

ORIGINAL ARTICLE

LPA signaling initiates schizophrenia-like brain and behavioral changes in a mouse model of prenatal brain hemorrhage

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Genetic, environmental and neurodevelopmental factors are thought to underlie the onset of neuropsychiatric disorders such as schizophrenia. How these risk factors collectively contribute to pathology is unclear. Here, we present a mouse model of prenatal intracerebral hemorrhage—an identified risk factor for schizophrenia—using a serum-exposure paradigm. This model exhibits behavioral, neurochemical and schizophrenia-related gene expression alterations in adult females. Behavioral alterations in amphetamine-induced locomotion, prepulse inhibition, thigmotaxis and social interaction—in addition to increases in tyrosine hydroxylase-positive dopaminergic cells in the substantia nigra and ventral tegmental area and decreases in parvalbumin-positive cells in the prefrontal cortex—were induced upon prenatal serum exposure. Lysophosphatidic acid (LPA), a lipid component of serum, was identified as a key molecular initiator of schizophrenia-like sequelae induced by serum. Prenatal exposure to LPA alone phenocopied many of the schizophrenia-like alterations seen in the serum model, whereas pretreatment with an antagonist against the LPA receptor subtype LPA₁ prevented many of the behavioral and neurochemical alterations. In addition, both prenatal serum and LPA exposure altered the expression of many genes and pathways related to schizophrenia, including the expression of *Grin2b*, *Slc17a7* and *Grid1*. These findings demonstrate that aberrant LPA receptor signaling associated with fetal brain hemorrhage may contribute to the development of some neuropsychiatric disorders.

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INTRODUCTION

Schizophrenia is a complex neuropsychiatric disorder with ~0.5% prevalence in the general population.¹ There is a growing body of literature to support a significant link between obstetric complications and risk for psychiatric disorders. These environmental risk factors include diabetes, Rhesus incompatibility, bleeding, pre-eclampsia, premature rupture of membranes and preterm birth.^{2–7} Prenatal brain bleeding/hemorrhage during pregnancy, reported generally as ‘cerebral trauma,’ was, in fact one of the first environmental risk factors identified for schizophrenia.^{8,9} There are currently no animal models of fetal brain hemorrhage related to schizophrenia, but there are models that utilize maternal infection. These maternal infection models exhibit several neurochemical and behavioral alterations relevant to schizophrenia,¹⁰ and immunostimulatory agents such as interleukin-6 and tumor necrosis factor alpha/beta have been implicated in these models.

A small lipid signaling molecule, lysophosphatidic acid (LPA), is known to be involved in immune activation and hemorrhage.^{11–13} Recently, a prenatal hemorrhage model using intraventricular injections of serum into mouse embryos was developed to study post-hemorrhagic hydrocephalus.¹⁴ In this model, a single, intraventricular injection of blood serum or high concentrations of LPA at embryonic day 13.5 (E13.5) was sufficient to induce severe and persistent hydrocephalus, characterized by ventriculomegaly, cortical mitotic displacement, ventricular-associated cadherin disruptions and cortical histological abnormalities such as neurosettes and third ventricle occlusions. Most importantly,

an LPA receptor antagonist was able to prevent all of these hydrocephalus characteristics when injected before LPA.

Prenatal hemorrhage, as well as infection, hypoxia and malnutrition, are epidemiologically linked to a variety of neurodevelopmental disorders that include hydrocephalus and schizophrenia.⁶ This linkage suggested that a spectrum of central nervous system effects might be initiated by common factors, whereby fetal hydrocephalus, with its alterations in brain morphology and severe ventriculomegaly, could represent one extreme outcome of prenatal hemorrhage. Conversely, a ‘hypomorphic’ outcome, which would not induce gross hydrocephalus but still affect the developing brain, might be produced by a milder insult. The identification of LPA as an initiating factor in fetal hydrocephalus suggested broader participation in other neurodevelopmental disorders.

As LPA is involved in both hemorrhagic events and immune activation,^{11–13} this suggested the potential involvement of LPA signaling in prenatal environmental insults. LPA acts through at least six currently identified cell surface G protein-coupled receptors, LPA₁–LPA₆.¹⁵ These receptors are widely expressed in the developing brain, and LPA signaling affects a range of important neurodevelopmental processes, including cell proliferation, migration, process outgrowth and apoptosis.¹⁶ LPA is present in the blood and can reach concentrations in serum of up to 20 μM,¹⁷ which is on the order of 20 000-fold over apparent LPA receptor K_Ds.¹⁶ LPA can also induce production of interleukin-6, tumor necrosis factor alpha/beta and other cytokines during immune activation or prenatal cerebral bleeding,^{18,19} providing a

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link between LPA signaling and previously identified prenatal environmental risk factors relevant to schizophrenia.

These observations led us to test the hypothesis that aberrant LPA receptor signaling during development could initiate schizophrenia-like deficits and represent a mechanism shared among environmental risk factors. Here we report the development of an embryonic environmental model of bleeding in the brain, which implicates aberrant LPA signaling as an environmental initiator of neurodevelopmental psychiatric disorders, particularly schizophrenia.

MATERIALS AND METHODS

Prenatal injections

All animal protocols were approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute and conform to National Institutes of Health guidelines. Serum was isolated via cardiac puncture from adult female C57BL/6J mice euthanized by CO₂. Serum was allowed to clot at room temperature for 1 h, spun down, diluted 1:4 with HBSS or 0.9% saline and frozen until use. LPA (18:1; 500 μM in HBSS or 0.9% saline; Avanti Polar Lipids, Alabaster, AL, USA) was prepared just before use. All solutions had trace amounts of blue food-coloring dye.

In utero intraventricular injections were performed as previously described.¹⁴ Briefly, timed-pregnant C57BL/6J dams were mated in-house and anesthetized with 10 mg kg⁻¹ nembutal on E13.5. Uterine horns were exposed through laparotomy and were kept moist throughout the surgery. A total 3 μl vehicle (HBSS or 0.9% saline), serum or LPA were slowly injected into the left lateral ventricle of each embryo. Every embryo in each pregnant dam was injected with the same treatment. The uterine horns were replaced into the dam and the incision was sutured closed. Dams regained full body control and movement within 2 h of being anesthetized. Most dams delivered normally, and there was no variation in survival between treatment groups. Pharmacological studies using Ki16425 (Kirin Brewing/DebioPharm Group, Lausanne, Switzerland) were performed using 1.5 μl of each ligand (0.5 μM Ki16425, 1:2 diluted serum in HBSS) to maintain consistent injection volumes.

Behavior testing

At 8 weeks of age, injected mice (2–5 per cage) were transferred to a reverse light-cycle box (0700–1900 h dark, 1900–0700 h light). After 2 weeks of acclimation, mice began behavior tests. Behavior tests were run sequentially between the hours of 0900 and 1700, with at least 1 day separating tests. The order of testing was as follows: prepulse inhibition (PPI), marble burying or stereotypy (data not shown), olfaction, light/dark, elevated plus maze, novel-object recognition, social interaction and open field. All the mice completed behavior tests in the same order and timeframe, except for a separate group that only underwent PPI testing, and no animals were deliberately excluded from analyses. Male and female mice from multiple litters per group were tested. All behavioral tests were videotaped (except PPI and open-field tests). All behavior tests were analyzed with two-way multivariate analysis of variance followed by Dunnett's *post hoc* correction for multiple comparisons (treatment and sex). See Supplementary Methods for full behavioral protocols.

