Behavioral genetic analysis of the role of NMDA receptors at intralaminar thalamic nuclei in brain higher-order functions

（脳の高次機能発現における視床髄板内核 NMDA 受容体の役割に関する行動遺伝学的研究）
## Contents

Abstract ............................................................................................................................. 3

General Introduction ......................................................................................................... 5

Chapter 1

Genetic dissection of intralaminar thalamic nuclei function in mice

  Introduction ......................................................................................................................... 10
  Materials and methods ...................................................................................................... 12
  Results ................................................................................................................................. 18
  Discussion ............................................................................................................................ 20

Chapter 2

A novel animal model of schizophrenia with NMDA receptor hypofunction in the intralaminar thalamic nuclei

  Introduction ......................................................................................................................... 23
  Materials and methods .................................................................................................. 25
  Results ................................................................................................................................. 32
  Discussion ............................................................................................................................ 37

Chapter 3

NMDARs in the intralaminar thalamic nuclei are a potential therapeutic target to enhance cognitive function

  Introduction ......................................................................................................................... 40
Abstract

The intralaminar thalamic nuclei (ILN) are thought to be important for controlling states of consciousness and awareness (Van der Werf et al., 2002). Dysfunction of the ILN might partly underlie the pathogenesis of psychiatric disorders, including schizophrenia, Parkinson’s disease, and Korsakoff’s psychosis (Henderson et al., 2000, Marenco et al., 2012, Vetreno et al., 2012). Patients with these psychiatric disorders exhibit cognitive impairments, such as working and long-term memory deficits, inattention, and inflexibility. While these findings suggest a crucial role for the ILN in cognitive function, this hypothesis has never been tested due to technical limitations (Brown et al., 2010, Kato et al., 2011, Bradfield et al., 2013).

To overcome the technical limitations and elucidate the ILN function, I utilized the Cre/loxP system to produce transgenic mice. In Chapter 1, I established an ILN-selective Cre transgenic mouse line, which allows for specific manipulation of the ILN. Using the novel transgenic mouse line, I established an ILN-NR1-cKO mouse strain in which the essential NR1 subunit of the N-methyl-D-aspartate receptor (NMDAR) was selectively eliminated in the ILN. NMDAR-mediated currents were lost in approximately 60% of the ILN neurons. The targeted ILN cells had a smaller number of dendritic spines, suggesting a crucial role of NMDARs in the synaptic plasticity of ILN neurons.

In Chapter 2, to further examine the role of NMDAR in the ILN, I extensively analyzed behavioral phenotypes of the ILN-NR1-cKO mice. The ILN-NR1-cKO mice exhibited deficits in spatial working and long-term memory in the Y-maze and Morris water maze tests, respectively. Furthermore, they exhibited attention deficits and enhanced impulsivity in the 5-choice serial reaction-time task. Moreover, the ILN-NR1-cKO mice exhibited deficits in the prepulse
inhibition test, representing deficits in sensory-motor gating. These findings suggest that NMDAR hypofunction in the ILN accounts for major endophenotypes of schizophrenia.

In Chapter 3, I demonstrated that restoring NMDARs in the ILN rescued the working memory deficits, even in adult mutant mice. The NMDAR restoration also rescued the diminished MK-801 induced hyperactivity in mutants. These findings support the notion that NMDAR signaling dysfunction in the mature ILN circuitry is causally linked to schizophrenia-related disorders, and that NMDA receptors in the ILN are a potential therapeutic target for enhancing cognitive function.
General Introduction

The mechanisms by which neuronal networks in the cortex generate several distinct oscillatory activity patterns depending on the state of consciousness and awareness has been a fundamental question since Hans Berger first recorded electrical activity generated by the human brain (Berger, 1929). Electroencephalography (EEG) is used to monitor and analyze cooperative electrical activities of large numbers of cortical neurons, the oscillatory activities of which dramatically change depending on the vigilance state of the subjects. The alpha rhythm (8-12 Hz) is typically recorded in awake subjects with eyes closed. Beta waves (12-30 Hz) are indicative of mental activity and attention. Theta (4-8 Hz) and delta (≤ 4 Hz) activities are associated with drowsiness and sleep. In awake subjects, sensory processing is accompanied by a transient oscillation at high (gamma) frequency (> 30 Hz) in related brain areas (Buzsaki, 2006). Despite intensive investigation over many decades, the neuronal substrate underlying the regulation of such synchronous oscillatory brain activity and its significance have not yet been elucidated.

One of the most prominent systems regulating the overall level of brain activity is the ascending arousal system, first proposed by Moruzzi and Magoun (Moruzzi and Magoun, 1949). Lesions of the putative origin of this system in the brainstem by intercollicular transection or electrolytic lesions produce behavioral stupor (Bremer, 1935, Lindsley et al., 1949). Electrical stimulation of a group of cholinergic neurons near the junction of the pons and midbrain, on the other hand, leads to a state of wakefulness and arousal, suggesting that these neurons comprise a major component of the ascending arousal system (Steriade et al., 1993a). Thus, by some unknown mechanism, excitation of these neurons is transformed into changes in the cortical oscillatory pattern.
Using a variety of neuroanatomic tracing techniques, the pathways of the ascending arousal system were identified (Jones et al., 1985, Parent and Descarries, 2008). One of the densest branches of the ascending arousal system enters the intralaminar thalamic nuclei (ILN), including the parafascicular, centrolateral, and paracentral subnuclei. The thalamus, a diencephalic structure located in the center of the brain, serves as the central relaying center for all sensory inputs except odors. For example, visual stimuli received at the retina enter the thalamic lateral geniculate nucleus and are then sent to the cerebral primary visual cortex (Saalmann and Kastner, 2011). In contrast, the ILN are not such relay nuclei, but are “non-specific” nuclei that send widespread connections throughout the cortex (Morison and Dempsey, 1941, Morison and Dempsey, 1943). Based on their anatomic and electrophysiologic features, the ILN are thought to participate in the control of the states of consciousness and awareness by regulating cortical oscillations (Saalmann, 2014).

In addition to widespread cortical connections, the ILN have abundant mutual connections with the prefrontal cortex and striatum – key structures regulating executive function (Aron et al., 2004, Smith et al., 2004). Although the exact role of the pathway from the thalamus to the striatum remains poorly characterized, the ILN were recently proposed to supply striatal neurons with information of attention values, namely internal information about the brain state, such as alertness, depending on the events occurring in the environment (Smith et al., 2011). A positron emission tomographic study in humans revealed that the ILN are activated when participants switch from a relaxed state to an attention-demanding reaction-time test (Kinomura et al., 1996). The ILN respond to a variety of sensory stimuli (auditory, visual, and somatosensory), and their inputs to the striatum are required for response execution acquired
through learning tasks (Matsumoto et al., 2001).

These anatomic and physiologic characteristics suggest that the ILN have important roles in the action-selection mechanisms of the basal ganglia (Kimura et al., 2004, Minamimoto et al., 2005, Varela, 2014). These roles of the ILN were recently evaluated by lesioning portions of the ascending arousal pathway, including the ILN (Brown et al., 2010, Kato et al., 2011, Bradfield et al., 2013), but behavioral evidence has remained inconclusive due to several technical limitations. For example, it is difficult to restrict lesion damage to specific subregions and cell types. In addition, achieving reproducible results with pharmacologic manipulations and lesion techniques is problematic. Therefore, the precise roles of the ILN in higher-order brain function have remained largely unknown.

ILN dysfunction may underlie the pathogenesis of psychiatric disorders, including schizophrenia, Parkinson’s disease, and Korsakoff’s psychosis (Henderson et al., 2000, Marenco et al., 2012, Vetreno et al., 2012). Patients with these psychiatric disorders exhibit cognitive impairment, such as working and long-term memory deficits, inattention, and inflexibility. Neuroimaging studies in patients with schizophrenia reveal differences in the morphology and metabolism of thalamic sub-nuclei, including the ILN (Hazlett et al., 1999, Kemether et al., 2003, Woodward et al., 2012). Furthermore, postmortem neurochemical studies in patients with schizophrenia imply reduced NMDAR expression in the ILN (Ibrahim et al., 2000, Clinton and Meador-Woodruff, 2004).

NMDARs are glutamate-gated ion channels crucial for neuronal communication (Paoletti et al., 2013). NMDARs, which are permeable to Ca^{2+} but blocked by Mg^{2+}, behave as molecular coincidence detectors (Tsien, 2000). The NMDAR channel opens only when two
events occur simultaneously: glutamate binds to the receptor and the postsynaptic cell is depolarized to relieve the Mg\(^{2+}\) block of the channel pore (Mayer et al., 1984, Nowak et al., 1984). The finding that NMDAR blockade leads to cognitive impairment in humans and rodents led to the NMDAR hypofunction hypothesis for the pathophysiology of psychiatric diseases (Javitt and Zukin, 1991, Mohn et al., 1999, Amitai and Markou, 2010, Neill et al., 2010, van den Buuse, 2010, Coyle, 2012).

Mice (\textit{Mus musculus}) are a primary animal research model for studying the function of neuronal circuits because of the wealth of molecular tools available for manipulating their gene function (Havekes and Abel, 2009). One powerful genetic tool is the Cre/loxP system, which allows for spatially restricted gene manipulation. Susumu Tonegawa and his colleagues successfully applied the Cre/loxP system to study the role of NMDARs, which are a specific type of ionotropic glutamate receptor, in distinct hippocampal subregions in learning and memory formation (Tsien et al., 1996). They obtained a mouse line in which Cre recombinase expression was restricted to area CA1 of the hippocampus. By achieving Cre expression in this very narrow region, they were able to demonstrate the role of NMDARs specifically within the hippocampal CA1 area for learning and memory.

