TERATOLOGY 61:305–313 (2000)

Ribonucleotide Reductase Subunit R1: A Gene Conferring Sensitivity to Valproic Acid-Induced Neural Tube Defects in Mice

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ABSTRACT Neural tube defects (NTDs), although prevalent and easily diagnosed, are etiologically heterogeneous, rendering mechanistic interpretation problematic. To date, there is evidence that mammalian neural tube closure (NTC) initiates and fuses intermittently at four discrete locations. Disruption of this process at any of these four sites may lead to a region-specific NTDs, possibly arising through closure site-specific genetic mechanisms. Although recent efforts have focused on elucidating the genetic components of NTDs, a void persists regarding gene identification in closure site-specific neural tissue. To this end, experiments were conducted to identify neural tube closure site-specific genes that might confer regional sensitivity to teratogen-induced NTDs. Using an inbred mouse strain (SWV/Fnn) with a high susceptibility to VPA- induced NTDs that specifically targets and disrupts NTC between the prosencephalon and mesencephalon region (future fore/midbrain; neural tube closure site II), we identified a VPA-sensitive closure site II-specific clone. Sequencing of this clone from an SWV neural tube cDNA library confirmed that it encodes the r1 subunit of the cell cycle enzyme ribonucleotide reductase (RNR). The abundance of *rnr-r1* mRNA was significantly increased in response to VPA drug treatment. This upregulated expression was accompanied by a significant decrease in cellular proliferation in the closure site II neural tube region of the embryos, as determined by ELISA cellular proliferation assays performed on BrdU-pulsed neuroepithelial cells in vivo. We hypothesize that *rnr-r1* plays a critical role in the development of VPA-induced exencephaly. *Teratology 61:305–313, 2000.*

INTRODUCTION

Neural tube defects (NTDs) are among the most common of all human congenital malformations, affecting 0.6 per 1,000 live births in the United States (Nakano, '73) and comparable numbers in England and Wales (Kadir et al., '99). Although easily diagnosed, NTDs possess an enigmatic etiology, most likely due to their multifactorial nature, comprised of both environmental and genetic components. Given that these malformations occur frequently and represent a significant public health problem, there is a tremendous incentive to identify genetic factors that contribute to NTD susceptibility, as well as to develop better prenatal screening methods for their detection and prevention. Despite exhaustive research efforts, little is known about the genetic mechanisms that govern neural tube closure (NTC), a complex process that occurs at four independent initiation sites in the mouse that coordinates multiple morphological and cellular events (Golden and Chernoff, '93; van Allen et al., '93). This multi-site NTC pattern provides an additional level of complexity to neural tube formation, making identification of NTD developmental origin- and tissue-specific classification critical.

Disorders of NTC involve abnormalities in the region-specific NTC junctures within the cranial and/or caudal levels of the neural tube, often resulting in the frank exposure of neural tissue. These defects range in severity, depending on the type and level of the lesion. The most severe and common of the cranial defects is anencephaly, which leads to partial or total secondary brain degeneration from a lesion caused by incomplete fusion of the neural folds in the second NTC site

Grant sponsor: National Institute of Dental and Craniofacial Research; Grant number: DE11303; Grant sponsor: National Institute of Environmental Health Sciences, National Institutes of Health; Grant number: ES07165; Grant number: P30 ES09106.

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Received 29 July 1999; Accepted 6 November 1999

(closure site II). This defect comprises approximately 50–65% of all human NTDs (Hunter, '93; Thomas et al., '94). Exposure of brain tissue without secondary degeneration is the analogous murine condition, commonly termed exencephaly. Spina bifida comprises the general category of caudal defects (below the level of T12) involving spinal cord tissue.

The anticonvulsant drug valproic acid (VPA) (Abbott Laboratories, Abbott Park, IL) has been directly implicated as a potent neural tube teratogen, producing a 1–2% spina bifida response frequency in exposed human fetuses (Lammer et al., '87). This represents a 10- to 20-fold increase in prevalence over the normal spina bifida rates observed in the general population (Bjerkedal et al., '82; CDC, '92). As only a small percentage of exposed fetuses present with spina bifida, the data suggest that those fetuses that are affected have some genetically determined predisposition that places them at increased risk of VPA-induced NTDs. In utero VPA exposure in humans also has been associated with craniofacial, cardiovascular, and skeletal defects (Bjerkedal et al., '82; Jäger-Roman et al., '86; Lindhout and Schmidt, '86), although the developing nervous system appears to be particularly sensitive to disruption after exposure to this drug.

