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TOLUENE DISRUPTION OF THE FUNCTIONS OF L1 CELL ADHESION MOLECULE AT CONCENTRATIONS ASSOCIATED WITH OCCUPATIONAL EXPOSURES

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Abstract

Background—Prenatal toluene exposure can cause neurodevelopmental disabilities similar to fetal alcohol syndrome. Both share neuroanatomic pathologies similar to children with mutations in L1 cell adhesion molecule (L1). L1 mediates neurite outgrowth (NOG) via signaling through ERK1/2 which require trafficking of L1 through lipid rafts. Our objective is to determine if (1) toluene inhibits L1-mediated NOG and (2) toluene inhibits L1 signaling at concentrations achieved during occupational exposure.

Methods—Concentrations of toluene reflective of blood concentrations achieved in solvent abusers and occupational settings are used. Cerebellar granule neurons (CGN) harvested from postnatal day 6 rat pups are plated on coverslips coated with poly-L-lysine (PLL) alone or PLL followed by laminin. L1 is added to the media of CGN plated on PLL alone. Toluene is added 2 hours after plating. Cells are fixed at 24 h and neurite length is measured. ERK1/2 activation by L1 in CGN is analyzed by immunoblot.

Results—Toluene significantly reduced mean neurite length of CGN exposed to L1 but not laminin. Toluene significantly reduced L1-mediated ERK1/2 phosphorylation.

Conclusion—Results suggest that toluene inhibits L1-lipid raft interactions at occupationally relevant concentrations and may lead to a fetal solvent spectrum disorder similar to fetal alcohol spectrum disorder.

INTRODUCTION

Toluene is a ubiquitous, volatile organic solvent. Its presence is widespread in the home and in industrial and outdoor environments. Toluene is found in household products (e.g. nail polish, paints, glues, and cleaners), is a component of octane-boosting gasoline, and is used industrially for its powerful dissolving properties(1, 2). Expectant mothers and children can

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be exposed to toluene inadvertently via the mentioned environmental/occupational exposures and/or intentionally through inhalant abuse(3).

Birth outcome studies(4-9) and animal models(6, 10, 11) have shown that prenatal toluene exposure is detrimental to neurodevelopment in fetuses and neonates. Children born to mothers with known prenatal toluene abuse can develop fetal solvent syndrome (FSS), showing traits similar to those born with fetal alcohol syndrome (FAS) (i.e. growth delays, impaired neurobehavioral development, and facial dysmorphia(12-16)). Both FAS and FSS have neuroanatomic pathologies similar to children with mutations in the L1 cell adhesion molecule (L1) (17-24). Thus, L1 has been implicated in the pathogenesis of prenatal ethanol and toluene teratogenicity. L1 has four cytoplasmic tyrosines whose phosphorylation status mediates L1 function, including aiding in axon fasciculation, the trafficking of neuronal precursors, cell-cell adhesion, and neurite outgrowth (NOG)(25).

Ethanol has been shown to disrupt NOG of cerebellar granule neurons (CGN) derived from postnatal day 6 rat pups (equivalent to third trimester human fetal cerebellum(26)) plated on L1 but not on laminin(27, 28). Laminin binds to the integrin receptor to promote NOG and is not dependent on lipid rafts, whereas L1-mediated NOG is dependent on lipid rafts(29, 30). This suggests that ethanol inhibits NOG through an effect on lipid rafts. Lipid rafts are specialized, dynamic microdomains of the plasma membrane(31). We have previously shown that ethanol increases the proportion of L1 in lipid rafts and inhibits L1 activation of a cascade of downstream events that normally initiates NOG(30, 32-34). These downstream events include activation of pp60src and ERK1/2, phosphorylation of L1 tyrosines, and the dephosphorylation of tyrosine 1176 in the cytoplasmic domain of L1(33-35). Pp60src is particularly interesting as it is a lipid raft associated protein(36).

