A systems-based computational model for dose-response comparisons of two mode of action hypotheses for 
etanol-induced neurodevelopmental toxicity

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Abstract

Investigations into the potential mechanisms for ethanol-induced developmental toxicity have been ongoing for over 30 years since Fetal Alcohol Syndrome (FAS) was first described. Eurodevelopmental endpoints are particularly sensitive to in utero exposure to alcohol as suggested by the more prevalent alcohol-related neurodevelopmental disorder (ARND). The inhibition of proliferation during neurogenesis and the induction of apoptosis during the period of synaptogenesis have been identified as potentially important mechanisms for ARND. However, it is unclear how these two mechanisms quantitatively relate to the dose and timing of exposure. We have extended our model of neocortical neurogenesis to evaluate apoptosis during synaptogenesis. This model construct allows quantitative evaluation of the relative impacts on neuronal proliferation versus apoptosis during neocortical development. Ethanol-induced lengthening of the cell cycle of neural progenitor cells during rat neocortical neurogenesis (G13-G19) is used to compute the number of neurons lost after exposure during neurogenesis. Ethanol-induced dose-dependent increases in cell death rates are applied to our apoptosis model during rat synaptogenesis (P0-P14), when programmed cell death plays a major role in shaping the future neocortex. At a human blood ethanol concentration that occurs after 3-5 drinks (~150 mg/dl), our model predicts a 20-30% neuronal deficit due to inhibition of proliferation during neurogenesis, while a similar exposure during synaptogenesis suggests a 7-9% neuronal loss through induction of cell death. Experimental in vitro and in vivo dose-response research and stereological research on long-term neuronal loss after developmental exposure to ethanol is compared to our model predictions. Our computational model allows for quantitative, systems level comparisons of mechanistic hypotheses for perturbations during specific neurodevelopmental periods.

Key words: computational model, ethanol, fetal alcohol syndrome, neurogenesis, apoptosis, neocortex
Introduction

Scientists have been investigating potential mechanisms for ethanol-induced developmental toxicity for over 30 years since Fetal Alcohol Syndrome (FAS) was first described (Jones and Smith 1973). Since then, researchers have discovered a dose-response relationship between alcohol consumption during pregnancy and a spectrum of disorders, with more subtle neurodevelopmental effects, termed alcohol-related neurodevelopmental disorder (ARND), occurring at the lowest exposure levels (Sampson et al. 1997). When FAS and ARND diagnoses are combined, it is estimated nearly 1 in every 100 live births is affected by alcohol exposure in utero (Sampson et al. 1997), making it a serious public health concern. Although the specific underlying mechanism of ethanol-induced CNS deficits is still a mystery, research advances in both normal and perturbed neurodevelopment have narrowed the possibilities to important modes of action (Goodlett and Horn 2001). In particular, the inhibition of proliferation during neurogenesis and the induction of apoptosis during the period of synaptogenesis have been identified as particularly important toxic endpoints for ARND (Ikonomidou et al. 2000; Miller 1986). However, it is unclear how these two modes of action quantitatively relate to the dose and timing of exposure.

A systems biology approach to neurodevelopmental research will greatly enhance our understanding of mechanisms of normal neurodevelopmental processes and perturbations that may lead to neurodevelopmental disorders such as ARND (Andersen et al. 2005; Cummings and Kavlock 2005). This approach is meant to provide quantitative models to integrate the growing amounts of molecular, cellular, anatomical, and behavioral data that is being generated (Kitano 2002; Waters et al. 2003). Here we develop computational models utilizing quantitative experimental data at the cellular level, specifically describing cell cycle kinetics and cell death in the developing brain. Our model links effects at the cell level to effects at the organ level by simulating neuron number acquisition in the adult through production and death of developing neurons in the developing organism.
This construct can also serve as a foundation for future application of data at the molecular and behavioral levels. Our model construct is based on the hypotheses that rapidly dividing, differentiating and dying cells within a developing organ represent a sensitive target for environmental insults (Faustman et al. 1999; Leroux et al. 1996). This framework is especially relevant for neurodevelopment, in which numerous experimental perturbations have shown disruption occurring during the discrete periods of neurogenesis, migration and synaptogenesis will result in specific malformations (Berger-Sweeney and Hohmann 1997; Monk et al. 2001; Rice and Barone 2000; Rodier 2004; Zamorano and Chuaqui 1979). For example, disruption of neurogenesis will most likely result in overall reduction of cell number. Manipulations that interrupt cell migration will likely result in ectopias, or abnormal locations of neurons; whereas manipulations that interrupt differentiation signals during synaptogenesis will likely result in apoptosis, or abnormalities in the connectivity of neurons. Considering synaptogenesis, a computational approach has been used to look at the in vitro effects of ethanol on the differentiation of neurons measured by dendritic growth, suggesting branching rather than elongation of dendrites is compromised (Granato and Van Pelt 2003).

Reduction in neuronal number is a salient feature of ethanol-induced developmental neurotoxicity (Maier and West 2001). Stereological investigations in animal models have attempted to characterize the long-term effects on cell number in sensitive brain regions, such as the neocortex, hippocampus, and cerebellum (Maier et al. 1997; Miller 1995, 1996; Miller and Potempa 1990; Mooney et al. 1996; Napper and West 1995; West et al. 1986). By focusing the exposure period to correspond with the most susceptible period for the particular brain region of interest, these studies have shown different developmental processes are more or less sensitive to ethanol exposure depending on the brain region and time of exposure (Gohlke et al. 2002).

Based on a review of stereological studies (Gohlke et al. 2002), we have focused on neocortical development as a sensitive target of ethanol induced neurotoxicity, starting with the generation of neurons, their subsequent migration to permanent locations within the cortex, and finally the formation of synapses and a period of naturally occurring programmed cell death (PCD). The rat neocortex is
particularly sensitive to neuronal loss following a relatively low exposure (approx. 150 mg/dl peak Blood Ethanol Concentration (BEC) which would be achieved in a pregnant woman after having 3-5 drinks) during the earlier periods of development including neurogenesis and migration (Miller and Potempa 1990). We have previously constructed computational models for normal neocortical development that simulates acquisition of adult neuron number through neurogenesis and synaptogenesis in the normal rat and mouse, corroborated by independent, stereologically determined neuron number data in the adult rodent (Gohlke et al. 2002; Gohlke et al. 2004).

Ethanol may cause neocortical developmental anomalies through inhibition of neuronal (Luo and Miller 1998) and glial proliferation (Guerri et al. 1990; Guizzetti and Costa 1996), induction of apoptosis (Dunty et al. 2001; Ikonomidou et al. 2000; Ward and West 1992), and perturbations during migration and synaptogenesis (Guerri 1998; Ward and West 1992). Various studies have shown ethanol to be a potent inhibitor of cellular proliferation, particularly in the developing brain (Pennington et al. 1984) (Dreosti et al. 1981; Laev et al. 1995). Effects seen include a reduction in the proliferating population or growth fraction (GF), and an increase in the length of the cell cycle, both contributing to fewer numbers of young neurons being generated (Guizzetti and Costa 1996; Miller 1989, 1992; Miller and Kuhn 1995). No increases in pyknotic cells have been detected when exposure occurs during neurogenesis, suggesting again that inhibition of proliferation in the progenitor population is the target (Miller and Muller 1989). When we applied ethanol-induced cell cycle perturbations to our neocortical neurogenesis model, our simulations accurately predicted independent stereological evidence of long-term neocortical neuronal loss after an in utero exposure of 150 mg/dl peak BEC per day in the rat (Gohlke et al. 2002).