Humans are not unique in their response to VPA, as this drug has been shown to induce exencephaly and spina bifida in rodents and other laboratory animal species (Nau and Hendrickx, '87; Finnell et al., '88). Murine model systems have been exploited in an effort to learn more about the genetic basis of susceptibility to VPA-induced NTDs. Such studies have demonstrated a strain-dependent hierarchy of NTD susceptibility to single maternal IP injections of 600 mg/kg VPA on gestational day (GD) 8:12 (8 days plus 12 hr; Finnell et al., '88). In these studies, SWV/Fnn mice demonstrated high sensitivity to exencephaly, LM/Bc/Fnn embryos demonstrated a more modest NTD response, and C57BL/6J and DBA/2J mice were completely resistant (Finnell et al., '88).

Several possible theories could explain a genetically regulated mechanism for susceptibility to VPA-induced NTDs, one of which involves the documented inhibition of folate metabolism by VPA (Wegner and Nau, '91, '92). Interference with selected steps in the folate pathway could potentially result in a decreased rate of methylation of essential, developmentally regulated genes during critical periods of embryogenesis. This would significantly enhance the sensitivity of the embryos to specific malformations. Such a difference in methylation patterns between embryos of several inbred strains might explain their differential sensitivity to VPA-induced NTDs. However, definitive interactions among folate metabolism, VPA therapy, and gene regulation remain to be documented.

The underlying pathogenesis of VPA-induced NTDs may also arise from alterations in neuroepithelial mitotic rates that drive the normal timing of neurulation. Thus, at discrete time points, VPA exposure may perturb mitosis, leading to insufficient neuroepithelial cellular proliferation that culminates in a failure of neural fold elevation and fusion. VPA exposure has been shown to inhibit the proliferation of neuronal cells in culture. At concentrations previously reported to be teratogenic to both humans and mice, VPA led to a 50% reduction in the proliferation rate of C6 glioma cells by impeding the cell cycle during the $G_{ä}$ phase (Nau and Hendrickx, '87; Martin and Regan, '91). If exposure of C6 glioma cells to VPA occurred after this specific cell cycle restriction point, the proliferation of these cells was not affected (Martin and Regan, '91). Furthermore, agents that inhibited cell proliferation in the C6 glial cell line, within twice their therapeutic dose, were consistently associated with major NTDs (Regan et al., '90). Collectively, these data illustrate the necessity for stable cellular proliferation within the developing neuroepithelia in order for NTC to occur, providing compelling evidence for a potential mechanism for VPA teratogenicity.

The present study was undertaken to identify those genetic components involved in conferring susceptibility to VPA-induced NTDs. To this end, we used a neural tube cDNA library to isolate a VPA-sensitive cDNA clone, subsequently identified as ribonucleotide reductase subunit R1 (RNR-R1), which was temporally restricted to the NTC site II region. *rnr-r1* mRNA encodes the larger of two subunits of a critical cell cycle regulatory enzyme (RNR) found in mitotically active cells. Altered function of this enzyme was previously linked to murine exencephaly (Sadler and Cardell, '77). Operating under the hypothesis that VPA-induced NTDs are caused by altered mitotic timing, we propose that altered expression of *rnr-r1* mRNA levels may contribute to VPA-induced exencephaly by decreasing the rate of neuroepithelial cellular proliferation in this targeted region of the neural tube. The data presented in this article suggest an exencephaly gene candidate, documented for the first time specifically in the closure site II region of the neural tube, which may confer sensitivity to VPA teratogenicity in the mouse.

MATERIALS AND METHODS

Teratogen treatment

Teratogenic doses of VPA were administered to pregnant SWV/Fnn dams for the cDNA library screening, genetic expression profiling, ribonucleotide protection assays (RPAs), and bromodeoxyuridine (BrdU) studies. No fewer than five dams were randomly assigned to each treatment group. Sodium valproate was dissolved in distilled water immediately before use and administered in volumes of 0.1 ml/10 g body weight. The dams received two intraperitoneal injections on GDs 8:12 and 8:18, consisting of either VPA (600 mg/kg) or distilled water. This VPA treatment regimen induced an exencephalic response frequency of 99.8%.