Receptor internalization assay

LPA₁-EGFP-B103 cells, which are rat B103 neuroblastoma cells with stable integration of an LPA₁-EGFP construct, were grown overnight on poly-L-lysine-coated 12-mm glass coverslips. The following night, cells were switched to serum-free medium for 16–24 h. The next day, cells were incubated with 10 nM, 1 μM and 10 μM concentrations of LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate; Avanti Polar Lipids, Alabaster, AL, USA) for 5 or 30 min and then fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS). Fixed cells were permeabilized in 0.1% Triton X-100/PBS for 15 min. F-actin was visualized by staining with 25 μg ml⁻¹ rhodamine-phalloidin (Sigma) in PBS/1% bovine serum albumin (BSA) for 30 min. Images were acquired using a Nikon N-SIM (structured illumination microscopy) super-resolution microscope with an Andor iXon3 back-illuminated high sensitivity EMCCD camera with single-photon detection capability (Nikon, Tokyo, Japan). Projections were rendered using 3D-SIM Elements software from Nikon.

Ca²⁺ imaging

Calcium signaling in LPA₁-EGFP-B103 cells were assessed as previously described.²⁰ Briefly, serum-starved cells were loaded with fura-2 AM (2 μM) in the presence of 1.5 μM pluronic acid F-127 and incubated for 30–45 min at 37° in the dark in Opti-MEM (Life Technologies, Carlsbad, CA, USA) buffer containing 2 mM CaCl₂ and either 0.1% BSA or 10 nM, 1 μM or 10 μM LPA. After 30 min, the coverslips were washed 1 × with Opti-MEM and secured to the bottom of a laminar flow perfusion chamber RC-25 (Warner Instruments, Hamden, CT, USA) with vacuum grease. Buffer (HBSS containing 20 mM HEPES pH4, 2 mM MgCl₂, 2 mM CaCl₂) was gently added and the chamber mounted on an Axiovert 200 M microscope. Clearly distinguishable individual cells with bright GFP signals were chosen for imaging. Cells were constantly perfused at room temperature. Fresh 0.1% BSA and 1 μM LPA-, and ionomycin-containing solutions were prepared immediately before use. The solutions were perfused through the chamber in the following order: 120 s BSA, 60 s LPA, 120 s BSA, 60 s ionomycin. A change in intracellular calcium level was scored as a response of 50% over baseline levels. Only cells responding to ionomycin were included in total cell number assessment. Population data were compared using a Student's paired *t*-test.

Immunohistochemistry

Adult brains from C57BL/6J male and female prenatally injected mice were collected at least 2 weeks after the last behavioral test was performed, fixed in formalin-alcohol-acetic acid solution, paraffinized and sectioned coronally (8 μm). Sections 24 μm apart were used for immunohistochemistry analysis. Sections were processed through antigen retrieval (boiled for 10 min in 10 mM sodium citrate buffer), blocked with species-appropriate serum and the following antibodies were used: tyrosine hydroxylase (Millipore, Temecula, CA, USA, rabbit 1:500), parvalbumin (Millipore, mouse, 1:500), DCX (Abcam, Cambridge, UK, rabbit, 1:1000), proliferating cell nuclear antigen (Oncogene, Cambridge, MA, USA, mouse 1:100), anti-rabbit Alexa 488 (Invitrogen, Carlsbad, CA, USA, 1:1000) and anti-mouse 568 (Invitrogen, 1:1000). For tyrosine hydroxylase (TH) analyses, the ventral tegmental area was designated as Bregma's area –3.0 to –3.8 mm and up to seven sections were stained and counted. Only those three sections containing the most tyrosine hydroxylase-positive (TH+) cells within the defined substantia nigra/ventral tegmental area (Bregma's –3.0 mm to –3.3 mm) were averaged for final assessment in each group to avoid bias. For parvalbumin (PV) analyses, PV+ cells from three sections from the prelimbic prefrontal cortex (PFC, Bregma's area 1.8–1.4 mm) were counted within a 2 × 2 mm fixed frame area. For neurogenesis analyses, cells in the dentate gyrus (Bregma's area –1.2 mm to –1.64 mm) were counted following unbiased stereological techniques. Statistical analyses were done using one- or two-way analyses of variance with Bonferroni's *post hoc* correction.

Microarray analysis

The brain specimens from 8-week-old prenatally exposed female mice were collected. Mice were deeply anesthetized with isoflurane, killed by decapitation and the brain was quickly isolated and placed into ice-cold PBS. The PFC (Bregma's area 2.5–1.3 mm) and ventral tegmental area (Bregma's area –3.0 to –4.2 mm) were isolated quickly from the brain via microdissection in sterile PBS using a standard dissection stereomicroscope. The specimens were placed into sterile tubes, flash-frozen with liquid N₂, and stored at –80° until use. RNA was isolated from tissue using an RNeasy Lipid Tissue Kit (Qiagen, Venlo Limburg, The Netherlands) as per instructions and frozen at –80° until use. RNA was first reverse transcribed into complementary DNA, and the template cDNA was then converted to Cy3-labeled complementary RNA by *in vitro* transcription using Agilent's Low Input Quick Amp Labeling Kit, one-color (Agilent, Santa Clara, CA, USA). The resulting cRNA (0.6 μg) was hybridized to an Agilent SurePrint G3 Mouse GE 8x60K Microarray (Agilent) as per manufacturer's instructions and scanned by an Agilent G2565CA DNA Microarray Scanner (Agilent). The functional relevance of microarray data generated from LPA and serum-treated animals was analyzed using Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). RT-PCR (PCR with reverse transcription) validation of select genes (see Supplementary Materials for primer sequences) was performed using a GoTaq RT-PCR kit (Promega, Madison, WI, USA) as per manufacturer's instructions and run on a RotorGene 3000 (Qiagen).

Statistical analyses

All the data were analyzed using a two-tailed students *t*-test for comparing means or parametric, one- or two-way analyses of variance with Bonferroni's *post hoc* correction, or two-way multivariate analysis of variance followed by Dunnett's *post hoc* correction for multiple comparisons (treatment and sex). Significance was set at $P < 0.05$ and all analyses were performed using the statistical software StatPlus (AnalystSoft, StatPlus:mac LE for the Mac OSX) or Prism (GraphPad). No data points were excluded as outliers. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications.^{14,21–24}

RESULTS

Prenatal serum exposure induces schizophrenia-like behavioral deficits

Intraventricular delivery of vehicle, LPA or diluted serum was administered to mouse embryos on E13.5. A concentration of 25% serum in HBSS was empirically determined not to produce hydrocephalus; higher concentrations resulted in ventriculomegaly and hydrocephalus (Supplementary Figure 1). Following intraventricular delivery of vehicle, LPA or serum, pups were born and developed postnatally for 10 weeks (Supplementary Figure 2). Between postnatal weeks 10 and 12, cohorts of adult animals treated at E13.5 were subjected to a battery of behavioral tests designed to test for a range of cognitive and negative symptom deficits associated with a variety of neuropsychiatric disorders. These tests were PPI, olfactory sensitivity, stereotypy or marble burying (data not shown), light/dark, elevated plus maze, novel-object recognition, social interaction and open field. At postnatal week 14, animals were killed for tissue isolation to perform immunohistochemical and gene expression analyses.

