho family members and furthermore that Mirk functions in myogenesis. Mirk is a member of the dyrk/minibrain family of arginine-directed serine/threonine protein kinases (7–9) and is identical to Dyrk1B (10). The roles of dyrk/minibrain/Mirk homologues in yeast (Yak1) and slime mold (YakA) suggest that this group of kinases regulates the transition from growth to differentiation (11, 12). During Dictyostelium growth, YakA regulates the intervals between cell division. Following nutrient depletion, YakA mediates growth arrest and an increase in protein kinase A activity, which regulates expression of adenyl cyclase and ultimately the induction of development. We have found a similar function for Mirk in myogenesis. Mirk is dramatically up-regulated when myoblasts are induced to differentiate, while depletion of endogenous Mirk by RNAi blocks myotube formation.

Mirk/dyrk1B functions as a transcription factor activator for hepatocyte nuclear factor 1 in endodermally derived tissues (13). Mirk is likely to fulfill a similar role in muscle differentiation, although the transcription factor which Mirk activates in myotubes is not yet known. Mirk itself is activated by co-expressed MKK3 (13), a MAP kinase kinase that can also activate p38 MAP kinase (14–16). Mirk activity is controlled by p38 through direct sequestration of Mirk (17). Since p38 has also been shown to be required for terminal muscle cell differ-
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EXPERIMENTAL PROCEDURES

Materials—Antibodies to myogenin, MyoD, troponin T, β-tubulin, and 21Clc1p1 were from Santa Cruz Biotechnology. Rabbit polyclonal antibody to a unique sequence at the C terminus of Mirk was raised as described previously (6). The MF 20 monoclonal antibody to MHC was developed by D. A. Fischman was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. Polyvinylidene difluoride transfer paper Immobolin-P was purchased from Millipore. PLUS reagent and LipofectAMINE were from Invitrogen; all radioactive materials were purchased from Amersham Biosciences, and tissue culture reagents from Mediatech (Fishers). All other reagents were from Sigma. Tissue from human skeletal muscle biopsies was kept in liquid N2 until use. The reagents for in vitro transcription were purchased from PerkinElmer Life Sciences, and tissue culture reagents from Mediatech (Fishers). For studies on differentiation, the cells were plated in GMP on dishes coated with E-C-L (entactin-collagen IV-laminin) cell attachment matrix (Upstream Biotechnology). After 24 h the GMP was replaced with differentiation medium (DM; Dulbecco’s modified Eagle’s medium containing 2% horse serum). Primary cultures of murine myoblasts were provided by Dr. Sten Rafaelsen, University of Texas Southwestern Medical Center and from tissue culture reagents from Mediatech (Fishers). The cell line MF 20 was developed by D. A. Fischman was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. Polyvinylidene difluoride transfer paper Immobolin-P was purchased from Millipore. PLUS reagent and LipofectAMINE were from Invitrogen; all radioactive materials were purchased from Amersham Biosciences, and tissue culture reagents from Mediatech (Fishers). All other reagents were from Sigma.

Cell Culture—C2C12 mouse myoblasts were obtained from the ATCC and L6/E9 rat myoblasts were a gift of Dr. S. M. Rosenthal (University of California San Francisco). Cells were maintained in growth medium (GM; Dulbecco’s modified Eagle’s medium containing 2% fetal bovine serum) and switching to differentiation medium (DM; Dulbecco’s modified Eagle’s medium containing 2% horse serum). Primary cultures of murine myoblasts were prepared from hindlimb muscles of adult mice by enzymatic dissociation using collagenase D and dispase (Roche Applied Science). The myoblasts were maintained in dishes coated with Vitrogen collagen (Coherence Technologies, Palo Alto, CA) in growth medium for primary culture. For maintenance of skeletal muscle biopsies, cells were lysed in boiling SDS-PAGE sample buffer, further homogenized with a Dounce homogenizer, centrifuged to remove insoluble material, and size-fractionated by SDS-PAGE. The cell line MF 20 was developed by D. A. Fischman was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA. Polyvinylidene difluoride transfer paper Immobolin-P was purchased from Millipore. PLUS reagent and LipofectAMINE were from Invitrogen; all radioactive materials were purchased from Amersham Biosciences, and tissue culture reagents from Mediatech (Fishers). All other reagents were from Sigma. Tissue from human skeletal muscle biopsies was kept in liquid N2 until use. The reagents for in vitro transcription were purchased from PerkinElmer Life Sciences, and tissue culture reagents from Mediatech (Fishers). For studies on differentiation, the cells were plated in GMP on dishes coated with E-C-L (entactin-collagen IV-laminin) cell attachment matrix (Upstream Biotechnology). After 24 h the GMP was replaced with differentiation medium for primaries (DM; Dulbecco’s modified Eagle’s medium containing 2% horse serum). The myoblasts were maintained in dishes coated with Vitrogen collagen (Coherence Technologies, Palo Alto, CA) in growth medium for primary culture. For maintenance of skeletal muscle biopsies, cells were lysed in boiling SDS-PAGE sample buffer, further homogenized with a Dounce homogenizer, centrifuged to remove insoluble material, and size-fractionated by SDS-PAGE.