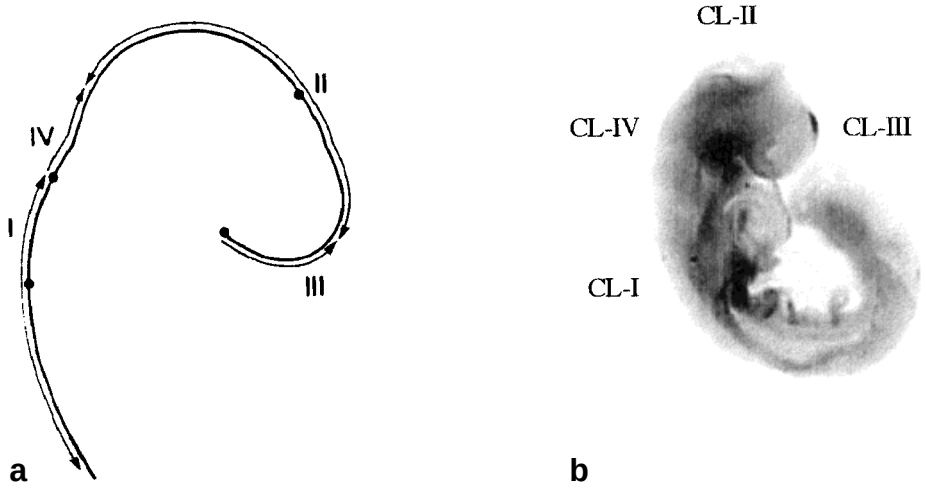

Fig. 1. a: Representative neural tube closure regions along the dorsal axis of a developing murine embryo. Right lateral view of the embryo, with sites and directions of closure indicated along the dorsal aspect. For closure site dissections, SWV/Fnn murine embryos were collected during peak activity of closure site II (gestational day [GD] 9:0). Neural tube tissue regions I, II, III, and IV were isolated from the areas indicated along the dorsal neural tube. **b:** Gestational day 9:0 SWV embryo lacking complete closure at site II (CL II).

Embryo collection and morphological staging

Pathogen-free, virgin females, 50–70 days of age were mated overnight with experienced males, and the dams were checked for the presence of a vaginal plug the next morning. The start of gestation was set at 10 p.m. of the previous evening (GD0), the midpoint of the light/dark cycle (Snell et al., '48). For the cDNA library construction, untreated control embryos were collected at GDs 8:12, 9:0, 9:12, 10:0, and 10:12, spanning the entire period of NTC. For the subsequent experimental procedures (differential cDNA library screening, genetic expression profiling, RPA, and BrdU assays), untreated control and VPA-treated embryos were collected at GD 9:0, the period representing peak NTC activity at closure site II. The pregnant dams were sacrificed by cervical dislocation, the abdomen opened, and the uterine contents removed. The location of all viable embryos and resorption sites was recorded. Watchmaker's forceps were used to dissect the embryos free of the decidual capsule, the chorion, and amnion, while in cold phosphate-buffered saline (PBS), pH 7.4, under a Wild M8 dissecting microscope (Heerbrugg, Switzerland). The gross morphology of the embryos was examined, and they were classified as to their stage of NTC, using previously described standardized staging criteria (Cole and Trasler, '80).

Removal of neural tube from NTC stage embryos

With the aid of watchmaker's forceps, the neural tissue was carefully isolated from the supporting paraxial mesodermal tissue under the dissecting microscope (Stemple and Anderson, '92, Taylor et al., '95). For the generation of radiolabeled probes for the primary and secondary differential screening procedures, and for performing the BrdU assays, neural tube tissue collection included (1) the area encompassing the closure site II region, and (2) the remainder of the neural tube (closure regions I, III, and IV) (Fig. 1). Probes used in the genetic expression profiles were made exclusively from tissue collected from the closure site II region of the neural tube. For the RPA procedures, tissue collection included the dorsal cranial region including, but not exclusive to, the closure site II region.

Total cellular RNA isolation

Cellular RNA was extracted from neural tube tissue for the cDNA library construction following the guanidinium thiocyanate method (Chomzynski and Sacci, '87), and with TriPure isolation reagent (Boehringer-Mannheim, Indianapolis, IN) for the RPA procedures. For cDNA library construction, total RNA was isolated from pooled tissue representing 358 nontreated neural tubes collected from SWV/Fnn embryos between GDs 8:12 and 10:12. Equivalent neural tube tissue amounts were obtained for each of the four active NTC stages. The number of somite pairs ranged from 0 to 25, with 0–8 representing embryos primarily in NTC I, 8–12 representing embryos primarily in NTC II, 13–15 representing embryos primarily in NTC III, and 15–25 representing embryos primarily in NTC IV. These somite numbers were used as a collection criterion for the embryos at each of the specified gestational time points.