Baseline locomotor activity was significantly decreased in serum- and LPA-exposed mice compared with the vehicle-exposed group (treatment $F_{3,76} = 23.42$, $P < 0.0001$) of both sexes (sex $F_{1,72} = 2.407$, $P = 0.1252$; Figures 1a and b, Supplementary Figure 3). Normally, treatment with amphetamine (AMPH), a stimulant that increases the neurotransmission of dopamine, induces mild hyperactivity in adult mice, which was observed in the vehicle cohort (Figures 1a and b), and significantly affects locomotor activity in schizophrenia models. AMPH treatment did induce hyperactivity (treatment $F_{3,40} = 5.728$, $P = 0.0023$), but only in serum-exposed female mice (time $F_{1,40} = 105.0$, $P < 0.0001$; treatment $F_{3,40} = 18.81$, $P < 0.0001$), fully ameliorating the baseline hypoactivity observed before AMPH treatment, whereas AMPH treatment had no effect on LPA-exposed females (Figure 1a) or males of either group (Figure 1b). Female serum- and LPA-exposed mice both displayed significantly decreased PPI at 100 dB, whereas males were unaffected (sex $F_{1,101} = 17.00$, $P < 0.0001$, Figures 1c and e, Supplementary Figure 4). There was no significant change in startle response by either sex (Supplementary Figure 5). Female serum- and LPA-exposed mice also showed significant deficits in exploratory behavior-induced anxiety, as evidenced by a decreased amount of time spent in the center block compared with vehicle-exposed mice (treatment $F_{3,40} = 5.124$, $P = 0.0043$; sex $F_{1,40} = 7.204$, $P = 0.0105$) during an open-field test (Figure 1f) and only serum-exposed female mice exhibited social interaction deficits (treatment $F_{3,57} = 6.831$, $P = 0.0005$; sex $F_{1,57} = 0.0689$, $P = 0.7939$) compared with vehicle-exposed mice (Figure 1g). The other behavioral tests did not reveal differences between vehicle and serum- or LPA-exposed mice (Supplementary Table 1).

Deficits in PPI, locomotion, anxiety and social interaction are common phenotypes of both genetic,²⁵ and especially other environmental,²⁶ animal models of schizophrenia, suggesting face validity for this serum-initiated environmental mouse model. These data also suggest that fetal brain exposure to exogenous

LPA, a known bioactive component of serum, can induce schizophrenia-related behavioral changes in adults and phenocopies several of the behavioral deficits seen in the serum model. In addition, sex-specific differences, similar to those observed here, are commonly reported in mouse schizophrenia models,^{23,24,27} and human symptoms and incidence rates of schizophrenia are known to vary between males and females.²⁸

Schizophrenia-like behavioral deficits in the serum model are primarily LPA₁ dependent

LPA has six known receptors, and one of these receptors, LPA₁ (gene name *Lpar1*), is highly relevant to neurodevelopment. *Lpar1* shows pronounced gene expression in the mouse E13.5 brain,²⁹ and was previously linked to major changes produced by serum and LPA in the initiation of hydrocephalus.¹⁴ We assessed the contribution of the LPA₁ receptor to our prenatal serum-exposure model using Ki16425, a short-acting, dual LPA_{1/3} receptor antagonist.³⁰ Although the dual-target effects of Ki16425 for LPA₁ and LPA₃ would normally be of concern, available genetic and tissue expression data have shown that LPA₃ is not expressed during development.^{20,29} Ki16425 was intraventricularly injected 10 min before serum exposure at concentrations previously determined to block LPA's effects on hydrocephalus.¹⁴

Pretreatment with Ki16425 did not affect the hypoactivity induced by prenatal serum exposure, but did prevent the hyperactivity induced by AMPH treatment in serum-exposed females (Figure 2a). Pretreatment with Ki16425 also completely prevented the PPI deficits (Figures 2b and c) and exploratory deficit-induced anxiety (Figure 2d). Ki16425 had no effect on social interaction deficits (Figure 2e), nor were other behavioral deficits induced by prenatal Ki16425 exposure (Supplementary Table 1). All statistics were done between the four groups (vehicle, LPA, serum, Ki+serum) and are the same as those reported in the above section. These data indicate that multiple behavioral deficits associated with prenatal serum exposure are LPA receptor dependent and primarily involve LPA₁ in the developing female brain.

Pretreatment with Ki16425 enabled short-term antagonism of LPA₁ during a critical developmental window. However, genetic deletion of LPA₁ had previously been linked to schizophrenia-like behavioral and neurodevelopmental deficits,^{31,32} and these reported behavioral deficits overlap with those seen in our prenatal serum and LPA exposure model, including increased anxiety, alterations in exploratory behavior and PPI deficits.^{32,33} These data seemed to be paradoxical, since both activation and constitutive deletion of LPA₁ appeared to mediate similar schizophrenia-like end points. However, prior receptor signaling studies on lysophospholipid receptors suggested an explanation via functional antagonism.

High levels of LPA produce agonism followed by functional antagonism of LPA₁

The signaling of another lysophospholipid could provide an explanation for the analogous effects of prenatal LPA receptor activation and constitutive deletion. A similar paradox was previously observed for the action of the sphingosine 1-phosphate receptor 1 (S1P₁). An S1P receptor agonist, fingolimod, was found to produce an initial agonism followed by prolonged functional antagonism with continued drug exposure.^{34–37} This raised the possibility that an excess of LPA might similarly result in initial super-activation followed by prolonged functional antagonism of LPA receptors, thereby explaining the similar effects seen through both genetic removal of LPA receptors and their apparent overactivation.

Normally, LPA₁ is internalized via endocytosis followed by recycling back to the cell surface. To test whether LPA exposure approximating the concentrations used in this model could

