Published in final edited form as:

Neurosci Lett. 2010 January 4; 468(2): 166–171. doi:10.1016/j.neulet.2009.10.091.

Neurochemical, behavioral and architectural changes after chronic inactivation of NMDA receptors in mice

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Keywords

NMDA receptor hypofunction; MK-801; schizophrenia

Schizophrenia is a psychotic illness characterized by problems in perception, learning, and memory. Post-mortem clinical data revealed abnormalities in neuronal organization, reduced soma and dendritic tree size. In rodents, reduction of glutamatergic neurotransmission by NMDA receptor antagonists mimics symptoms of schizophrenia. However, the dosage, treatment and species used in previous studies have not been consistent, leading to a lack of correlation between the findings reported in low-dose, long term treatment models and the results in acute or chronic high dose administration. Thus, the present study investigates whether long term, low dose blockade of NMDA receptors with MK-801 in the early postnatal period results in molecular, cellular, morphological and behavioral changes in the mouse, alterations that have been singly described by using different drugs and dosages in either mice or rats. We found that early postnatal administration of 0.1mg/kg MK-801 for 15 days altered protein translation, synapse formation, hippocampus-dependent learning and neuronal development, resembling findings reported in schizophrenia. These results suggest that there are strong parallels between this animal model and schizophrenia, which validates it as an animal model for this condition and lends further strength of the NMDA receptor hypofunction as a useful model for the study of psychosis.

Schizophrenia is a human mental disorder affecting 1% of the population. Problems in perception, learning, and memory begin during early childhood or adolescence and are lifelong [12,42]. Cognitive impairments [6] as well as anatomical alterations affecting brain connectivity [42] are one of the persistent manifestations of the disease observed in patients.

The NMDA receptor plays a significant role in the development of cognitive functions, as well as in brain maturation [12]. In rodents, inactivation of the NMDA receptor produces memory deficits and biochemical changes in the brain [16,19] similar to the ones reported in schizophrenia [6]. Previous studies indicate that treatments with NMDA receptor antagonists affect learning and memory [31,40] as well as dendritic morphology in rodents [39]. However, most of these studies have not been consistent regarding species, chemicals and doses used and do not address whether the observed behavioral or morphological changes are occurring

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together or with any other biochemical/cellular changes. Thus, the main aim of this study was to investigate whether chronic inactivation of the NMDA receptor with MK-801 (a non-competitive and selective NMDA receptor blocker) in early postnatal period produces changes in synaptic plasticity in mice. Correlation among molecular, morphological and behavioral changes were investigated and linked to the effects observed in schizophrenia. Learning and memory functions that affect synaptic plasticity [31,33] require both initiation of protein synthesis (through AKT [RAC-alpha serine/threonine-protein kinase or PKB] and mTOR [mammalian target of rapamycin] signaling) and actin remodeling (through the small GTPase, Rac [Ras related C3 botulinum toxin substrate]) [18,28,37]. Thus, alterations in the expression level of both or either of these molecules may elicit critical changes for the brain [26]. Because schizophrenia develops in general during puberty, early administration of MK-801 is believed to better represent this animal model. We hypothesized that early postnatal NMDA receptor hypofunction would result in deregulation of the signaling pathways involved in synaptic plasticity, likely leading to aberrant neuronal morphology, which also will correlate with learning impairment. We chose to examine the mouse hippocampus because it is a brain structure crucial for learning and memory functions.

For this study, animals were handled in compliance with institutional and national policies. The protocols were approved by the IACUC-University of Houston. A daily dose of MK-801 (0.1mg/kg b.w) was administered intraperitoneally to C57BL/6J mice pups for a period of 15 days (P3-P17). Control animals were administered a NaCl 0.9% solution. Mice remained with the mother until weaning (P21) (Figure 1A).

For neurochemical measurements, mice were sacrificed by cervical dislocation once treatment (MK-801 or saline) and weaning were completed. The hippocampus was extracted, sonicated (HBC; 200mM HEPES, 2.5mM NaCl, 100mM EDTA, 100mM EGTA, 200mM Na₄P₂O₇, 50mM NaF, 1mM sodium orthovanadate, 10ug/ml leupeptin, 2ug/ml aprotinin, 1uM microcystin-LR, 200nM calyculin A), and frozen until analysis.