For the RPA procedures, total RNA was isolated from no fewer than 10 dorsal cranial neural tubes from untreated control and VPA-treated SWV/Fnn embryos for each of the three RPA replications. The dorsal

cranial neural tube region was chosen for these experiments because obtaining adequate amounts of tissue specifically from closure site II for each RNA isolation procedure was not feasible. Furthermore, since the differential screening and genetic expression profiling procedures determined closure site II expression of RNR-R1, and the objective of RPA was to verify altered levels of expression of this clone, it was reasoned that strict NTC site specificity was not vital to these particular experiments.

mRNA isolation

mRNA for cDNA library construction was recovered by column chromatography from the total cellular RNA after five cycles of adsorption and elution from an oligodeoxythymidylate [oligo(dT)]-cellulose column, precipitated using ethanol and 3M sodium acetate (NaOAC), and lyophilized. A λ cDNA library construction was performed using a Zap II cDNA synthesis kit (Stratagene, La Jolla, CA), with some minor modifications to the protocols provided.

Antisense RNA probes

Radiolabeled amplified anti-sense RNA (aRNA) probes were generated from the two untreated tissue sources described above (closure site II and the remaining neural tube) (Eberwine et al., '92). For the gene expression profiling procedures, aRNA probes were generated exclusively from closure site II tissues. cDNA templates were covalently coupled to reusable magnetic porous glass (MPGLCA) beads (CPG, Lincoln Park, NJ) to permit the generation of multiple aRNA probes from each sample for the screening and expression profiling procedures, as well as the reduction of variability associated with sampling error. This procedure entailed priming the poly(A)$^+$ RNA population from each of the tissue sources with an oligo(dT)+T7 primer sequence (5′-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC$(T)_{24}$-3′) attached to the MPGLCA beads, following the manufacturer's protocols. First- and second-strand cDNA synthesis and probe generation utilized in situ transcription and anti-sense RNA amplification (IST/aRNA) procedures as described previously (Eberwine et al., '92). The resulting cDNA templates were used to produce aRNA by the addition of T7 RNA polymerase (Epicentre Technologies, Madison, WI) in the presence of nonradiolabeled dNTPs (2.5 mM dATP, dTTP, and dGTP and 100 μM dCTP) and 2 μl [^{32}P] αCTP (3,000 Ci/mmol). The aRNA from each of the two tissue sources was generated from 10 cDNA samples, each of which represented no fewer than five litters of mice.

Membrane preparation and probe hybridization

For the cDNA library screening procedures, Hybond-N$^+$ nylon membranes (Bio-Rad, Richmond, CA), lifted against the SWV/Fnn neural tube cDNA library were prehybridized at 50°C for 2 hr in the following: 2 × 1,4-piperazine-diethanesulfonic acid (PIPES) buffer, 50% deionized formamide, and 0.5% (w/v) sodium dodecyl sulfate (SDS) and hybridized overnight at 42°C in the same solution containing 100 μg/ml denatured sonicated salmon sperm DNA and 1×10^6–5×10^6 cpm of radioactive probe (aRNA probes from closure sites I, III, and IV, or closure site II) per ml. The membranes and probes were hybridized overnight (approximately 12–14 hr) at 42°C with rotation. After hybridization, the membranes were washed three separate times, each for 15–30 min with 0.1× SSC buffer and 0.1% (w/v) SDS solution at 65°C with rotation. The membranes were exposed to X-OMAT AR film (Eastman Kodax, New Haven, CT).

Differential clone selection

Selection of closure site II-specific clones was accomplished by superimposing autoradiographs from the closure site II membrane lifts onto those derived from the remaining neural tissue (closure sites I, III, and IV). Clones representing common positive hybridization to transformants from both tissue sources were disregarded in favor of clones with unique hybridization to closure site II transformants. Plaques representing these clones were selected for further characterization. A secondary library screen was performed on the replated plaques, in order to reduce background contamination by adjacent transformants. Following the secondary screening procedures, selected plaques were subcloned by using ExAssist helper phage (Stratagene) to excise the pBluescript phagemid, according to the provided protocols. The identity of each clone was confirmed by DNA sequencing.

Screening of clones with VPA

Genetic expression profiling procedures were performed to identify closure site II clones that were sensitive to VPA exposure. The aRNA obtained from the untreated control and VPA-treated closure site II tissue was hybridized to the closure site II clones that were immobilized to nylon membranes, in order to quantify relative changes in gene expression as previously described (Eberwine et al., '92). After construction, these membranes were washed three times with increasingly stringent solutions (final solution contained 0.1× SSC and 0.1% SDS at 42°C), dried, wrapped in plastic wrap, exposed to a phosphorimaging plate, and stored in the dark for approximately 2 hr. The exposed plate was subsequently imaged by a Fujix Bas2000 Phosphorimaging System (Fuji Medical Systems, Stamford, CT). The values for each slot of the imaged arrays were generated with a MacBass System (Fuji Medical Systems) for statistical analysis. The individual signal values were normalized to the expression of cyclophilin gene, thus enabling comparisons between different arrays. This cDNA was selected as an internal standard because it is expressed constitutively and is found in high abundance in the mouse brain, thymus and embryo (Danielson et al., '88). These qualities make cyclophilin an excellent internal standard for use in