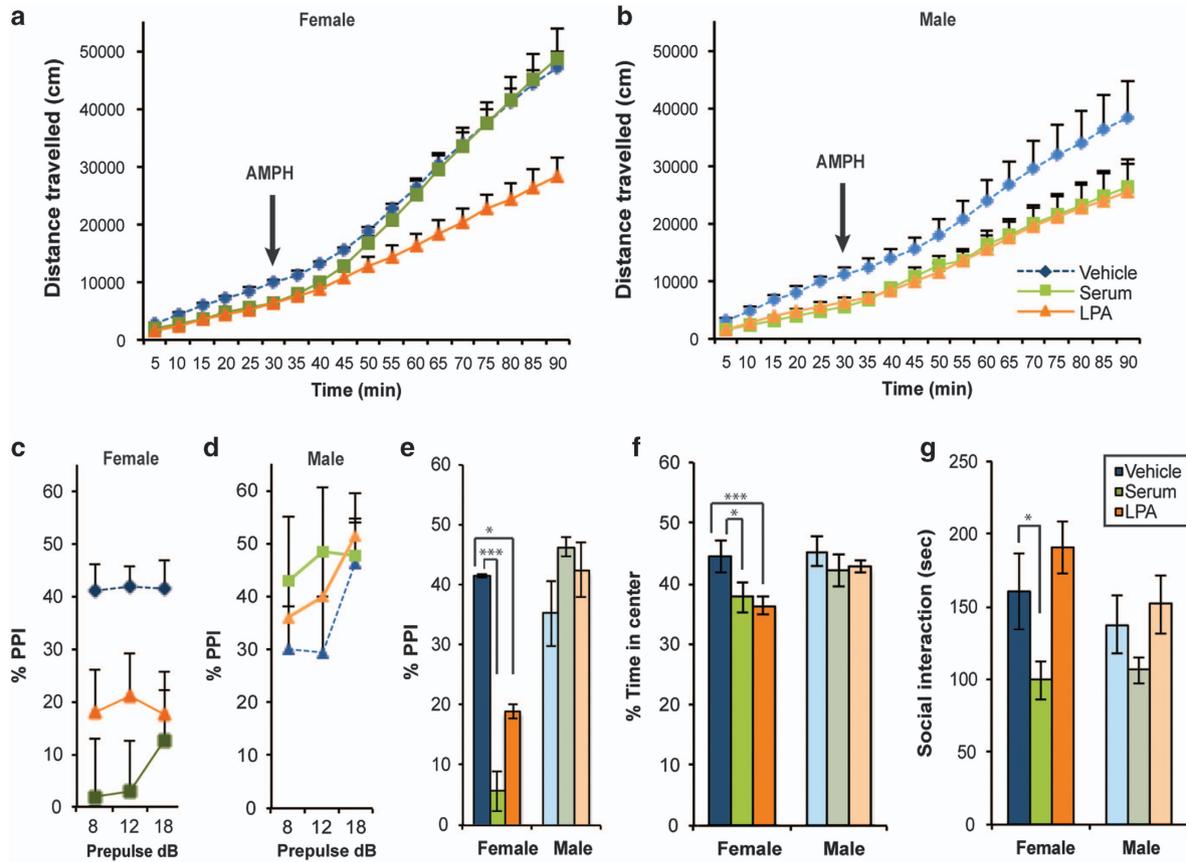


Figure 1. Prenatal serum and LPA exposure induces similar schizophrenia-like behavioral deficits in females. **(a and b)** Serum- and LPA-exposed adult mice displayed hypoactivity during an initial 30-min period. Amphetamine (AMPH) treatment completely ameliorated the hypoactivity in serum-exposed female mice, but not LPA-exposed female or male mice of either group. **(c–e)** PPI was significantly decreased in female serum- and LPA-treated mice at 100 dB compared with controls. Three prepulse tones (8, 12 and 18 dB) were assessed. **(f and g)** Female serum- and LPA-treated mice spent a decreased amount of time in the center of the open-field box over a 30-min period **(f)** and female serum-exposed mice spent significantly less time interacting with a sex- and strain-matched stranger mouse **(g)**. For each experiment, data were collected simultaneously for vehicle, serum, LPA and serum+Ki treatment group (see Figure 2, Supplementary Figure S1, Supplementary Figure S3 and Supplementary Figure S4). $n = 7–17$ per sex and group, mean \pm s.e.m., * $P < 0.05$, *** $P < 0.001$. LPA, lysophosphatidic acid; NS, not significant; PPI, prepulse inhibition.

produce functional antagonism, LPA₁-EGFP-B103 neuroblastoma cells were briefly pretreated with either BSA or increasing concentrations of LPA (10 nM–10 μ M) and imaged using super-resolution microscopy. Control (BSA) and lower concentrations of LPA allowed receptor recycling back to the cell surface (Figures 3a and d), whereas increasing concentrations of LPA induced prolonged receptor internalization (after ligand washout, Figures 3e and h). Following pretreatment with 10 μ M LPA, a loss of LPA-activated Ca²⁺ signaling was observed after subsequent challenge with 1 μ M LPA (30 min after initial exposure, Figures 3i and l). Therefore, increasing levels of LPA produced prolonged functional antagonism of LPA₁ and resulted in a loss of LPA₁ signaling (Figure 3m), which could in part explain the paradox of similar phenotypes seen in genetic LPA₁ nulls and the fetal hemorrhage/LPA models involving high concentrations of exogenous LPA.

Prenatal serum or LPA exposure induces immunohistochemical changes in the adult brain that are partially dependent on LPA₁. Within our prenatal mouse models, the number of dopaminergic cells was assessed using TH+ immunolabeling in the midbrain of female animals exposed to prenatal serum, LPA or LPA₁ antagonist (Ki16425+serum). Both prenatal serum- and LPA-exposed females displayed significantly increased numbers of TH+ cells in the midbrain (treatment $F_{3,30} = 14.35$, $P < 0.0001$). Importantly, these changes in TH+ cell number were completely prevented with

Ki16425 pretreatment (Figure 4a). The number of GABAergic, PV+ cells in the medial PFC of adult females was also assessed and, contrasting with dopaminergic cells, only prenatal serum-exposed mice displayed decreases in PV+ cells in the medial PFC (treatment $F_{3,25} = 4.913$, $P = 0.0078$), with no change seen in the LPA or Ki16425+serum models (Figure 4b).

Previous studies suggested a contribution of hippocampal neural stem cell proliferation to the pathophysiology of schizophrenia.³⁸ Yet, no evidence of altered hippocampal neurogenesis in either model (treatment $F_{2,29} = 2.289$, $P = 0.123$) was observed in proliferating (proliferating cell nuclear antigen) cells identified by the neural cell marker doublecortin (DCX) in the hippocampus (Figure 4c).

Significant gene expression changes overlap between prenatal models and schizophrenia

Genome-wide transcriptome analyses were used to identify genes, pathways and networks associated with prenatal serum and LPA exposure models, as compared with genes linked to schizophrenia. The PFC and the midbrain (ventral tegmental area) of serum-, LPA- or vehicle-exposed female adult mice were compared, since females showed the most robust phenotypic changes. In total, there were between 1500 and 2100 differentially expressed messenger RNA gene transcripts ($P \leq 0.01$) between female adult prenatal serum- or LPA-exposed brains versus vehicle-exposed

