Published in final edited form as:

Schizophr Res. 2015 March ; 162(0): 216–221. doi:10.1016/j.schres.2014.12.034.

Subchronic Pharmacological and Chronic Genetic NMDA Receptor Hypofunction Differentially Regulate the Akt signaling pathway and Arc Expression in Juvenile and Adult mice

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Abstract

NMDA receptor (NMDAR) hypofunction is a compelling hypothesis for the pathophysiology of schizophrenia, because in part, NMDAR antagonists cause symptoms in healthy adult subjects that resemble schizophrenia. Therefore, NMDAR antagonists have been used as a method to induce NMDAR hypofunction in animals as a pharmacological model of schizophrenia. Serine racemase-null mutant (SR^{-/-}) mice display constitutive NMDAR hypofunction due to the lack of D-serine. SR^{-/-} mice have deficits in tropomyosin-related kinase receptor (TrkB)/Akt signaling and activity regulated cytoskeletal protein (Arc) expression, which mirror what is observed in schizophrenia. Thus, we analyzed these signaling pathways in MK801 sub-chronically (0.15 mg/kg; 5 days) treated adult wild-type mice. We found that in contrast to SR^{-/-} mice, the activated states of downstream signaling molecules, but not TrkB, were increased in MK801 treated mice. Furthermore, there is an age-dependent change in the behavioral reaction of people to NMDAR antagonists. We therefore administered the same dosing regimen of MK801 to juvenile mice and compared them to juvenile SR^{-/-} mice. Our findings demonstrate that pharmacological NMDAR antagonism has different effects on TrkB/Akt signaling than genetically-induced NMDAR

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Conflict of Interest

The authors have nothing to disclose.

Contributors

Shunsuke Takagi oversaw all aspects of the design and implementation of the study. Dr. Balu, and Coyle contributed to the design of the study and interpretation of the data. All authors contributed to and have approved the final manuscript.

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hypofunction. Given the phenotypic disparity between the MK801 model and schizophrenia, our results suggest that SR^{-/-} mice more accurately reflect NMDAR hypofunction in schizophrenia.

Keywords

schizophrenia; D-serine; serine racemase; tropomyosin receptor kinase B (TrkB); MK801

1. Introduction

N-methyl-D-aspartate receptor (NMDAR) hypofunction is a compelling hypothesis for the pathophysiology of schizophrenia (Coyle et al., 2010). This hypothesis has been developed partially by the observation that NMDAR antagonists, such as phencyclidine and ketamine, reproduce all of the schizophrenia symptom domains in healthy human subjects including hallucinations, negative symptoms and cognitive symptoms (Itil et al., 1967; Javitt, 2009). Therefore, NMDAR antagonists have been used as a tool to induce NMDAR hypofunction in experimental animals as a pharmacologic model of schizophrenia (Adell et al., 2012; Eyjolfsson et al., 2006; Javitt and Zukin, 1991; Wiescholleck and Manahan-Vaughan, 2013). These pharmacological models exhibit hyperlocomotion, stereotypies (Hoffman, 1992) and social withdrawal (Zou et al., 2008), as well as long-term potentiation (LTP) and learning deficits (Manahan-Vaughan et al., 2008).

There is also genetic and biochemical evidence to support NMDAR hypofunction as a key etiological component of schizophrenia. Recent large-scale, exome sequencing (Fromer et al., 2014) and genome-wide association (GWAS) studies (Timms et al., 2013; Ripke et al., 2014) have identified *de novo* mutations and genetic loci, respectively, in genes encoding proteins involved in glutamatergic transmission, including NMDAR subunits, with increased risk for schizophrenia. A single nucleotide polymorphism (SNP) in the enzyme serine racemase (SR), which produces D-serine, the forebrain NMDAR co-agonist, was among the risk alleles significantly associated with schizophrenia (Morita et al., 2007; Ripke et al., 2014). Furthermore, SR and D-serine are reduced in schizophrenia (Bendikov et al., 2007; Hashimoto et al., 2003; Nishikawa, 2011). Thus, our laboratory generated serine racemase-null mutant (SR^{-/-}) mice that display constitutive NMDAR hypofunction due to the lack of D-serine (Basu et al., 2009).