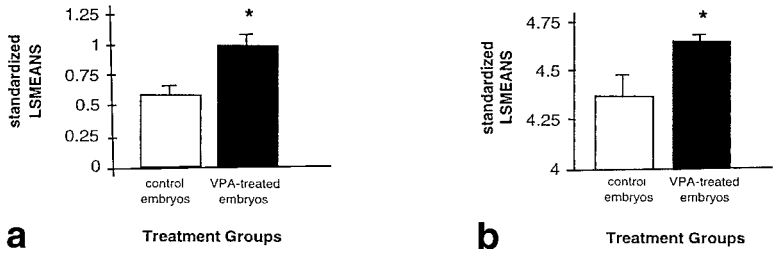

Fig. 2. VPA-induced alterations in RNR-R1 gene expression at GD 9:0 as determined by genetic expression profiling (a) and ribonuclease protection assays (b). The expression of RNR-R1 in closure site II tissue (**a**) or cranial neural tube tissue (**b**) obtained from embryos under control conditions (unfilled bars) and teratogenic VPA treatment (filled bars) are indicated as normalized mean cpm values. *Significant differences from control values. The increase of VPA-treated versus control RNR-R1 expression in the genetic expression profiles is 39.5% (a), while that in the RPA is 44.5% (b).

multiprobe assays, when examining low abundance messages.

Ribonucleotide protection assay procedure

For these experiments, anti-sense riboprobes were synthesized. An RPA II™ kit (Ambion) was used, with minor modifications to the provided protocols. Approximately 1×10^4–1×10^5 cpm of the purified *rnr-r1* and 28S riboprobes, obtained by the in vitro transcription reactions, were added to the total RNA isolated from embryonic neural tissue from both of the treatment groups. The co-precipitation reactions underwent electrophoresis. The RPA gel was then exposed to X-OMAT AR film (Kodak) for approximately 12 hr. Three replicate experiments were conducted on three independently isolated RNA samples from the treatment groups. Values for the hybridization signals representing the protected fragment bands were generated using MacBass System (Fuji Medical Systems) and standardized to 28S for subsequent statistical analysis.

Bromodeoxyuridine pulse labeling and detection procedures

Pregnant SWV/Fnn dams were injected with VPA or saline at GDs 8:12 and 8:18, followed by a single-bolus intraperitoneal injection of 5-bromo-2′-deoxyuridine (BrdU, 100 µg/gm body weight) in sterile H_2O at GD 9:0. For this study, BrdU incorporation was only measured in the developing neuroepithelia of control and VPA-exposed embryos. However, closure site II was isolated from the remaining neural tissue (closure sites I, III, and IV) and analyzed separately using an enzyme-linked immunosorbent assay (ELISA). Changes in cell proliferation were quantified using a colorimetric ELISA assay for incorporated BrdU, using a kit (5-Bromo-2′-deoxy uridine Labeling and Detection Kit III-Boehringer Mannheim) and the manufacturer's instructions. Briefly, culture medium was aspirated and the cells fixed for 30 min with 200 µl/well of the kit-supplied fixation/denaturation solution. Cells were incubated for 120 min with anti-BrdU antibody conjugated to peroxidase, 100 µl/well. Cells were washed and then exposed to chromogenic substrate solution (tetramethylbenzidine) for 30 min. The absorbance was measured at 340 nm against a reference wavelength of 490 nm, using a microtiter plate reader (ELx808, Biotek Instruments). The values were standardized to embryonic somite number and tissue weight. These assays were performed in triplicate, using 10 dams and 10 embryos from each litter for each treatment group.

Statistical analysis

Simple statistical tests were performed to determine treatment differences at GD 9:0 for the closure site II clones, in all the above experimental procedures. These comparisons were evaluated by analysis of variance (ANOVA) and by the least-square means (LSMEANS) option in the general linear models (GLM) procedure. Statistical significance for analyses was set at the $\alpha = 0.05$ ($P < 0.05$) level.