Viral vectors have also received much attention and are powerful tools for manipulating gene function in mice. In combination with Cre transgenic mice, Cre-activated adeno-associated viral vectors enable high-level, stable, and cell-type specific gene expression (Kuhlman and Huang, 2008). Viral gene transduction provides substantial advantages, such as extremely high efficiency and spatial resolution (Cetin et al., 2006).
The mouse genetics should also greatly contribute to delineate the precise roles of the ILN in higher-order brain function. Therefore, in my doctoral thesis, by establishing a novel ILN-specific Cre mouse line and manipulating NMDAR function in the ILN cells, I aimed for the first time to genetically dissect the role of the ILN in cognitive function. Through behavioral analysis of newly developed mutant mice, I aimed to elucidate the neural and molecular mechanisms underlying the pathophysiology of psychiatric disorders.


Chapter 1

Genetic dissection of intralaminar thalamic nuclei function in mice

Introduction

The intralaminar thalamic nuclei (ILN) are thought to have an important role in controlling states of consciousness and awareness (Van der Werf et al., 2002). Anatomic and electrophysiologic findings suggest that the ILN also have a role in other brain functions, such as selective attention (Matsumoto et al., 2001, Smith et al., 2004, Smith et al., 2011). Behavioral evidence of these roles, however, is inconclusive due to several technical limitations, such as difficulties in restricting damage to specific subregions and cell types when using pharmacologic manipulations and lesion techniques.

To overcome these problems and elucidate the functions of the ILN, I utilized the Cre/loxP system. This technique allows for gene expression and knockout in selective brain regions or cell-types. The system is based on the ability of Cre DNA recombinase to induce homologous recombination between the 34-base pair (bp) loxP recognition sites. The loxP sequences can be inserted into the mouse genome using a gene-targeting method to surround one or more exons of a target gene (referred to as a floxed gene). Mice homozygous for the floxed gene are then crossed with a transgenic mouse line that expresses Cre recombinase under the
control of a spatially restricted promoter. In offspring that are homozygous for the floxed gene and carry the Cre transgene, the floxed gene will be excised out in a selective population of cells.

To genetically dissect ILN function, I first generated a Cre transgenic mouse line expressing Cre recombinase selectively in ILN cells. The ILN-Cre mice were then crossed with mice in which the NMDAR subunit gene \( NR1 \) was floxed to obtain ILN-selective conditional knockout (cKO) mice for NR1. Immunohistochemistry and quantitative reverse transcription-polymerase chain reaction (RT-PCR) studies confirmed the decreased expression of NR1 in the ILN. Electrophysiologic experiments provided further evidence for the lack of NMDAR-dependent currents in the targeted ILN cells. Moreover, cells lacking functional NMDARs had smaller numbers of dendritic spines, which contribute to excitatory synaptic transmission (Engert and Bonhoeffer, 1999, Toni et al., 1999), providing evidence for a role of NMDARs in the synaptic plasticity of ILN cells.
Materials and methods

All experimental protocols were approved by the RIKEN Institutional Animal Care Committee.

Generation of ILN-specific NR1 knockout mice. A bacterial artificial chromosome clone (RP23-116A1) containing Lypd6B (LY6/PLAUR domain containing 6B) gene was used to construct a Cre expression vector according to a previously described procedure (Kobayashi et al., 2013), and microinjected into fertilized eggs from C57BL6J mice. One line was selected by characterizing Cre-mediated recombination patterns by crossing with Rosa26-STOP-nls-LacZ (RNZ) reporter mice (Kobayashi et al., 2013).

The heterozygous ILN-Cre mice were crossed with mice homozygous for the loxP-flanked NR1 allele (NR1 flox/flox) (Tsien et al., 1996) to obtain double heterozygous mice for the Cre transgene and floxed-NR1 allele (ILN-Cre; NR1 flox/+), and these mice were further crossed with NR1 flox/flox mice to produce ILN-Cre; NR1 flox/flox mice and littermate control mice NR1flox/flox. The ILN-Cre; NR1 flox/flox mice are referred to as ILN-NR1-cKO mice. For electrophysiologic experiments, ILN-NR1-cKO mice were crossed with NR1 flox/flox; Rosa26-STOP-enhanced yellow fluorescent protein (EYFP) to obtain ILN-NR1-cKO; Rosa26-STOP-EYFP mice, and ILN-Cre mice were crossed with Rosa26-STOP-EYFP mice to obtain ILN-Cre mice; Rosa26-STOP-EYFP control mice.

DNA extraction from mouse tail and genotyping. Tail clippings were placed in a 1.5-ml tube with 200 μl tail lysis solution and 0.2 mg ml⁻¹ Proteinase K/Lysis buffer containing 100 mM Tris [hydroxymethyl] aminomethane, pH8.5; 5 mM EDTA, 200 mM NaCl, and 0.2% sodium dodecyl
sulfate. After incubating overnight at 55°C, the tail samples were homogenized by vortexing and centrifuged for 5 min at 12,000 rpm. Each supernatant was transferred to 100 μl MeOH in a 0.2 ml 8-strip tube, and mixed through by vigorously inverting 20 times. After 5-min centrifugation at 12,000 rpm, MeOH was replaced with 100 μl EtOH to wash the extracted tail DNA, and the tubes were again centrifuged at 12,000 rpm for 5 min. EtOH was removed before Tris-EDTA (pH8) was added to elute the tail DNA.

Mouse genotype was determined by PCR. PCR was performed in a final volume of 12 μl using 0.1 μl of each primer (50 μM), 0.07 μl Extaq (TAKARA, RR001A), 12 μl of 10 × ExTaq buffer, 0.96 μl of 2.5 mM dNTP, 8.47 μl H2O, and finally 1 μl of the extracted tail DNA. PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems) with a 2-min pre-incubation at 94°C followed by 35 cycles of 30 s at 94°C (denaturation), 30 s at 60°C (annealing), and 30 s at 72°C (amplification), using the following primers: CW-Cre2, 5’ - ACC TGA TGG ACA TGT TCA GGG ATC G – 3’ and CW-Cre3, 5’ - TCC GGT TAT TCA ACT TGC ACC ATG C – 3’, producing a 108-bp fragment from the Cre allele; NR-ER5, 5’ – TGT GCT GGG TGT GAG GGT TG – 3’ and NR-ER10, 5’ – GTG AGC TGC ACT TCC AGA AG -3’, producing 220-bp and 280-bp fragments from wild-type and knock-in NR1 alleles, respectively.

**LacZ staining.** Mice were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PB), and post-fixed by the same fixative for 3 h. Brain sections (50-μm thick) were prepared on a Vibrotome (Pro7, DOSAKA) and collected in phosphate buffered saline (PBS). After rinsing in PBS, the sections were then incubated at 37°C overnight in X-gal staining solution (0.1 M PB [pH 7.5], 20 mM Tris–HCl [pH 7.5], 5 mM K3Fe(CN)6, 5 mM
K4Fe(CN)6, 2 mM MgCl2, 1 mg/ml X-gal).

**Immunohistochemistry.** Mice were transcardially perfused with 4% PFA in 0.1 M PB and post-fixed in the same fixative overnight. For NeuN, β-galactosidase, or NR1 immunostaining, brain sections were prepared on a Vibratome (Pro7, DOSAKA) and collected in PBS. Free floating sections were first treated with PBST (100 mM Tris-HCl, 140 mM NaCl, and 0.2% Triton-X), then with 0.8% Block Ace (DS Pharma Biomedical Co. Ltd) for 1 h. For β-galactosidase and NeuN staining, vibratome sections (50-μm thick) were incubated with primary antibody (mouse anti-NeuN, 1/100, Chemicon, NAB337; rabbit anti-β-galactosidase, 1/10000, MP Biomedicals, 55976) diluted in 0.4% blocking solution at 4°C overnight, and then incubated with secondary antibodies (Alexa 488-conjugated anti-mouse IgG, 1/2000, Molecular Probes, A-21041; anti-rabbit IgG, 1/2000, Molecular Probes, A-21045) diluted in 0.4% blocking solution for 1 h at room temperature. For NR1 immunostaining, vibratome sections (60-μm thick) were incubated with primary antibody (Rabbit anti-NR1, 1/200, Frontier Institute, GluRz1-Rb-Af720) diluted in 0.4% blocking solution at 4°C for 1 d, and then incubated with secondary antibodies (Alexa 546-conjugated anti-rabbit IgG, 1/1000, Molecular Probes, A-11030) diluted in 0.4% blocking solution for 5 h at room temperature. After rinsing with PBST, the sections were mounted on glass slides. Images were obtained with a confocal microscope (Fluoview, Olympus).