Similar to schizophrenia, SR^{-/-} mice have reduced cortico-hippocampal volume and ventricular enlargement that is accompanied by decreased dendritic spine density and complexity in these regions (Balu et al., 2013; Puhl et al., 2014). Further investigation revealed that SR^{-/-} mice have impaired neurotrophic signaling that parallels what is observed in schizophrenia, including brain-derived neurotrophic factor (BDNF) / tropomyosin receptor kinase B (TrkB)/Akt/glycogen synthase 3 kinase (GS3K) cascade (Balu et al., 2013). In addition, we found that activity-regulated cytoskeleton-associated protein (Arc), which is genetically associated with schizophrenia (Kirov et al., 2012; Ripke et al., 2014), is reduced in the hippocampus of adult SR^{-/-} mice (Balu and Coyle, 2014). Because BDNF expression, Akt signaling and Arc levels are regulated by NMDAR activity,

we therefore analyzed this pathway and Arc in a pharmacological NMDAR hypofunction model.

Among NMDAR antagonists, (+)-MK801 hydrogen maleate (MK801) has a favorable profile because it has extremely high (10-100 fold higher than PCP and ketamine) affinity to (Kornhuber and Weller, 1997), and a high selectivity for the PCP binding site of the NMDAR (Wong et al., 1986) whereas PCP also binds to the dopamine D2 receptor (Seeman et al., 2005). Furthermore, there is a notable age-dependent change in the behavioral response of people to NMDAR antagonists. In children, PCP and ketamine do not produce psychosis, which are typical for these drugs in adult (Spear, 2000). This age dependency of NMDAR antagonists' effects is also interesting because schizophrenia typically has its symptomatic onset in early adulthood. Although there are some studies that analyzed the age-dependent difference of NMDAR antagonists on rodent behavior (Boulay et al., 2013; Sircar and Soliman, 2003), there are few that examined intracellular signaling.

Thus, we analyzed TrkB /Akt/GS3K signaling pathways and Arc in MK801 sub-chronically (0.15 mg/kg; o.d; 5 days) treated adult wild-type mice and SR^{-/-} mice to elucidate these two models difference on the TrkB signaling, Akt signaling and Arc. We administered the same dosing regimen of MK801 to juvenile mice (3-4 weeks old) and compared them to juvenile SR^{-/-} mice.

2. Materials and Methods

2.1. Animals

Wild-type (WT) and constitutive SR^{-/-} mice were generated as previously described (Basu et al., 2009). The serine racemase null mutation of the first coding exon has been backcrossed for over 10 generations onto a C57BL/6J background. SR^{+/-} parents were bred to produce WT and SR^{-/-} offspring. Male mice were used for all experiments as they exhibit a much more robust phenotype than females. 3-7 months old mice were utilized for adult mice, and 3-4 weeks old mice were for juvenile mice. The animals were housed in a temperature- (22 °C) and humidity-controlled facility with a 12/12 h light/dark cycle and provided with food and water *ad libitum*. All animal procedures were approved by the McLean Hospital Institutional Animal Care and Use Committee.

2.2. Drug Treatment

WT mice were administered either vehicle (saline) or MK801 via intraperitoneal (i.p.) injection at a volume of 10 ml/kg body weight once daily for 5 days at 10 to 11 A.M.. MK801 was obtained from Sigma-Aldrich (M107, St. Louis, MO, USA). MK801 was dissolved in sterile isotonic saline at a dosage of 0.15 mg/kg.