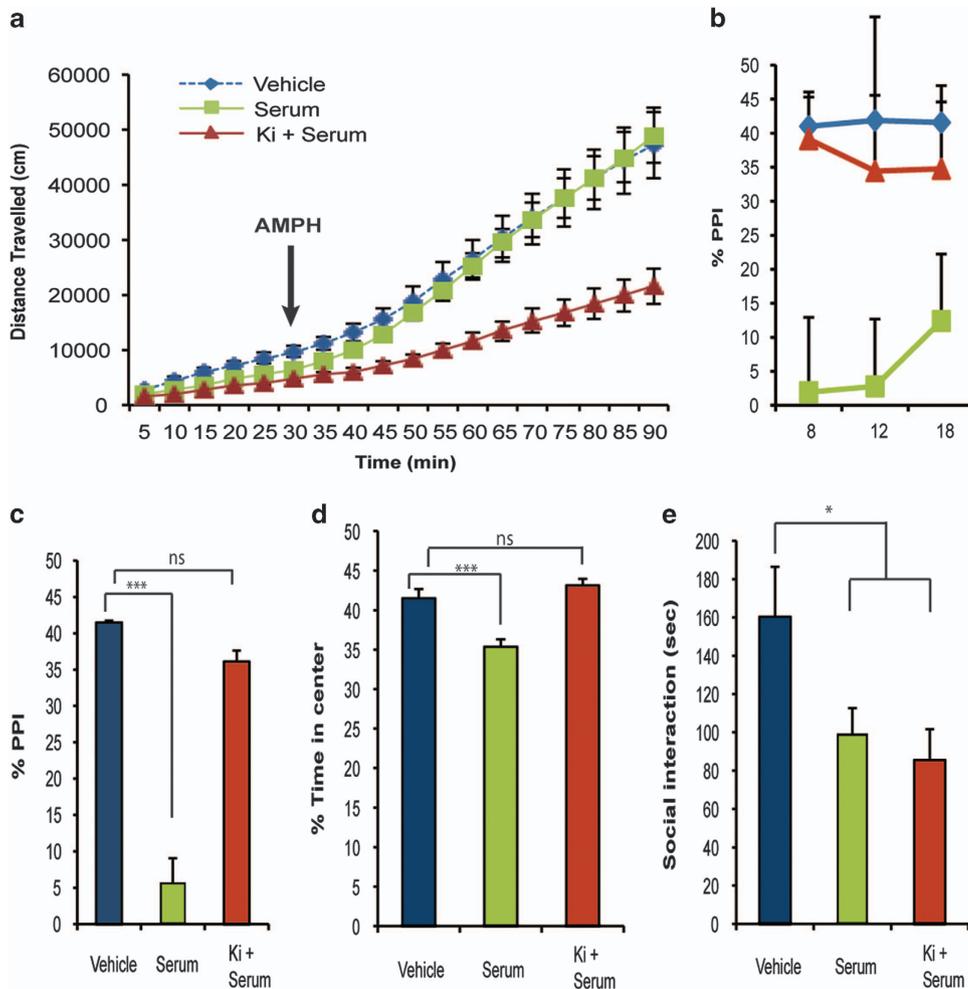


Figure 2. LPA₁ antagonism prevents serum-induced schizophrenia-like behavioral deficits in females. (a) LPA₁ antagonism through Ki16425 treatment did not prevent prenatal serum-induced decreases in locomotor activity, but did prevent AMPH-induced hyperlocomotion. (b and c) Treatment with Ki16425 prevented prenatal serum-induced deficits in PPI at 100 dB. (d–e) Ki16425 treatment also prevented the decreased percentage of time female serum-exposed mice spent in the center of the open-field box over a 30-min period (d), but had no effect on serum-induced social interaction deficits (e). Data for vehicle and serum are as in Figure 1. All data were analyzed with multivariate two-way analysis of variance comparisons between groups (vehicle, serum, LPA and Ki16425+serum). $n=6-17$ per group, mean \pm s.e.m., * $P < 0.05$, *** $P < 0.001$. LPA, lysophosphatidic acid; NS, not significant; PPI, prepulse inhibition.

control brains (PFC serum=1815, PFC LPA=2129, midbrain serum=1828 and midbrain LPA=1467). There was ~50% overlap between the prenatal serum- and LPA-exposed groups, with most genes showing decreased expression compared with controls (Supplementary Figures 6a and b).

Pathway and network analyses were assessed with Ingenuity Pathways Analysis software, using only those genes concordantly dysregulated by both prenatal serum and LPA exposure (genes changed in the same direction and at a significance level of $P < 0.05$). Notably, alterations in several top canonical pathways relevant to schizophrenia—including calcium signaling, dopamine-DARPP32 feedback in cAMP signaling and synaptic long-term potentiation—were identified (Supplementary Tables 2a and b). Several relevant networks of highly connected genes related to developmental disorders, gene expression, lipid metabolism and synaptic activity, among others, were also identified (Figure 5a, Supplementary Table 3). In particular, glutamatergic and GABAergic neurotransmitter pathways were affected by both prenatal serum and LPA exposure, emphasizing the global nature of neurotransmission-related alterations in our models.

Many genes previously implicated in the pathology of schizophrenia³⁹ were common to both models ($P < 0.05$; Supplementary Table 4). In particular, 52 out of 278 genes

previously identified in human genetic studies as strongly associated with schizophrenia⁴⁰ were among the list of altered genes in both serum and LPA models, compared with only 13 of 278 genes selected at random from the entire list of genes present on the microarray. This number reflected a statistically significant overrepresentation of schizophrenia-associated genes concomitantly regulated by both serum and LPA exposure (Fisher's exact probability test, two-side probability threshold; $P < 0.0001$).

Gene expression data were also compared with a list of the most reproduced, differentially expressed genes in human postmortem brain samples from schizophrenia patients. This list contained 679 distinct genes identified from a meta-analysis of 12 individual gene expression data sets involved in 988 arrays from the Stanley Medical Research Database.⁴¹ Serum exposure resulted in 127 of 679 (18.7%) differentially expressed genes, while LPA exposure resulted in 143 of 679 (21.1%), with an overlap between serum and LPA of 53 genes (Figure 5b, Supplementary Table 5). Quantitative PCR validation of selected genes verified the decreased expression of several glutamatergic-related genes (Figures 5c and d). Collectively, these data indicate that prenatal serum or LPA exposure induces significant and overlapping alteration in gene expression, particularly in genes associated with neurotransmission.