**Quantitative RT-PCR.** Circular tissue punches (1-mm diameter) were obtained from 140-μm thick frozen coronal brain sections from 10-month-old mice (4 females/group) and stored at
−80°C until assayed. Circular tissue punches was lysed with QIAzol Lysis Reagent (QIAGEN, 79306), and total RNA was extracted and purified with miRNeasy Mini Kit (QIAGEN, 217004) following the manufacturer’s instructions. The mRNA level of individual genes was analyzed using a SuperScript III Two-Step qRT-PCR kit (Invitrogen, 11734-050) and SYBR Premix Ex Taq II (Takara, RR081A). Real-time PCR was performed in a final volume of 25 μl using 0.5 μl of each primer (10 μM), 12.5 μl of the supplied enzyme mix, 6 μl H2O, and 5 μl of the template cDNA. PCR was performed in a 7500 Real-Time PCR System (Applied Biosystems) with a 2-min pre-incubation at 95°C followed by 40 cycles of 15 s at 95°C (denaturation), and 5 s at 53°C (annealing and amplification). The PCR products were subjected to melting curve analysis using the 7500 Real-Time PCR System to exclude the amplification of non-specific products. The amount of mRNA of the target genes, normalized to that of an endogenous control (glyceraldehyde-3-phosphate dehydrogenase, GAPDH), was calculated with a standard curve. The standard curve was generated using a 4-log spanning serial dilution of cDNA samples derived from circular tissue punches of wild-type mice. The following primers were used: NR1-F, 5’ – TAC AAG CGA CAC AAG GAT GC – 3’ and NR1-R, 5’ – TCA GTG GGA TGG TAC TGC TG – 3’, producing 98-bp fragments; GAPDH-F, 5’ – GGG TTC CTA TAA ATA CGG ACT GC -3’ and GAPDH-R, 5’ – CCA TTT TGT CTA CGG ACT GA -3’, producing 112-bp fragments.

Electrophysiologic experiments and dendrite morphology analysis. ILN-Cre; Rosa26-EYFP mice and ILN-Cre; NR1 flox/flox; Rosa26-EYFP mice were used for Control and cKO experiments, respectively, at postnatal day 27–30. The brains were removed under deep anesthesia with isoflurane. Acute parasagittal slices (300-μm thick) were prepared using a
vibratome (Leica Microsystems) in ice-cold artificial cerebrospinal fluid (ACSF) containing the following (in mM): 126 NaCl, 3 KCl, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, 26 NaHCO3, and 10 glucose; and bubbled with 95% O2 and 5% CO2. The slices were recovered in a submerged-type chamber filled with ACSF at 32°C for 30 min, and then maintained at room temperature for at least 30 min before recording.

During recording, the slices were perfused with ACSF containing 10 or 50 μM bicuculline methiodide and 10 μM strychnine hydrochloride (Abcam) at 26°C to 30°C. An infrared differential interference contrast video microscope (BX-51; Olympus) with a 40×, 0.8 NA water immersion lens was used to visualize neurons. Patch pipettes (3–8 MΩ) were filled with an intracellular solution containing (in mM): 130 potassium gluconate, 8 KCl, 1 MgCl2, 0.6 EGTA, 10 HEPES, 3 Na2ATP, 0.5 Na2GTP, 10 Tris-phosphocreatine, and 0.2% biocytin (pH adjusted to 7.35 with KOH). Whole-cell patch-clamp recordings were performed on EYFP(+) neurons just anterior to the fasciculus retroflexus. Current signals were recorded using a MultiClamp 700B amplifier (Molecular Devices), low-pass filtered at 10 kHz, digitized at 20 kHz, and stored using pClamp10 (Molecular Devices). Data were analyzed using MATLAB (MathWorks). Membrane potentials are given with a correction for the liquid junction potential of -13 mV. A series resistance compensation of up to 50% was used for some recordings.

A glass stimulating electrode was placed at the slice surface above the recorded cell. Electrical stimulation of 100-μs duration was applied at 0.2 Hz to evoke excitatory postsynaptic currents (EPSCs) while the voltage of the recorded cell was held at -70 mV, 0 mV, and +40 mV. Stimulation was typically repeated 20 to 50 times at each holding potential. Cells for which stimulation elicited outward inhibitory postsynaptic currents at the holding potential of 0 mV were
discarded from the analyses. The decay time constant of the EPSCs was calculated by fitting between 10% and 90% of the current peak and baseline. A decay time constant of 8 ms was used to judge the existence of NMDA currents (Fig. 2C). For blocking experiments, the cells were held at +40 mV and stimulation was applied at 0.2 Hz. The α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid (AMPA) receptor blocker, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX), and the NMDAR receptor blocker, D-(-)-2-amino-5-phosphonopentanoic acid (APV), were obtained from Tocris Bioscience.

Morphologies of recorded neurons were visualized after staining with biocytin and streptavidin-Cy5 (GE Healthcare). For quantification, spine density from segments (20 μm) of branches of ILN neurons was measured in z-stacks.
Results

本項の内容は、学術雑誌論文として出版する計画があるため公表できない。5年以内に出版予定
NMDARs in the targeted cells, I induced the expression of EYFP in a Cre-dependent manner by crossing ILN-NR1-cKO or ILN-Cre mice with a loxP-flanked EYFP line. Then, in collaboration with Dr. Nao Nakagawa, we made parasagittal brain slices at around postnatal 4 weeks for whole-cell patch-clamp recording (Fig. 4). In control mice, electrical stimulation induced EPSCs with a longer duration at a holding potential of +40 mV and a shorter duration at -70 mV (Fig. 4B), indicating that the EPSCs were mediated by both AMPA receptors and NMDARs. We detected a loss of NMDAR-mediated current in 36 of 56 cells (64.3%) from 6 cKO mice, while we detected NMDAR currents in all 33 cells from 4 control mice (Figs. 3B, C, and D).

Amplitudes and frequencies of spontaneous EPSCs (sEPSCs) were increased in the ILN cells without NMDARs (Fig. 3E). The ILN cells with NMDARs in the mutants also showed increased sEPSC frequency, but not increased sEPSC amplitude (Fig. 3E). These findings confirm that NMDAR was functionally eliminated from the Cre-targeted ILN cells.

Loss of NMDAR function led to abnormal spine structures. NMDARs have crucial roles in synaptic plasticity in several circuits (Tsien et al., 1996, Iwasato et al., 2000). To determine if NMDAR loss leads to abnormalities in synaptic plasticity in the ILN cells, I analyzed the dendritic spine structures of cells using patch clamp analysis (Fig. 4). Spine density was reduced in the ILN-NR1-cKO mice (unpaired t-test, *P = 0.0453, Fig. 4). These findings suggest a crucial role of NMDARs in the structural plasticity of the ILN neurons.
Discussion

The ILN, long thought to be a non-specific arousing system in the brain (Jones and Leavitt, 1974, Kinomura et al., 1996, Siegel, 2004), are thought to be involved in cognitive, sensory, and motor functions (Berendse and Groenewegen, 1991, Van der Werf et al., 2002, Smith et al., 2004). Supporting this notion, radio-frequency lesions, selective immunotoxin lesions, and pharmacologic inactivation of the ILN disrupt spatial working and long-term memory, reinforcement learning, and behavioral flexibility (Savage et al., 1997, Brown et al., 2010, Kato et al., 2011, Bradfield et al., 2013). The behavioral evidence supporting this notion, however, is inconclusive due to several technical limitations, such as difficulty in restricting damage to specific thalamic subregions. In addition, the molecular mechanisms underlying ILN function remain unknown.

To overcome these obstacles, I developed genetic methods to dissect ILN functions in mice. The Cre/loxP system allows for gene knockout in particular brain regions to help determine the causal relationship between changes in the target gene and behavior deficits. Thus, I first generated ILN-specific Cre transgenic mice. LacZ staining in RNZ reporter mice revealed that Cre-mediated recombination was efficiently induced in the ILN within the thalamus of the ILN-Cre mice, although Cre-mediated recombination was also detected at a low frequency outside of the thalamus, such as in the cortex, hippocampus, and pons. This expression pattern of Cre recombinase is almost identical to that of Lypd6b mRNA (Lein et al., 2007). By crossing the ILN-Cre mice to NR1 floxed mice, I developed mutant...
ILN-NR1-cKO mice selectively lacking NMDARs in the ILN. Loss of NR1 in the ILN of the mutant mice was confirmed by immunohistochemistry and quantitative RT-PCR. Consistent with these findings, electrophysiologic experiments revealed the loss of functional NMDARs in approximately 60% of ILN cells. The loss of NMDAR function was associated with increases in sEPSC amplitudes and sEPSC frequencies of the targeted ILN cells. Interestingly, the non-targeted ILN cells in the mutants also showed physiologic phenotypes similar to those of the targeted ILN cells, probably reflecting changes in local network activities. NMDAR-mediated signaling, as a coincident detector, has a crucial role in activity-dependent synaptic plasticity (Tsien et al., 1996, Iwasato et al., 2000). Because synaptic plasticity is generally associated with structural changes of synapses (Lamprecht and LeDoux, 2004), we examined dendritic spine structures representing excitatory synapses. The number of dendritic spines and their morphology contribute to alterations in excitatory synaptic transmission during learning (Engert and Bonhoeffer, 1999, Leuner et al., 2003). Cells lacking functional NMDARs had decreased dendritic spine density, suggesting deficits in synaptic plasticity.

Postmortem neurochemical studies in patients with mental disorders suggest reduced NMDAR expression in the ILN (Ibrahim et al., 2000, Clinton and Meador-Woodruff, 2004). The ILN-NR1-cKO is expected to provide clues to clarify the pathophysiology of these mental illnesses. Behavioral studies using the ILN-NR1-cKO mice will help to elucidate the role of the ILN in cognitive function and provide insight into the NMDAR hypofunction hypothesis for schizophrenia.