The goals of the current study are to determine (1) at what concentrations does toluene, like ethanol, inhibit L1-mediated NOG and (2) if toluene inhibits L1 signaling. We investigate the effect of toluene (0.02 to 2.0 mM) on L1- and laminin-mediated NOG of CGN harvested from postnatal day 6 rat pups. The selected range of toluene concentrations is representative of blood toluene concentrations achieved by solvent abusers and occupationally exposed workers (Table 1). Furthermore, we evaluate the effect of 0.1 mM toluene on the activation of ERK1/2 by L1.

RESULTS

Neurite length of CGN plated on a substrate of poly L-lysine (PLL), L1 alone, L1 with toluene (0.02 to 2.0 mM), and L1 with ethanol (25 mM) (used as a positive control) was determined. PLL is a synthetic cell adhesive polymer which is permissive for neurite outgrowth(37). The neurite length promoting effect of L1 and laminin can be determined by comparing the neurite lengths of CGN grown on PLL to that of CGN grown on PLL either coated with laminin, or treated with L1. Shown in Figure 1 are the neurite lengths of one representative experiment using one cell preparation and, for this experiment, toluene concentrations of 0.02 – 1.0 mM. Neurite lengths were plotted as the percent of neurites longer than each neurite so that each curve can be compared although the numbers of neurites per condition varied. As can be seen, increasing concentrations of toluene reduced

the lengths of the longest neurites without shifting the lengths of the shortest neurites. To summarize the effect of toluene on neurite length, the mean neurite length from each condition for each cell preparation was calculated, then the mean of the mean neurite length from each experiment was determined as shown in Figure 2. As previously shown, L1 significantly increased the mean neurite length over PLL alone(27, 30, 38). Ethanol significantly decreased the mean neurite length of CGN treated with L1. Toluene at all tested concentrations significantly decreased mean neurite length of CGN treated with L1.

To determine if the decrease of neurite length by toluene was specific to L1 or was a general effect on neurite length, we plated CGN on laminin alone, laminin with toluene (0.02 to 2.0 mM), and laminin with ethanol (25 mM). As previously shown, laminin significantly increased neurite length over PLL alone(27). Ethanol and toluene at all tested concentrations did not have a significant effect on the length of neurites of CGN grown on laminin (Figure 3).

Next, we tested the capability of toluene to inhibit L1 activation of ERK1/2. Because ERK1/2 is activated through its phosphorylation, we measured the relative densitometric units of phosphoERK1/2 normalized to total ERK1/2 after exposing CGN to toluene. We used a concentration of 0.1 mM toluene as it was within the range of blood concentrations which have been measured (Table 1). As previously shown, phosphoERK1/2 was significantly increased following addition of a cross-linked monoclonal antibody (clASCS4, which activates L1) over PLL alone(34). However, the clASCS4-induced increase in phosphoERK1/2 was significantly less in the presence of 0.1 mM toluene at 10 minutes (Figure 4).

DISCUSSION

The goals of the current study were to determine if (1) toluene, like ethanol, inhibits L1 mediated NOG. Our results suggest that toluene inhibits L1 mediated NOG, which may be caused via effects of toluene on lipid rafts. The present study is the first report of toluene-induced inhibition of L1-mediated NOG in CGN, as initially shown for ethanol(27, 28). Toluene significantly reduced neurite length of CGN plated on L1 (Figure 1, 2). However, toluene did not reduce neurite length of CGN plated on laminin (Figure 3). Because L1-mediated NOG is dependent on lipid rafts whereas laminin is not(29), our results suggest that toluene disrupts L1-lipid raft interactions.

Normal L1-lipid raft interactions that lead to L1-mediated NOG involve a cascade of downstream events, including L1-mediated activation of both pp60src and ERK1/2, phosphorylation of L1 tyrosines, and the dephosphorylation of tyrosine 1176 of L1, all of these actions of L1 are inhibited by ethanol(33, 34). In the present study, our results suggest that toluene disturbs this cascade at least at the level of ERK1/2 activation by inhibiting the L1-mediated phosphorylation of ERK1/2. PhosphoERK1/2 was significantly reduced in the presence of 0.1 mM toluene.