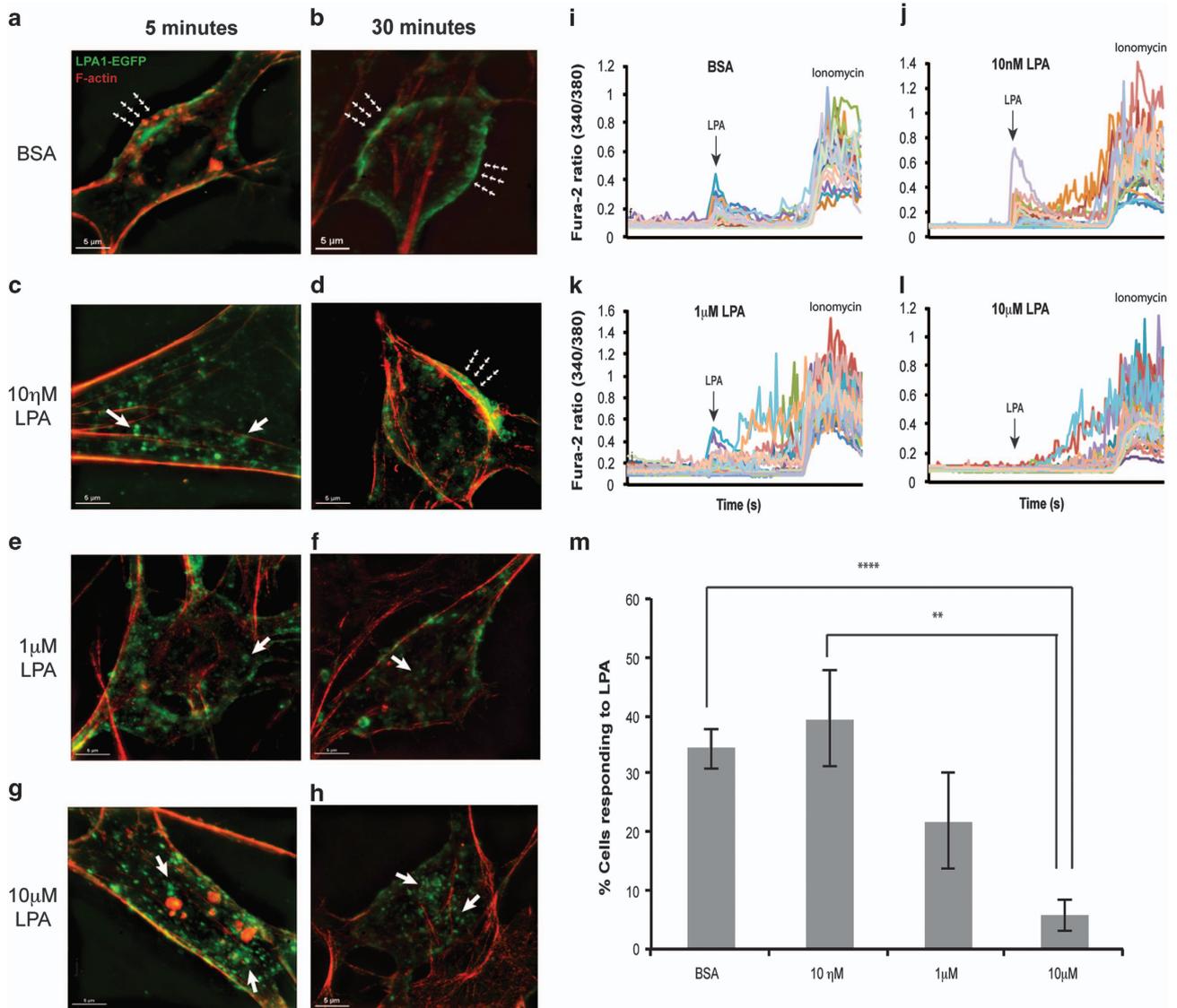


Figure 3. High levels of LPA produce initial agonism followed by functional antagonism of LPA_1 after LPA exposure. Receptor internalization was monitored in EGFP- LPA_1 -B103 neuroblastoma cells combined with fluorescence microscopy and functional calcium imaging. (**a**, **c**, **e** and **g**) Receptors localized on the cell surface (green signal, dashed arrowheads, **a**) are rapidly internalized with increasing LPA concentration (green vesicles, arrows, **c**, **e** and **g**). (**b** and **d**) After 30 min, cells exposed to BSA and low concentrations of LPA (10 nM) exhibit LPA_1 receptor recycling that returns receptors to the cell surface (dashed arrowheads). (**f** and **h**) In contrast, exposure to high concentrations of LPA (1 and 10 μ M) resulted in prolonged and sustained receptor internalization (arrows), effectively removing receptors from the cell surface. (**i–m**) To assess calcium signaling, cells were pretreated with BSA or increasing concentrations of LPA for 5 min, washed and after 30 min were challenged with 1 μ M LPA followed by assessment of cell signaling viability using ionomycin. Normal levels of LPA-stimulated intracellular Ca^{2+} (**i** and **j**) contrasted with reduced (**k**) and absent (**l**) Ca^{2+} release in cells exposed to higher LPA pretreatment concentrations, indicating functional antagonism of the internalized receptors (**m**, BSA $n = 7$, 10 nM LPA $n = 6$, 1 μ M LPA $n = 5$, 10 μ M LPA $n = 6$, mean \pm s.e.m.). Data were analyzed with Student's two-tailed t -test, ** $p < 0.01$, **** $p < 0.0001$. BSA, bovine serum albumin; LPA, lysophosphatidic acid.

DISCUSSION

In this study, a new environmental model of prenatal brain hemorrhage was developed and found to display both face (Table 1) and construct validity (through modeling a validated schizophrenia risk factor) as a schizophrenia-like model for neuropsychiatric disease.⁴² LPA_1 receptor signaling was identified as a common molecular pathway initiated by prenatal serum exposure. In particular, overactivation with subsequent functional antagonism of LPA_1 during prenatal hemorrhage appears to produce specific dopaminergic and glutamatergic signaling-related behavioral deficits such as PPI, altered exploratory behavior-induced anxiety and sensitivity to dopaminergic signaling-induced behaviors.

As increases in LPA concentrations are present in many of the environmental risk factors associated with schizophrenia, such as infection,¹² bleeding¹³ and hypoxia,⁴³ LPA_1 receptor overactivation followed by prolonged functional antagonism during neurodevelopment could represent a common link between different environmental risk factors. In addition to LPA, other factors present in serum include immune-related signaling molecules, such as interleukin-6, tumor necrosis factor alpha and interleukin-1 beta, which are elevated during bleeding, immune activation and other environmental insults. It is likely that one or more of these factors mediate non-LPA induced changes by serum, such as the observed social interaction deficits in the prenatal hemorrhage model.

