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Ketamine Exposure in Early Development Impairs Specification of the Primary Germ Cell Layers

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Abstract

Preclinical and clinical evidence implicates N-methyl-D-aspartate receptor (NMDAr) signaling in early embryological development. However, the role of NMDAr signaling in early development has not been well studied. Here, we use a mouse embryonic stem cell model to perform a step-wise exploration of the effects of NMDAr signaling on early cell fate specification. We found that antagonism of the NMDAr impaired specification into the neuroectodermal and mesoendodermal cell lineages, with little or no effect on specification of the extraembryonic endoderm cell lineage. Consistent with these findings, exogenous NMDA promoted neuroectodermal differentiation. Finally, NMDAr antagonism modified expression of several key targets of TGF- β superfamily signaling, suggesting a mechanism for these findings. In summary, this study shows that NMDAr antagonism interferes with the normal developmental pathways of embryogenesis, and suggests that interference is most pronounced prior to neuroectodermal and mesoendodermal cell fate specification.

Keywords

Mouse embryonic stem cells; NMDA; Ketamine; Neurogenesis; Mesoendoderm; Differentiation

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Author Contributions

O.A performed all cell culture experiments, analyzed the data and wrote the initial draft of this manuscript. B.D analyzed the RNA sequencing experiments. S.C performed confocal microscopy experiments. J.I generated the RNA sequencing samples. O.A and K.E contributed to the experimental design, and revised the manuscript.

1. Introduction

Ketamine is a widely used anesthetic, analgesic, and sedative agent that is also currently being investigated as a new chronic treatment for major depressive disorder (Dong and Anand, 2013; Lapidus et al., 2013). Multiple lines of evidence implicate ketamine in the alteration of neural stem cell differentiation (Cuevas et al., 2013; Dong and Anand, 2013; Felix et al., 2013; Kanungo et al., 2013). However, the effects of intrauterine ketamine exposure during early gestation are not clear. In humans, ketamine abuse during pregnancy has been reported to result in the birth of an infant with intrauterine growth retardation, remarkable hypotonia, and poor reflex responses (Su et al., 2010).

Ketamine's principal molecular target is the N-methyl-D-aspartate (NMDA) receptor (NMDAr), a major postsynaptic, ionotropic receptor for the excitatory neurotransmitter glutamate. (Brown et al., 2011; Sinner and Graf, 2008). This pharmacologically defined receptor has an obligatory NR1 subunit and a modulatory NR2 subunit (Brown et al., 2011). Channel opening requires that glutamate or NMDA binds to the NR2 subunit and glycine binds to the NR1 subunit (Brown et al., 2011). Ketamine antagonizes the NMDAr by uncompetitive binding at a location other than the glutamate or glycine sites (Brown et al., 2011).

Since mammalian development proceeds in a relatively inaccessible manner, laboratory investigations modeling time points that correspond to *in utero* ketamine exposure or NMDAr antagonism has been challenging. However, cellular populations representing early developmental stages accessed with mouse embryonic stem cells (ESC) provides an attractive approach for addressing the molecular and cellular underpinnings of chronic intrauterine NMDAr antagonism. A recent report described a role for early alcohol exposure in significantly diminishing the differentiation potential of ESC in an apoptosis independent manner. Of note, the effects of alcohol are due in large part to either NMDAr antagonism or activation of γ -Aminobutyric acid receptors (GABA_Ar) (Ikonomidou et al., 2000). At the receptor level, GABA_Ar modulation has been shown to function in ESC proliferation (Andang et al., 2008). However, it remains untested whether NMDAr modulation has an effect on early development or cell fate-specification, and if so, what that effect might be.

The underlying mechanisms of NMDAr signaling are complex. Experimental evidence suggests that NMDAr activation causes a Ca^{2+} influx. This influx is responsible for cAMP-response-element-binding-protein and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) mediated gene expression (Hardingham and Bading, 2003). The catalytic subunit of NF- κ B, I κ B kinase, is a critical coregulator of transforming growth factor-beta (TGF- β) signaling (Descargues et al., 2008). Given the central role of TGF- β signaling in early embryonic development (Oshimori and Fuchs, 2012), we hypothesized that perturbation of NMDAr signaling in the developing embryo might impair normal cell fate specification. Thus, the objective of this study was to use an ESC model to perform a step-wise exploration of the effects of NMDAr signaling on early cell fate specification.