Exposure to ethanol has also been shown to cause alterations in the natural waves of PCD during synaptogenesis, which occurs postnatally in the rat or during the 3rd trimester in humans (Climent et al. 2002; Ikonomidou et al. 2000). Ikonomidou et al. (2000) suggests that by blocking NMDA glutamate receptors and activating GABA receptors, ethanol triggers widespread increases in apoptosis during the period of synaptogenesis of many brain regions including the hippocampus, thalamus and frontal, parietal, cingulate, and retrosplenial cortex. A discreet window of time, coinciding with the synaptogenesis period
of each region tested, occurring anywhere from E19 to P14 depending on the region, was found to be the most susceptible period (Ikonomidou et al. 2000). Cell death was measured by DeOlmos silver staining in this study and was confirmed with Caspase 3 activation in a more recent study (Olney et al. 2002). Twenty-four hours after a subcutaneous injection of ethanol (2.5 g/kg x 2) resulting in a peak BEC of 500 mg/dl, forebrain tissue contained 15 times the amount of apoptotic, silver stained neuronal cells of control tissue (Ikonomidou et al. 2000). Different acute exposures producing diverse blood ethanol profiles were also analyzed and a dose-response relationship suggesting doses producing a peak BEC of 200 mg/dl or above for more than 4 hours can significantly increase apoptotic neurons. The effect became progressively more severe in proportion to the length of time the BEC exceeded 200 mg/dl (Ikonomidou et al. 2000). An earlier wave of apoptosis was subsequently identified using Caspase 3 activation at lower peak BECs of approximately 120 mg/dl (Olney et al. 2002; Olney et al. 2004; Tenkova et al. 2003). Caspase 3 is a key component in a series of caspases that are activated in apoptosis to carry out the programmed degradation of proteins and DNA within the cell. Caspase 3 activation is frequently used to identify toxicant-induced apoptosis (Robertson and Orrenius 2000), whereas DeOlmos silver stain does not differentiate between apoptosis and other forms of cell death, such as necrosis.

The differential contribution of each of these mechanisms, namely inhibition of proliferation and induction of cell death, to the final spectrum of neurodevelopmental disorders attributed to in utero exposure to ethanol remains to be elucidated. Therefore, the construct of our computational model allows for direct quantitative linkage of these cellular mechanisms to a final outcome on neuronal number in the adult, and can estimate the potential relative contributions of these mechanisms across dose ranges and developmental life stages.

The research presented in this paper builds upon the mechanistic work described above and quantitatively evaluates ethanol-induced cell death in the developing neocortex using a computational modeling approach. Our extended computational model for normal neocortical development includes PCD during the period of neocortical synaptogenesis, postnatal day 0 to postnatal day 14 (P0-P14) in the rodent and is described elsewhere (Gohlke et al. 2004). We apply data of ethanol-induced cell death
during the synaptogenesis period using both DeOlmos silver staining and Caspase 3 activation to identify dying cells in the rat to our model of neocortical development (Ikonomidou et al. 2000; Tenkova et al. 2003). We compare our simulations with those of our previous model looking at exposure during neocortical neurogenesis (G13-G19) (Gohlke et al. 2002). To further evaluate our mode of action based dose-response functions for ethanol-induced perturbations of proliferation and cell death, we also compare them to independent in vitro and in vivo studies. The resultant biologically based dose response (BBDR) models can facilitate improvements in risk assessment practices by quantitating the relative importance of temporally specific effects during the critical stages of neocortical neurogenesis and synaptogenesis.

Methods

Our model framework including neocortical neurogenesis and programmed cell death (PCD) during the synaptogenesis period is illustrated in Figure 1. Our generalized stochastic model construct has been described in detail previously (Leroux et al. 1996). The key model parameters include cell cycle rates ($\lambda$), differentiation rates ($\nu$), and cell death rates ($\mu$) within a critical time period specific to the cell population, organ, or tissue of interest (Fig. 1E). The mathematical construct tracks the X and Y cell populations through time using probabilities of transition from one state to another (i.e. division, transformation or death), using a Kolmogorov forward equation:

$$DP(x,y,t)/dt = (x - 1)\lambda_1(t)P(x - 1,y,t) + (x + 1)\mu_1(t)P(x + 1,y,t) + (y - 1)\lambda_2(t)P(x,y - 1,t) + (y + 1)\mu_2(t)P(x,y + 1,t) + (x + 1)\nu(t)P(x+1, y-1,t) - [x\lambda_1(t) + x\mu_1(t) + y\lambda_2(t) + y\mu_2(t) + x\nu(t)] P(x,y,t)$$

The initial time $t_0$ represents the beginning of the particular developmental process under study, so that the number of type Y cells initially present will be $y_0 = 0$. When the number of X cells
initially present is large, the distribution of \((X(t), Y(t))\) is approximately bivariate normal by the central limit theorem. Therefore, the moments, or the numbers of \(X\) and \(Y\) cells at time \(t\) can be derived through a first-order differential equation and can be approximated through a solution matrix:

\[
A(t) = \begin{pmatrix}
\varepsilon_1(t) & 0 & 0 & 0 \\
\nu_1(t) & \varepsilon_2(t) & 0 & 0 \\
\lambda_1(t) + \mu_1(t) + \nu_1(t) & 0 & 2\varepsilon_2(t) & 0 \\
\nu_1(t) & \lambda_2(t) + \mu_2(t) & 0 & \nu_1(t) \\
-\nu_1(t) & 0 & \nu_1(t) & \varepsilon_1(t) + \varepsilon_2(t)
\end{pmatrix}
\]

*Model of neocortical development*

In this application, the \(X\) cell represents progenitor cells in the ventricular epithelium where one of three outcomes is possible: division, differentiation into a \(Y\) cell, or death (Figure 1E) whereas the \(Y\) cell has two potential outcomes: division or death. For our neocortical model, the \(Y\) division rate is set to zero, as \(Y\) cells are defined as postmitotic neurons migrating to the cortical plate and subsequently differentiating. Therefore, the founder cell population of neurons in the neocortex is tracked through neurogenesis and synaptogenesis simulating the acquisition of final adult neocortical neuronal cell number. Our model results have compared favorably with independent stereological estimates of adult neocortical cell number in the mouse and rat (Bondolfi *et al.* 2002; Calhoun *et al.* 1998) (Duffell *et al.* 2000; Moller *et al.* 1990; Mooney *et al.* 1996; Strange *et al.* 1991).