In summary, in this study, ILN-specific Cre mice were created and characterized, and can be widely used in studies aimed at understanding the functions of the ILN as well as the
functions of genes expressed within this brain area. In the next chapter, I describe my extensive analysis of the ILN-NR1-cKO mice with a particular focus on the role of NMDAR hypofunction in the pathophysiology of schizophrenia.
Chapter 2

A novel animal model of schizophrenia with NMDA receptor hypofunction in the intralaminar thalamic nuclei

Introduction

Schizophrenia is a mental disorder that affects 1% of the global population. Patients with schizophrenia exhibit positive symptoms (hallucinations and delusions), negative symptoms (social deficits and anhedonia), as well as cognitive symptoms, such as working and long-term memory deficits, inattention, and inflexibility (Marder, 2006, Geyer et al., 2012, Pratt et al., 2012, American Psychiatric Association, 2013). Cognitive impairments also occur in humans or rodents following administration of NMDA receptor (NMDAR) antagonists, lending support to the NMDAR hypofunction hypothesis in the pathophysiology of schizophrenia (Javitt and Zukin, 1991, Amitai and Markou, 2010, Neill et al., 2010, van den Buuse, 2010, Coyle, 2012). Little is known about the brain area in which the NMDAR hypofunction is responsible for such symptoms, although it is thought that NMDAR hypofunction during development of inhibitory neurons might be crucial in the pathophysiology of schizophrenia (Nuechterlein et al., 2004).

Dysfunction of the thalamus is a putative candidate underlying pathology of schizophrenia (Andreasen et al., 1998). Neuroimaging studies in patients reveal differences in the morphology and metabolism of the thalamic sub-nuclei, including the intralaminar thalamic
nuclei (ILN) (Hazlett et al., 1999, Kemether et al., 2003, Woodward et al., 2012). While the precise function of these nuclei is not well understood, some ILN neurons have rich reciprocal connections with the prefrontal cortex and striatum (Berendse and Groenewegen, 1991, Van der Werf et al., 2002, Vercelli et al., 2003), both of which are key structures involved in the control of executive function (Aron et al., 2004, Smith et al., 2004). The thalamocortical connectivity from the ILN to the prefrontal cortex, which is linked to working memory task performance, is also altered in schizophrenia (Marenco et al., 2012). Furthermore, postmortem neurochemical studies imply reduced NMDAR expression in the ILN (Ibrahim et al., 2000, Clinton and Meador-Woodruff, 2004). These findings led me to hypothesize that NMDAR hypofunction in the ILN is causally related to the cognitive impairments observed in schizophrenia. This hypothesis, however, has not been tested.

To test the hypothesis, I generated ILN-selective conditional knockout (cKO) mice for NR1, which encodes an essential subunit of the NMDAR. Using a novel transgenic mouse line expressing Cre selectively in this brain area (ILN-Cre), I directly examined the role of NMDAR in the ILN. Comprehensive behavioral examination of the conditional mutant mice for NR1 in the ILN revealed abnormalities resembling the cognitive and positive symptoms of patients with schizophrenia. These findings support the notion that NMDAR hypofunction in the ILN has a crucial role in the pathophysiology of schizophrenia.
Materials and methods

Breeding of mice. Heterozygous ILN-Cre mice were crossed with mice homozygous for loxP-flanked NR1 allele (NR1 flox/flox) (Tsien et al., 1996) to obtain double heterozygous mice for the Cre transgene and floxed-NR1 allele (ILN-Cre; NR1 flox/+). These mice were further crossed with NR1 flox/flox mice to produce ILN-Cre; NR1 flox/flox mice and littermate control mice, NR1 flox/flox. The ILN-Cre; NR1 flox/flox mice are referred to as ILN-NR1-cKO mice. DNA extraction from mouse tails and genotyping were performed as described in Chapter 1.

The mouse genotype was determined by polymerase chain reaction (PCR). PCR was performed in a final volume of 12 μl using 0.1 μl of each primer (50 μM), 0.07 μl Extaq (TAKARA, RR001A), 12 μl of 10 × ExTaq buffer, 0.96 μl of 2.5 mM dNTP, 8.47 μl H2O, and 1 μl of the extracted tail DNA. PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems) with 2-min pre-incubation at 94°C followed by 35 cycles of 30 s at 94°C (denaturation), 30 s at 60°C (annealing), and 30 s at 72°C amplification, using the following primers: CW-Cre2, 5’ - ACC TGA TGG ACA TGT TCA GGG ATC G – 3’ and CW-Cre3, 5’ - TCC GGT TAT TCA ACT TGC ACC ATG C – 3’, producing a 108-bp fragment from the Cre allele; NR-ER5, 5’ – TGT GCT GGG TGT GAG GGT TG – 3’ and NR-ER10, 5’ – GTG AGC TGC ACT TCC AGA AG –3’, producing 220-bp and 280-bp fragments from wild-type and knock-in NR1 alleles, respectively.

Behavioral analysis. Mice were group-housed in individually ventilated cages. The light cycle was 8:00 ON and 20:00 OFF. Water and foods were provided ad libitum, unless otherwise stated. All behavioral tasks were performed during the light phase by an investigator blind to the mouse
Prepulse inhibition test. Prepulse inhibition (PPI) testing was conducted in four startle chambers (O’Hara & Co.) as described previously with some modification (Sano et al., 2009). The test comprised 45 trials. Nine different trial types were presented: non-stimulus trials, trials with an acoustic stimulus (40-ms white noise, either 80, 90, 100, 110, or 120 dB) alone, and trials in which the prepulse stimulus (20-ms white noise, either 70, 75, or 80 dB) had an onset 100 ms before the onset of the acoustic startle stimulus (40-ms white noise, 120 dB). The different trial types were presented in blocks of nine, in randomized order in each block, with a mean inter-trial interval of 15 s (range: 10–20 s). The percent startle response was recorded by measuring the amplitude of the startle reflex in response to the prepulse stimulus and the startle stimulus, and calculated as 100 - [response amplitude of the prepulse stimulus + response amplitude of the startle stimulus]/response amplitude of the startle stimulus] at each prepulse sound level (70, 75, and 80 dB).

5-Choice serial reaction-time task. The 5-choice serial reaction-time test (5-CSRTT) was performed as previously described with some modifications (Kobayashi et al., 2013). Male and female mice were food-restricted to reduce their body weight to approximately 85% of their free-feeding weight. After the mice were trained to consume food pellets delivered automatically at 45-s intervals from a pellet dispenser for 15 min daily for 3 d (Food training, aperture), the mice were trained to associate nose-pokes with food delivery for 30 min during the next 10 d (Nose-poke training, 100 trials/d). In the Nose-poke training, the stimulus duration of green
signal lights behind all of the holes was 30 s. In the following sessions (spatial 1 - 8), mice were trained to respond to presentations of a green signal light that was pseudorandomly displayed in one of five apertures. Each trial commenced with an inter-trial interval, during which any response to a hole was recorded as a premature response. The stimulus was then displayed, followed by a limited holding period. In spatial stages 2 - 8, when a mouse did not perform a nose-poke within the limited holding period (omission) or performed a nose-poke into a wrong hole (incorrect), the house light was extinguished for 2 s. When a mouse continued nose-pokes in any hole after a correct response, but before collecting food pellets, the responses were recorded as perseverative responses. The inter-trial interval, stimulus duration, and limited holding periods were 2 s, 30 s, and 30 s (spatial stage 1, 14 sessions); 10 s, 30 s, and 30 s (spatial stage 2, 5 sessions); 10 s and 10 s, and 10 s (spatial stage 3, 3 sessions); 15 s, 10 s, and 10 s (spatial stage 4, 3 sessions); 15 s, 5 s, and 5 s (spatial stage 5, 5 sessions); 15 s, 2 s, and 2 s (spatial stage 6, 5 sessions); 15 s, 1 s, and 2 s (spatial stage 7, 2 sessions); and 15 s, 0.6 s, and 2s (spatial stage 8, 2 sessions), respectively.

Mice were required to complete 100 trials in a 30-min session. When an animal reached a performance criterion (completed 100 trials, > 80% correct response, < 20% omissions for 3 out of consecutive 5 d) at spatial stage 5, it was then tested in the following spatial stages in which the stimulus duration was reduced to 2.0 (stage 6), 1.0 (stage 7), and 0.6 s (stage 8).

**Y-maze tests.** A Y maze with three identical arms made of transparent Plexiglass (39.5 × 3 × 12 cm) 120° apart was placed in the center of a diffusely illuminated room (30 lx). Each mouse was placed at the end of one arm facing the center and allowed to freely explore the apparatus
with the experimenter out of sight. All sessions were video-recorded through a camera mounted above the maze and evaluated using EthoVision XT 8.0 (Noldus). Entry into each arm was scored for 5 min beginning from the first entry, as described previously (Belforte et al., 2010). Alternation behavior was defined as consecutive entries into each of the three arms without repetition (i.e., ABC, BCA, CAB, ACB, CBA, or BAC). We defined the percentage of spontaneous alternations as the actual alternations divided by the possible alternations (total arm entries – 2) × 100.

An alternative test (forced Y-maze test) for evaluating working memory comprised two phases: habituation and testing, as described previously (Redrobe et al., 2009). In phase 1 (habituation), the mouse was placed at the end of the start arm and then forced to enter one of the exploration arms in the maze, while the other arm was closed. After the mouse entered the arm, access to the start arm was blocked, and the mouse was allowed to explore the arm (termed the familiar arm) for a period of 5 min. The arm that was designated the familiar arm was counter-balanced for each genotype. Immediately thereafter, in phase 2 (testing), the mouse was allowed to explore both the familiar and the unfamiliar arms, but not the start arm, for a period of 2 min. All sessions were video-recorded by a camera mounted above the maze and evaluated using EthoVision XT 8.0 (Noldus). The cumulative time spent in each arm and total distance traveled were recorded during the test session. A discrimination index was calculated for each mouse: discrimination index = Time spent in unfamiliar arm/ (Time spent in familiar arm + Time spent in unfamiliar arm).