2.3. Western blot analysis

Immunoblotting was performed as modification of the previously described method (Balu and Coyle 2011). MK801-treated animals were sacrificed 90 min after the last injection, and their brains were quickly removed. Both lobes of the hippocampus were collected. The tissue was flash frozen and stored at -80 °C until homogenizing. Brain tissue was briefly

homogenized by sonication (>10 s) in extraction buffer (60-mM Tris buffer, 2% sodium dodecyl sulfate, 0.1% phosphatase inhibitor, pH 6.8). Protein content was determined by a colorimetric assay based upon the Bradford method using Bio-Rad dye reagent (Bio-Rad Life Sciences, Hercules, CA). Prior to gel loading, samples were heated to 95 °C for 5 minutes. Samples were electrophoretically separated on an SDS–12.5% polyacrylamide gel. Nitrocellulose membranes (Bio-Rad, Hercules, CA) were blocked with 5% nonfat dry milk (Shaw's; Boise, ID) in 0.05% Tween-20/Tris-buffered saline and then incubated with primary antibody overnight at 4 °C. The primary antibodies (Cell Signaling Technologies; Danvers, MA) raised in rabbit were used at the following dilutions: p-AKT1 (ser) (1:1000), AKT1 (1:1000), p-GS3K (1:1000), GS3K (1:1000), p-TrkB (1:2000), and β -actin (1:8000; Abcam; Cambridge, MA). Arc (Santa Cruz Biotechnology; Santa Cruz, CA), and TrkB (Millipore; Temecula, CA) was detected using a mouse monoclonal primary antibody (1:200, 1:2000, respectively). After incubation with goat anti-rabbit (1:5000; Abcam, Cambridge, MA) or rabbit anti-mouse (1:1500; Abcam, Cambridge, MA) horseradish peroxidase-conjugated secondary antibodies, immunocomplexes were visualized by chemiluminescence using Western Lightning-ECL (Perkin Elmer; Waltham, MA). Semi-quantitative assessment of protein bands was executed by computerized densitometry using Image Lab Software (Bio-Rad, Hercules, CA). Chemiluminescent values of the protein of interest were divided by its corresponding β -actin chemiluminescent values.

2.4. Statistical analyses

Results were compared using unpaired Student t-test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Constitutive genetic NMDAR hypofunction and sub-chronic pharmacologic NMDA Receptor antagonism have opposite effects on TrkB/Akt/GS3K signaling in adult mice

We first measured the phosphorylation status of the TrkB receptor, which is upstream of the Akt/GS3K pathway, as a way to determine the activation state of the receptor. In agreement with our previous findings, adult SR^{-/-} mice showed a 20% reduction of phosphorylated TrkB (pTrkB; Fig. 1A; $p < 0.001$), without a change in the total amount of TrkB. In contrast, MK801-treated mice did not show a significant change in the phosphorylation state of the TrkB receptor compared to vehicle treated mice (Fig. 1B; $p = 0.4$).

The amount of phosphorylated-Akt1 (p-Akt) and phosphorylated-GS3K (p-GS3K), which are the active states of the enzymes, were reduced in the hippocampus of adult SR^{-/-} mice compared to WT mice (Fig. 1C, E; pAkt1, $p < 0.05$; pGS3K α : $p < 0.05$; pGS3K β , $p < 0.01$), in line with our previous results. There was no difference in the total amounts of Akt or GS3K (Fig. 1C, E). However, in adult MK801 treated mice, the phosphorylation states of these proteins were increased compared to saline treated mice (Fig. 1D, F; p-Akt1, $p < 0.05$; p-GS3K α , $p < 0.05$; p-GS3K β , $p < 0.05$), with no difference in the total amounts of Akt and GS3K (Fig. 1D,F).

3.2. Juvenile SR^{-/-} mice Showed Reduced TrkB activation and Akt/GS3K signaling

Because we were interested in whether the reductions in TrkB and Akt/GS3K signaling were age-dependent, we examined the phosphorylation states of these proteins in juvenile SR^{-/-} mice. Similar to adult SR^{-/-} mice, juvenile SR^{-/-} animals exhibited deficits of equal magnitude in pTrkB (Fig. 2A; $p < 0.05$), pAkt1 (Fig. 2B; $p < 0.05$), pGS3K α (Fig. 2C; $p < 0.05$), and pGS3K β (Fig. 2C; $p < 0.05$), with no changes in the total amount of these proteins.