RESULTS

The primary library screen resulted in the isolation of 85 closure site II-specific clones, each of which was subjected to secondary library screens of approximately of 5×10^4 pfu. The secondary screening procedures indicated that, on average, 0.5% of the plaques were positive for closure site II hybridization. DNA sequencing reactions and similarity comparisons of these clones revealed representation of several regulatory gene groups. Gene expression profiling studies established statistically significant alterations ($P < 0.05$) for six of the 85 closure site II clones. Two of these clones were novel, as determined by sequence similarity searches, and are being characterized further. Of the remaining four clones, only one, ribonucleotide reductase subunit R1 (RNR-R1), was an attractive candidate gene from a developmental perspective. The RNR-R1 cDNA sequence encodes a developmentally regulated mRNA, which was significantly upregulated (by approximately 67%) in response to VPA exposure, as compared with controls (Fig. 2a). This sequence demonstrated 100% similarity to the published murine cDNA sequence, and 83% similarity to the human *rnr-r1* cDNA sequence.

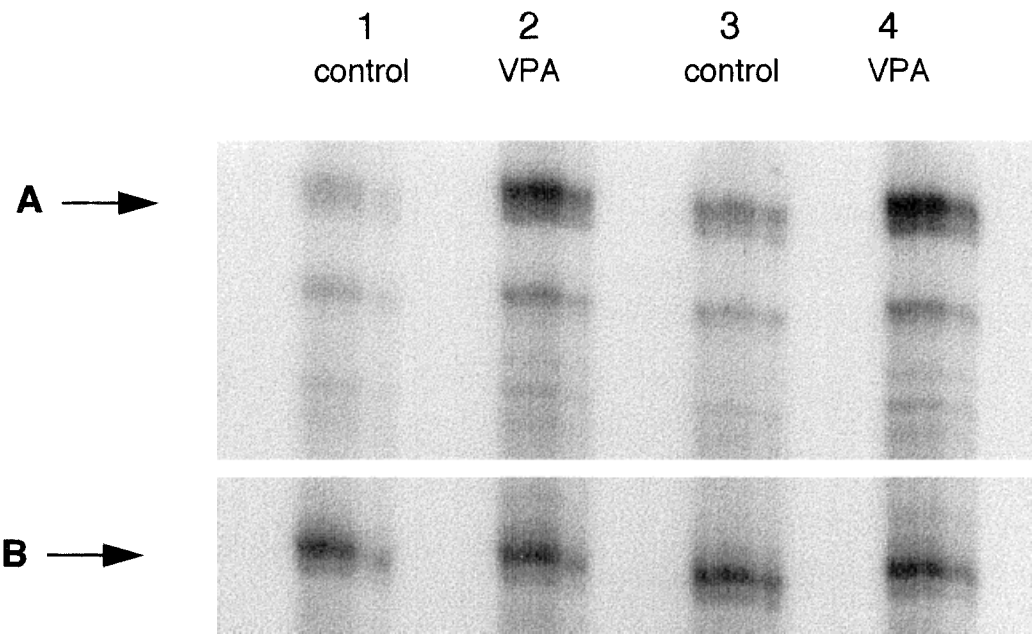

Fig. 3. Representative ribonuclease protection assay (RPA) gel. For this assay, an in vitro transcribed antisense RNA probe was generated from the cloned 3′ cDNA of the rnr-r1 transcript and hybridized against approximately 10 μg of total RNA from untreated control and VPA-treated SWV/Fnn anterior neural tissue. **A:** The rnr-m1 protected fragment (~220 bp). **B:** Expression of the 28S ribosomal subunit, included as an internal control for RNA loading (115 bp). **Lanes 1,3**, rnr-r1 expression for the untreated control neural tissue; **lanes 2,4**, rnr-R1 expression for the VPA-treated neural tissue. A total of three separate RPAs were conducted.

Since *rnr-r1* represented the only developmental sequence obtained from the library screen, we focused our efforts on learning about its role in VPA-mediated alterations in neural tube cell cycling.

The RPAs consistently verified the genetic expression profiling results by demonstrating a statistically significant upregulation of *rnr-r1* levels in response to teratogenic doses of VPA, as compared with controls in the developing neural tube tissue ($P < 0.05$) (Fig. 2b). A representative RPA gel is illustrated in Figure 3.

Given that altered RNR enzyme activity has been linked previously to NTDs (Sadler et al., '77), and because of its critical role in cell cycle maintenance, we sought to determine whether VPA treatment could lead to decreased mitotic activity in closure site II tissue. This would suggest a role for RNR subunit expression in the development of VPA-induced exencephaly. This possibility was tested by the use of a BrdU cell proliferation determination assay, the results of which indicated a statistically significant decrease in cellular proliferation in the VPA-exposed closure site II tissue ($P < 0.05$) (Fig. 4). A slight but nonsignificant decrease in the proliferation rate was observed in the remainder of the neural tube tissue (closure sites I, III, and IV) from VPA-exposed embryos, when compared to their corresponding controls ($P > 0.05$) (Fig. 4). In addition, there was no significant difference in cellular proliferation levels between the untreated control closure site II region and caudal neural tissue ($P > 0.05$), indicating substantial cell cycle activity in the closure site II region in control samples.