**Morris water maze.** The Morris water maze test was performed as previously described with
some modifications (Tanaka et al., 2013). The water pool used was 150 cm in diameter. The
temperature of the water was held constant at 25 ± 1°C. Mice were given 4 trials per day for 9 d
in the hidden platform task. During the acquisition period, four different starting points were used
and the starting positions were varied pseudorandomly over the trials. A probe test was performed on
the final day of the acquisition session. In the probe test, the platform was removed from the
maze and each mouse was allowed to swim for 90 s. The visible platform test was performed for
4 d. In the visible platform task, a black cubic landmark was attached to the platform. Mouse
movement in the water maze was recorded by a camera and analyzed using software (O’hara &
Co.)

**Open field locomotor activity.** Each mouse was placed in the center of an open field (50 x 50
x 50 cm, 70 lx) and allowed to freely explore the apparatus for 10 min. The activity of the mice
was recorded by a camera and analyzed using software (O’hara & Co.) to assess the total
distance moved (cm), time in the center (s), and rearing activity (count).

**Home cage activity and MK-801 induced hyperactivity.** To measure locomotor activity in
the home cage, each mouse was placed in a transparent cage (20.7 [W] x 36.5 [L] x 14 [H]
containing bedding materials, and horizontal and vertical activities were monitored for
continuous 5 days and using infrared area sensors (SCANET Behavioral Analysis System;
Melquest), as described previously (Sano et al., 2009). To examine MK-801 induced
hyperactivity, mice were injected with a noncompetitive NMDAR antagonist, MK-801 ((+)-MK
801 maleate [TOCRIS], 0.3 mg per kg intraperitoneal in 0.9% saline), or solvent (0.9% saline).
Mice were tested in a home cage placed in the SCANET Behavioral Analysis System (Melquest). The activity of the mice was measured for 240 min through infrared beam breaks and analyzed every 1 min. The session began with a 30-min habituation period, and mice were subsequently injected with MK-801 or solvent by an experimenter.

**Three-chamber social interaction test.** The three-chamber social interaction test consisted of three phases. In the first phase, mice were placed the three-chambered apparatus, in which partitions divided the apparatus into three equal chambers (42 x 21 x 22.5 cm), and allowed to explore freely for 5 min for habituation. After 5 min, the mice were gently guided to the center chamber, and the doors to the two side chambers were blocked while a stranger mouse (stranger 1; 7-month-old C57BL/6J male) was introduced into a cage (right triangular prism, base: 15 x 13 x 9 cm, height: 15 cm) that was placed in one of the side chambers. An identical empty cage was placed in the other side chamber. In the interaction phase, the doors to the two side chambers were opened to allow the mouse in the center chamber to explore the cage containing stranger 1 or the empty cage for 5 min. In the third phase, another stranger mouse (stranger 2) was placed into the second cage. Mice were allowed to explore the stranger mice for 5 min. Time spent in nose contact in each of the cages was measured using EthoVision XT 8.0 (Noldus). The preference index represents the numerical difference between time spent exploring each target (stranger 1 vs. empty, or stranger 2 vs. stranger 1) divided by total time spent exploring both targets.

**Viral injection for NR1 local deletion.** I used the adeno-associated virus,
AAV.DJ-CMV-mCherry-iCre virus, for virus-mediated local deletion of NR1 in the ILN. The technical details and in vitro viral production are described in Chapter 3. To make sure that the virus vector mediated local Cre recombination, Rosa26-STOP-EFYP mice were injected bilaterally with 0.2 µl of AAV.DJ-CMV-mCherry-iCre. For behavioral experiments, the NR1 floxed homozygous mice (5 month-old male) were anesthetized with isoflurane and positioned in a stereotaxic frame (Angle two, Leica). They were injected bilaterally with 0.2 µl AAV.DJ-CMV-mCherry-iCre or AAV.5-CamK2-mCherry (1.0 × 10^{13} genome copy ml^{-1}, Vector Biolabs) using the following coordinates; from bregma: ML, ±0.75 mm; DV, -3.5 mm; AP, -2.18 mm. After making a small craniotomy using a drill, a glass capillary (30 um) was placed at each site, and 0.2 µl virus was delivered to each site (0.02 µl/min). After the injection, the capillary was kept in place for 5 additional minutes to allow the virus to diffuse in the brain. All animals were allowed 3 weeks to recover before behavioral testing began. Mice were deeply anesthetized with 2,2,2-tribromoethanol (Avertin, Sigma-Aldrich), and tissue samples were assayed to locate the virus injection sites by detecting mCherry expression. Images were obtained with a confocal microscope (Fluoview, Olympus). A single mouse injected with AAV.DJ-CMV-mCherry-iCre was excluded from analysis because mCherry was detected outside of the ILN.
Results

ILN-NR1-cKO mice exhibit cognitive impairments. Prepulse inhibition (PPI) is a measure of sensorimotor gating (Geyer and Braff, 1987), which is reduced in patients with schizophrenia and in rodent models of schizophrenia (Geyer et al., 2001, Brigman et al., 2010, van den Buuse, 2010). ILN-NR1-cKO mice had intact auditory responses, but displayed impaired PPI of the startle reflex (Figs. 5A and B). Deficits in sensorimotor gating, which represent the inability to filter out irrelevant information, can lead to sensory overload and consequent cognitive fragmentation in schizophrenia (Geyer and Braff, 1987).

To explore the cognitive deficits in the mutant mice, I used the 5-CSRTT (Robbins, 2002, Chudasama and Robbins, 2004) to measure sustained attention: in this task, the animal must identify by a nose poke which of the five apertures is illuminated to obtain a reward (Fig. 6A). The 5-CSRTT simultaneously assesses multiple cognitive modalities, including attention, impulse control, cognitive flexibility, and processing speed (Amitai and Markou, 2010, Kobayashi et al., 2013). The 5-CSRTT is particularly relevant because of its similarity with the continuous performance task used to assess humans (Powell and Miyakawa, 2006, Pratt et al., 2012), which reveals attention impairments in patients with schizophrenia (Young et al., 2013), and because 5-CSRTT performance is also disrupted in rodent models treated with NMDAR antagonists, such as phencyclidine and MK-801 (Paine and Carlezon, 2009, Amitai and Markou, 2010). In the initial stage, in which mice were first trained to respond to a stimulus displayed for 30 s, and after they acquired a suitable performance level, the stimulus duration was decreased to

32
impact task difficulty or attentional load. No difference in learning speed was detected between control and mutant mice performing the initial reinforcement learning (Fig. 6B). In the task with a shorter stimulus duration, ILN-NR1-cKO mice had increased errors compared with control siblings (Fig. 6C), indicating that the mutant mice had attention deficits. In addition, ILN-NR1-cKO mice displayed more premature and perseverative responses, a measure of impulsivity and inflexibility, respectively (Figs. 6D and E). The mutant mice showed intact processing speed, indicated by the latency to make a correct response (Fig. 6F). These findings suggest that NMDAR deletion in the ILN impairs attention, impulse control, and cognitive flexibility.

To test for working memory deficits, which are well-documented in schizophrenia, I assessed the mutant mice in the Y-maze spontaneous alternation task, which is based on the natural tendency of rodents to explore a novel environment, and thus alternate visits between recently visited and other arms. The control mice showed reliable alternation, while the mutant mice displayed reduced alternation (Fig. 7A). Mutant mice and control mice had similar numbers of arm entries (Fig. 7B). These findings indicate that NMDAR deletion in the ILN impairs spatial working memory.

To test for spatial reference memory, I tested mutant mice in the Morris water maze. ILN-NR1-cKO mice showed deficits in learning to find a hidden platform using distal cues compared to control siblings (Fig. 8A). In the probe test, during which the platform was removed from the maze, control and mutant mice equally and preferentially swam in the quadrant in which the platform had been located (Fig. 8B). Whereas control mice preferentially crossed over the previous platform location, however, mutant mice did so less frequently, suggesting poor
memory for the location of the platform (Fig. 8C). Mutant mice exhibited intact visual ability and motivation to escape in the visible platform test (Fig. 8D). These findings suggest that deletion of NR1 in the ILN impairs spatial learning and memory.

ILN-NR1-cKO mice exhibit positive symptom-like, but not negative symptom-like, behavior. While the positive symptoms of schizophrenia, such as auditory hallucinations and delusions, may be unique to humans, locomotive hyperactivity is considered a measure of psychosis-like behavior in rodents (van den Buuse, 2010). Spontaneous locomotor activity was measured for 10 min in a novel open field chamber. The mutant mice were not different from controls in the total distance traveled and in the time spent in the center region of the open field (Fig. 9). The mutant mice, however, had significantly less vertical activity, suggesting deficits in general attention (Fig. 9).

Next, I investigated the effects of the NMDAR antagonist MK-801 on the mutant mice. Subcutaneous administration of MK-801 induced locomotor hyperactivity sustained over 3 h after injection in control animals (Figs. 10A and B). The MK-801 induced hyperactivity, however, was largely diminished in ILN-NR1-cKO mice (Figs. 10A and B). The findings suggest that NMDAR in the ILN have a major role in hyperactivity induced by MK-801.