3.3. MK801 Treatment does not affect TrkB activation and Akt/GS3K signaling in juvenile mice

In human subjects, administration of NMDAR antagonists to juveniles does not produce psychosis. We hypothesized that this lack of effect in juvenile MK801 treated mice reflects differences in downstream intracellular signaling, potentially involving the TrkB receptor and the Akt/GS3K pathway. In contrast to what was observed in adult mice treated with MK801, there were no significant changes to p-TrkB (Fig. 3A), p-Akt1 (Fig. 3B), or pGS3K α/β (Fig. 3C) in juvenile MK801-treated mice. In addition, MK801 did not affect the total amounts of these proteins.

3.4. Differential effects of genetic and pharmacologic NMDA receptor hypofunction on Arc expression

The immediate early gene Arc is enriched in the postsynaptic density (PSD) where it interacts with the NMDAR to regulate synaptic plasticity. Arc mRNA, which is induced by calcium influx through voltage-gated calcium channels and NMDARs, is trafficked to dendrites and synthesized at synaptic sites (Korb and Finkbeiner, 2011). In accordance with our previous findings, hippocampal Arc protein was reduced in both adult (Fig. 4A; $p < 0.001$) and juvenile (Fig. 4B; $p < 0.05$) SR^{-/-} mice. In adult MK801-treated mice, which had significantly increased p-TrkB and Akt/GS3K activation, there was no change in Arc expression (Fig. 4C; $p = 0.61$). However, in juvenile mice, MK801-treatment had the opposite effect and increased Arc protein expression (Fig. 4D; $p < 0.05$).

4. Discussion

In the present study, we replicated the findings of our previous research demonstrating reduced TrkB/Akt/GS3K phosphorylation and Arc protein in adult SR^{-/-} mice (Balu et al., 2013), and now show that these intracellular signaling abnormalities are also present in the hippocampus of juvenile SR^{-/-} mice. Furthermore, we sub-chronically administered MK801 to juvenile and adult mice, to determine whether short-term NMDAR antagonism produces similar deficits in Akt/GS3K signaling and Arc expression. Surprisingly, we found that MK801 differentially affected TrkB/Akt/GS3K phosphorylation and Arc levels compared to SR^{-/-} mice in an age-dependent manner.

Our findings demonstrate that sub-chronic, pharmacological NMDAR antagonism in adulthood has different effects on TrkB/Akt/GS3K signaling and Arc protein when compared to adult SR^{-/-} mice, a genetic model of NMDAR hypofunction. Many of the pharmacological NMDAR hypofunction models utilize sub-chronic NMDAR antagonist administration of 5-7 days (Daya et al., 2014; Eyjolfsson et al., 2006; Hawken et al., 2013),

or even shorter (Khella et al., 2014; Lopez-Gil et al., 2007; Manahan-Vaughan et al., 2008; Park et al., 2014) durations. In this study, we employed a 5-day NMDAR antagonist administration paradigm and found that it increased phosphorylation in the Akt/GS3K pathway, opposite to what we observed in SR^{-/-} mice. Interestingly, MK801 administration did not affect the phosphorylation of TrkB, which is an upstream component of the Akt/GS3K pathway. Future work will be needed to determine whether other TrkB phosphorylation sites or upstream signaling pathways contribute to the positive effect of MK801 on Akt/GS3K signaling.