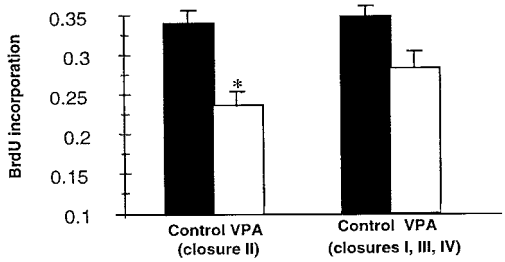

Fig. 4. Bromodeoxyuridine (BrdU) incorporation for cellular proliferation enzyme-linked immunosorbent assay (ELISA). Incorporation is represented by mean immunofluorescence values. Comparisons were made between untreated control and VPA-treated region-specific neural tube tissue from the SWV/Fnn embryos. *Significant mean difference ($P < 0.05$), as compared with controls.

DISCUSSION

This study demonstrates that teratogenic doses of VPA administered to SWV/Fnn dams during critical periods of embryonic development resulted in both the upregulated expression of ribonucleotide reductase subunit R1 mRNA, and a concomitant decrease in neuroepithelial cellular proliferation in the closure site II neural tube region of the embryos. The abundance of *rnr-r1* mRNA was significantly increased, as determined by genetic expression profiling and RPA procedures ($P < 0.05$) (Fig. 2). This finding was accompanied by a significant decrease in cellular proliferation in the closure site II neural tube region of the embryos, as determined by ELISA cellular proliferation assays

(Boehringer Mannheim) performed on BrdU pulsed neuroepithelial cells in vivo ($P < 0.05$) (Fig. 4). We hypothesize that *rnr-r1* plays a critical role in the development of VPA-induced exencephaly.

The function of RNR activity in cell proliferation has been well established, with a primary role in reducing all four ribonucleoside diphosphates to their respective deoxyribonucleoside diphosphates, before their incorporation into DNA (Thelander and Reichard, '79). Thus, this enzyme coordinates DNA synthesis, DNA repair, and cellular proliferation in mitotically active tissues (Hurta and Wright, '95). The structure and function of *rnr* mRNA are conserved across almost all species and are expressed in most mammalian tissues, supporting an important role for this gene product in cellular function. Given the function of RNR in DNA synthesis and cellular proliferation, it is not surprising that RNR activity is highly regulated throughout the cell cycle, virtually absent in nonproliferating and/or quiescent cells (Wright et al., '90). The enzyme is composed of two nonidentical protein subunits (R1 and R2) that are distinct in size and regulation as well as protein and gene structures. The activities of the subunits are also distinct, as the large effector binding subunit, RNR-R1, is solely responsible for the allosteric feedback regulation of enzyme activity, while the smaller subunit, RNR-R2 contains the tyrosyl radicals necessary for catalysis (Larsen et al., '92).

Regulation of the *rnr-r1* and *rnr-r2* subunit mRNAs occurs at both the transcription and post-transcriptional levels. Tied to the post-transcriptional regulatory system of RNR is a downstream stoichiometric effect that represents a novel control point for malignancy determination, which might possibly influence embryogenesis. Specifically, this mechanism involves deregulation of subunit-specific protein binding at the 3′ UTR of either *rnr-r1* or *rnr-r2*. This effect leads to a disruption in the balance of subunit mRNA abundance and protein ratios, decreased RNR holoenzyme formation and function, and suppression of tumor growth (Huizhou et al., '96). For example, it has been shown that overexpression of *rnr-r1* leads directly to marked tumor suppression through decreased cellular proliferation, as demonstrated in mouse 10 T1/2 cells, malignant mouse RMP-6 cells, human HeLa cells, and BALB/c nu/nu mice (Fan et al., '96, '97). A similar effect was observed in the present study, whereby overexpression of *rnr-r1* was correlated with a significant decrease in neuroepithelial cellular proliferation secondary to VPA exposure. Although VPA has not been considered as a cancer therapeutic agent, nor has it been previously shown to alter *rnr* mRNA expression, its antiproliferative effects have been documented in human neuroblastoma cells, in which VPA dramatically suppressed tumorigenicity, decreased expression of oncoproteins, and induced differentiation and apoptosis (Cinatl et al., '96). Furthermore, the cell cycle arresting effects of VPA have been correlated with a concomitant onset of differentiation in cell lines and in the developing neural tube (Courage-Maguire et al., '97). Therefore, the identification of the RNR 3′ UTRs as regulators of biological characteristics may have important medical implications for abnormal embryonic development, and possibly VPA-induced exencephaly.