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The Rho Family of GTPases Induces Mirk Expression—The Rho GTPases RhoA, Rac1, and Cdc42 differentially regulate skeletal muscle cell differentiation. RhoA and serum response factor function together to induce MyoD (3, 4). In contrast, while expression of exogenous Rac1 and Cdc42 can activate promoter constructs of certain muscle-specific genes (4), they also activate the kinases JNK and p38MAPK, which, respectively, inhibit and enhance myogenesis (23). Precise temporal regulation of these Rho GTPases and the kinases that they activate must occur during myogenesis. We tested the ability of Rho GTPases to activate the Mirk promoter after first defining the minimal Mirk promoter. A 6.2-kb pMP-luc Mirk promoter construct was fused to a luciferase reporter gene. Assessment of the reporter activity of several deletion mutants revealed that activity was lost when the deletion creating the 111-bp construct removed the putative CAAT box. Thus the 364-bp pΔXE47-luc Mirk promoter construct contains the minimal promoter region (Fig. 2A). After transient transfection into C2C12 cells, this reporter construct was activated when cells were placed into differentiation medium and exhibited increased activity with time in DM (Fig. 2B). Therefore, the Mirk promoter reporter construct is activated by transcription factors active in differentiating C2C12 cells.

The vertebrate muscle maturation program is controlled by the myogenic basic helix-loop-helix (bHLH) transcription factor family, which acts in a sequential fashion. Two of these bHLH transcription factors, MyoD and Myf5, are expressed in proliferating undifferentiated myoblasts and upon growth factor withdrawal are activated to initiate myoblast maturation and fusion into myotubes. RhoA together with serum response factor activates MyoD (3), so we next tested the activity of Rho family members on the Mirk promoter construct in differentiating C2C12 cells. Co-expression of the Mirk reporter construct with the constitutively active constructs RhoA-QL or Cdc42-QL induced an 8-fold increase in reporter activity, while co-expression with constitutively active Rac1-QL led to a smaller 4-fold increase (Fig. 2C). Thus, each of these Rho GTPases activated the Mirk promoter construct in C2C12 cells. The activity of the Mirk promoter construct was increased more each day in DM (0–2 days) by co-transfected constitutively activated RhoA-QL (Fig. 2D). After 2 days in DM, Mirk induction by endogenous RhoA was inhibited 20% by co-transfected dominant negative RhoA-N19, and 40% by the exoenzyme C3 transferase (Fig. 2D). C3 inactivates RhoA, RhoB, and RhoC proteins but not Cdc42 or Rac. Therefore, RhoA induced Mirk expression, while dominant negative RhoA and the RhoA inhibitor C3 inhibited Mirk expression. To confirm that Rho family members induced endogenous Mirk expression, constitutively active Cdc42-QL, Rac1-QL, and RhoA-QL were transiently expressed in C2C12 cells. Even though the transfection efficiencies were on average about 30%, an increase in Mirk protein over control levels was seen after 2 days of expression in DM. Compared with cells transfected with vector DNA, the Mirk to tubulin ratio increased about 1.7–2.3-fold in cells expressing activated Cdc42, Rac1, or RhoA (Fig. 2E), which translates into 6-fold increases when the efficiency of transfection is factored in. Transient expression of the Rho inhibitor C3 transferase inhibited endogenous Mirk expression (Western blotting data not shown). Thus, the Rho family members RhoA, Cdc42, and to a lesser extent Rac1, induced Mirk during C2C12 cell maturation.