Our model of neocortical neurogenesis has been previously described (Gohlke *et al.* 2002). Briefly, experimental data describing normal murine and rat neocortical neurogenesis were used to determine parameter values for our model of murine and rat neocortical neurogenesis under normal development (Miller and Kuhn 1995; Takahashi *et al.* 1995, 1996, 1997). In our model, progenitor cells making up the pseudostratified ventricular epithelium (PVE) located in the developing rostral neural tube
are referred to as “X cells”, and postmitotic young neurons leaving the PVE to migrate and subsequently populate the cortical plate are labeled “Y cells” (Figure 1A-B). The X cells have a time-dependent division and differentiation rate, and each subsequent cycle contributes a greater percentage of G1 cells to the leaving population that begin migration and eventually form the cortical layers, therefore leaving fewer cells to replenish the proliferating population (Figure 1B).

The period of natural cell death for the rodent neocortex occurs between postnatal days 1 and 14 (P1-P14), with a peak between P4 and P7 and has been quantified by stereological examination of stained brain sections (Figure 1C-D) (Ferrer et al. 1990; Spreafico et al. 1995; Thomaidou et al. 1997; Verney et al. 2000). In our previous investigation we analyzed these four datasets for estimating cell death through the period of synaptogenesis (Gohlke et al. 2004). Here we use a baseline time-dependent postnatal cell death rate based on the Thomaidou et al. (1997) dataset estimating the TUNEL+ and/or pyknotic neurons on P0, P7 and P14 (Table 1). We chose the Thomaidou et al. (1997) dataset as our baseline, as this dataset was the most complete dataset analyzing neuronal death in the whole neocortex through the synaptogenesis period using the TUNEL technique, the technique used to estimate the clearance time of dying cells (see Gohlke et al. 2004).

During synaptogenesis (P0 to P14), each time step has a Y cell death rate ($\mu_2$) based on regressions of the Thomaidou et al. (1997) experimental study analyzing % TUNEL+ and % pyknotic cells at a given slice in time. The death rate ($\mu_2$) is calculated by the following equation:

$$\mu_2(t) = -\left[\ln(1-(% \text{ TUNEL}^+)/100)\right]24\ h/2.5\ h.$$  

where 2.5 h. represents the experimentally determined clearance time of TUNEL+ cells in the subventricular region of the neocortex at PND0 (Thomaidou et al. 1997). The subventricular zone is a secondary proliferative zone that produces some neurons, but mostly glial cells after the ventricular zone has been depleted. The X and Y cell division rates ($\lambda$) and the transformation rate ($\nu$) are set to zero in our model of cell death during synaptogenesis, as the Y cell population represents postmitotic, growth factor dependent young neurons forming synapses in the cortical plate.
We previously performed a sensitivity analysis of the growth fraction (GF), or percentage of cells actively cycling, an important experimental parameter in our neurogenesis model (Gohlke et al. 2002), that varies between 80 and 100% depending on the study (Gohlke et al 2004). Here we use the mid-range experimental estimate of 93% during rat neocortical neurogenesis (Cai et al. 1997; Miller and Kuhn 1995; Miller and Nowakowski 1991; Miyama et al. 1997; Takahashi et al. 1995).

In the current rat model we are using the experimentally derived mouse founder cell population \( (X_0) \) increased by 20% based on the larger fetal brain of the rat. Previously, we performed a sensitivity analysis of this parameter in which the founder cell population \( (X_0) \) is increased in the rat model by 20 and 40%, respectively, and demonstrated that a 20% increase in the founder population leads to more accurate model predictions of neuronal number (Gohlke et al 2004).

_Dose-response analyses_

We have fit a Weibull dose response function to our models for ethanol-induced cell death during synaptogenesis using the following equations to determine the predicted fractional decrease in neurons at a given dose:

\[
\begin{align*}
(2) \quad \text{Fractional decrease in neurons (DeOlmos silver model)} &= 0.03188 \times e^{0.00447 \times \text{dose}} \\
(3) \quad \text{Fractional decrease in neurons (Caspase 3 model)} &= 0.01653 \times e^{0.004883 \times \text{dose}}
\end{align*}
\]

_Results_

_Model construct for ethanol-induced cell death_

We simulate ethanol-induced changes to the cell death rate during the period of synaptogenesis \( (\mu_2) \), based on the data of Ikonomidou et al. (2000 and 1999) using DeOlmos silver staining and Tenkova et al. (2003) using Caspase 3 activation immunohistochemistry (Table 1 and 2). Ikonomidou et al. (2000 and 1999) uses DeOlmos silver staining and TUNEL to identify dying neurons. Although neither TUNEL nor DeOlmos silver staining is specific for apoptosis (Carriaut-Marlangue and Ben-Ari 1995; Collins et al. 1992), ethanol-induced cell death has been shown to fit the criteria of apoptosis by
identifying the ultrastructural changes that characterize apoptosis (Olney et al. 2000b). Furthermore, the DeOlmos silver staining control data of Ikonomidou et al (2000) on P8 matches well with other published data using TUNEL and pyknotic nuclei such as Ferrer et al (1992) and Thomaidou et al (1997), varying between 0.2 and 1% of cells labeled in neocortical regions.

Because the data of Ikonomidou et al. (2000) is only looking at silver stained neuronal cells at one time-point (24 hrs.), we use time-course data from the same group looking at MK801, an NMDA antagonist, which has been shown to induce apoptosis in the same manner (Ikonomidou et al. 1999). We use the time-course of the MK801-induced apoptosis fit to the ethanol 24 hr. time-point to simulate the time-course of ethanol-induced cell death. To look at dose-dependence, we increase each time point by the % increase that is shown at the 24 hr. timepoint at the different dose levels. Therefore, the % cell death labeled neurons at a given time-point after a given dose of ethanol on P7 is determined using the following equations:

\[
\text{(4) } \% \text{ cell death labeled neurons}(t) = \text{MK801fi}(t) \times \text{df} \times 0.27\% + 0.27\% \\
\]

where MK801fi(t) is the MK801-induced fractional increase of apoptotic neurons over baseline at timepoint (t) after exposure (see Table 2 for values) and df is the dose factor given as:

\[
\text{(5) } \text{df} = \text{ethanolfi}(d) / \text{MK801fi}(24 \text{ hrs.}) \\
\]

where ethanolfi(d) is the ethanol-induced fold increase at a peak BEC dose (d) and the MK801fi(24 hrs.) is the MK801-induced fold increase in cell death labeled neurons at 24 hrs after the dose was given (see Table 2 for values) and 0.27% is the baseline cell death based on linear regression of data from Thomaidou et al 1997 (see Gohlke et al 2004 for the complete analysis of this dataset used to model normal cell death during synaptogenesis).