Identifying toluene as well as ethanol as disruptors of L1-lipid raft interactions suggests a common neurotoxic mechanism of FAS and FSS. In a review of literature reporting toluene

concentrations in different populations, it was found that toluene blood concentrations differ by orders of magnitude between the general population (median values from 0.085 to 0.50 μg/L), occupationally exposed workers (median values of 1.2 – 1470 μg/L in occupations with increasing exposure) and two different groups of inhalant abusers (median values of 16,600 – 25,400 μg/L)(Table 1). Differences in the median values of these populations can be due to the number of subjects in each study, the differing levels of exposure even within each group, and the time of blood sampling relative to the time of exposure. Toluene concentrations used in this study were reflective of blood toluene concentrations easily achieved by solvent abusers and in highly exposed workers in certain occupational settings (i.e. print work) (Table 1). Toluene significantly reduced NOG of CGN at all tested concentrations and significantly reduced L1 activation of ERK1/2 in CGN at 0.1 mM toluene, suggesting that toluene is toxic to CGN not only at high blood toluene concentrations achieved by solvent abusers but also at an order of magnitude lower concentrations that are achieved in occupational settings. The low concentrations at which toluene can reduce NOG and inactivate ERK1/2 implies that FSS is not a diagnosis reserved for children born only to heavy solvent abusing women, but is indeed a spectrum disorder, similar to fetal alcohol spectrum disorder.

CONCLUSIONS

Our work suggests that toluene, similar to ethanol, disrupts L1-lipid raft interactions at blood toluene concentrations achieved not only in substance abusers but also in individuals in occupational settings.

METHODS AND MATERIALS

Antibodies

Antibodies used in the NOG assay were mouse monoclonal anti-beta III tubulin obtained from Sigma (St. Louis, MO) and goat anti-mouse IgG (heavy and light chain) conjugated to Alexa 488 which is obtained from Invitrogen (Grand Island, NY). Antibodies used in the ERK1/2 activation and immunoblot assays were the following: rabbit polyclonal antibody against dually phosphorylated, activated ERK1/2 (New England Biolabs, Ipswich, MA, USA), rabbit polyclonal antibodies against ERK1/2 (anti-ERK1/2), goat polyclonal antibodies to rabbit IgG (H+L) conjugated to hydrogen peroxidase (Jackson Immuno-Research, West Grove, PA, USA), and goat anti-mouse IgG conjugated to Oregon Green (Molecular Probes, Eugene, OR, USA).

Mouse monoclonal antibodies to rat L1 (ASCS4) were produced from a hybridoma cell line developed by P. H. Patterson and obtained from the Developmental Studies Hybridoma Bank (The University of Iowa) as previously described(34). L1-Fc, a chimeric protein consisting of the extracellular domain of L1 and Fc domain of IgG, was obtained from R&D systems in Minneapolis, MN.

Cell Cultures

CGN from 6 day-old Sprague-Dawley rat pups were prepared as previously described(27, 30) and approved by the University of Maryland School of Medicine Institutional Animal

Care and Use Committee (IACUC). This is an animal only study, therefore institutional review board approval was not necessary. Viability of CGN was assessed with trypan blue and is routinely >90 %. These cells have been characterized as >90% CGN(29, 39, 40).

Preparation of coverslips

Cover slips obtained from Fisher Scientific (Hanover Park, IL) were cleaned and placed in the bottom of each of a 24 well Costar tissue culture flat bottom plate. PLL coated plates were prepared as follows: One ml ice cold 0.1% PLL was placed into each of the 24 wells. The wells were sealed and placed in the refrigerator at 4°C overnight. Laminin containing wells were prepared as follows: PLL containing wells were washed in ice cold phosphate buffered saline (PBS) 3 times, then 1 ml of a 2 mg/ml laminin solution in PBS was added to each well. The plate was sealed and placed in the refrigerator overnight at 4°C. L1 containing wells were prepared as follows: just prior to the addition of CGN, each PLL containing well was washed in ice cold PBS 3 times. CGN in tissue culture media were added to the well in a final volume of 1 ml. L1-Fc was added following CGN to give a final concentration of 0.2 μg/ml.