The deficits in TrkB/Akt/GS3K signaling were apparent in juvenile SR^{-/-} mice and equal in magnitude to the reductions observed in adult mutants. This suggests that the changes in these signaling pathways are directly due to reduced NMDAR activity as a consequence of D-serine deficiency, rather than compensatory adaptations to NMDAR hypofunction in adulthood. The age (3-4 weeks postnatal) at which these signaling pathways were examined in juvenile mice corresponded to the developmental time-point when SR protein expression is peaking (Miya et al., 2008). Unlike adult mice, MK801 treatment did not statistically augment Akt/GS3K signaling and increased Arc expression in juvenile mice. This age dependent difference might contribute to the lack of psychoactive effects of NMDAR antagonists in juvenile subjects. In schizophrenia, the relationship between intracellular signaling pathways and psychotic symptoms is largely unknown. The psychosis of schizophrenia and that induced by ketamine in normal volunteers is associated with elevated forebrain dopamine release (Howes et al., 2012; Rabiner, 2007). Thus, alterations in hippocampal intracellular signaling in SR^{-/-} mice likely reflect the cortical hippocampal pathology of schizophrenia including neuronal atrophy, which is already present at first episode (Lui et al., 2009).

The time course of NMDAR hypofunction is one major difference between MK801-treated and SR^{-/-} mice. In the MK801 experiments, NMDAR antagonism occurred for only 5 days, while SR^{-/-} mice, which lack D-serine, have NMDAR hypofunction from conception. Moreover, in contrast to MK801, which blocks all open NMDARs, SR^{-/-} mice exhibit hypofunction only on NMDARs in which D-serine is the primary co-agonist. It was recently shown that D-serine is the preferential co-agonist at synaptic, NR2A-containing NMDARs in the hippocampus (Papouin et al., 2012). Thus, the duration of NMDAR hypofunction and the population of NMDARs being affected likely contribute to the differential effects on signaling cascades.

The age-dependent differences in TrkB/Akt/GS3K signaling and Arc expression in MK801-treated mice might be due to differences in NMDAR subunit composition. The NMDAR is a tetrameric ion channel comprised of two obligatory NR1 subunits and a combination of NR2 and/or NR3 subunits. Importantly, NMDAR subunit composition is developmentally regulated. NR1 is essential for the NMDAR and is expressed ubiquitously throughout pre- and postnatal development. In the rodent brain, NR2B, NR2D and NR3A subunits are highly expressed during the neonatal and juvenile periods, while NR2A, NR2C, and NR3B are highly expressed in adulthood (Okabe et al., 1998; Henson et al., 2010). These different subunits change the biophysical properties of the NMDAR and also have unique intracellular protein binding partners based on their intracellular carboxyl-terminal domain

(Traynelis et al., 2010). Therefore, this developmental transition in NMDAR subunit expression might underlie the age-dependent effects of MK-801 on intracellular signaling and protein expression changes.

Even though SR^{-/-} mice and MK801 NMDAR hypofunction models share some common characteristics relevant to individuals with schizophrenia, including deficits in LTP and spatial learning (Balu et al., 2013; Basu et al., 2009; Manahan-Vaughan et al., 2008), there are substantial differences between the two. While TrkB/Akt/GS3K signaling and Arc expression is reduced in SR^{-/-} mice and schizophrenia, the converse is observed following MK801 administration. Furthermore, SR^{-/-} mice have many of the same brain morphological abnormalities observed in schizophrenia, such as reduced cortico-hippocampal volume, decreased dendritic spine density (Balu et al., 2012; Balu et al., 2013) and increased lateral ventricles (Puhl et al., 2015). Pharmacological NMDAR hypofunction models produce hyperlocomotion and stereotypy (Hoffman, 1992), as well as social withdrawal (Zou et al., 2008), behavioral abnormalities that are not observed in SR^{-/-} mice (Basu et al., 2009; DeVito et al., 2011). Thus, the SR^{-/-} mice exhibit a forebrain NMDA receptor hypofunction endophenotype with cortical neuronal atrophy associated with negative symptoms and cognitive but not with several behavioral abnormalities linked to psychosis. The above findings highlight the complex nature of schizophrenia and how different animal models of reduced NMDAR activity recapitulate different phenotypes of the disease (Wiescholleck and Manahan-Vaughan, 2013).