The data used in these calculations are shown in Table 2. An example calculation of % cell death labeled for the peak BEC dose of 500 mg/dl at each time-point evaluated is shown in Table 3. Figure 2 shows these time- and dose-dependent calculations transformed into a cell death rate (see Equation 1) for each time-step in which ethanol affects the cell death rate in our model. Although the MK801 time-course shows a significant drop below baseline of apoptotic neurons at the 48 hr. time-point, we chose to
keep the death rate at baseline at 48 hrs (see Table 3). If ethanol does indeed cause a decrease in cell
death at 48 hrs., our simulations overestimate the cell death caused by ethanol.

We use peak Blood Ethanol Concentration (BEC) as our dose metric as this has been shown to be
the most relevant metric for ethanol-induced neurodevelopmental effects in animal and human models
(Bonthius et al. 1988; Bonthius and West 1990; Driscoll et al. 1990; Maier and West 2001). Because
only time-points before the peak of cell death labeled neurons at 24 hrs. are evaluated in detail, we
developed a second model with death rates that mirror those as they approach the 24 hr. time-point on the
way back down to 48 hrs., assuming a more Gaussian-like distribution instead of an abrupt cut off at 24
hrs (Figure 2). Here we use the Thomaidou clearance time estimate of 2.5 hrs. for all simulations,
however it should be noted Olney et al. (2002) suggests the silver staining method may have a longer
clearance time (as much as 24 hrs.) than other cell death markers, such as TUNEL or Caspase 3 activation
(Olney et al. 2002). If this were true, our cell death rates based on the silver staining method would over
predict the amount of neurons lost.

Ethanol may cause more acute apoptosis at lower levels of exposure than previously documented
using the DeOlmos silver staining methodology. Using Caspase 3 activation immunohistochemistry to
identify dying cells, Tenkova et al. (2003) suggests ethanol can induce apoptosis at doses causing peak
BECs of approximately 122 mg/dl. To compare this Caspase 3 activation data to the data using silver
staining techniques we utilize the quantitative data presented in Tenkova et al. (2003), showing significant
increases of cell death labeled neurons in the ganglion cells of the retina and the superior colliculus
(Tenkova et al. 2003). Although this data is not in neocortical structures, similar results in other brain
regions including neocortical structures have been discussed (Olney et al. 2002; Olney et al. 2004);
however quantitative data has not been published. Controls for this dataset on P8 matches well with other
published data using TUNEL and pyknotic nuclei such as Ferrer et al (1992) and Thomaidou et al (1997),
showing only occasional isolated Caspase 3 activated neuronal profiles (Olney et al. 2002). To model
this data, we determined the % cell death labeled neurons at each time point after a given dose of ethanol
on P7 using the following equations:
\[
(6) \quad \% \text{ cell death labeled neurons} (t) = \text{ethcaspfi}(500)(t) \times \text{df(casp)} \times 0.27\% + 0.27\%
\]

where ethcaspfi(500)(t) is the ethanol-induced fractional increase of apoptotic neurons over baseline at time-point \(t\) after exposure resulting in a peak BEC of 500 mg/dl (see Table 2 for values) and df(casp) is the dose factor given as:

\[
(7) \quad \text{df(casp)} = \frac{\text{ethanolfi}(122)(4 \text{ hrs.})}{\text{ethanolfi}(500)(4 \text{ hrs.})}
\]

where ethanolfi(122)(4 hrs.) is the ethanol-induced fold increase in cell death labeled neurons at 4 hrs after the dose resulting in a peak BEC of 122 mg/dl was given and ethanolfi(500)(4 hrs.) is the ethanol induced fold increase at a peak BEC dose of 500 mg/dl at the 4 hr. timepoint after exposure and 0.27\% is the baseline cell death based on linear regression of data from Thomaidou et al (1997).

The data used in these calculations are described in Table 1 and shown in Table 2. Our analysis approach is similar to that of the silver staining dataset (see Equations 4-5). Since only 3 time-points were evaluated, we assume the peak labeling occurs at the 8 hr. timepoint and the 10 and 12 hr. timepoints are similar in death labeled neurons as the 4 and 6 hour timepoints (Figure 2). The resulting calculation of \% cell death labeled for the peak BEC dose of 500 mg/dl at each time-point evaluated is shown in Table 3. Figure 2 shows these time- and dose-dependent calculations used in the cell death rate equation (see Equation 1) between P7 and P9. Again we use the clearance time of 2.5 hours based on Thomaidou et al. (1997), which is supported by a recent study looking at ethanol-induced cell death using Caspase-3 activation in the developing cerebellum (Light et al. 2002).

Model simulations of ethanol-induced cell death during synaptogenesis

We have simulated dose-dependent ethanol-induced neocortical neuron death in the rat using DeOlmos silver staining data of Ikonomidou et al (2000)(Figure 3). This plot shows predicted number of neocortical neurons after various in utero exposures to ethanol reaching specific peak BECs. As shown here and detailed further elsewhere (Gohlke et al. 2004), our model for unexposed rats predicts adult neocortical number well compared to independent, stereologically determined mean estimates between 1.5 - 2.1 x 10^7 neurons (Duffell et al. 2000; Moller et al. 1990; Mooney et al. 1996; Stewart et al. 1997;
Strange et al. 1991). We have shown here our most conservative (predicting the largest amount of cell
death) model of cell death which incorporates predicted cell death rates beyond 24 hrs. post exposure
mirroring those prior to the 24 hr. peak seen in the experimental research. Our predicted dose-dependent
decreases in neurons at the peak blood ethanol concentrations reached in the study by Ikonomidou et al.
(2000) are shown with error bars representing the predicted standard deviation of neocortical neurons in
adult rats based on stereological studies in unexposed rats (Duffell et al. 2000; Moller et al. 1990;
Mooney et al. 1996; Stewart et al. 1997; Strange et al. 1991). Furthermore, our model predicts
significant neuronal loss only at the highest dose reaching a peak BEC of 500 mg/dl.

In order to evaluate our predictions of apoptosis using an additional marker for apoptosis, we
applied Caspase-3 activation data from Tenkova et al. (2003) and compared it with the model using the
DeOlmos silver staining methods to identify dying neurons (Figure 4). We show a Weibull (exponential)
dose-response function fit to our simulations (Equation 2-3), providing an estimate of neuronal loss at
doses not tested, but based on the relationship found in Ikonomidou et al. (2000) and Tenkova et al.
(2003)(Figure 4). These results are simulations using the calculated death rates shown in Figure 2b. and
c., assuming a Gaussian-like distribution of dead cells after the peak percentage of dead cells at 24 hrs.
after exposure, based on the experimental data available for timepoints before 24 hrs. From this analysis
it is evident that our model based on the DeOlmos silver staining methodology of identifying dying
neurons predicts a steeper dose-response relationship when compared to our model using Caspase 3
activation to identify dying neurons. This result is important because although the Caspase 3 activation
data suggests larger increases in cell death labeled neurons (see Figure 2), the time in which this occurs is
of much shorter duration, therefore producing less overall cell death than the model using the DeOlmos
silver staining methodology. Furthermore, one must keep in mind Caspase 3 activation is a method
to quantitate apoptosis as a mode of cell death in multiple cell types in the brain (eg, glia,
neuron), whereas silver staining is not specific to the mode of cell death, therefore it stains both
apoptotic and necrotic neurons. This is particularly important when interpreting Figure 4, which
shows a steeper dose-response prediction based on the silver staining data. We suggest this may reflect higher levels of ethanol exposure may cause increases in necrosis, which is picked up by silver staining, but not Caspase 3 activation.