Neurite Outgrowth Assay

CGN were prepared in Neurobasal media (Gibco, Rockville, MD) with the following additions: 2% B27 supplement (Gibco, Rockville, MD), 20 mM L-glutamine, 6 g/L glucose, 20 mM HEPES, Ph 7.2, penicillin/streptomycin, and added to the tissue culture wells containing the prepared coverslips. CGN cultures were incubated for 2 hours at 37°C in 5% CO_2 to allow for cell adhesion. 2 hours after plating, plates were treated with toluene dissolved in medium. First, a 2 mM toluene stock solution was made, from which a serial dilution was performed to attain smaller concentrations of toluene, and added to cell cultures. Two plates of CGN were kept as untreated controls with PLL alone, two plates of CGN were treated only with laminin, and two plates were treated only with L1-Fc. Cells were grown for 24 hours in a humid atmosphere of 90% air, 5% CO_2 at 37°C. After 24 hours, the media was removed, and cells were washed with ice cold PBS 3 times. The cells were then fixed in 4% paraformaldehyde for 30 min at room temperature, followed by 3 more washes of PBS. Blocking solution (3% BSA/0.2%Triton X-100/PBS) was added to each coverslip for 1 hour at 37°C, or overnight at 4°C. Blocked cells were exposed to mouse monoclonal anti-tubulin beta III for 1 hour at 37°C and washed 3 times with PBS followed by Alexa 488 anti-mouse IgG for 1 hour at 37°C, washed 3 times with PBS, and mounted on glass slides. Eligible neurites were identified by a masked investigator in an *a priori* design. The eligible neurons were visualized using a Zeiss Observer Z1 fluorescence inverted microscope, and images were captured using Axiovision camera software (Carl Zeiss Oberkochen, Germany). Neurite length was measured using Image J software (National Institutes of Health, Bethesda, USA). Only neurons containing neurites that meet the following criteria were measured: 1) The neurite is as long as the width of the soma, 2) The neurite is not in contact with another neuron, 3) The neuron must be single and not in a cluster. At least 30 neurites from each coverslip were measured.

ERK1/2 Assay

CGN (6×10^5 cells) were plated on PLL-coated 60-mm tissue culture dishes in DMEM with 10% FBS and 20 mM HEPES, pH 7.2, and grown overnight. One hour prior to the addition of toluene, media was removed and replaced with DMEM, 20 mM HEPES, pH 7.2 to reduce background phosphoERK1/2 (18, 26). After 1 hour, toluene dissolved in media was added to the cell cultures. One dish was kept as a control, treated only with mouse IgG (MsIgG). To form multimeric complexes, ASCS4 was cross-linked by mixing with goat anti-mouse IgG (1:2.5 g/g). The mixture was incubated for 1 hour at 4°C prior to addition to cells. One hour after addition of toluene, cross-linked ASCS4 (clASCS4) was added to the cell culture. Ten min after addition of the clASCS4 or MsIgG, the cells were placed on ice, the media was removed, the cells were washed with ice-cold PBS, and cell lysates are prepared.

Preparation of cell lysates

All procedures were at 4°C. Cells were extracted in lysis buffer consisting of phosphatase inhibitor cocktail I (Sigma) in RIPA buffer (Sigma) at 1:100. Cell extracts were incubated for 30 min and then centrifuged in a microfuge at maximum speed for 10 min. The cell lysate supernatants were transferred to clean microfuge tubes and stored in −4°C.