2. Materials and Methods

2.1. NMDAr modulators

Ketamine (Ketamine hydrochloride, 50 mg/mL) was obtained from Bioniche Pharma (Lake Forest, IL). MK-801 and NMDA were both obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Cell Culture

All cell cultures were maintained at 37°C, 5% CO₂. ESC were cultured on irradiated mouse embryonic fibroblasts (MEFs) with ESC medium, which consisted of Knockout DMEM (Invitrogen) supplemented with 15% Hyclone (Gibco), 1×10^3 units mL⁻¹ recombinant murine leukemia inhibitory factor (LIF), 1× GlutaMax, (Invitrogen), 1× nonessential amino acids (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), and 0.1 mM 2-mercaptoethanol (Sigma). Media was changed every 24 hours and cells were passaged every third day as a single cell suspension using 0.25% trypsin/EDTA (Invitrogen). We thank T. Jessell for *Hb9::GFP* ESC (Wichterle et al., 2002), A. Smith for *Sox1::GFP* ESC (Aubert et al., 2003), G. Keller for *Brachyury::GFP* ESC (Fehling et al., 2003), K. Hochedlinger for *Nanog::GFP* ESC (Maherali et al., 2007), and S. Morrison for *Sox17::GFP* ESC (Kim et al., 2007).

2.3. Embryoid Body Formation and differentiation

Embryoid bodies (EBs) were generated from ESC after MEF depletion (Cocouvanis and Martin, 1995). In order to form EBs, ESC was grown in ultra low cluster 6-well plates (Corning). For spinal motor differentiation, approximately 1×10^6 cells were suspended in 3mL of knock-out serum replacement (KOSR) media and media was changed every 48 hours. KOSR media consisted of DMEM/F12 (Invitrogen) supplemented with 10% knockout serum replacement (Invitrogen), 1× GlutaMax (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen), and 0.1 mM 2-mercaptoethanol (Sigma). After 48 hours from the time of initial suspension, EBs were induced towards spinal motor neuron identity for 5 days using 0.01 M Retinoic Acid (Sigma) and 1.3M Smoothed Agonist (Calbiochem). At day 7, EBs were dissociated to single cells with Papain/DNase (Worthington Bio). The resulting single cells were then washed with D-MEM/F12 and plated on culture slides or analyzed by flow cytometry. For plating, cells were first resuspended in motor neuron media supplemented with neurotrophic factors (GDNF, BDNF, CNTF; 10ng/mL, R&D Systems) and an equal number of cells were then plated onto poly-lysine laminin-coated chamber slides (BD Biosciences) coated with Matrigel (BD Biosciences). Motor neuron media consisted of F12 (Invitrogen), 1× GlutaMax (Invitrogen), 5% Horse serum (Invitrogen), 1× N2 supplement (Invitrogen), 1× B27 supplement (Invitrogen). For, mesoendodermal and extraembryonic endodermal differentiation, cells were cultured in EB media (Niakan et al., 2010; Vigneau et al., 2007). EB media consisted of ESC media described above, without LIF and NEAA.

2.4. Flow cytometry

The LSRFortessa cell analyzer (BD Biosciences) was used for flow cytometry. Data was analyzed in FlowJo (Version 9.4.10).

2.5. Immunohistochemistry

EBs were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, equilibrated in 30% sucrose, embedded and cryosectioned (25µm) prior to antibody staining. Neuronal cell cultures were fixed in 4% PFA for 15 minutes. Cells were permeabilized with 0.2% Triton-X in PBS for 45 minutes and incubated in blocking solution for 1 hour (10% donkey serum, Triton-X 0.1%). Cells were then incubated in primary antibody overnight and secondary antibodies for 1 hour in blocking solution after several washes in between. DNA was visualized by a Hoechst stain. The following primary antibodies were used: TUJ1 (1:1000, Sigma, T2200), Pax6 (1:100, DSHB, Pax6), Nkx2.2 (1:100, DSHB, 75.5A5), Nkx6.1 (1:100, DSHB, F55A10), Nestin (1:100, DSHB, Rat-401), Olig2 (1:250, Millipore, AB9610). Secondary antibodies used were AlexaFluor (1:1000, Life echnologies; 488, 555, 594, and 647).

2.6. Imaging

Confocal immunofluorescence pictures of EB cryosections were taken with a Zeiss LSM 700 confocal. Epifluorescence images were performed on a Zeiss AX10 microscope using and an Axiocam HRC camera.