Comparison of ethanol-induced inhibition of proliferation versus induction of cell death

We compare our model for ethanol-induced cell death during the period of synaptogenesis to our model of ethanol-induced inhibition of the cell cycle during neurogenesis (Figure 5). For this comparison, we use our most conservative apoptosis model described above, applying the DeOlmos silver stain data of Ikonomidou et al. (2000). Our model for ethanol-induced inhibition of the cell cycle is explained in detail elsewhere (Gohlke et al. 2002). Comparison of our models suggest ethanol-induced inhibition of the cell cycle during neurogenesis produces greater deficits in adult neocortical neuron number than ethanol-induced cell death during the period of synaptogenesis. For example, at a peak BEC of approximately 150 mg/dl, which would occur in a human female after approx. 3-5 drinks, our neurogenesis model predicts long-term deficits in neuron number of 35-40%, whereas our model of cell death during synaptogenesis predicts approximately 7-9% neuronal loss at this dose level. Although not shown in this figure, our model of ethanol-induced cell death using the Caspase 3 activation dataset (Fig. 4) predicts a shallower dose-response curve.

Our neurogenesis model is based on the chronic exposure paradigm of Miller and Kuhn (1995), in which peak blood levels of ethanol are reached once each day throughout the neurogenesis period (GD13-GD19). Our model of cell death during synaptogenesis, based on data from Ikonomidou et al. (2000), simulates an acute dose of ethanol reaching the peak BEC only once on P7. Ikonomidou et al. (2000) reports P7 is a discreet window of susceptibility for ethanol-induced apoptosis for neocortical structures, and shows continued exposure either before or after this window does not result in increases in cell death. Therefore, our neurogenesis model simulates cell loss after a larger total dose of ethanol over time.
We compare results of simulations from our neurogenesis model to independent stereological evidence on long-term neocortical neuronal loss in the somatosensory region of the neocortex after an exposure regimen with peak BECs reaching approx. 140 mg/dl once during each day of neurogenesis showing a 33% neuronal cell deficit (Miller and Potempa 1990) (Fig. 5). Stereological analysis of the medial prefrontal cortex also shows a 30% reduction in neurons after a chronic exposure paradigm through neurogenesis with peak BECs of approximately 117 mg/dl (Mihalick et al. 2001). This study also showed generalized learning impairment consistent with prefrontal damage. Furthermore, an analysis of DNA content of the entire neocortex shows a similar long-term neuronal loss after exposure during neurogenesis (Miller 1996). Conversely, when binge ethanol exposure reaching a peak of approx. 300 mg/dl is given during the period of synaptogenesis, no long-term neuronal loss was found, although glial cell loss and overall brain volume and weight reductions were evident (Mooney et al. 1996).

As very little is known about dose-response relationships in vivo, we broadened our analysis by comparing effects on proliferation versus effects on cell death in in vitro model systems and compared these with our model predictions (Figure 5). As a model of neocortical neurogenesis, primary cultures of neuroepithelial cells dissociated from the embryonic rat telencephalon on E13 were kept in serum free medium containing basic fibroblast growth factor (bFGF) for 5 days then switched to bFGF-free medium (Ma et al. 2003). Ethanol exposure for 24 hrs. dose-dependently blocked neuroepithelial expansion measured by reduced \(^{3}\)H-thymidine incorporation. To model neocortical synaptogenesis, organotypic explant cultures of the developing rat cerebral cortex (P2) were maintained for 6 days in vitro (Cheema et al. 2000). Apoptosis was measured using enzyme-linked immunosorbent assay for DNA fragmentation and flow cytometric analysis of Annexin-V binding to phosphatidylinerine externalized to the outer leaflet of the plasma membrane. Ethanol exposure (on day 4 and 6) produced a dose-dependent induction of apoptosis on Day 6. Another study corroborates the above study, looking at ethanol-induced neuronal death in organotypic cultures of the rat cerebral cortex and showing no effects until concentrations of 400 mg/dl are reached (Mooney and Miller 2003). These in vitro model systems support our model
simulations suggesting ethanol-induced inhibition of neurogenesis may be a more sensitive endpoint for ethanol-induced developmental neocortical toxicity than induction of apoptosis during synaptogenesis.

**Discussion**

Here we have developed a novel computational model for ethanol-induced neocortical neuronal cell death during the synaptogenesis period in the rat. Our model predicts a 20-35% reduction in neocortical neurons after a binge dose of ethanol reaching a peak BEC of 500 mg/dl. Comparison of this model with our previous model describing ethanol-induced inhibition of neocortical neurogenesis (Gohlke *et al.* 2002) suggests inhibition of proliferation during neurogenesis may be a more sensitive endpoint, as a peak BEC of 150 mg/dl is estimated to produce a long-term neocortical neuronal deficit of approximately 35-40%. Our models are supported by results in independently-derived stereologically determined neuron number data in the rat neocortex, along with in vitro model systems showing dose-response effects on both proliferation and apoptosis.

Other studies also suggest inhibition of proliferation may be a more important mechanism than induction of cell death in the etiology of ethanol neurotoxicity. A time-course study of neuron number, $[{}^3{}H]$-thymidine incorporation, and percent dying neurons in the principal sensory nucleus of the trigeminal nerve suggests the decreases in neuron number are more dependent on anti-proliferative effects than on induction of cell death at a chronic dosing regimen reaching a peak BEC of approximately 143 mg/dl (Miller 1999). Furthermore, a recent mathematical model comparing ethanol-induced cell death versus inhibition of proliferation in primary cortical cultures and B104 neuroblastoma cells at media concentrations reaching 400 mg/dl also suggests the inhibition of proliferation is a larger contributor to the loss of cells when compared to cells lost from induction of cell death (Miller 2003). However, induction of cell death prenatally during neurogenesis is most likely dose-dependent in that higher doses of ethanol may induce cell death prenatally as well as postnatally. In vivo analyses
at lower levels of exposure (150 peak daily BEC) shows no increases in cell death during the prenatal neurogenesis period (Miller 1989; Miller 1999).