Immunoblot for phosphoERK1/2 and ERK1/2

For immunoblot analysis, cell lysate supernatants were analyzed for their protein concentration. 5 μg of each sample was measured out in clean microfuge tubes and boiled for 5 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. The samples were separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (10% gel) and transferred to a polyvinylidene fluoride membrane. The membrane was blocked in Tris-buffered saline containing 2% BSA and 0.1% Tween-20. The membrane was incubated with antibodies to dually phosphorylated ERK1/2, washed, probed with horseradish peroxidase conjugated-goat anti-rabbit IgG, and reactive proteins were visualized with chemiluminescence. Blots were stripped and re-probed with anti-ERK1/2 antibodies to assess protein loading. The relative intensity of the bands was quantified using transmittance densitometry using Image J software (NIH, Bethesda, MD). The phosphoERK1/2 band densities were normalized for the amount of total ERK1/2 protein for all quantitative analyses. Relative densitometric units were determined by calculating the ratio of the normalized band densities to that of the MsIgG control.

Statistical analysis

The mean neurite length was determined for each condition from each cell preparation. Descriptive statistics determined the mean +/− standard error of the mean (SEM) neurite length from multiple cell preparations. Mean neurite length was compared between PLL and L1 or laminin alone by paired t-test to determine if L1 or laminin significantly increased neurite length. Mean neurite length of each EtOH or toluene treated culture was compared to L1 or laminin alone by paired t-test to determine if each concentration of toluene significantly reduced L1 or laminin mediated neurite length. The relative amount of phosphoERK1/2 of clASCS4 treated cultures was compared to cultures treated with MsIgG alone by paired t-test to determine if clASCS4 significantly increased phosphoERK1/2. The

relative amount of phosphoERK1/2 of toluene and clASCS4 treated cultures was compared by paired t test to clASCS4 treated cultures to determine if 0.1 mM toluene significantly reduced L1 activation of ERK1/2. $p < 0.05$ was set as significant.

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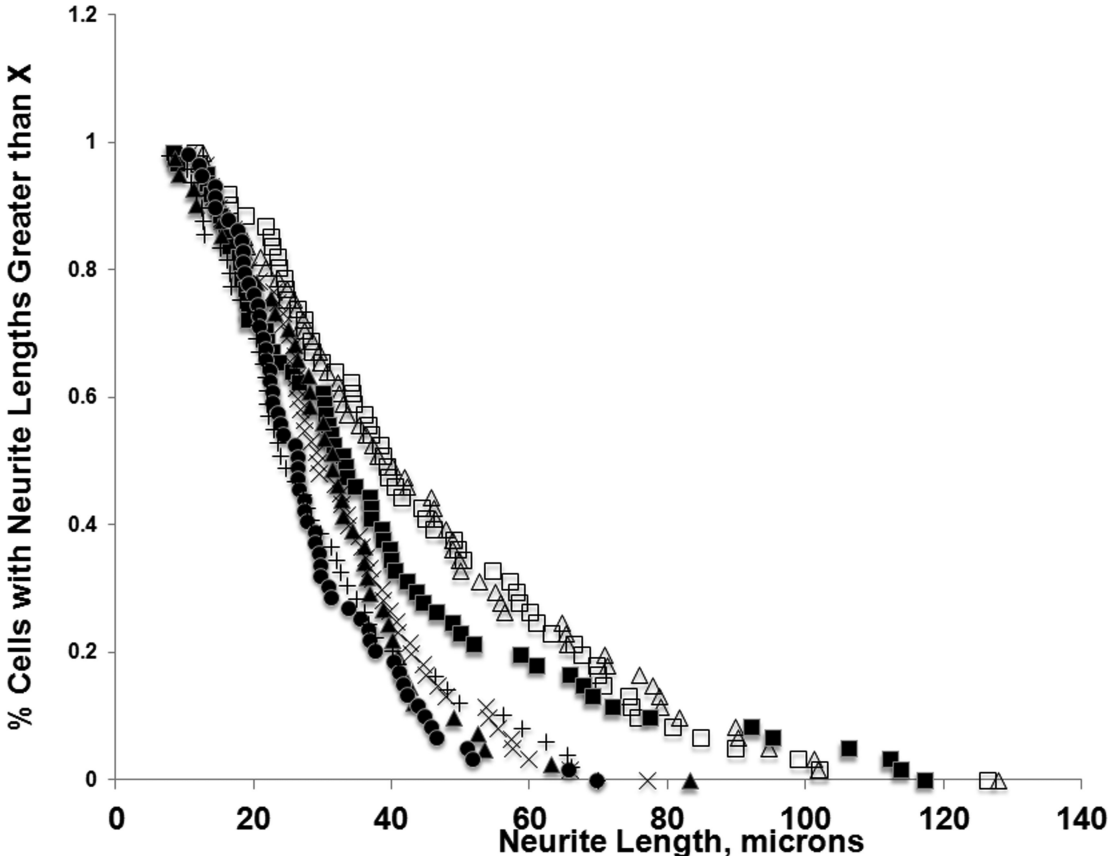