2.7. iPS generation, Embryonic Motor Neuron and RNA sequencing

MEFs were harvested from *Hb9::GFP* E12.5 embryos under a dissection microscope (Leica). To generate mouse induced pluripotent stem cells (iPSCs), MEFs were transduced with retroviruses (pMXs vector) encoding *Oct4*, *Sox2*, and *Klf4*. Cells were cultured in ESC media and colonies were picked, expanded, and verified by Nanog immunostaining. Embryonic motor neurons were harvested from *Hb9::GFP* E13.5 embryos. Briefly, whole spinal cords were washed in F-12 (Invitrogen) and incubated in 10 ml of 0.025% trypsin with DNase for 45 min with gentle agitation every 15 min. Media was added to the dissociated spinal cords and the cells were triturated, spun down at 1,000 rpm for 5 min, and resuspended in DMEM/F-12 with glutamax and penicillin/streptomycin prior to flow purification of *Hb9::GFP*⁺ motor neurons directly into Trizol. Following harvesting of RNA from indicated sources, RNA quality was determined using BioAnalyzer (Agilent). RNA integrity numbers above 7.5 were deemed sufficiently high quality to proceed with library preparation. RNA sequencing libraries were generated from ~250 ng total RNA using the illumina TruSeq RNA kit v2, according to the manufacturers' directions. Libraries were sequenced at the Harvard Bauer Core Sequencing facility on a HiSeq 2000. Libraries were generated from at least two independent biological replicates and 20–40 million, 100 base pair, paired end reads were obtained for each sample. Reference files of the genome build mm9 (mouse), as well as ensembl transcript annotations, were obtained from iGenomes (http://support.illumina.com/sequencing/sequencing_software/igenome.ilmn). Reads were aligned to the genome using the split read aligner Tophat (v2.0.7) and Bowtie2 (v2.0.5)16 using default parameters. Transcript assembly and isoform-specific quantitation was performed using Cufflinks (v2.1.1). Abundance of individual isoforms is reported as fragments per kilobase of transcript per million mapped reads (FPKM). Computations were performed on the Odyssey cluster supported by the FAS Science Division Research Computing Group at Harvard University.

2.8. Microarray

For microarray analysis, total RNA from four biological replicates of control and ketamine (200 μ m; day 0–2) treatment was harvested at day 2. RNA was amplified and biotin labeled using Illumina TotalPrep RNA Amplification kit (Ambion). The illumina MouseRef-8 v2.0 Expression BeadChip Kit was used and the cRNA was analyzed with an in-house Illumina BeadArray Reader. The quality of the raw data generated by Illumina Beadstudio was evaluated using Bioconductor packages. The normalized gene expression data was used to identify significant differentially expressed genes using the empirical Bayes moderated *t*-test in the Bioconductor package Linear Models for Microarray Data (LIMMA). A Multiple test corrected False Discovery Rate *P* value < 0.05 and greater than 2 fold expression difference was used as a cutoff to identify differentially expressed genes.

2.9. RT-PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen) and reverse transcribed using the iSCRIPT kit (Bio-rad). Quantitative RT-PCR was then performed using SYBR green (Bio-Rad) and the iCycler system (Bio-rad). Quantitative levels for all genes were normalized to endogenous GAPDH and expressed relative to the control samples using the Ct method.

2.10. Statistical analysis

Statistical significance (comparisons to control) was assessed with a two-tailed Students T-test with a Bonferroni adjusted *P* value to account for the multiple comparisons. For the Nanog experiments, statistical significance was assessed by the Tukeys HSD test. Statistical analysis was performed using the JMP 10.0.0 software (SAS Institute Inc.). Error bars represent $\pm$ standard error of the mean (s.e.m.)

3. Results

3.1. NMDAr antagonism (ketamine and MK-801) Impairs Specification of ESC into Neurons

We tested the effect of NMDAr antagonism on a well-characterized EB stem cell differentiation strategy for producing spinal motor neurons (Fig. 1A). This strategy was attractive for our studies because it generates neurons through a step-wise process recapitulating many aspects of normal embryological development (Di Giorgio et al., 2007; Wichterle et al., 2002). We found that when we exposed differentiating cultures to the selective NMDAr antagonist's ketamine (200 μ m, day 0–7) or MK-801 (200 μ m, day 0–7) throughout the motor neuron differentiation experiment, there was a reduction in the number of Tuj1+ and *Hb9*::GFP+ cells with a neuronal morphology (Fig. 1B, C)