When home cage activity was monitored for 5 d, control mice increased their activity after the onset of the dark phase (Fig. 11A), and this response gradually decreased over 5 d after transfer to a new home cage (Figs. 11A and B). In contrast, ILN-NR1-cKO mice showed no such adaptive changes during the observation period (Mixed between-within subjects ANOVA, genotype × day interaction, F (4, 52) = 3.2816, P = 0.0179, Figs. 11A and B). The maladaptation...
may represent a positive symptom-like phenotype. Schizophrenia is also associated with negative symptoms, such as social deficits (Marder, 2006, Neill et al., 2010). I examined whether the mutant mice exhibited negative symptom-like behavior in the three-chamber sociability and social novelty tests. ILN-NR1-cKO mice preferred to explore the first novel mouse introduced (stranger 1) over an empty cage, similar to control animals (Fig. 12A). When another novel mouse (stranger 2) was introduced, the mutant mice preferred to explore stranger 2 over stranger 1, suggesting a normal level of social novelty recognition (Fig. 12B). These findings suggest that the mutant mice did not have social deficits.

A AV-Cre mediated local deletion of NR1 in the ILN induced working memory deficits. As discussed in Chapter 1, recombination may occur at a low frequency in the ILN-Cre transgenic mice in areas other than the ILN. Thus, to further explore the causal relationship between NMDAR loss in the ILN and the behavioral deficits of ILN-NR1-cKO mice, I deleted NR1 locally by injecting A AV-DJ-CMV-mCherry-iCre vectors into the ILN of NR1 floxed homozygous mice. Local injection of AA V.DJ-CMV-mCherry-iCre vectors in Rosa26-STOP-enhanced yellow fluorescent protein (EYFP) reporter mice revealed successfully induced expression of EYFP in a Cre-dependent manner. In the Rosa26-STOP-EYFP; A AV.DJ-CMV-mCherry-iCre, the EYFP signal was observed most densely in the ILN, with substantial expression in other thalamic areas (Figs. 13A and B).

To evaluate the behavioral consequences of the local deletion of NR1 in the ILN, A AV.DJ-CMV-mCherry or A AV.5-CaMKII-mCherry was injected as a control into the ILN.
of the NR1 flox/fox mice, and the mice were tested in the forced Y-maze test (Fig. 13C). The discrimination index was significantly decreased in the ILN-NR1-deleted mice compared to control mice (Fig. 13D), although there was no significant group difference in distance traveled during the test (Fig. 13E). These findings suggest that the NR1 deletion in the ILN leads to working memory deficits.
Discussion

The ILN, long thought to be a non-specific arousing system in the brain (Jones and Leavitt, 1974, Kinomura et al., 1996, Siegel, 2004), was recently proposed to be involved in cognitive, sensory, and motor functions (Berendse and Groenewegen, 1991, Van der Werf et al., 2002, Smith et al., 2004). Radio-frequency lesions, selective immunotoxin lesions, and pharmacologic inactivation of the ILN disrupt spatial working and long-term memory, reinforcement learning and behavioral flexibility (Savage et al., 1997, Brown et al., 2010, Kato et al., 2011, Bradfield et al., 2013). These methods are associated with several technical limitations, however, such as difficulty in restricting damage to specific thalamic subregions and a lack of molecular information underlying phenotypes.

In the present study, I characterized ILN-specific NR1 cKO mice, and provided evidence that NMDARs in the ILN have a role in multiple cognitive functions. ILN-NR1-cKO mice exhibited deficits in spatial working and long-term memory, implying the importance of NMDAR-dependent synaptic plasticity in the ILN during these memory tasks. Unlike previous intervention studies (Savage et al., 1997), the ILN-NR1-cKO mice displayed intact auditory and visual responses in the startle response test and the visible platform test of the Morris water maze, respectively. Reinforcement learning in the ILN-NR1-cKO mice was also not impaired during an initial reinforcement learning stage of the 5-CSRTT, which contradicts previous lesion studies (Burk and Mair, 2001, Kato et al., 2011). The results presented in this thesis suggest that NMDAR signaling in the ILN has a crucial role in cognitive function, whereas it has little effect...
on sensory processing and motor control.

The anatomic and physiologic characteristics of the ILN led to the hypothesis that the ILN play an important role in action-selection mechanisms of the basal ganglia (Kimura et al., 2004, Minamimoto et al., 2005, Varela, 2014). The ILN have abundant connections with the prefrontal cortex and striatum (Van der Werf et al., 2002, Smith et al., 2004). ILN neurons in primates supply the striatum with information about behaviorally significant sensory events (Matsumoto et al., 2001), and their thalamostriatal afferents are thought to suppress ongoing motor behavior and allow for the selection of a different action under changing conditions (Ding et al., 2010, Smith et al., 2011, Smith et al., 2014). In support of this hypothesis, I found that a lack of NMDARs in the ILN caused deficits in attention, impulse control, and cognitive flexibility. Thus, I propose that NMDARs in the ILN mediate information processing that is critical for shifting attention and behavior in the face of changing events.

NMDAR dysfunction is suggested to contribute to the pathology of schizophrenia (Javitt and Zukin, 1991, Amitai and Markou, 2010, Neill et al., 2010, van den Buuse, 2010, Coyle, 2012). Neuroimaging and neurochemical studies in schizophrenic patients show alterations in the ILN (Hazlett et al., 1999, Ibrahim et al., 2000, Kemether et al., 2003, Clinton and Meador-Woodruff, 2004, Woodward et al., 2012). These studies in patients, however, cannot draw causal relationships between thalamic NMDAR hypofunction and specific symptoms. The findings of present study, by using genetic approaches, clearly demonstrate that NMDA hypofunction in the ILN is causally related to cognitive deficits relevant to schizophrenia. Cognitive deficits are core symptoms in schizophrenia, and most are resistant to currently available medication (Geyer et al., 2012). The ILN-NR1-cKO mice are expected to provide new
In Chapter 3, I examined the possibility that NMDARs in the ILN are potential therapeutic targets. In addition to cognitive impairments, patients with schizophrenia exhibit positive and negative symptoms. The mutant mice demonstrated deficits in PPI and maladaptation to a new home cage. These abnormalities may in part represent endophenotypes closely associated with positive symptoms in schizophrenia. Given that the PPI deficits are thought to represent the inability to filter out irrelevant information (Geyer and Braff, 1987), the maladaptation observed in the mutants might reflect excessive or distorted perception, which is relevant to hallucinations and delusions in schizophrenia. Systemic administration of MK-801 induced long-lasting locomotor hyperactivity in control mice, resembling a state of positive symptoms (Carlen et al., 2012, Kocsis, 2012). The ILN-NR1-cKO mice clearly showed insensitivity to MK-801, suggesting a crucial role of the ILN in positive symptom-related behaviors. Recent findings that administration of a hallucinogen activates the ILN in a maternal immune activation mouse model of schizophrenia also support the notion that the ILN play a role in the pathogenesis of positive symptoms (Malkova et al., 2014). On the other hand, the mutant mice showed no phenotypes representing negative symptoms, as assessed by a social interaction test. Taken together, these findings suggest that ILN-NR1-cKO mice have endophenotypes of schizophrenia, particularly in some of the cognitive domains.
Chapter 3

NMDARs in the intralaminar thalamic nuclei are a potential therapeutic target to enhance cognitive function

Introduction

Cognitive impairment in schizophrenia is a core deficit that is not satisfactorily improved by current medications (Geyer et al., 2012). Clinical and preclinical research has focused on psychosis, which is only one of the hallmarks of schizophrenia, after the fortuitous discovery of the first antipsychotic drug. Several new antipsychotics have been developed to address the serious side effects of the initial drugs, although they are ineffective toward normalizing cognitive dysfunction (Geyer et al., 2012, Pratt et al., 2012). Thus, we need to consider a novel therapeutic approach to enhance cognition based on the theoretical concept of the illness.

In Chapter 2, I developed a novel schizophrenia mouse model with NMDAR hypofunction in the ILN, based on the hypothesis that hypofunction in this brain area causes the observed cognitive symptoms. In support of this hypothesis, the mutant mice displayed cognitive impairments related to the cognitive symptoms in schizophrenia. They also exhibited positive symptom-like behaviors. These findings led us to propose that NMDARs in the ILN are a potential therapeutic target for schizophrenia. To test this idea, I performed selective restoration of NMDARs in the ILN in adult ILN-NR1-cKO mice using the adeno-associated virus (AAV) expression system.
Materials and methods

Virus production and purification. The turboRFP-coding sequences were polymerase chain reaction (PCR)-amplified along with Ascl and NheI linkers from a plasmid, TiTMPV-Neo (shRen) (Addgene, Plasmid #27993), using the following primers: turboRFP forward: 5’- GCT AGC TAG CCA CCA TGA GCG AGC TGA TCA AGG AG -3’, turboRFP reverse: 5’- TTG GCG CGC CTC ATC TGT GCC CCA GTT TGC - 3’. PCR was performed in a final volume of 100 μl using 5 μl of each primer (10 μM), 20 μl of 5× Phusion HF buffer (New England Biolabs, M0531S), 8 μl of 2.5 mM dNTP, 59 μl H2O, and 1 μl of the template DNA. The PCR products were digested with Ascl and NheI. The fragments containing turboRFP were ligated in the antisense direction into Ascl and NheI sites of pAAV-EF1a-DIO-EYFP-WPRE-HGHpA (Addgene, Plasmid #20296), to yield the plasmid pAAV-DIO-turboRFP.