Figure 1. Neurite Length Distribution

Neurite lengths are shown of a single preparation of rat postnatal day 6 cerebellar granule neurons plated on PLL and treated with or without L1-Fc (L1) and/or different concentrations of toluene (this preparation did not include an L1 + 2.0 mM toluene condition). Each measured neurite is plotted as its length versus the percent of neurites longer than that particular length. + - no additions, Δ - L1 only, □ - L1 + 0.02 mM toluene, ■ – L1 + 0.05 mM toluene, ▲- L1 + 0.1 mM toluene, x - L1 + 1.0 mM toluene, ● – L1 + 25 mM Ethanol

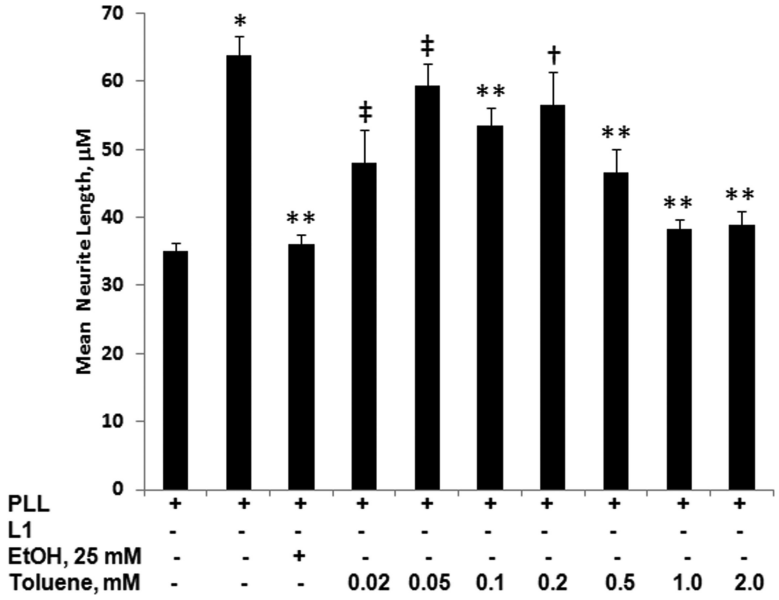

Figure 2. Mean Neurite Length of Cerebellar Granule Neurons Treated with L1

Rat postnatal day 6 cerebellar cells were isolated and plated on poly-L-lysine (PLL). L1 was added to some cultures following plating. Cells plated on PLL were not treated further. Cells plated on PLL and treated with L1, following a 2 h incubation, were treated with one of the following conditions: 25 mM ethanol (EtOH) or 0, 0.05, 0.02, 0.1, 0.2, 0.5, 1, or 2 mM toluene. Cells were incubated for an additional 24 h and then fixed, and neurite length was measured. The error bars indicate ± SEM. Plating cells on L1 significantly increased mean neurite length over cells plated on PLL alone, and toluene at all concentrations significantly reduced mean neurite length (PLL vs L1, *$p<10^{-8}$, L1 vs L1 + toluene or ethanol, **$p<0.0005$, † $p=0.02$, ‡ $p<0.05$, 2 tailed paired t-test).