Muscle-restricted basic helix-loop-helix transcription factors such as MyoD form heterodimers with other ubiquitous helix-loop-helix factors and bind to DNA elements called E boxes, which are found in the promoter regions of genes expressed in muscle. Two putative E boxes (CACGTT) were found in the pΔXE47 Mirk promoter construct at bp +12 (E1) and bp −245 (E2) from the Mirk/dyrk1B start site. Mutation of E1 did not alter basal transcription activity (data not shown), whereas
Mirk is induced in differentiating C2C12 cells by Rho family GTPases. A, deletion mapping to determine the minimal Mirk promoter. Deletions of the 6.2-kb region upstream of Mirk exon I, the putative promoter region, were made by appropriate restriction enzymes; the Mirk promoter regions were coupled to a luciferase reporter gene and transiently transfected into U9 colon carcinoma cells in triplicate, and 24 h later the activity of the promoters was assayed. Data were normalized against co-transfected β-galactosidase. Mean ± S.E. is shown (S.E. bars shown if greater than 5%). Similar activity of the 6.2- and 2.8-kb promoter constructs is not shown. Promoter length shown includes 67 bp of exon I. B, Mirk promoter activation in differentiating C2C12 cells and effect of E2 mutation on promoter activity; C2C12 cells were transiently transfected with the wild-type 364-bp pΔXE47 Mirk promoter-luciferase reporter construct or one containing mutations in both E1 and E2 E boxes. Luciferase activity was assayed 24 and 48 h after transfer to DM. Mean ± S.E. of the luciferase activity was normalized against the activity of a co-transfected β-galactosidase encoding expression plasmid. C, the 364-bp Mirk promoter-luciferase reporter construct was transiently co-transfected into C2C12 cells with expression plasmids for constitutively active Cdc42-QL, RhoA-QL, or Rac1-QL or with expression plasmids for E47, MyoD, or Myf5. Cells were then cultured in DM for 2 days. Mean ± S.E. of the luciferase activity was normalized against the activity of a co-transfected β-galactosidase encoding expression plasmid. Data shown are the mean of two independent experiments, each performed in triplicate. E, C2C12 cells were transiently transfected with expression plasmids for constitutively active Cdc42-QL, RhoA-QL, or Rac1-QL and then placed in DM for 2 days before lysis and Western blotting. The relative protein level of Mirk compared with the tubulin internal control is given under each lane.
mutation of E2 alone or both E2 and E1 resulted in a 3–4-fold decrease in basal transcription of the pAXE47 Mirk promoter after 1–2 days in DM (Fig. 2B). Therefore, the Mirk promoter reporter construct is activated by transcription factors active in differentiating C2C12 cells, and moreover, mutation of the E2 E box blocks this response.

Since putative E boxes were present in the mirk promoter, we next tested transcription factors that might bind to these promoter elements. MyoD binds to an E box element in the myogenin promoter and induces its transcription (24). However, co-transfection with expression plasmids for either MyoD, Myf5, or the E box-binding protein E47 (25) had no effect on Mirk promoter construct activity (Fig. 2C). Co-expression of MyoD and E47 were likewise ineffective (not shown).

**Mirk Expression Increased following Inhibition of MEK**—We next considered the possibility that, in addition to up-regulation by Rho-mediated differentiation signals, Mirk transcription might be blocked by growth signals. Growth medium contains high levels of serum mitogens including IGFs, which activate the Ras-MEK-Erk system leading to cell proliferation. Switching colon carcinoma cells to serum-free medium had caused a marked increase in Mirk protein levels in earlier studies (6). Myoblasts responded in a similar fashion. Mirk protein levels increased 10-fold (Fig. 1, B and C), and the Mirk promoter construct (Fig. 2) was activated when myoblasts were transferred from high serum GM to low serum DM. While Erks can transactivate several nuclear transcription factors by phosphorylation, there are also a number of examples of Erk-mediated transcriptional down-regulation (26).