Viruses were produced using a triple-transfection, helper-free method (Samulski et al., 1989), and purified using an ultracentrifugation protocol (Zhang et al., 2010). The 293FT cells (Invitrogen) were cultured in 30 ml Complete Medium (Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate solution, 0.075% sodium bicarbonate solution, 1% penicillin-streptomycin-L-glutamine solution) in 225 cm² cell culture flasks (Nunc, 159934) at 37°C/5% CO2, until 293FT cells reached approximately 70% to 80% confluence. They were transfected with pHelper, pAAV-DJ/8 (Cell Biolabs), and pAAV-DIO-turboRFP or pAAV-CBA-fsNR1 (provided by Dr. Richard D. Palmiter of the University of Washington) (Parker et al., 2011). Plasmids (20 μg each) were mixed with 293fectin™ transfection reagent (Invitrogen) in 300 μl Opti-MEM (Invitrogen, 11058021) and incubated for 30 min at 25°C before the mixture was transferred to the 70% to 80% confluent
293FT cells. After 3 d incubation at 37°C/5% CO₂, each lysate was collected into 50-ml conical tubes and spun down by centrifugation for 30 min at 2500 rpm, followed by filtration. Each cleared lysate was moved into ultracentrifuge tubes and 20% sucrose/phosphate buffered saline (PBS) solution was added to the bottom of the tubes before centrifuging in a Beckman SW-28 rotor at 22,000 rpm at 4 ºC for 2 h. After pouring out the supernatant and drying the side of each tube with a Kimwipe, viruses at the bottom were eluted with 100 µl of cold PBS by gently pipetting the PBS up and down. The final purified viruses were stored at −80°C.

**Virus titration using quantitative PCR.** Genomic AAV titers were determined using a modification of a published protocol (Rohr et al., 2002). PCR was performed using the FastStart SYBR Green Master (Roche Molecular Biochemicals). PCR was performed in a final volume of 50 μl using 0.5 μl of each primer (50 μM), 25 μl of the supplied enzyme mix, 23 μl H2O and 1 μl of the template. PCR was performed in a 7500 Real-Time PCR System (Applied Biosystems) with a 2 min pre-incubation at 95°C followed by 40 cycles of 15 s at 95°C (denaturation) and 5 s at 53°C (annealing and amplification). PCR products were subjected to melting curve analysis using the 7500 Real-Time PCR System to exclude the amplification of non-specific products. Finally, the PCR products were analyzed by conventional agarose gel electrophoresis. Primers were synthesized by Life Technologies Japan. The following primers were used: EF1a forward: 5’-GAG TTT CCC CAC ACT GAG TG-3’, EF1a reverse: 5’-GAG GCT TGA GAA TGA ACC AAG A-3’. A fragment length of 201 base pairs of the quantitative PCR product is expected using the primers.

In each quantitative PCR run, a standard curve was generated using a 4-log spanning
serial dilution of the vector plasmid pAAV-EGFP containing one EF1a promoter per plasmid molecule. Serial dilution ranged from 1 to 1000 pg of the vector plasmid. Each dilution step was measured in triplicate per Real-Time PCR run. The standard curve was calculated by the 7500 Real-time PCR software (7500 system SDS, Version 1.3.1, Applied Biosystems) by regression of the crossing points of the PCR curves from the dilution series of the vector plasmid.

Recombinant AAV-DJ/8 containing fractions were pre-treated with DNase. For DNase digestion, 5 μl of the viral sample were incubated with 1 μl of DNase I (7500 U/ml; QIAGEN) in a final volume of 50 μl at 37°C for 30 min. DNase I was inactivated by incubation at 65°C for 10 min. A set of serial dilution of viral sample was prepared (1:100, 1:1000, 1:10,000). One microliter of the sample was then applied to PCR using the PCR conditions described above. Specificity of the PCR products was analyzed by melting curve analysis. To quantitate the template number in unknown AAV vectors, the 7500 system software compared the PCR result of the unknown sample to the standard curve derived from the plasmid dilution series. The single-stranded nature of the AAV genome as well as the double-stranded plasmid standard curve values were taken into consideration. The value from each sample was multiplied by the dilution factor (1:100, 1:1000, 1:10,000) and by 1000 to obtain the titer per milliliter (1 μl × 1000=1 ml). Assuming that one AAV particle contained one single DNA copy and that 1 pg DNA of the 6324 bp vector plasmid corresponded to $2.88 \times 10^5$ plasmid molecules, the viral AAV titer was calculated.

**Viral injection for NR1 rescue.** For virus-mediated rescue of NR1 into the ILN of conditional knockout (cKO) mice, we used AAV.DJ/8-fsNR1 virus, which expresses NR1 after Cre-mediated
recombination. The ILN-NR1-cKO mice (2 month-old males) were anesthetized with isoflurane and positioned in a stereotaxic frame (Leica Angle two). They were injected bilaterally with 0.3 μl AAV.DJ/8-fsNR1 (1.2 × 10^9 genome copy ml^-1) or AAV.DJ/8-DIO-turboRFP (1.8 × 10^13 genome copy ml^-1) at the following coordinates; from bregma: ML, ±0.75 mm; DV, -3.5 mm; AP, -2.18 mm. After making a small craniotomy using a drill, a glass capillary (30 μm) was placed at each site, and 0.3 μl purified virus was delivered to each site (0.1 μl/min). After the injection, the capillary was held in place for 5 additional minutes to allow the virus to diffuse in the brain. All animals were allowed 3 weeks to recover before behavioral testing began. All mice were deeply anesthetized with 2,2,2-tribromoethanol (Avertin, Sigma-Aldrich), and tissue samples were assayed by immunohistochemistry.

**Immunohistochemistry.** Mice were transcardially perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer and post-fixed by the same fixative overnight. For hemagglutinin (HA)-tag immunostaining, brain sections (50-μm thick) were prepared on a Vibratome and collected in PBS. Free-floating sections were first treated with PBST (100 mM Tris-HCl, 140 mM NaCl 0.2% Triton-X), followed by treatment with 0.8% Block Ace (DS Pharma Biomedical Co. Ltd) for 1 h. The sections were then incubated at 4°C overnight with primary antibody (mouse anti-HA, 1/500, ABM, G036) diluted in 0.4% blocking solution. After rinsing with PBST, the sections were incubated with secondary antibodies (Alexa 594-conjugated anti-mouse IgG, 1/2000, Molecular Probes) for 2 h at room temperature. After rinsing with PBST, the sections were mounted on a glass slide. Images were obtained with a confocal microscope (Fluoview, Olympus).
**Y-maze spontaneous alternation test.** A Y maze with three identical arms made of transparent Plexiglass (39.5 × 3 × 12 cm) 120° apart was placed in the center of a diffusely illuminated room (30 lx). Each mouse was placed at the end of one arm facing the center and allowed to freely explore the apparatus with the experimenter out of sight. All sessions were video-recorded through a camera mounted above the maze and evaluated by EthoVision XT 8.0 (Noldus). Entry into each arm was scored for 5 min beginning from the first entry, as described in Chapter 2. Alternation behavior was defined as consecutive entries into each of the three arms without repetition. We defined the percentage of spontaneous alternations as the actual alternations divided by the possible alternations (total arm entry – 2) × 100.
Results

In ILN-NR1-cKO mice, the NR1 gene is thought to be disrupted during the development including juvenile stages. To rule out the possibility that the lack of NMDAR function during development is critical for performance in the working memory test, I used a viral rescue strategy in adult animals (Parker et al., 2011) that allows for conditional re-expression of the NR1 subunit of the NMDA receptor in a Cre-dependent manner (Fig. 14A). Histologic analysis revealed the specific expression of tRFP and restoration of HA-tagged NR1 restricted to the ILN (Fig. 14B). To test the extent to which the restoration of NR1 in the mutant mice rescued the working memory deficits, I assessed virally injected ILN-NR1-cKO mice in the Y-maze spontaneous alteration task. While mutants with control vectors exhibited a reduced alteration index to chance levels, the virally rescued mutant mice showed reliable alternations (Fig. 14C). There was no difference between mutant and control mice in distance traveled (Fig. 14D). These findings suggest that restoration of NMDAR signaling to the ILN of adult ILN-NR1-cKO mice was sufficient to rescue the working memory deficit.

Next, to test the extent to which the restoration of NR1 in the mutant mice rescued the function of NMDARs in the ILN, I investigated the effect of NMDAR antagonist MK-801 on virally injected ILN-NR1-cKO mice (Fig. 15A). While MK-801 induced hyperactivity was largely diminished in the ILN-NR1-cKO mice with a control vector, the hyperactivity was restored in the virally rescued mutants to the control level (Fig. 15B). These results suggest that the NMDAR blockade in the ILN was partly responsible for the MK-801 hyperactivity.
Discussion

本項の内容は、学術雑誌論文として出版する計画があるため公表できない。5年以内に出版予定

Viral rescue of NMDARs enhanced working memory performance of the ILN-NR1-cKO mice in the Y-maze test. These findings suggest that NMDARs in the ILN are a potential therapeutic target for schizophrenia.

Cognitive impairment in schizophrenia is not satisfactorily improved by the currently available medications. To develop precognitive therapies for schizophrenia, it is important to identify the neural circuit and molecular changes that underlie the cognitive deficits. The findings of the present study indicated that restoring the NMDARs in the ILN of adult mutant mice rescued working memory deficits, suggesting that post-maturation NMDA hypofunction leads to the cognitive dysfunction.

Acute administration of NMDAR antagonists induces schizophrenia-like states in healthy humans and mice (Javitt and Zukin, 1991, Amitai and Markou, 2010, Neill et al., 2010, van den Buuse, 2010, Coyle, 2012). A previous study, however, showed that post-adolescent deletion of NR1 on inhibitory neurons does not cause schizophrenia-related cognitive deficits in mice, although early-postnatal NMDAR deletion results in abnormalities (Belforte et al., 2010). In addition, hypofunction of NMDAR in prefrontal and cortical excitatory neurons does not contribute to positive and negative symptoms (Rompala et al., 2013). In the present study, I found that a lack of NMDARs in the ILN led to cognitive deficits, and that NR1 expression induced by viral injection into the ILN restored spatial working memory in the Y-maze. In addition, the viral restoration of NMDARs in the ILN also rescued the attenuated MK-801
induced hyperactivity in mutants. These findings indicate that the NMDAR-dependent synaptic mechanisms in the ILN in adults are required for appropriate function of processes underlying working memory and probably other cognitive functions as well.