3.2. Early NMDAr antagonism Impairs Specification of ESC into Neurons

To quantify the effect of NMDAr antagonism on differentiation, we utilized an ESC line harboring an *Hb9*::GFP transgene. Since this transgene is selectively activated in motor neurons (Wichterle et al., 2002), it enables for a more precise flow cytometry based analysis. We exposed cultures to ketamine (200 μ m) for distinct time periods, and then quantified the number of *Hb9*::GFP+ cells by flow cytometry (Fig. 1D, E). When we treated cultures from

days 1–7, we found that there was a significant reduction in motor neuron differentiation from 16% to 2% *Hb9*::GFP+ cells ($P < 0.0001$, Bonferroni adjusted $P = 0.0038$). Similarly, treating cultures with ketamine for overlapping periods of time that accounted for most of differentiation (days 2–7, 0–6, 0–5, 0–4; $P < 0.0001$ for all conditions, Bonferroni adjusted $P = 0.0038$) resulted in a significant reduction of motor neuron differentiation. Treatment at a very early time point (day 0–1; $P = 0.8212$, Bonferroni adjusted $P = 0.0038$) had no effect on motor neuron differentiation. Likewise, treatment at later time points (days 3–7, $P = 0.0088$; 4–7, $P = 0.0029$; 5–7, $P > 0.0176$; 6–7, $P = 0.0328$; Bonferroni adjusted $P = 0.0038$) had only a modest effect on motor neuron differentiation. However, shorter treatment at relatively early time points (days 0–3; $P < 0.0001$; days 0–2, $P = 0.0004$; Bonferroni adjusted $P = 0.0038$) recapitulated the effects of longer treatment (Fig. 1D, E). These results suggest that ketamine was most likely exerting an influence during early stages of neural specification rather than later events such as patterning of neuronal precursors or neuronal survival.

3.3. Impaired Specification of ESC into Neurons is dose dependent

When we treated differentiating cultures with ketamine (day 0–2) and quantified *Hb9*::GFP expression at day 7, we found that there was a significant dose dependent reduction in *Hb9*::GFP+ cells (Fig. 2A; 50 μ m, $P = 0.0056$; 100 μ m, $P = 0.0004$; 200 μ m, $P < 0.0001$; Bonferroni adjusted $P = 0.0125$). Similarly, when we treated cultures from days 1–2 with ketamine and quantified *Hb9*::GFP expression at day 7, we found that there was also a significant dose dependent reduction in *Hb9*::GFP+ motor neuron differentiation (Fig. 2B; 100 μ m, $P = 0.0094$; 200 μ m, $P = 0.0054$; Bonferroni adjusted $P = 0.0125$). When we directly fixed, sectioned and stained EBs treated with ketamine (200 μ m, day 0–2), we confirmed that *Hb9*::GFP+ fluorescence was reduced (Fig. 2C). We also found a significant reduction in the number of Tuj1+ neurons after ketamine treatment, suggesting that NMDAr antagonism was more generally influencing neuronal differentiation (Fig. 2C).

3.4. NMDAr receptor subunits are expressed in ESC and differentiating neurons

To experimentally determine the presence and relative abundance of NMDAr subunits, we performed RNA sequencing on ESC, iPSC, ESC derived *Hb9*::GFP motor neurons, iPSC derived *Hb9*::GFP motor neurons, *Hb9*::GFP purified embryonic motor neurons, and MEFs. NMDAr subunits (NR1, NR2a, NR2b, NR2C, NR2d, NR3a, and NR3b) were most highly expressed in mouse embryonic motor neurons. In ESC and iPSC, the NR1 and NR2c were more abundant relative to the modest expression levels of the other subunits (NR2a, NR2b, NR2d, NR3a, and NR3b). Directed differentiation of ESC and iPSC into motor neurons resulted in increased expression of NR2a, NR2c, NR2d and NR3b compared to their ESC and iPSC progenitors. For comparison, we also profiled the expression pattern of MEFs. We found that although MEFs expressed NR1, NR2d and NR3a subunits, the expression levels were lower than that detected in motor neurons or pluripotent stem cells. Taken together, these results confirm that NMDAr subunits are expressed in ESC, iPSC and the resultant spinal motor neurons obtained from their directed differentiation.