Total protein in samples was quantified as described [4] and equivalent amounts of protein were resolved by electrophoresis and blotting [31]. Blots were incubated with appropriate primary antibodies [Rac (1:10,000; Millipore), mTOR, p-mTOR, AKT, p-AKT, tubulin (1:1,000; Millipore)] and secondary antibodies [HRP-anti-mouse or anti-rabbit IgG (1:5,000-10,000; Promega)]. NIH image software was used for densitometry analysis. To determine activation of Rac (Rac-GTP) an affinity purification assay was performed [37]. Additionally, neuronal morphology of dendrites and spines was assessed using Golgi-Cox impregnation procedure (FD Rapid GolgiStain™ Kit, FD Neurotechnologies) following manufacturer's instructions.

To measure changes in behavior, a second group of identically-treated mice were subjected to several tests (P22-P30) in the following order. For the open field test (P22), the mouse was allowed to explore a novel environment in a clear chamber for 30 min while being monitored (OptoMax, Columbus Instruments). For the rotarod test (P23), the mouse was placed on a horizontal accelerating rod (4-40 rpm) and subjected to 4 trials (5-minutes/trial) per day for 2 consecutive days. To test for fear conditioning (P25), animals were placed into a chamber with 19 metal rods equally spaced on the floor, housed in a sound-attenuating cubicle. During each 7-minutes session, a footshock (2-seconds, 0.75 mA) preceded by a 30 sec tone was presented to the animal at 120, 240, and 360 seconds. To test conditioning to the context, the animals were returned to the chamber 1 and 24 hours after training for a 7-minute test with no tone/shocks presented. To test conditioning to the tone, the chamber was modified with respect to tactile, spatial, visual, and olfactory properties. The animals were placed into the modified chamber for a 7-minute test session 1 and 24 hours after training and the tone was presented continuously during the second 3-minutes period. Freezing was measured during the testing

sessions. In the Morris Water Maze (MWM), mice (P27) were trained to locate a hidden platform in a circular pool, using visual cues outside the pool. Each mouse was given 4 trials/day for 4 consecutive days. Time to find the platform was measured for a maximum of 60-seconds. After day 4, the platform was removed and animals were allowed to search the pool for 60-seconds (probe test). Search time in each quadrant was assessed. Mice also were trained on the visible platform task locating a platform marked by a large black cube extending out of the water. A computer software (Noldus) was used to assess behavior.

Statistics were performed using Student's *t*-tests to determine significant differences between control and experimental groups ($p \leq 0.05$ was considered statistically significant).

NMDA receptor hypofunction is involved in the neurodevelopmental pathogenesis of schizophrenia [34], inducing neuronal apoptosis and long-term behavioral changes in the developing brain. It is well established that learning and memory functions require protein synthesis (through AKT and mTOR signalling) and actin remodeling (through the small GTPase Rac) [18,28,37]. Thus, alterations in the expression level of these molecules could result in critical changes for the brain [26]. To determine whether MK-801 affects actin remodeling, protein translation, and synaptic plasticity in the brain (Figure 1B), we first investigated the expression and activation state of Rac. While total Rac was unchanged, active Rac (Rac-GTP) in the hippocampus of MK-801-treated mice was significantly lower than in saline-treated controls (Figure 1C, D, $p < 0.05$). Since protein synthesis is also required for neuronal plasticity [8,24], we next examined the status of AKT and mTOR. Total AKT, mTOR and p-AKT were not significantly different in the hippocampus of control or MK-801-treated mice, while p-mTOR was significantly upregulated in the MK801 treatment (Figure 1C, D, $p < 0.05$). Overall, our findings suggest that inactivation of the NMDA receptor by MK-801 might alter protein translation and actin remodeling, both involved in synapse formation [7, 17,31,37]. Thus, dysregulation of Rac-GTP and p-mTOR might lead to abnormal plasticity. These dysfunctions in the developing brain could lead to problems severe enough to cause structural damage in the neurons, influencing the functional developmental process and inducing long-lasting behavioral changes.