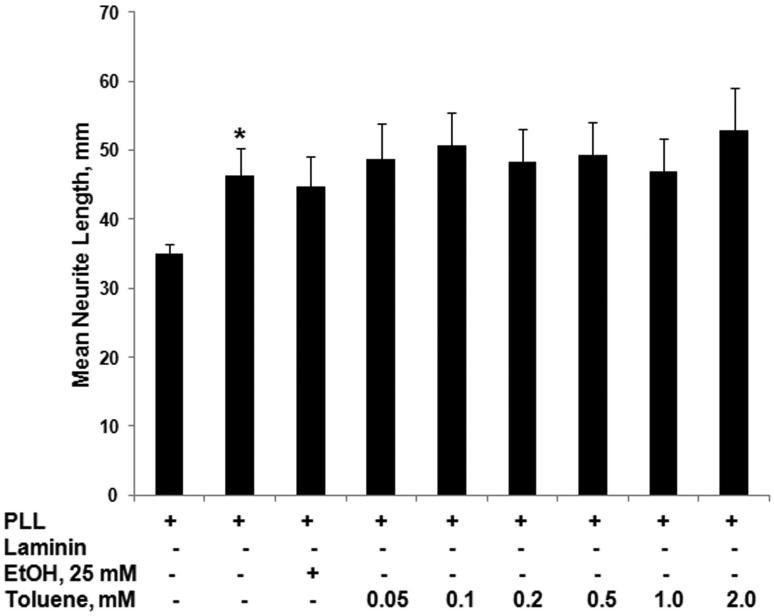

Figure 3. Mean Neurite Length of Cerebellar Granule Neurons Plated on Laminin

Rat postnatal day 6 cerebellar granule cells were isolated and plated on PLL or laminin. Cells plated on PLL were not treated further. Cells plated on laminin, following a 2 h incubation, were treated with one of the following conditions: 25 mM EtOH or 0, 0.05, 0.1, 0.2, 0.5, 1 or 2 mM toluene. Cells were incubated for an additional 24 h and then fixed, and neurite length was measured. Plating cells on laminin significantly increased mean neurite length over cells plated on PLL alone (*$p<0.02$, paired t-test). Ethanol or toluene at any dose did not significantly reduce mean neurite length of CGN plated on laminin ($p>0.2$, 2 tailed paired t test). The error bars indicate ± SEM.