3.5. Ketamine and MK-801 impair ESC differentiation into Neural Stem Cell Progenitors

Motor neuron differentiation unfolds in a step-wise manner in which early *Sox1*⁺ neural ectodermal progenitors, give rise to more lineage restricted neural progenitors that express factors such as *Pax6*, *Nkx6.1* or *Nkx2.2* depending on their dorsal-ventral identity (Wichterle et al., 2002). In the case of motor neurons, progenitors transit through expression of *Pax6* then *Nkx6.1* and ultimately *Olig2* under the influence of proper levels of sonic hedgehog activity. These *Olig2*⁺ progenitors then activate *Hb9* and *Hb9::GFP* expression at the time of mitotic exit. We found that treatment with ketamine (day 0–2) led to a dose dependent reduction in *Olig2*⁺ cells (Fig 4A, B; 50 μ m, $P = 0.0057$; 100 μ m, $P < 0.0001$; 200 μ m, $P < 0.0001$; Bonferroni adjusted $P = 0.0125$). We also found that treatment with MK-801 (day 0–2) led to a dose dependent reduction in *Olig2*⁺ cells (Fig 4C; 100 μ m, $P = 0.0005$; 200 μ m, $P < 0.0001$; Bonferroni adjusted $P = 0.017$). When we directly fixed, sectioned and stained EBs treated with ketamine (200 μ m) from day 0–2, we confirmed that *Olig2* expression was decreased (Fig. 4D; 200 μ m ketamine). We found that ketamine treatment (200 μ m; day 0–2) also decreased the expression of *Nkx2.2* and *Nkx6.1* in differentiating EBs. This suggested that ketamine was acting early in neural specification to influence dorsal-ventral patterning (Fig. 4E). Lastly, we found that ketamine treatment (200 μ m; day 0–2) decreased the expression of *Pax6* and *Nestin* (Fig. 4F), suggesting that ketamine was regulating very early phases of neural specification rather than dorso-ventral patterning.

3.6. NMDAr activation promotes ESC differentiation into Neuronal Progenitor Cells

We found that treatment with NMDA (10 μ m, day 0–2) led to a modest but significant increase in the number of *Olig2* progenitors (Fig. 4G; 10 μ m, $P = 0.006$; Bonferroni adjusted $P = 0.0125$). To test whether the NMDAr was regulating early neural specification, we utilized a mouse embryonic stem cell line in which GFP was targeted to the endogenous *Sox1* locus (*Sox1::GFP*). Consistent with the idea that NMDAr modulation acts either at or before the level of the *Sox1* progenitor, we found that ketamine treatment (day 0–2) inhibited the accumulation of *Sox1*⁺ cells in a dose dependent manner (Fig. 4H and I; 50 μ m, $P = 0.0017$; 100 μ m, $P = 0.0003$; 200 μ m, $P = 0.0001$; 300 μ m, $P < 0.0001$; Bonferroni adjusted $P = 0.007$). We also found that the combination of RA and 10 μ m of NMDA significantly led to a significant increase of *Sox1*⁺ cells from 28% to 45% suggesting a synergistic effect (Fig. 4H, I, $P < 0.0001$; Bonferroni adjusted $P = 0.007$). These results suggest that NMDAr antagonism impairs neuroectodermal specification and that NMDAr activation functions alone and synergistically with RA to promote neuroectodermal specification.

3.7. Ketamine Impairs ESC specification into Mesoendodermal Progenitor Cells

As our results suggested that NMDAr modulation modified neuroectodermal specification, we reasoned that NMDAr might also regulate progenitors of the embryonic germ layers obtained from ESC (Fig. 5). To test this hypothesis, we examined the influence of NMDAr modulation on expression of the T-box transcription factor *Brachyury*. EBs display a significant degree of self-organization manifested by establishment of anterior-posterior polarity and the emergence of cells with properties of the primitive streak (ten Berge et al., 2008). In EBs, *Brachyury* is transiently expressed in cells with properties of the primitive

streak (ten Berge et al., 2008). *In vitro* lineage tracing studies show that both differentiating mesoderm and endoderm populations derive from these *Brachyury*⁺ cells (Fehling et al., 2003; Kubo et al., 2004). To test the role of ketamine in this context, we used a transgenic *Brachyury*::GFP mouse ESC line and found that ketamine (200μm, day 0–2) treatment caused a significant decrease in *Brachyury* expression (Fig. 6A, B; day 4, $P = 0.0003$; day 5, $P = 0.0011$; Bonferroni adjusted $P = 0.017$).

3.8. Ketamine does not Impair ESC specification into Extraembryonic Endoderm Cells

Sox17 has been shown to be an important mediator of extraembryonic endoderm (ExEn) differentiation (Niakan et al., 2010; Shimoda et al., 2007). Therefore, we tested whether NMDAr modulation played a role in the earliest phases of differentiation from the pluripotent state, corresponding to specification of the extraembryonic endoderm (ExEn) from the inner cell mass. By monitoring *Sox17*::GFP during mouse ESC differentiation, we found that NMDAr antagonism with ketamine (200μm, day 0–2) did not influence the specification of ExEn (Fig. 6C, D). We also found that NMDA (10μm, day 0–2) treatment had little or no effect on specification of ExEn (Fig. 6C, D). These results suggest that NMDAr modulation did not affect specification into the ExEn cell lineage.