Because of these observations, the hypothesis that the MEK1-Erk pathway could inhibit Mirk transcription was tested. The Mirk minimal promoter construct pAXE47-luc was activated 6-fold when C2C12 cells were treated with the MEK1 inhibitor PD98059 (Fig. 3A). A similar 6-fold activation of the E box mutant promoter was induced by PD98059 treatment, confirming that this transcriptional down-regulation was not mediated through E box elements. The MEK1 signaling pathway also blocked Mirk transcription in colon carcinoma cells, showing the generality of this response. Both the Mirk minimal promoter and the longer 6.2-kb putative promoter construct were activated by treatment of U9 colon carcinoma cells with increasing concentrations of the MEK1 inhibitor (Fig. 3B). Northern analysis confirmed the reporter assay findings. Treatment with the MEK1/2 inhibitor PD98059 increased Mirk mRNA levels 6-fold (Fig. 3, C and D, upper panel), and a parallel increase in Mirk protein level was observed (Fig. 3, C and D, lower panel). The induction of Mirk mRNA by serum-free culture was increased by concurrent treatment with cycloheximide to block protein translation (data not shown). Therefore, Mirk expression was negatively controlled on the transcriptional level by the Ras-Erk pathway, and protein synthesis was required to block Mirk transcription. Erk activation temporally preceded Mirk down-regulation in U9 colon carcinoma cells deprived of serum for 16 h, then released into medium containing fetal bovine serum. Serum caused a slow onset of Erk activation 3–6 h after addition, which persisted for
several hours in these colon carcinoma cells, leading to a decrease in Mirk protein levels after 21–24 h (Fig. 3R). These data together show that blocking the MEK-Erk pathway increases Mirk abundance by up-regulating Mirk transcription.

Time Course of Mirk Induction during Myoblast Differentiation—The time course for Mirk induction in two models of myoblast differentiation, rat L6E9 cells and mouse C2C12 cells, was determined as a first step in studying the biological effects of up-regulating or down-regulating Mirk levels. Cell lysates were examined for expression of Mirk and other markers of muscle cell differentiation by Western blotting for 0–4 days after switching the cells from growth medium to differentiation medium. Mirk levels were very low in growth medium, but increased 100-fold over 4 days in DM, with a large increase seen after 1 day (Fig. 4). In both cell types myogenin was rapidly induced after 1 day in DM, while both muscle MHC and fast twitch troponin T were induced after 2 days in DM. In contrast to MHC and troponin T, the levels of which were very low or undetectable in growth medium, levels of the Cdk inhibitor p21 were already elevated when cells were switched to DM and continued to rise about 2–3-fold. Therefore, in both myoblast cell types, Mirk has a similar time course for appearance as myogenin. Mirk appears before the induction of contractile protein genes like MHC and troponin T (4), and Mirk is induced later than the MyoD-induced p21 (27–29). This pattern is very similar to that seen in primary cultured muscle cells (Fig. 1C).

The vertebrate muscle maturation program is controlled by the myogenic bHLH transcription factor family, which acts in a sequential fashion. Two of these bHLH transcription factors, MyoD and Myf5, are expressed in proliferating undifferentiated myoblasts and upon growth factor withdrawal are activated to initiate myoblast maturation and fusion into myotubes. Myogenin controls the differentiation process and functions later than MyoD and Myf5. The time course analysis indicates that Mirk, like myogenin, mediates muscle differentiation, not commitment to this lineage.

Stable Overexpression of Mirk—Multiple experiments demonstrated that stable C2C12 Mirk transfectant cells fused more rapidly when placed in DM (Fig. 5A). Analysis by Western blotting demonstrated that more myogenin was induced at each time point up to 3 days in DM, when Mirk transfectants were compared with control transfectants (Fig. 5B). For example, on day 2, 25% more myogenin was seen in mirk transfectant cultures (p < 0.05, Student’s t test). Thus overexpression of Mirk enhanced the skeletal muscle differentiation program.

Mirk Function in Muscle Differentiation Shown by RNAi—To confirm the role of Mirk in muscle development, endogenous Mirk levels were depleted with RNAi directed to a region of the mouse Mirk coding sequence. Initially three sequences within the Mirk coding domain were targeted (data not shown). The sequence S11 was most effective so it was used for all subsequent experiments. C2C12 cells were co-transfected with either RNAi to Mirk or vector control DNA together with an expression plasmid for GFP. The transfected cultures were sorted for the positive transfectants by the presence of GFP and then placed in growth medium for 1 day, then switched to DM. Cell lysates were analyzed after 1 and 2 days in DM by Western blotting, with blotting and transfer controlled by measurement of tubulin. Mirk levels doubled in the vector control cultures between 1–2 days in DM, while parallel cultures treated with RNAi expressed 6% as much Mirk after 1 day and 25% as much Mirk after 2 days (Fig. 6A). The decrease in RNAi effect was due to loss of Mirk RNAi containing cells. Levels of myogenin and muscle MHC were very low to undetectable after 1 day in DM but increased substantially after 2 days of differentiation. However, RNAi to Mirk limited these increases to 25% of vector control levels. A smaller 20% decrease in p21 protein levels was seen in repeated experiments, but no decrease in the phosphorylation of pRb at Thr821 could be detected (data not shown).