Our model prediction for neuronal loss after moderate to heavy exposures (below peak BEC 300 mg/dl) during the period of synaptogenesis is relatively small (approx. 10-15% neuronal loss), taking into account normal variability of rodent neocortical neuronal number is between 10 and 13 percent (Duffell et al. 2000; Moller et al. 1990; Mooney et al. 1996; Stewart et al. 1997; Strange et al. 1991). Although a stereological analysis validates our simulations showing no statistically significant permanent decrease in neocortical neuronal number after postnatal exposure (peak BEC = 251-387 mg/dl) (Mooney et al. 1996), it should be noted that neuronal loss in other brain regions such as the cerebellum and hippocampus after a postnatal exposure scenario are well documented (see (Livy et al. 2003) and (Maier and West 2001) for review). Furthermore, although long-term neocortical neuronal loss is not evident after exposure during synaptogenesis, this exposure scenario has been shown to cause long-term reduction in the glial cell number and total DNA content as well as decreased volume and weight of neocortical structures (Maier et al. 1997; Mooney et al. 1996). It is postulated that ethanol-induced effects on glial proliferation during this period may account for these findings.

There are over 12 million glial cells in the mature rat neocortex (Mooney et al. 1996), and they are produced almost exclusively during the brain growth spurt coinciding with synaptogenesis (Guerri 1998; Parnavelas 1999; Rakic 1991). Ethanol is thought to target astrogliogenesis (Costa and Guizzetti 2002). As a model of astrogliogenesis, Guerri et al. (1990) found a significant increase in cell cycle length of primary cortical astrocyte cultures from newborn rat fetuses exposed to ethanol (Guerri et al. 1990). Cellular doses equivalent to a mildly inebriating in vivo dose (BEC of 92-115 mg/dl) were found to inhibit muscarinic receptor mediated primary rat cortical astrocyte (E21) and human astrocytoma proliferation by as much as 70% (Guizzetti and Costa 1996). The dose-response relationship found in this study (Guizzetti and Costa 1996), and other studies looking at ethanol-induced inhibition of rat glial cell proliferation mediated through PKC signal transduction (Kotter et al. 2000), including fetal human
astrocyte cultures (Guizzetti et al. 2003), is much steeper than dose-response relationships found in
neuronal cell lines or primary neuronal cultures (Luo and Miller 1997, 1998; Ma et al. 2003) (Figure 6).
This data suggests that astrogliogenesis, coinciding with the synaptogenesis period, may be particularly
sensitive to ethanol, explaining the reduced brain mass and loss of glial cells in the mature neocortex after
ethanol exposure during this period (Maier et al. 1997; Mooney et al. 1996). 

Several mechanistic hypotheses have been put forth to explain ethanol-induced developmental
neurotoxicity including growth factor inhibition, oxidative stress, and inhibition of retinoic acid
metabolism (Goodlett and Horn 2001; Guerri 1998; Zachman and Grummer 1998). The research
describing ethanol-induced apoptosis during the period of synaptogenesis suggests ethanol acts as a
 glutamate antagonist through the NMDA receptor as well as a GABA agonist (Ikonomidou et al. 2001;
 Ikonomidou et al. 1999; Olney et al. 2001). Several other studies have also implicated a role of the
 glutamate NMDA signaling system in ethanol-induced neurodevelopmental toxicity (for review see
(Kimura et al. 2000) and (Costa et al. 2000)). Furthermore, other known NMDA antagonists or
GABA mimics commonly used in pediatric and obstetric surgery such as phencyclidine, ketamine, nitrous
oxide, barbiturates, benzodiazepines, halothane, isoflurane, and propofol, have been shown to cause a
similar pattern of neurodegeneration after exposure during synaptogenesis, and may result in persistant
learning deficits in rats when drugs are maintained at anesthetic dose levels for 6 hrs. or more (Jevtovic-
Todorovic et al. 2003; Olney et al. 2000a). Recently, a systems level approach has been described for
evaluation of NMDA antagonist induced apoptosis, highlighting the importance of this mechanism in the
risk assessment of several developmental neurotoxicants (Slikker et al. 2005).

Ethanol’s abilities to cause cell death and inhibit proliferation may be dependent on interactions
with the GABA and glutamate neurotransmitter systems. Ethanol has been shown to inhibit glutamate
transmission through both NMDA and AMPA receptors (Fischer et al. 2003; Gruol et al. 1998; Li and
Kendig 2003). Recent studies have implicated GABA and glutamate in the regulation of neocortical
neural progenitor proliferation (Haydar et al. 2000; LoTurco et al. 1995; Monk et al. 2001). These
neurotransmitters stimulate proliferation in the ventricular zone while inhibiting proliferation in the
subventricular zone through the GABA_A and AMPA/kainate receptors (Haydar et al. 2000; LoTurco et al. 1995). Furthermore, there is evidence that ethanol actually induces subventricular proliferation while inhibiting ventricular proliferation in the developing neocortex (Miller 1989; Miller and Nowakowski 1991), which may be explained by the opposing effects of GABA and glutamate on these two different proliferative populations (Haydar et al. 2000). Therefore, ethanol’s ability to both inhibit proliferation and induce apoptosis during different stages of neocortical neuronal development may be explained by its glutamate antagonist and GABAmimetic properties. Interestingly, ethanol-induced inhibition of astroglial proliferation may be mediated through another neurotransmitter system, namely the cholinergic muscarinic receptor (Balduini et al. 1991; Guizzetti and Costa 1996; Guizzetti et al. 2003).

Although these time-dependent effects of ethanol on neocortical development may be explained by the same mechanism of action, it is important to differentiate the sensitivity of these periods to ethanol-induced effects. Our current model suggests ethanol’s ability to inhibit proliferation during neurogenesis may have a greater long-term impact on neuronal number than the induction of apoptosis during the period of synaptogenesis, although the duration of exposure is longer to encompass neurogenesis than that simulated to encompass the sensitive window during synaptogenesis. Future linkage of our current model with a physiologically based pharmacokinetic (PBPK) model may allow for more detailed analysis of the tissue dose and exposure duration components of ethanol-induced developmental neurotoxicity. We have utilized this methodology to analyze methylmercury neurodevelopmental toxicity (Lewandowski et al. 2002).

Our current analysis involves the extrapolation to lower doses. However the current lack of experimental data bars comparison of our model construct at doses below approximately 100 mg/dl peak daily BEC. There is empirical evidence suggesting the sensitivity of neurogenesis in the Rhesus monkey, as low daily alcohol exposure (peak BEC approx. 20 mg/dl) as it relates to neurobehavioral outcomes is especially sensitive to exposure during earlier neurodevelopmental processes (gestational days 0-50) when compared to later exposure scenarios in these same studies (gestational days 50-135) (Schneider et al. 2001).
Our current model does not take into account potential compensatory actions of the developing neocortex, therefore simulation of exposure throughout neurogenesis and synaptogenesis would predict an additive response. For example, at a chronic dose of approximately 3-5 drinks/day (peak BEC of 150 mg/dl per day) our model would predict a 35-40% neuronal deficit at the end of neurogenesis and an additional 7-15% neuronal deficit at the end of synaptogenesis. The research described below suggests compensatory mechanisms may come into play.