3.9. NMDAr modulation affects Nanog Expression in ESC differentiation and not self renewal conditions

We tested the effect of NMDAr modulation on the differentiation of *Nanog*⁺ epiblast progenitors that give rise to the primitive streak and neuronal ectoderm. Although not statistically significant, treatment of differentiating cultures with ketamine (200μm, day 0–2) caused an increase in the percentage of *Nanog*⁺ cells from 12% to 15% (Fig. 6E, F). Conversely, treatment with NMDA (10μm, day 0–2) reduced the percentage of *Nanog*⁺ cells from 12% to 9% (Fig. 6E, 6F). When we compared ketamine exposed *Nanog*⁺ cells to NMDA exposed *Nanog*⁺ cells in differentiating conditions, this difference was statistically significant (Fig. 6E, F; $P = 0.04$, Tukeys HSD). We also tested the effect NMDAr modulation on *Nanog*⁺ epiblast progenitors in self-renewal conditions (ESC media). When we compared the ketamine (200μm, day 0–2), NMDA (10μm, day 0- and control groups, there was no difference between all groups (Fig. 6G; $P > 0.05$ for all comparisons, Tukeys HSD). These results suggest that NMDAr modulation regulates exit from a pluripotent state in ESC.

3.10. Microarray and RT-PCR of Embryoid Bodies Exposed to Ketamine

In order to gain mechanistic insight into how NMDAr antagonism impacts lineage specification, we compared the transcriptional profiles of control EBs ($n = 4$) and ketamine treated EBs ($n = 4$; 200μm, day 0–2) and also performed functional and pathway enrichment analysis (Tables S2 and S3). Expression changes were deemed significant only if they showed a twofold change in expression compared to the controls, and had false discovery rate corrected P value less than 0.05 (Fig. 7A, B). We then confirmed significant alterations in transcription by qRT-PCR (Fig. 7C, Fig. S1). These studies identified *Otx2*, *Lefty1* and *Pitx2*, critical downstream targets of the TGF- β signaling pathway as genes whose transcription were depressed by NMDAr antagonism (Acampora et al., 2009; Beddington

and Robertson, 1999; Shen, 2007; Takaoka et al., 2011; Takaoka et al., 2006; Yamamoto et al., 2004).

Discussion

Here, we use a combination of stem cell and reprogramming approaches to perform a step-wise exploration of the effects of NMDAr signaling on early cell fate specification. We found that NMDAr antagonists impaired specification into neuronal, neuronal progenitor, and neuroectodermal cells. Consistent with these findings, exogenous NMDA promoted neuronal progenitor and neuroectodermal specification. Furthermore, we found that NMDAr antagonism impaired specification into the mesoendodermal cell lineages with little or no effect on specification of the extraembryonic endoderm cell lineage. When we studied the effects of NMDAr antagonism on *Nanog* expression in the ESC, we found that NMDAr modulation had no effect on ESC maintained in self-renewal conditions. However, in differentiation conditions, NMDAr antagonism increased the number of *Nanog*⁺ cells while NMDA decreased the number of *Nanog*⁺ cells suggesting that NMDAr antagonism impaired the transition to *Nanog*⁻ intermediates. Finally, ketamine exposure during ESC differentiation modified expression of several key targets of TGF- β superfamily signaling, suggesting a mechanism for these findings. Altogether, our results suggest a role for the development of experimental models using NMDAr modulation to study and potentially explain the fetal abnormalities and decreased fecundity in women exposed to NMDAr antagonists (Kesmodel et al., 2002; Rowland et al., 1995; Rowland et al., 1992; Windham et al., 1997).

The three key targets of the TGF- β signaling pathway identified by our genetic profiling experiments as being downregulated by ketamine are *Otx2*, *Lefty*, and *Pitx2*. In the developing mouse embryo, *Otx2* null mutants exhibit a headless phenotype with severe gastrulation impairment (Acampora et al., 2009). Embryological and genetic studies show that *Otx2* activates expression of *Lefty1* in cells representing the distal visceral endoderm (Acampora et al., 2009). These *Lefty1*⁺ cells are necessary for the required movement of the anterior visceral endoderm to the future anterior side of the embryo, an event necessary for primitive streak positioning and forebrain development (Kimura et al., 2000; Kimura-Yoshida et al., 2005; Perea-Gomez et al., 2001). Furthermore, *Pitx2*, acting early in the TGF- β signaling pathway is also essential for normal germ cell layer formation (Faucourt et al., 2001). We noted that the phenotypes that result from genetic manipulation of these TGF- β targets parallel those observed in our model.