Because symptoms of schizophrenia begin in late adolescence, NMDA receptor hypofunction was induced during early postnatal development, a period critical for the differentiation of neurons. The NMDA receptor plays a crucial role in neuronal spine formation and learning [10], possibly through the activation of Rac. Post-mortem studies revealed that schizophrenia results in decreased spine density in certain brain regions [15]. Herein, since activation of Rac was altered in MK-801-treated mice, we wanted to determine whether neuronal spine morphology was modified. While no abnormalities were found in the gross morphology of the hippocampus (data not shown), pyramidal neurons on MK-801-treated mice (P 21) appeared disorganized and lacked complexity (Figure 2A, B). The length and number of basal dendrites was diminished, while apical segments remained intact. Moreover, MK-801-treated mice presented reduced branching, lower mature spine density and increased spine length (Figure 2C), strongly suggesting that actin remodeling in dendrites was affected [26], probably shrinking the number of synaptic contacts. These changes may have important consequences, since hippocampal pyramidal neurons are engaged in the processing of cognitive functions [21]. The same changes in the length of basilar dendrites have been observed in rats [39] as well as in humans suffering from schizophrenia [15].

Because NMDA receptor activation [6] and mature spine density [2,15,26] play a significant role in the impaired cognitive functions of schizophrenics, we next examined the performance of MK-801-treated mice on two hippocampus-dependent learning and memory tasks. Using an associative fear conditioning paradigm (Fig 3A, B) we assessed contextual conditioning (hippocampally-dependent task) and conditioning to a discrete cue (hippocampally-

independent task) [30]. Fear behavior during training was comparable in both groups, demonstrating that MK-801-treated mice perform similarly during initial exploration and exposure to the cue–shock pairing. When tested over time (1 and 24 hours post-training) for the retention of contextual information (context test), MK-801-treated mice showed increased freezing response compared to controls. Conversely, previous studies using a single dose of MK-801 revealed that the NMDA receptor is required for acquisition of memories [31]. Since NMDA receptors in the hippocampus have been reported to increase after antagonist administration (PCP, and others) [13,35], chronic blockade with MK-801 might also lead to *de novo* NMDA receptor synthesis. Additionally, freezing of the MK-801-treated mice was significantly higher during the presentation of the auditory cue 1 hour post-training (Fig 3C, $p < 0.01$). Because cue fear conditioning is amygdala-dependent [9], MK-801 systemically administered could have superior effects on the amygdala, resulting in increased levels of fear.

In psychosis, working memory and cognitive control are deficient [1,5]. Thus, we also tested mice on a spatial learning paradigm (MWM), which is a complex behavior that relies primarily on the hippocampus, and requires protein synthesis. The ability of MK-801 treated mice to find the platform during training, swimming speed (to rule out motor impairments) and escape latency to the platform by providing a visual cue (to rule out visual impairment) showed no differences compared to saline control (Figure 4A, B, C respectively). Physiologically, both groups seem to be comparable, presenting equivalent visual capabilities, muscular development and strength. During a probe trial on day 4, in which the platform was removed from the pool and the mice were allowed to search for 60 seconds, MK-801 mice spent significantly less time in the target quadrant where the platform was located compared to saline (Figure 4C, $p < 0.05$). Thus, our results suggest that consistent with the deficiencies reported in schizophrenia patients, treatment with MK-801 during adolescence may have an effect on spatial learning and memory. Other laboratories have also found spatial learning deficits after withdrawal from repeated injections of NMDA antagonists [29,32,35]. However, other treatment plans showed that NMDA receptor blockade after weaning does not produce spatial learning deficits [27].