After in utero exposure to ethanol, irradiation, or ethylnitrosourea (ENU) there is evidence suggesting compensatory alterations in proliferation and death rates following the initial insult (Ferrer et al. 1992; Miller 1999; Mooney and Miller 2001; Oyanagi et al. 1998). For example, after the initial inhibition of proliferation in the developing rodent brain immediately following ENU administration, a subsequent increase in proliferation is seen at later timepoints (Oyanagi et al. 1998). Repair mechanisms may also take place during synaptogenesis by inhibiting PCD. Data presented in Mooney and Miller (2001) indicate reduced Caspase 3 expression during synaptogenesis after exposure during neurogenesis in the rat neocortex (Mooney and Miller 2001). In line with this finding, PCD during synaptogenesis in the cerebral cortex has been shown to decrease in microencephalic rats produced after prenatal X-irradiation during neurogenesis, suggesting postnatal cell death may be correlated to the total number of cortical neurons present after neurogenesis (Ferrer et al. 1992).

Recent studies have implicated that adolescent and adult neurogenesis in the hippocampus is also acutely sensitive to ethanol-induced inhibition (Herrera et al. 2003; Jang et al. 2002; Nixon and Crews 2002). For example, Jang et al. (2002) shows a dose-dependent reduction of BrdU-positive cells in the rat dentate gyrus starting at a peak BEC reaching only 4 mg/dl. However, some studies also suggest enhanced proliferation of these progenitor granule cells up to 3 weeks after the exposure, thus regeneration of the lost neurons may occur (Pawlak et al. 2002; Zharkovsky et al. 2003). It should be noted that adult generated neurons most likely have specific functional capabilities and therefore cannot replace those generated during development (Gould and Gross 2002).
We have discussed two mode of action hypotheses, namely inhibition of proliferation vs. induction of apoptosis, and how they relate to our model and implications for risk assessment. For example, exposure assessment is an important part of risk assessment. We have evaluated human exposure levels and how they relate to our model of organ level effects in the rodent. Another key component of risk assessment is risk characterization and an understanding of the mechanisms by which agents can cause effects and interact with susceptibility. In this paper we have evaluated temporal susceptibility and the key components of dose response assessment and risk characterization for ethanol effects on neocortical development. Our computational modeling approach specifically highlights the importance of a critical evaluation of temporal sensitivity in the risk assessment of developmental neurotoxicants.

Our computational model uncovers an important concept for assessing risk based on transient developmental perturbations. Although ethanol induces a measurably intense spike in apoptotic neurons (on average 15 times that of the baseline), this transient response may not confer to a significant long-term neuronal loss. In contrast, a relatively small, potentially harder to detect, lengthening of the cell cycle (less than 2 times the baseline) at the beginning of neurogenesis, can result in massive neuronal deficits in the mature neocortex. Therefore, transient impacts during development must be analyzed within the context of the critical underlying process in which they occur. Our modeling efforts suggest this type of context leads to more robust predictions of long-term impacts.

The rich database for ethanol developmental neurotoxicity found in the literature is an excellent source for evaluation of a systems biology computational model approach. At the cellular level, ethanol-induced developmental neurotoxicity is characterized by a range of effects depending on the dose and time of exposure (see (Maier and West 2001) for review). However, reduction in cell number may be a sufficient explanation to describe the key toxic effects of ethanol for risk assessment purposes. This mode of action hypothesis has been used to quantitatively evaluate methylmercury neurodevelopmental toxicity in a biologically based model framework (Lewandowski 2000; Lewandowski et al. 2003). Integration of other neurodevelopmental toxicants such as organophosphate pesticides, fungicides such as
benomyl, and irradiation induced effects into an overall model for developmental neurotoxicology is also being explored (DeFrank et al. 2004; Faustman et al. 2004). A mode of action modeling methodology has the potential to vastly improve the usage of scientific data in the developmental toxicology risk assessment arena by providing a quantitative framework in which cellular and eventually molecular effects can be linked to an adverse neurodevelopmental outcome.
Figure 1. Mechanism based framework for evaluation of neocortical development. The anatomy of neocortical neurogenesis, apoptosis, and differentiation and how it relates to model building. A. A mammalian nervous system at the beginning of neurogenesis at the 5 vesicle stage in lateral and dorsal views. B. During neurogenesis progenitor cells are generated in the pseudostratified ventricular epithelium (PVE), which is part of the ventricular zone. During G1 newly generated cells either stay in the proliferative population (P fraction) or become postmitotic (Q fraction) and begin migration through the intermediate zone (IZ) to the cortical plate (CP). In the mouse, the neurogenesis period is six days long and traverses eleven cell cycles (CC1-CC11) C. Differentiation along with ubiquitous apoptosis of the new neuronal population takes place postnatally (P0-P14) in the mouse and rat neocortex. D. Neuronal cell death was modeled using studies performing TUNEL+ immunocytochemistry or identification of pyknotic nuclei in sectioned neocortex. E. Basic model framework from Leroux (1996), which was modified as a model for neocortical neurogenesis where Type X cells represent neuronal progenitor cells in the PVE and Type Y cells, represent postmitotic neurons leaving the PVE and emphasizing that the transformation rate is dependent upon the division rate.
Figure 2  Time and dose-dependent cell death rates used to model ethanol-induced neocortical cell death during synaptogenesis.  
A.  Death rate determined using DeOlmos silver staining data from Ikonomidou et al. (2000) and Ikonomidou et al. (1999).  
B.  Death rates simulating similar increases in cell death between 24 and 48 hrs. as is experimentally determined between 0 and 24 hrs (shown in A.) based on Ikonomidou et al. 2000 and Ikonomidou et al. (1999).  
C.  Cell death rates using Caspase-3 activation for cell death determination based on Tenkova et al. (2003).  
See Table 1 and equations 1-5 for specifics on how the cell death rates are determined.
Figure 3  Predicted adult neocortical neuron number after exposure during synaptogenesis.