In summary, our results show reduced TrkB/Akt/GS3K signaling activity and reduced Arc expression in both juvenile and adult SR^{-/-} mice, a life-long genetic model of NMDAR hypofunction. Divergent effects on the same signaling pathways were observed following sub-chronic, pharmacologic NMDAR antagonism. These findings demonstrate that the duration and nature of NMDAR hypofunction affect its impact on neuroplasticity pathways, and suggest that genetic models of impaired NMDAR function more appropriately model the neurochemical and morphological aspects of schizophrenia.

Acknowledgement

We thank Dr. Toru Nishikawa for advice and support, and Alexandra Berg for animal colony maintenance and genotyping.

Role of funding source

Shunsuke Takagi was supported in part by Strategic Young Researcher Overseas Visits Program for Accelerating Brain Circulation from the Japan Society for the Promotion of Science (S2301). A Phyllis & Jerome Lyle Rappaport Mental Health Research Scholars Award and 1K99MH099252-01A1 (DTB), as well as grants R01MH05190 and P50MH060450 (JTC) supported this work.

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Fig. 1. Constitutive genetic NMDA receptor hypofunction and subchronic pharmacologic NMDA receptor antagonism have opposite effects on TrkB/Akt/GS3K signaling in adult mice

Protein levels of (A) TrkB/p-TrkB, (C) Akt1/p-Akt1 and (E) GS3K a/b; p-GS3K α /b were measured in the hippocampus of adult WT (n = 10; white bars) and adult SRKO (n = 9; black bars) mice. Protein levels of (B) TrkB/p-TrkB, (D) Akt1/p-Akt1 and (F) GS3K α /b; p-GS3K α /b were measured in the hippocampus of vehicle treated adult mice (n = 5; white bars) and MK801 treated adult mice (n = 6; striped bars). Values are expressed as the optical density (OD) normalized to WT or vehicle treated values (% WT or % vehicle). Asterisk (*) indicates significant differences from the WT or vehicle treated group (p < 0.05). All values represent the mean $\pm$ SEM.

Fig. 2. Phosphorylated TrkB, Akt, and GS3K are also reduced in juvenile SR^{-/-} mice

Protein levels of (A) TrkB/p-TrkB, (B) Akt1/p-Akt1 and (C) GS3Kα/b; p-GS3Kα/b were measured in the hippocampus of juvenile WT (n = 9; white bars) and juvenile SR^{-/-} (n = 7; black bars) mice. Values are expressed as the optical density (OD) normalized to WT values (% WT). Asterisk (*) indicates significant differences from the WT group (p < 0.05). All values represent the mean ± SEM.

Fig. 3. Phosphorylated TrkB, Akt, and GS3K are not modulated in juvenile MK801 treated mice Protein levels of (A) TrkB/p-TrkB, (B) Akt1/p-Akt1 and (C) GS3K α/β ; p-GS3K α/β were measured in the hippocampus of juvenile WT ($n = 8$; white bars) and juvenile SR $^{-/-}$ ($n = 8$; stripe bars) mice. Values are expressed as the optical density (OD) normalized to vehicle treated values (% vehicle). Asterisk (*) indicates significant differences from the vehicle treated group ($p < 0.05$). All values represent the mean $\pm$ SEM.

Fig. 4. D-Serine deficiency and MK801 treatment have different effects on Arc expression
Protein levels of Arc were measured in the hippocampus of adult (A; WT: n = 10, white bars; SRKO: n = 9, black bars) and juvenile (B; WT: n = 10, white bars; SRKO: n = 7, black bars) mice. Following drug treatment, hippocampal Arc protein was measured and adult (C; saline: n = 5, white bars; MK801: n = 6, black bars) and juvenile (D; saline: n = 8, white bars; MK801: n = 8, stripe bars). Values are expressed as the optical density (OD) normalized to WT or vehicle treated values (% WT or % vehicle). Asterisk (*) indicates significant differences from the WT or vehicle treated group ($p < 0.05$). All values represent the mean $\pm$ SEM.