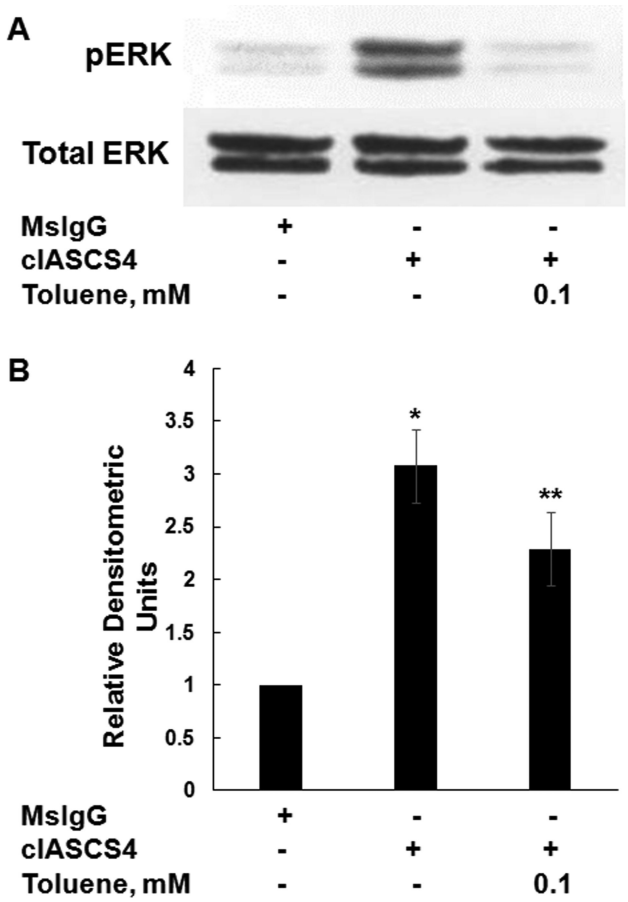

Figure 4. Toluene Inhibits L1 Mediated ERK1/2 Activation

A. Representative images of ERK1/2 and phosphoERK1/2 immunoblots. B. Densitometric quantification of ERK1/2 phosphorylation corrected for total ERK1/2, plotted as relative densitometric units relative to the MsIgG control. The bars indicate the mean ± SEM. The single asterisk (*) indicates a statistically significant increase in ERK1/2 activation in the presence of clASCS4 versus MsIgG control alone (*$p<0.002$, two tailed paired t-test). The double asterisk (**) indicates a statistically significant decrease in phosphorylated ERK1/2 following toluene treatment of 0.1 mM (**$p<0.01$, 2 tailed paired t-test).

Table 1

Blood toluene concentrations from various modes of exposure.

Ref	Exposure	N	Toluene, μg/L (mM) Median	Toluene, μg/L (mM) Maximum value reported	Metric of maximum value reported
(41)	General population (NHANES: females, 2003-2004)	689	0.085 (0.92×10^{-6})	0.58 (6.92×10^{-6})	95 th%
(41)	General population (NHANES: females, 2005-2006)	1609	0.110 (1.01×10^{-6})	0.69 (6.35×10^{-6})	95 th%
(41)	General population (NHANES: females, 2001-2002)	504	0.15 (1.63×10^{-6})	0.81 (8.79×10^{-6})	95 th%
(42)	General population (NHANES III, 1988-1994)	604	0.28 (3.04×10^{-6})	1.5 (1.6×10^{-5})	95 th%
(43)	Lower Rio Grande Valley Residents	18	0.17 (1.8×10^{-6})	0.7 (7.6×10^{-6})	maximum
(44)	Office work, non-smoker, no ETS[a]	42	0.274 (2.97×10^{-6})	0.578 (6.27×10^{-6})	90 th%
(44)	Office work, non-smoker, ETS[a]	27	0.348 (3.78×10^{-6})	0.891 (9.67×10^{-6})	90 th%
(44)	Office work, smoker	31	0.547 (5.94×10^{-6})	1.225 (1.33×10^{-5})	90 th%
(45)	Woodworking, non-smoker	15	0.428 (4.65×10^{-6})	6.04 (6.56×10^{-5})	maximum
(45)	Woodworking, smoker	10	0.78 (8.47×10^{-6})	5.148 (5.59×10^{-5})	maximum
(46)	General public (Rome)	269	0.500 (5.43×10^{-6})	7.1 (7.7×10^{-5})	98 th%
(47)	Street vending (end of shift)	6	0.51 (5.53×10^{-6})	4.6 (5.0×10^{-5})	maximum
(42)	Office workers	12	0.53 (4.88×10^{-6})	1.2 (1.11×10^{-6})	95 th%
(47)	Office work (end of shift)	8	0.61 (6.62×10^{-6})	7.4 (8.0×10^{-5})	maximum
(47)	Service station attendants (end of shift)	25	1.2 (1.3×10^{-5})	4.7 (5.1×10^{-5})	maximum
(48)	Print work (end of shift)	771	266.3 (0.003)	2529 (0.027)	maximum
(46)	Print work (plastification) (end of shift)	14	1181 (0.013)	2120 (0.023)	maximum
(49)	Print work (rotogravure) (end of shift)	20	1470[b] (0.016)	5910 (0.064)	maximum
(50)	Solvent abuse (prisoners)	6	25,400 (0.276)	31400 (0.341)	maximum
(51)	Solvent abuse (homeless adolescents in Brazil)	57	16,600 (0.181)	83700 (0.908)	maximum

[a] environmental tobacco use

[b] average