Exposed model simulates cell death after an acute exposure producing a peak blood ethanol concentration (BEC) reached on P7 in the rat (●). This model applies the DeOlmos silver staining data of Ikonomidou et al. (2000) and Ikonomidou et al. (1999) extended to 48 hrs. after exposure (see Fig. 2B). For comparison, independent stereological studies indicating neocortical neuron number in the unexposed adult rat are shown (□)(Bondolfi et al. 2002; Duffell et al. 2000; Moller et al. 1990; Mooney et al. 1996; Strange et al. 1991), where the error bars represent reported STD. Error bars shown for model predictions
represent predicted 95% population intervals based on reported coefficients of variability in the stereological studies shown (10-13%).
Figure 4  Dose-response comparisons of models using DeOlmos silver staining or Caspase 3 activation to determine cell death labeled neurons. Lines shown are exponential (Weibull) fits to the data (See equations 6 and 7). Ethanol-induced cell death was modeled based on Ikonomidou et al. (2000) and Ikonomidou et al. (1999) using DeOlmos silver staining or Tenkova et al. (2003) using Caspase –3 activation immunohistochemistry to determine percent of cells labeled at various doses. Error bars shown for model predictions represent predicted 95% population intervals based on reported coefficients of variability in the stereological studies for unexposed adult rat neocortical neuronal number (a CV of approx. 10-13%).
Figure 5  Dose-response simulations of predicted neuronal loss after exposure during neurogenesis (solid line) versus synaptogenesis (dashed line). We compare predicted neuronal loss based on ethanol-induced inhibition of neurogenesis with ethanol-induced induction of cell death during synaptogenesis. Dotted lines represent estimated 95% population intervals based on intraspecies variability reported in stereological studies of normal neocortical neuronal number in the rat (Duffell et al. 2000; Pakkenberg and Gundersen 1997). Dose-response simulations are exponential fits to model results (See Gohlke et al. 2002 for neurogenesis model and equation 6 for synaptogenesis model). For comparison, stereologically determined long-term neocortical neuronal loss in the somatosensory region (▲) and the medial prefrontal region (■) are shown when exposure occurs during neocortical neurogenesis (Mihalick et al. 2001; Miller and Potempa 1990) or in the total neocortex after exposure during synaptogenesis (□)(Mooney et al. 1996). In vitro dose-response relationships are shown for inhibition of BrdU incorporation in primary neuroepithelial cells dissociated from the embryonic rat telencephalon (E13) (♦)
(Ma et al. 2003) and induction of Annexin V binding to phosphatidylserine on the outer leaflet of the plasma membrane (measure of apoptosis) in an organotypic explant culture of the developing cerebral cortex established from postnatal day 2 rats and maintained 6 days in vitro (Cheema et al. 2000). Error bars represent standard errors reported for responses or ranges reported for peak blood ETOH concentrations.
Figure 6  Dose-response comparisons of inhibition of proliferation in astrocytes versus neuronal progenitor cells. Dose-response relationships shown as percent inhibition of $^3$H-thymidine incorporation after 24 hrs. exposure to ethanol in carbachol (muscarinic agonist) stimulated proliferation in human fetal astrocytes (▲) from (Guizzetti et al. 2003), in carbachol mediated (♦) or phorbol ester (PKC activator)(■) stimulated proliferation in rat primary astrocytes from (Guizzetti and Costa 1996) and (Kotter et al. 2000) respectively, and in primary neuroepithelial cells dissociated from the embryonic rat telencephalon (E13) expanded with bFGF then switched to bFGF-free medium overnight before inclusion of ethanol from (Ma et al. 2003). Inhibition of bFGF mediated growth in human peripheral (□) and rat CNS (○) neuroblastoma cell lines from (Luo and Miller 1997) and (Luo and Miller 1998) are also shown for comparison.
Table 1. Description and source for parameters used to determine cell death rates in rat neocortical synaptogenesis models

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ethanol Exposure Status</th>
<th>Data</th>
<th>Model</th>
<th>Tissue</th>
<th>Developmental Period*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>% death labeled cells</td>
<td>Unexposed</td>
<td>% TUNEL+ staining</td>
<td>Baseline</td>
<td>Neocortex</td>
<td>P0, P7, and P14</td>
<td>Thomaidou et al. 1997</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td>Exposed</td>
<td>DeOlmos silver stain</td>
<td>Silver stain</td>
<td>Neocortex</td>
<td>24 hrs. after exposure on P7</td>
<td>Ikonomidou et al. 2000</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exposed</td>
<td>Caspase 3 activation</td>
<td>Caspase 3</td>
<td>Retina</td>
<td>4, 6, and 8 hrs. after exposure on P1</td>
<td>Tenkova et al. 2003</td>
</tr>
<tr>
<td>Clearance time</td>
<td>Unexposed</td>
<td>TUNEL+ staining relative to cell cycle status</td>
<td>All</td>
<td>Neocortex</td>
<td>P0</td>
<td>Thomaidou et al. 1997</td>
</tr>
</tbody>
</table>
Table 2. Dose and time-dependent fold increases in cell death in neocortical neurons after exposure to ethanol (ETOH) or another NMDA antagonist (MK-801) on P7 in the rat.

<table>
<thead>
<tr>
<th></th>
<th>4 hrs.</th>
<th>6 hrs.</th>
<th>8 hrs.</th>
<th>12 hrs.</th>
<th>16 hrs.</th>
<th>24 hrs.</th>
<th>48 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ETOH(silver stain)</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>230 mg/dl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>250 mg/dl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.1</td>
<td>-</td>
</tr>
<tr>
<td>290 mg/dl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>500 mg/dl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td><strong>ETOH(Caspase-3)</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>122 mg/dl</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>500 mg/dl</td>
<td>11</td>
<td>15</td>
<td>39</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>MK-801(silver stain)</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.27</td>
<td>-</td>
<td>0.67</td>
<td>2.67</td>
<td>4.00</td>
<td>4.53</td>
<td>-0.53</td>
</tr>
</tbody>
</table>

<sup>a</sup> ETOH-induced fold increase in silver stained neurons over baseline at each dose (given as peak blood ethanol concentration) at 24 hrs. after exposure (Ikonomidou et al. 2000).

<sup>b</sup> ETOH-induced fold increase in Caspase-3 positive cells over baseline at each dose and timepoint analyzed (Tenkova et al. 2003).

<sup>c</sup> MK801-induced fold increase in silver stained neurons over baseline at each timepoint (t) after exposure (Ikonomidou et al. 1999).
Table 3: Estimated percent of cells labeled for cell death at selected timepoints after dose causing peak BEC of 500 mg/dl.

<table>
<thead>
<tr>
<th></th>
<th>4 hrs.</th>
<th>6 hrs.</th>
<th>8 hrs.</th>
<th>12 hrs.</th>
<th>24 hrs.</th>
<th>48 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline(^a)</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
</tr>
<tr>
<td>Silver stain Model(^b)</td>
<td>0.49</td>
<td>-</td>
<td>0.83</td>
<td>2.49</td>
<td>3.61</td>
<td>4.05</td>
</tr>
<tr>
<td>Caspase 3 Model(^c)</td>
<td>3.24</td>
<td>4.32</td>
<td>10.80</td>
<td>3.24</td>
<td>0.27</td>
<td>0.27</td>
</tr>
</tbody>
</table>

\(^a\) Baseline % cells death labeled in normal rats from Thomaidou et al. (1997).
\(^b\) Estimated % silver labeled neurons at timepoints before 24 hrs. is calculated by applying the timecourse seen in MK801-induced cell death through the following equation: % death labeled (t) = MK801 fold inc(t) * prop. dose factor * 0.27% +0.27% where MK801 fold inc(t) is the MK-801 fold increase over baseline at timepoint(t), the proportional dose factor = ETOH fold inc.(24 hrs.) / MK801 fold inc.(24 hrs.), and .27% is the baseline % cells death labeled in normal rats. See Table 2 for fold increase values.
\(^c\) Estimated % Caspase-3 labeled neurons at dose causing peak BEC of 500 mg/dl = ETOH casp fold inc(500)(t) * 0.27% +0.27% where ETOH casp fold inc(500)(t) is the ethanol-induced fold increase after the 500 mg/dl dose at timepoint (t) (See Table 2 and Equations 4 and 5 in text).
Acknowledgements:

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