In conclusion, I propose that NMDAR-mediated signaling in the ILN can be a therapeutic target for psychiatric diseases related to schizophrenia.
Concluding remarks

To the best of my knowledge, these data obtained using genetic approaches are the first to demonstrate that the ILN are critical for cognitive function. Deletion of NR1 in the ILN was sufficient to cause multiple schizophrenia-like cognitive impairments. Restoring NMDA signaling in the ILN rescued working memory deficits in adult mutant mice. Together, the findings of the above-described studies provide evidence supporting the notion that NMDAR-mediated signaling in the ILN is a potential therapeutic target for psychiatric disorders, including schizophrenia.

The newly established ILN-specific Cre mouse line enabled me to genetically dissect the role of the ILN in higher-order brain functions. Although the ILN are thought to be a major component of the "non-specific" arousal system (Morison and Dempsey, 1941, Steriade et al., 1993b), the findings of the present study suggest that NMDARs in ILN cells have specific roles in cognitive function in mice. Based on their anatomic features, the ILN can be considered as an interface between the brainstem arousal system and the cortico-striatal system. The ILN receive monoaminergic and cholinergic inputs from the pons and midbrain (Jones et al., 1985, Steriade et al., 1991, Krout et al., 2002, Van der Werf et al., 2002), which may convey information about arousal and reward signals (Kinomura et al., 1996, Hong and Hikosaka, 2014). They also have mutual connections with the prefrontal cortex and striatum (Berendse and Groenewegen, 1991, Vercelli et al., 2003), which is critical for behavioral control. In this study, ILN-NR1-cKO mice showed impairments in 5-CSRTT, which assesses sustained attention and impulse control. These
findings suggest that NMDAR signaling in the ILN process information regarding the level of arousal and the reward value of response options, which are coded by the ILN in human and monkey (Kinomura et al., 1996, Minamimoto et al., 2005).

By characterizing the behavioral abnormalities of the ILN-NR1-cKO mice, I demonstrated a causal relationship between hypofunction of NMDARs in the ILN and cognitive impairments related to psychiatric disorders, such as schizophrenia. The loss of NMDAR in the ILN led to cognitive deficits as well as positive symptom-like behavior. NMDAR deletion in the ILN, however, had little effect on negative symptom-like behavior, suggesting that the neural and neurochemical mechanisms of cognitive and positive-like symptoms differ from those underlying negative-like symptoms, as demonstrated previously in human patients with schizophrenia (Callicott et al., 2000).

Accumulating evidence suggests that the pathophysiology of schizophrenia includes abnormal functional interactions between the thalamus and cortex (Marenco et al., 2012, Pratt et al., 2012, Woodward et al., 2012, Dawson et al., 2013, Kubota et al., 2013). The lack of NMDARs in the ILN reduces sensitivity to the effects of NMDAR antagonists, which induce generalized cortical synchrony in healthy human subjects (Malhotra et al., 1997, Lahti et al., 2001) and animal models (Hakami et al., 2009). NMDAR deletion in cortical inhibitory neurons in mice (Belforte et al., 2010, Carlen et al., 2012), but not excitatory neurons (Rompala et al., 2013), also diminished MK-801-induced hyperlocomotion, supporting the idea that NMDARs in cortical inhibitory neurons have an important role in network oscillatory activity (Saalmann, 2014). To my knowledge, the present study is the first molecular genetic approach to demonstrate the relationship between NMDARs in the ILN and MK-801-induced hyperactivity.
Although further studies are needed to characterize their physiologic abnormalities using EEG or multi-tetrode electrophysiologic recordings, the present study complements findings of previous studies suggesting that the reduced network activities caused by NMDAR hypofunction are associated with schizophrenia-like behaviors (Belforte et al., 2010, Carlen et al., 2012, Suh et al., 2013).

The behavioral and pharmacologic abnormalities of the ILN-NR1-cKO mice partially support the hypothesis that the ILN participate in the control of states of consciousness and awareness by regulating cortical oscillation (Van der Werf et al., 2002, Saalmann, 2014). Such an ILN-mediated oscillation mechanism may give rise to the large-scale integration of information across widespread cortical circuits (Saalmann, 2014) that supports the unity of cognitive experience in the awake and rapid-eye-movement (REM) sleep or dream states (Llinas and Ribary, 1993, Braun et al., 1997, Llinas et al., 2002). This hypothesis is further supported by the finding that neuronal death in the ILN is most typically observed in disorders of consciousness (Schiff, 2010). On the other hand, deep brain stimulation of the central thalamus, including the ILN, promotes behavioral recovery from severe traumatic brain injury in patients (Schiff et al., 2007, Shah and Schiff, 2010) and enhances cognitive performance in rodents (Shirvalkar et al., 2006). Further studies with the ILN-NR1-cKO mice are expected to elucidate the molecular and neural circuit mechanisms underlying consciousness and awareness, and also demonstrate the "loss of inner unity" of consciousness ("orchestra without a conductor") (Sass and Parnas, 2003) as a core feature of psychiatric disorders.
Acknowledgements

I would like to express my devout gratitude to my supervisor, Dr. Masayoshi Kuwahara, Professor of Laboratory of Veterinary Pathophysiology and Animal Health, Department of Animal Resource Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, for his outstanding support and warm encouragement.

I would like to express my sincere appreciation to Dr. Shigeyoshi Itohara, Senior Team Leader of Laboratory for Behavioral Genetics, RIKEN Brain Science Institute, for his constructive comments and hearty encouragement, and for providing me the opportunity to study in such a splendid environment.

I am grateful to Dr. Yu Hayashi, Junior Principal Investigator of International Institute for Integrative Sleep Medicine, University of Tsukuba, for his invaluable technical support and patient guidance throughout the project as a collaborator. I greatly appreciate the generosity and cooperation of Dr. Arthur Huang (Laboratory for Circuit and Behavioral Physiology, RIKEN Brain Science Institute) and Dr. Nao Nakagawa (Laboratory for Local Neuronal Circuits, RIKEN Brain Science Institute) for their generous collaboration. My grateful thanks are also extended to other members of the Laboratory of Behavioral Genetics for benign support and valuable discussions.

Finally, I wish to thank my parents for their heartfelt support throughout my study.
References


Amitai N, Markou A (2010) Disruption of performance in the five-choice serial reaction time task induced by administration of N-methyl-D-aspartate receptor antagonists: relevance to cognitive dysfunction in schizophrenia. Biological Psychiatry 68:5-16.


Burk JA, Mair RG (2001) Effects of intralaminar thalamic lesions on sensory attention and motor intention in the rat: a comparison with lesions involving frontal cortex and...


Kemether EM, Buchsbaum MS, Byne W, Hazlett EA, Haznedar M, Brickman AM, Platholi J,


Lindsley DB, Bowden JW, Magoun HW (1949) Effect upon the EEG of acute injury to the brain stem activating system. Electroencephalography and Clinical Neurophysiology 1:475-486.


Marenco S, Stein JL, Savostyanova AA, Sambataro F, Tan HY, Goldman AL, Verchinski BA,


thalamic nuclei. Journal of Comparative Neurology 511:678-691.
Parker JG, Beutler LR, Palmiter RD (2011) The contribution of NMDA receptor signaling in the
corticobasal ganglia reward network to appetitive Pavlovian learning. Journal of
Neuroscience 31:11362-11369.
Powell CM, Miyakawa T (2006) Schizophrenia-relevant behavioral testing in rodent models: a
uniquely human disorder? Biological Psychiatry 59:1198-1207.
Pratt J, Winchester C, Dawson N, Morris B (2012) Advancing schizophrenia drug discovery:
optimizing rodent models to bridge the translational gap. Nature Reviews Drug discovery
11:560-579.
nicotinic acetylcholine receptor activation ameliorates scopolamine-induced behavioural
changes in a modified continuous Y-maze task in mice. European Journal of
Robbins TW (2002) The 5-choice serial reaction time task: behavioural pharmacology and
recombinant adeno-associated virus type-2 using quantitative real-time PCR. Journal of
receptor hypofunction in prefrontal and cortical excitatory neurons to schizophrenia-like
Saalmann YB (2014) Intralaminar and medial thalamic influence on cortical synchrony,
viruses: normal integration does not require viral gene expression. Journal of Virology
63:3822-3828.
Sano Y, Ornthanalai VG, Yamada K, Homma C, Suzuki H, Suzuki T, Murphy NP, Itohara S
(2009) X11-like protein deficiency is associated with impaired conflict resolution in mice.
Journal of Neuroscience 29:5884-5896.
29:427-444.


Steriade M, Amzica F, Nunez A (1993a) Cholinergic and noradrenergic modulation of the slow (approximately 0.3 Hz) oscillation in neocortical cells. Journal of Neurophysiology 70:1385-1400.


Young JW, Geyer MA, Rissling AJ, Sharp RF, Eyler LT, Asgaard GL, Light GA (2013) Reverse translation of the rodent 5C-CPT reveals that the impaired attention of people with schizophrenia is similar to scopolamine-induced deficits in mice. Translational Psychiatry 3:e324.