Several drugs that adversely affect cell proliferation are known to have significant teratogenic potentials. The anticancer drug, hydroxyurea, has been shown experimentally to induce abnormal embryonic development through an RNR-mediated premature cell cycle arrest. One such study performed in mouse embryos revealed a wide range of hydroxyurea-induced exencephalic response frequencies (4–90%), and associated necrotic or apoptotic neuroepithelial cell death in the cranial neural tube region (Sadler and Cardell, '77). Although the mechanism of hydroxyurea-induced exencephaly remains unknown, cancer studies suggest that this drug targets RNR subunit mRNA stability through a PKC-mediated post-transcriptional modification of *rnr-r1* expression levels. This is thought to occur by a hydroxyurea-induced disruption of the binding between the ribonucleotide reductase R1 mRNA binding protein and the rnr-r1 mRNA (R1BP-RNA) in mammalian cells, leading to mRNA degradation and an imbalance of *rnr* subunit mRNA and protein levels (Chen et al., '94). This tumor-suppression effect may play a similar role in embryogenesis, during which significant decreases in RNR enzyme activity may cause an inappropriate or premature reduction in the rates of cellular proliferation.

The expression of *rnr-r1* may be affected by VPA through one or both of the transcriptional and post-transcriptional regulatory control mechanisms mentioned above. VPA has been shown to interact with PKC activity, and PKC-associated molecules such as the cyclic nucleotides and the activator protein 1 (AP-1) in murine neural tissues and cell lines, in a manner similar to lithium (Ferrendelli and Kinscherf, '79; Ogawa et al., '84; Babcock-Atkinson et al., '89; Nosek, '85; Manji and Lenox, '94; Chen et al., '94, '96; Lenox et al., '96). Furthermore, since activated PKC translocates to the nucleus to phosphorylate a number of protein transcription regulators, including AP-1, in a cell cycle-dependent manner, it follows that VPA can induce selective regulation of gene expression (Boulikas, '95). These effects were not directly observed in the present study. However, VPA has been shown to alter the transcription of AP-1-affiliated genes (*Tgfβ-1*, c-*fos*, and c-*jun*) in the neuroepithelium of developing SWV/Fnn embryos (Finnell et al., '97). At least one of these genes (*Tgfβ-1*) regulates the transcription of *rnr-r1*. In addition, VPA has been shown to modulate the PKC-specific myristoylated alanine-rich C kinase substrate (MARCKS) in hippocampal cells (Lenox et al., '96; Watson et al., '98). Disruption of MARCKS and the MARCKS-like protein MacMARCKS, induced high frequencies of murine exencephaly (35%), as well as associated anomalies, suggesting a direct role for PKC

in VPA-induced NTDs (Slack and Tannahill, '92; Blackshear et al., '96; Chen et al., '96).

In conclusion, we suggest that the *rnr-r1* gene is an excellent candidate for determining NTD susceptibility in SWV/Fnn embryos. Whether the observed VPA-induced upregulation in *rnr-r1* was due to increased mRNA expression or to increased mRNA stability is uncertain and, without having determined protein levels, it is not yet possible to state conclusively that overexpression of this gene directly inhibited neuroepithelial cellular proliferation. However, taken in the light of the existing literature, our data do suggest two scenarios by which a VPA-induced overexpression of *rnr-r1* mRNA and decreased closure site II cellular proliferation may culminate in the observed exencephalic lesions. The first possibility is that increased *rnr-r1* mRNA abundance is caused by increased *rnr-r1* stability triggered directly by VPA through a PKC-mediated post-transcriptional disruption in R1BP/r1mRNA binding at the 3′ UTR. The second possibility is that increased *rnr-r1* mRNA abundance is caused by *rnr-r1* mRNA overexpression triggered indirectly by VPA as a consequence of altered expression levels of *Tgf*β*-1* via PKC and AP-1. This latter effect would have a negative impact on the availability of this growth factor for binding to a *Tgf*β*-1* recognition site in the RNR-R1 promoter region. Either of these situations could potentiate an imbalance of *rnr* subunit mRNA, such that *rnr-r1* overexpression, relative to *rnr-r2* levels, leads to a subsequent decrease in closure site II cell proliferation via decreased RNR enzyme function. In either case, our findings concur with those of previous studies that observed RNR- and VPA-induced tumor suppressing and anti-proliferative capabilities, and provide a potential link between VPA, RNR and exencephaly.

ACKNOWLEDGMENTS

The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS. The authors express their appreciation to Dr. James H. Eberwine, University of Pennsylvania, and to Ms. M. Davda, for their helpful advise and technical expertise.

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