The results of this study are consistent with alteration in the differentiation potential of ESC that was recently demonstrated for alcohol in an ESC model (Sanchez-Alvarez et al., 2013). In that study, it was reported that early exposure of differentiating ESC to alcohol significantly alters the differentiation potential of ESC in an apoptosis independent manner (Sanchez-Alvarez et al., 2013). Interestingly, the effects of alcohol are due in large part to antagonism of the NMDAr and activation of GABA_AR. Since GABA_AR activation has been shown to limit ESC proliferation but not differentiation potential (Andang et al., 2008), we suggest that the described effects from modeling studies of fetal alcohol toxicity are likely due to NMDAr antagonism.

Our results using the ESC model are suggestive of decreased neural stem cell proliferation (Fig 4D, 4E and 4F). A recent study using a human embryonic stem cell (hESC) model found that ketamine increased neural stem cell proliferation without inducing apoptosis (Bai et al., 2013). This difference can be explained by the different developmental stages represented by ESC and hESC. hESC represent a later stage of embryonic development than ESC. Studies have likened hESC to epiblast stem cells which have recently been isolated from post-implantation stage mouse embryos (Brons et al., 2007; Nichols and Smith, 2009; Rossant, 2008; Tesar et al., 2007). At the stage of embryonic development represented by hESC, inhibition of the TGF- β pathway promotes differentiation along the neuronal lineage. Numerous hESC neuronal differentiation strategies make use of small molecule antagonists of the TGF- β signaling pathway to induce a rapid and very efficient neural conversion of hESCs (Chambers et al., 2009; Davis-Dusenbery et al., 2014; Zhou et al., 2010). Conversely, TGF- β inhibition at the earlier time point presented by ESC impairs normal gastrulation (Tam and Loebel, 2007).

We believe that our findings were mainly mediated through developmental effects rather than toxicity because when we examined the parent gated populations in our experiments (*Hb9*, *Olig2*, *Sox1*, *Brachyury*), there was no difference between NMDAr antagonist exposed and control groups (Fig. S2). At present, we can only speculate on the molecular mechanism responsible for this phenotype (Fig. 7D). Accordingly, an alternative explanation for our results is that ketamine and MK-801 both alter ESC differentiation through a non-NMDAr target. This seems unlikely given that exogenous NMDA administration increased ESC differentiation into neuronal cells. Genetic silencing experiments of the NMDAr subunits we have identified in this study may provide further insight. Since we took advantage of an *in vitro* ESC model, *in vivo* animal models that account for the rapid drug metabolism and excretion present in laboratory animals are necessary to complement our study.

In exploring the potential patterning deficits implicated with NMDAr antagonism, we found and describe a previously unrecognized role of NMDAr signaling in regulating key TGF- β responsive genes necessary in embryological development. Altogether, our results show that NMDAr regulates early cell-fate specification. We suggest that this is a previously unrecognized dynamic potentially contributing to embryotoxicity and we propose a model that strongly implicates alteration of TGF- β superfamily signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. NMDAr antagonism Impairs Specification of mouse embryonic stem cells (ESC) into Neurons

(A) Schematic of spinal motor neuron differentiation protocol.

(B) Representative immunofluorescence images of day 0–7 ketamine (200μm) exposed EBs derived from *Hb9::GFP* ESC. Scale bars represent 500μm.

(C) Immunofluorescence analysis of Tuj1 from control and day 0–7 ketamine (200μm) and MK-801 (200μm) exposed EBs. EBs were dissociated into single cells and plated on a monolayer. Nuclei were stained with Hoechst. Scale bars represent 5μm.

(D–E) Representative FACS plots showing gating of *Hb9::GFP* cells and quantification of *Hb9::GFP* cells after exposure to ketamine (200μm) during differentiation into spinal motor neurons. Data are shown as mean ± s.e.m., $n = 2$. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$ (Bonferroni adjusted $P = 0.0038$).

Figure 2. Early NMDA receptor antagonism decreases in differentiation potential of mouse embryonic stem cells (ESC) into neurons

(A) Quantification of Hb9::GFP cells after day 0–2 exposure to ketamine during differentiation into spinal motor neurons. Data are shown as mean $\pm$ s.e.m., $n = 3$. * $P < 0.0125$, ** $P < 0.001$, *** $P < 0.0001$ (Bonferroni adjusted P value = 0.0125).