Impaired performance on learning tasks may be alternatively due to non-cognitive functions such as sensory-motor, and/or emotional-motivational processes [14,41]. To minimize the error on interpretation of our results, basic measures of these parameters were analyzed. The open field activity tests the spontaneous exploratory activity of mice, hyperactivity, and the ability to habituate to a novel environment [9]. Both MK-801 and saline treated groups presented similar exploratory behavior (Figure 5A). Other studies have reported that MK-801 lowers overall hyperactivity and stereotypic behaviors, but only when hyperactivity was induced by drug treatment or ischemia and followed immediately by administration of MK-801 [20,25]. Also, the dose of MK-801 administered is a determinant factor, with low doses showing no effect in exploratory behavior [25,29]. To determine whether these animals had motor impairment or lack of motivation, we measured motor learning, coordination, and balance on a rotarod. MK-801-treated mice performed similarly to the saline group (Figure 5B); only extremely high doses of antagonists seem to affect this learning task [36].

The neurodevelopmental hypothesis of schizophrenia assumes that this disorder affects the pre-developmental brain but the disease manifests usually in early adulthood [12]. We wanted to know whether our treatment affected the physical development of these animals. Although the drug administration was not lethal to any of the animals, MK-801-treated mice showed significantly lower body weight from P6 to P17. However, once the injection period stopped, MK-801-treated animals attained the same developmental state as controls (inset in Figure 5C, $p < 0.05$), indicating that this effect was most likely due to the drug treatment. The same effect has been reported using PCP and MK-801 in rats [3,22]. NMDA receptor inhibition has been shown to inhibit puberty by reducing the release of gonadotropin-releasing hormone (GRH)

[38], and to hinder body weight gain [11]. Furthermore, densely rich glutamate receptors of the hippocampus are involved in regulating motivated ingestive behavior [23]. Therefore, MK-801 treatment might delay puberty by affecting hormonal function as well as appetite.

In conclusion, MK-801-induced hypoactivity of the NMDA receptor had numerous effects on the neonatal brain. These results simulate phenotypes of patients with schizophrenia, mimicking at least several important aspects of the disease, and corroborating the association of the NMDA receptor hypofunction theory with schizophrenia.

Acknowledgments

Supported by NS48037, University of Houston-I098045 (M.V.T.S), the University of Houston SURF, PURS, and the Houston-AGEP (M.E.).

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Figure 1.

(A) Flow chart of the protocol followed for dosing and testing. (B) Immunoreactivity of Rac-GTP, p-AKT, and p-mTOR in the mouse hippocampus. Biochemical pathways involved in synaptic plasticity. (C) Representative western blot analysis. (D) Group data (mean $\pm$ SEM, $n=7$) of quantified immunoreactivity in saline and MK-801-treated animals. Data were normalized to total protein levels and expressed as % relative to control. * $p < 0.05$.

Figure 2. Neuronal morphology in MK-801 treated mice (P21). (A) 4x hippocampal coronal view of a saline and MK-801-treated mouse. (B) 10x view of the CA1 region of saline and MK-801-treated mouse. (C) 100x view of dendritic spines in saline and MK-801-treated mouse.

Figure 3. Associative fear conditioning. **(A)** Protocol followed for testing. **(B)** Training test. **(C)** Mean freezing behavior for contextual and cued fear conditioning tests 1 and 24 h after training (mean $\pm$ SEM, $n=15$). ** $p < 0.01$.

Figure 4. Morris Water Maze. **(A)** Escape latency in MK-801 and saline treated mice. **(B)** Swimming speed in MK-801 and saline treated mice. **(C)** Escape latency during the visual cue. **(D)** Platform searching. (mean $\pm$ SEM, $n=15$). * $p < 0.05$. ** $p < 0.01$.

Figure 5.

Performance in non-cognitive functions. **(A)** Anxiety levels and exploratory behavior measured by the open field activity (OFA). (MOVE) = Total time moving, (STEREO) = time moving in stereotypic fashion, (REST) = time at rest, (MARGIN) = time in the margin, (REARING) = time rearing. **(B)** Motor coordination, balance, and motor learning using an accelerating Rotarod. **(C)** Weights from MK-801 and saline treated animals during the injection (P3-17), testing and post-testing (P26-44) periods. (mean $\pm$ SEM, $n=15$). * $p < 0.05$.