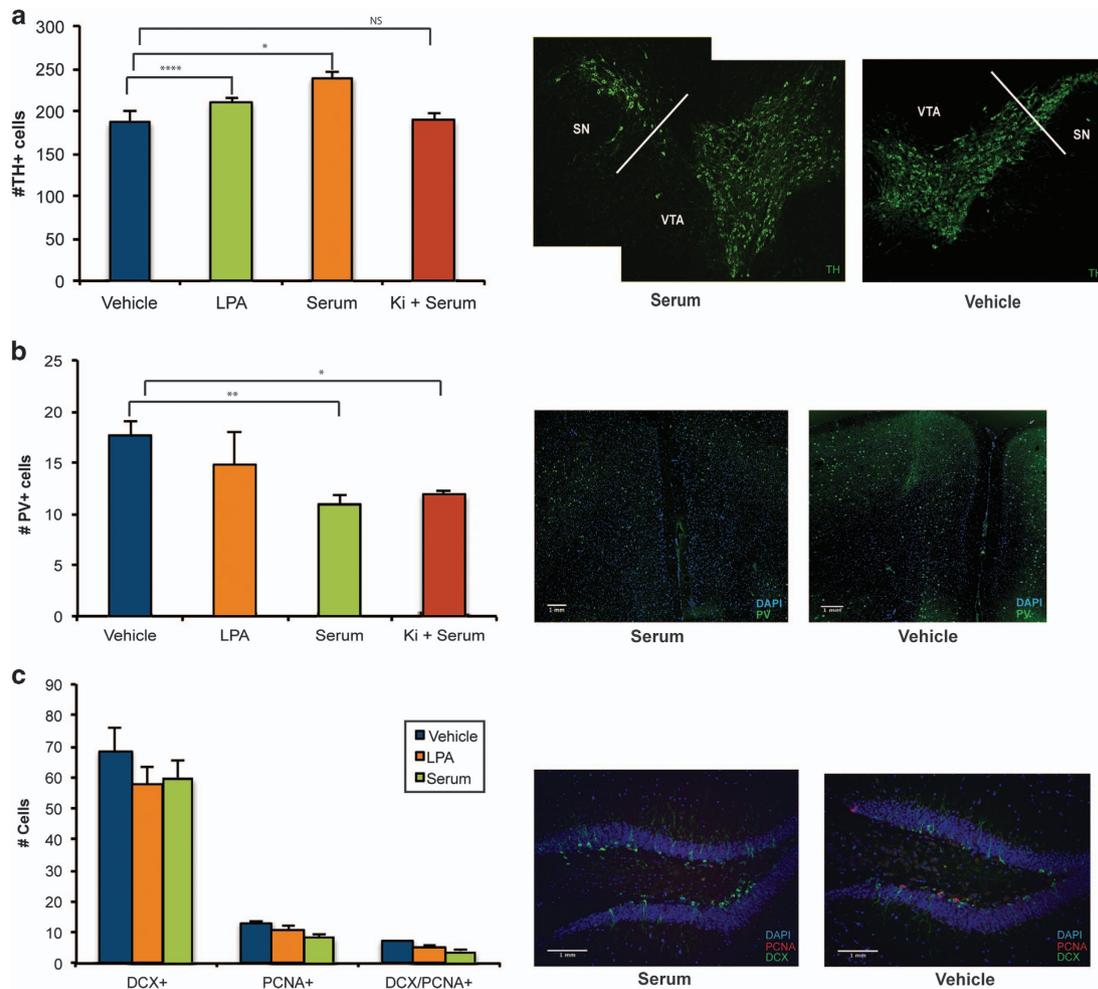


Figure 4. Prenatal serum exposure induces stereotypical dopaminergic and GABAergic immunohistochemical alterations partially dependent on LPA₁ signaling. (a) In adult female mice, serum and LPA exposure increased dopaminergic cell numbers in the midbrain (Bregma coordinates -3.00 to -3.80 ; tyrosine hydroxylase (TH+, green). Ki16425 treatment (Ki+serum) prevents TH+ cell number increases. (b) Serum exposure, but not LPA, reduces parvalbumin cell number (PV+, green) in the prelimbic medial prefrontal cortex (Bregma coordinates 1.80 – 1.40) independent of LPA₁ signaling. (c) Hippocampal neurogenesis (number of DCX+/PCNA+ cells) was not affected (Bregma coordinates -1.20 to -2.00). All data were analyzed with two-way multivariate analysis of variance comparisons between groups (vehicle, serum, LPA and Ki16425+serum when applicable). $n = 4$ – 7 per group, mean \pm s.e.m. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. LPA, lysophosphatidic acid; NS, not significant; PCNA, proliferating cell nuclear antigen; VTA, ventral tegmental area.

Both the LPA and hemorrhage/serum models of both sexes display an interesting baseline hypoactivity. When these mice are exposed to amphetamine, male and female LPA-exposed and male serum-exposed mice are insensitive to amphetamine-induced hyperactivity. LPA₁ receptor antagonism does not prevent this hypoactivity, suggesting the action of an additional LPA receptor. The amphetamine insensitivity may be a result of altered glutamatergic neurotransmission or even monoaminergic transmission, which has been shown in other rodent models of decreased amphetamine sensitivity.^{44,45} However, the serum-exposed females are significantly affected by amphetamine exposure. The baseline hypolocomotion serum-exposed females exhibit is not only treated upon amphetamine exposure, bringing the values up to those of vehicle-exposed female mice, but also induces prolonged hyperactivity in serum-exposed female mice (Supplementary Figures 3c and d). Importantly, this is an LPA-dependent hypersensitivity to amphetamine. LPA₁ inhibition, therefore, appears to prevent amphetamine-induced hyperlocomotion in serum-exposed females, though does not affect the baseline hypoactivity.

There have been many previous reports on sex differences in the development of neuropsychiatric symptoms in both humans²⁸ and mouse models.^{23,27} Disease onset and time course are different

between male and female schizophrenia patients,²⁸ and a recent study suggested that males and females have different molecular profiles, with females exhibiting dysregulation in inflammation-related pathways.⁴⁶ Estrogen is known to increase the immune response, whereas androgens decrease it.⁴⁷ Consequently, females are known to display stronger antibody responses, increased resistance to foreign grafts and an increased risk for autoimmune diseases.⁴⁸ Some maternal infection mouse models of schizophrenia (MIA models) also display sex-specific deficits,²⁷ and LPA and hemorrhage events are known to induce immune activation.⁴⁹

A recent report described sex-specific S1P₂ expression increases in multiple sclerosis female patients and in a mouse model for multiple sclerosis.⁵⁰ S1P is a bioactive lipid similar to LPA, and these data suggest the possibility that differential timing and location-specific expression of LPA₁ in females during development could drive the sex-specific behavioral deficits seen in our models. In addition, one of the original reports on *Lpar1*^{-/-} mice described a female-specific PPI deficit,³² whereas subsequent reports using a modified *Lpar1*^{-/-} line showed behavioral deficits in males (females were not tested).^{31,51–53} Further assessment of LPA receptor deletion in strain- and sex-specific populations will provide a greater understanding of potential behavioral modifier