(B) Quantification of Hb9::GFP cells after day 1–2 exposure to ketamine during differentiation into spinal motor neurons. Data are shown as mean $\pm$ s.e.m., $n = 3$. * $P < 0.01$, ** $P < 0.0001$ (Bonferroni adjusted P value = 0.0125)

(C) Immunofluorescence analysis of cryosectioned control and ketamine exposed (200um) EBs at day 5 (Retinoic Acid and Smoothed Agonist patterned) with labeled markers. Nuclei were stained with Hoechst. Scale bars represent 100um.

Figure 3. NMDAr receptor subunits are expressed in mouse embryonic stem cells (ESC) and differentiating neurons

The expression of NMDAr subunits in selected cell types are shown. FPKM, fragments per kilobase of transcript per million fragments mapped. Mouse embryonic fibroblasts (MEFs), mouse induced pluripotent stem cells (iPSC), mouse embryonic stem cells (ESC).

Figure 4. NMDA receptor modulation is involved in specification of neuronal precursor cells (A–B) Representative FACS plots showing gating of *Olig2::GFP* cells and dose response quantification of *Olig2::GFP* cells exposed to ketamine (days 0–2). Data are shown as mean $\pm$ s.e.m., $n = 3$. * $P < 0.0125$, ** $P < 0.0001$, (Bonferroni adjusted $P = 0.0125$). (C) Dose response quantification of *Olig2::GFP* cells exposed to MK-801 (days 0–2). Data are shown as mean $\pm$ s.e.m., $n = 3$. * $P < 0.001$, ** $P < 0.0001$ (Bonferroni adjusted P value = 0.017).

(D–F) Immunofluorescence analysis of cryosectioned day 5 EBs with labeled neural progenitor markers. Nuclei were stained with Hoechst. Scale bars represent 100µm.

(G) Dose response quantification of *Olig2::GFP* cells to NMDA. Data are shown as mean $\pm$ s.e.m., $n = 6$. $*P < 0.0125$, $**P < 0.0001$ (Bonferroni adjusted $P = 0.0125$).

(H–I) Representative FACS plots showing gating of *Sox1::GFP* cells and dose response quantification of *Sox1::GFP* cells to ketamine and NMDA. Data are shown as mean $\pm$ s.e.m., $n = 3$. $*P < 0.007$, $**P < 0.001$, $***P < 0.0001$ (Bonferroni adjusted $P = 0.007$).

Figure 5. Early Development pathway from mouse embryonic stem cells
Cell lineage and transcription factor relationships from embryonic stem cells and the earliest stages of differentiation.

Figure 6. NMDA receptor modulation is involved in specification of mesoendodermal cell lineage but not the extraembryonic cell lineage

(A–B) Representative FACS plots showing gating of *Brachyury*::GFP cells and dose response quantification of *Brachyury*::GFP cells exposed to ketamine (200μM). Data are shown as mean ± s.e.m., $n = 3$. * $P < 0.017$, ** $P < 0.001$, *** $P < 0.0001$ (Bonferroni adjusted $P = 0.017$).

(C–D) Representative FACS plots showing gating of *Sox17*::GFP cells and dose response quantification of *Sox17*::GFP cells exposed to ketamine (200μm). Data are shown as mean ± s.e.m., $n = 3$. * $P < 0.017$, ** $P < 0.001$, *** $P < 0.0001$ (Bonferroni adjusted $P = 0.017$).

(E–F) Representative FACS plots showing gating of *Nanog*::GFP cells and quantification of *Nanog*::GFP cells exposed to ketamine (200μm; day 0–2) and NMDA (10μm; day 0–2) in differentiation media. Cells were cultured as EBs in KOSR media. Data are shown as mean ± s.e.m., $n = 3$. * $P < 0.05$, Tukeys HSD.

(G) Quantification of *Nanog*::GFP cells exposed to ketamine (200μm; day 0–2) and NMDA (10μm; day 0–2) in self-renewal media. Data are shown as mean ± s.e.m., $n = 3$.

Figure 7. NMDA antagonism phenotype is associated with downregulation of key genes regulated by TGF-β superfamily signaling

The genes were identified in a supervised analysis using an absolute fold change of 2 and P value < 0.05.

(A) Hierarchical clustering of gene expression profiles of ketamine treated and control EBs on day 2 of differentiation. $n = 4$ replicates each. The columns represent samples and rows represent the genes. Gene expression is shown with pseudocolor scale (−3 to 3) with red denoting high gene expression levels and green denoting low gene expression levels of genes.

(B) Sample relations of experimental replicates based on 10980 genes.

- (C) Qualitative PCR confirming candidate downregulated genes and select upregulated genes.
- (D) Proposed model of NMDA antagonism mediated disruption of embryonic development implicating alteration in TGF- β superfamily signaling.