Multiple RNAi experiments were performed without cell sorting and confirmed that decreases in myogenin, MHC, and to a lesser degree p21, occurred following depletion of endogenous Mirk with RNAi (data not shown). Another component of the skeletal muscle differentiation program is fast twitch troponin T. The large increases in fast twitch troponin T isoforms seen after 2 and 3 days of DM treatment were reduced by half after endogenous Mirk levels were decreased by RNAi (Fig. 6B).
No decreases in MyoD were observed in multiple experiments. We conclude that depletion of endogenous Mirk by RNAi treatment prevented myoblast differentiation by blocking the induction of several muscle-specific proteins, in particular myogenin, but not MyoD. Thus Mirk must function following the commitment stage of myogenesis when MyoD is induced but prior to the induction of myogenin.

The biochemical analysis of myoblast differentiation was extended by visual examination. C2C12 cells were co-transfected with RNAi to Mirk or vector DNA along with an expression plasmid for DsRed and switched to DM. After 1 day in DM, cells with the DsRed marker were seen throughout both cultures and no myoblast fusion was seen in phase photomicrographs of the same fields (Fig. 7). However, by 5 days in DM numerous myotubes, which expressed DsRed in the vector control cultures, were observed. In contrast, the DsRed-expressing Mirk RNAi-transfected cells remained predominantly unfused single cells, with a rare small myotube. Nontransfected cells in the same culture formed normal-appearing myotubes (see phase contrast photomicrograph), demonstrating that cells in which Mirk levels were unperturbed retained the ability to undergo normal maturation, even in the presence of nonfusing cells with low Mirk levels. However, in multiple experiments, the largest myotubes were found in the control cultures. This is reasonable because in the Mirk RNAi-transfected cultures the percentage of adjacent cells capable of fusing was decreased. Cultures examined after 3 and 4 days in DM showed the same inhibition of myoblast fusion (data not shown). These biochemical and morphological studies confirm that the RhoA-induced protein kinase Mirk plays a necessary role in muscle development in these cell line models.

**DISCUSSION**

Muscle cell differentiation is mediated by muscle regulatory factors (MRFs) belonging to the basic helix-loop-helix protein family, including MyoD, Myf5, myogenin, and MRF4 (30). Only MyoD and Myf5 are expressed in dividing myoblasts, indicating that they orchestrate early steps in muscle determination. Myogenin and MRF4 are not expressed in dividing myoblasts, but their expression is induced during terminal differentiation of myotubes (reviewed in Ref. 30). The Rho family of small GTPases plays a role both in muscle determination and in later steps of muscle maturation. RhoA, together with serum response factor is required for the expression of MyoD, but the related molecules, Rac or Cdc42, cannot take the place of RhoA (3). Inhibition of RhoA has been shown to block induction of myogenin, MRF4, and muscle-specific contractile proteins, while constitutively activated forms of Rho family proteins stimulated their transcription (4). In the current study we have identified the serine/threonine kinase Mirk/dyrk1B as another RhoA-induced gene involved in muscle cell differentiation. Mirk must function following the commitment stage of myogenesis when MyoD is induced but prior to the induction of myogenin.

Induction of Mirk transcription also requires a block, or at least a diminution, of the MEK-Erk pathway. In myoblast differentiation, this block is provided by the switch from medium containing high levels of serum growth factors, to low
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Mirk is blocked by p38MAPK isoforms α, β but not isoforms γ, δ, which sequester Mirk and prevents its activation by MKK3 (17). This unusual kinase to kinase interaction suggests that Mirk and p38 (18) mediate opposite functions in myogenesis or that the activity of Mirk is temporally regulated by p38. Mirk is a member of the multigene minibrain/dyrk family. The related kinase, Yak1, acts as a growth attenuator in response to stress and nutrient condition in yeast (20). Another Mirk-related kinase, Yak1, regulates stress responses in Dictyostelium discoideum in response to nutrient starvation (12). Mirk induction in myoblasts in response to growth factor deprivation, as described in the current study, is an interesting parallel.

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