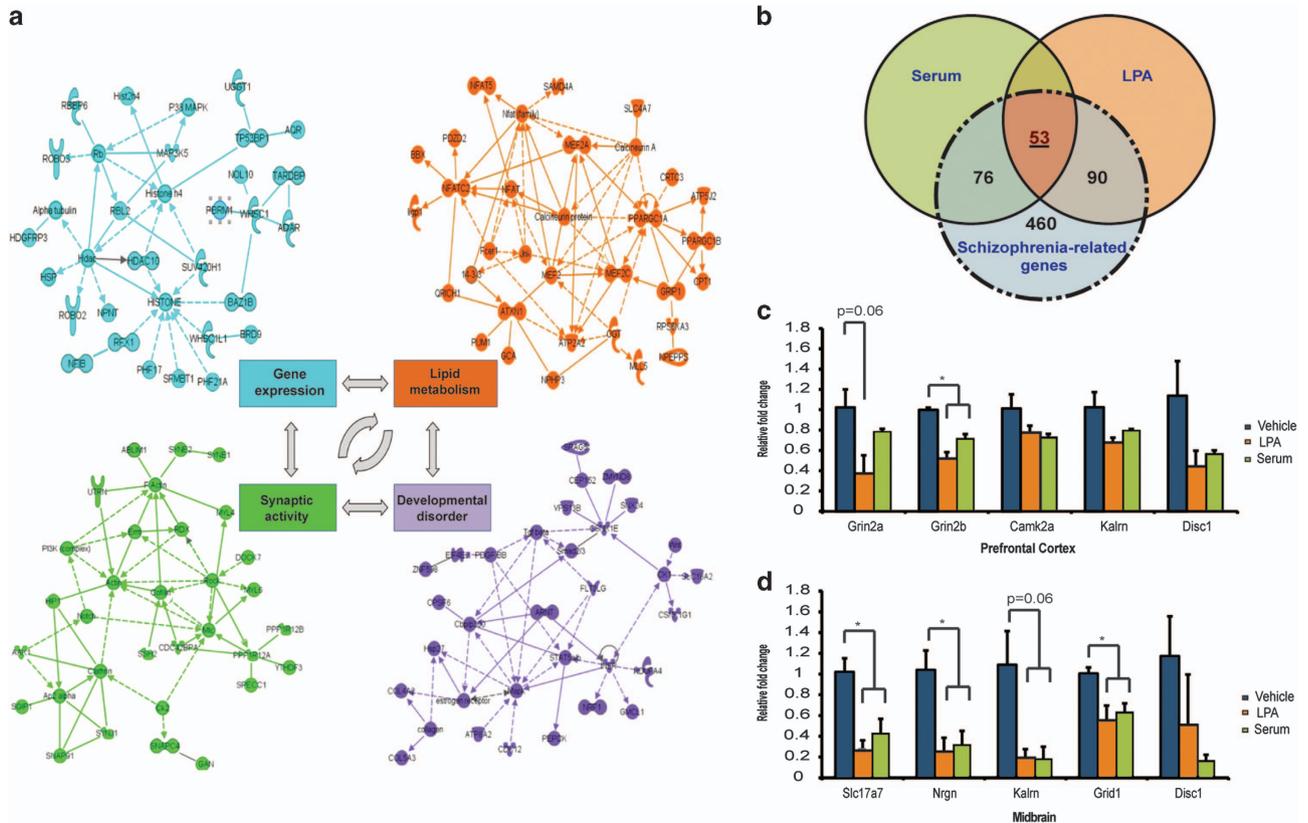


Figure 5. Prenatal serum- and LPA exposure alters gene expression profiles relevant to schizophrenia in adult animals. RNA was isolated from female adult prefrontal cortex (Bregma coordinates 2.50–1.30) and midbrain (Bregma coordinates –3.00 to –4.20) and gene expression changes were assessed by microarray analyses ($n=5$ per group). **(a)** Gene networks of greatest relevance were related to developmental disorder, gene expression, lipid metabolism and synaptic activity in the prefrontal cortex (as determined by Ingenuity Pathway Analysis software). **(b)** Venn diagram illustrating overlap between genes altered with serum/LPA exposure and schizophrenia-related genes in the combined midbrain and prefrontal cortex. **(c)** and **(d)** RT-PCR (PCR with reverse transcription) validation of selected genes ($n=3$, mean \pm s.e.m., Student's t -test, $*P < 0.05$). LPA, lysophosphatidic acid.

Table 1. Summary of the effects that prenatal exposure to serum, LPA and LPA₁ antagonist produced in adult female mice

Schizophrenia-related deficits	Serum	LPA	LPA ₁ antagonist
PPI	↓	↓	↑
Induced hyperactivity	↑	—	↓
Anxiety	↑	↑	↓
Social interaction	↓	—	—
Dopaminergic alterations	↑	↑ ^a	↓
GABAergic alterations	↓	± ^a	— ^a
Glutamatergic alterations	↓	↓	NA

Abbreviations: LPA, lysophosphatidic acid; NA, not assessed; PPI, prepulse inhibition; PV+, parvalbumin-positive. Prenatal serum exposure induces several schizophrenia-related behavioral and neurochemical deficits that are primarily dependent on LPA receptor signaling. These models display good face validity (through validated schizophrenia-like deficits) and construct validity (through the modeling of prenatal hemorrhaging, a validated schizophrenia risk factor). ^aPV+ cell numbers were not decreased in prenatal LPA-exposed females. However, the expression of many GABAergic-related genes was altered in the PFC and midbrain of female mice prenatally exposed to LPA.

genes associated with LPA receptor signaling. Ultimately, our findings suggest that prenatal hemorrhage may impact females more severely than males, at least in the C57BL/6J background utilized here. It is also possible that differences in the timing and duration of prenatal insults are necessary to observe prenatal hemorrhage-induced schizophrenia symptoms in males. In

addition, at least one LPA receptor (LPA₄) is X-linked, which may contribute to the sexual dimorphism observed here.

Receptor and timing mechanisms may also explain the effects of high concentrations of LPA that produce functional antagonism of LPA₁. A relatively brief functional antagonism within a 'critical period' of susceptibility appears to be sufficient to produce a phenotypic change. Notably, functional antagonism is the mechanism used by a highly related agonist-receptor combination of S1P and one of its cognate receptors, S1P₁.³⁶ S1P, like LPA, is a lysophospholipid, which also signals through related G protein-coupled receptors (historically, S1P₁ was first thought to be an LPA receptor).⁵⁵ An S1P receptor modulator, fingolimod,^{56–58} was found to produce an initial agonism followed by prolonged functional antagonism with continued drug exposure.^{34–37} In fact, recovery of S1P₁ expression required 2–8 days following an initial incubation with fingolimod.³⁴ Therefore, the functional antagonism of LPA₁ observed here by calcium imaging and receptor internalization, during a critical developmental window could account for the overlapping behavioral phenotype seen in the constitutive LPA₁ receptor deletion mutant. Functional antagonism and the resultant influence on NPCs likely takes place during a comparatively brief critical period of susceptibility, however, many cells expressing LPA₁ at that stage of development would likely be affected.

Disturbances in dopaminergic and glutamatergic neurotransmission, as well as deficits in GABAergic regulation, have frequently been observed in schizophrenia patients and models.^{59,60} Alterations in cell numbers—like the TH+ dopaminergic cell increases seen not only in our prenatal hemorrhage model but also in MIA²¹ schizophrenia-like

models—frequently portend disruptions in the balance between excitatory and inhibitory neurotransmission.⁵⁹ Glutamatergic signaling alterations, both through NMDA and AMPA receptors, are particularly implicated in schizophrenia-like behavioral deficits,⁶¹ and LPA is known to initiate glutamate uptake.⁶² Modification of LPA₁ expression has also been specifically linked to altered miniature excitatory postsynaptic potential kinetics and frequency, inhibitory postsynaptic potential amplitude and gamma oscillations.^{63–65} These data implicate LPA signaling-induced neurotransmission alterations as a possible mechanism for the specific glutamatergic and dopaminergic-related neural network and behavioral deficits manifested by our mouse model of prenatal hemorrhage. Future studies will characterize LPA's effect on neurotransmission within this model, particularly in relation to antipsychotic drug therapy and predictive validity for schizophrenia-related behaviors.

Schizophrenia is a disease dependent on synergistic interactions between multiple environmental, genetic and epigenetic risk factors. LPA has been linked to many of the underlying pathways involved in these risk factors. Glycogen synthase kinase 3 (Gsk3)—a signaling network node affected by several schizophrenia-related risk factors such as *Disc1*, *Nrg1*, thymoma viral proto-oncogene 1 (*Akt1*) and *reelin*—can be regulated by LPA.^{66–69} Disturbances in neural precursor cell migration, proliferation and cortical layer formation are strongly linked to several environmental and genetic risk factors, and LPA receptor modulation is known to be capable of disrupting these neurodevelopmental processes.¹⁵ Alterations in LPA receptor signaling could represent a shared, proximal modulator of these molecular and cellular events; specifically those events initiated by hemorrhage or related environmental risk factors for schizophrenia. Notably, gene expression analyses identified a significant overlap with a highly validated schizophrenia-related gene set in both serum- and, especially, LPA exposure models.

The relationship of our model to a more severe phenotype, hydrocephalus, is consistent with the heterogeneity seen in other schizophrenia models. Prenatal exposure to high concentrations of LPA (10 mM in 3 μ l) during peak neurogenesis (E13.5) induces severe hydrocephalus and ventricular dilation,¹⁴ whereas low concentrations (500 μ M in 3 μ l) initiate schizophrenia-like deficits. A similar relationship is seen in the MIA model, which displays more severe, schizophrenia-like deficits when induced during early gestation (E9), but displays autism-like deficits when induced during late gestation (E16.5).⁷⁰ The involvement of LPA signaling in neurological and neuropsychiatric diseases as diverse as hydrocephalus and schizophrenia suggests that a range of neurological effects could be initiated by aberrant LPA receptor signaling. Several other neurodevelopmental disorders, including cerebral palsy,⁷¹ bipolar disorder,⁷² depression⁷³ and autism,⁷⁴ have also been epidemiologically associated with prenatal bleeding. It is possible that the timing, severity and/or duration of aberrant LPA receptor signaling during prenatal bleeding could also contribute to the development of a number of these disorders. Considering the tractability of this receptor family for therapeutics, as demonstrated by the FDA's approval of fingolimod for the treatment of multiple sclerosis,⁵⁸ targeting LPA receptors may offer a therapeutic approach to some forms of neurological and neuropsychiatric disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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