SPECIFIC AIMS:

The administration of glutamatergic NMDA receptor (NMDAR) antagonists to human subjects elicits core symptoms of schizophrenia, implicating hypofunction of NMDARs in the disease process. However, identifying sensitive periods and potential cell-types of NMDAR hypofunction has proven elusive. The first strong evidence that NMDAR hypofunction occurs in cortical GABAergic interneurons during postnatal development was provided by our transgenic mice that lacked an indispensable subunit, NR1(Grin1), of NMDARs in cortical and hippocampal parvalbumin (PV)-positive interneurons (Belforte et al, 2010). The mutant mice displayed several schizophrenic-like phenotypes, including deficits of prepulse inhibition (PPI) and spatial working memory (cognitive-like symptoms), exacerbated amphetamine-induced dopamine release in ventral striatum (positive-like symptoms), and impaired evoked-gamma band local field potential (LFP) oscillation in the cortex. Interestingly, NR1 deletion in the same GABA neurons failed to produce such phenotypes when the deletion was introduced after adolescence, suggesting existence of a 'sensitive period' for NMDAR hypofunction. Thus, it appears that the dysregulation of GABAergic neurons is not solely attributable to NR1 deletion. Rather, the NR1 deletion may impair the postnatal maturation of GABAergic neurons and, in the absence of proper GABAergic inhibition, the refinement of cortical circuitry may be impeded. However, a more rigorous measure of spatial (cell-types) and temporal windows of NMDAR hypofunction remains to be determined. Further, the cellular and neuronal events downstream from NMDAR hypofunction during the sensitive period responsible for the elaboration of schizophrenia pathophysiology need to be delineated.

Our *long-term goal* is to identify the critical mechanism(s) that regulate postnatal maturation of corticolimbic interneuron circuitry and to specify how abnormalities in these systems lead to mental illnesses. The *objective of this application*, which is the next step in pursuit of that goal, is to determine the cell-types (e.g., excitatory neurons or parvalbumin (PV)-positive interneurons) in which NMDAR hypofunction is critical for the emergence of schizophrenia-like phenotypes in mice. Furthermore, we plan to determine the downstream cellular and network events that are critical for this phenomenon. The *central hypothesis* is that NMDAR hypofunction in PV neurons alters their presynaptic GABA release property, thereby inducing deficits in coordinated inhibition of postsynaptic principal neurons. This may in turn result in impaired synchronized firing of the cortical networks, impaired gamma oscillations, and subsequent subcortical dopamine dysregulation. Therefore, it is postulated that certain cellular mechanisms following NMDAR hypofunction in PV neurons prior to maturation is crucial for the later development of schizophrenia. The *rationale* for the proposed research is that identifying cellular events that follow NMDAR hypofunction in specific cell-types during the sensitive period will deepen our understanding of the schizophrenia disease process and help understand why symptoms arise mainly after adolescence. This information might play an important role in developing new therapeutic targets and define the requisite time of intervention.

We plan to test our central hypothesis and accomplish the objective of this application by pursuing the following two *specific aims*:

Aim 1: Define the cell-types and sensitive period for NMDAR hypofunction critical for the manifestation of schizophrenia-like phenotypes

Our working hypothesis is that NMDAR hypofunction in corticolimbic PV neurons during the sensitive period is responsible for the majority of schizophrenia-like phenotypes manifest in the adult. Therefore, we postulate that a NR1 knockout selectively in cortical excitatory neurons (in G35-3-Cre/NR1 KO mutant mice) would not show robust schizophrenia-like phenotypes (Study #1.1). Furthermore, we hypothesize that NR1 deletion specifically in PV neurons by postnatal 4th week (in PV-Cre/NR1 KO mutant mice) will also trigger robust schizophrenia-like phenotypes (Study #1.2).

Aim 2: Determine the cellular events that follow as a consequence of NMDAR hypofunction during the sensitive period

Our *Preliminary Results* suggest that uncoordinated firing of cortical pyramidal neurons is a key event in the development of schizophrenia-like phenotypes (in Ppp1r2-Cre/NR1 KO mutant nice). We *hypothesize* that this event could be caused by an impaired evoked GABA release at presynaptic terminals of NMDAR-deleted interneurons during the sensitive period.

This project is *innovative*, because it employs unique transgenic animal models to provide new knowledge about the NMDAR hypofunction theory of schizophrenia pathophysiology. It is our *expectation* that the findings derived from this work will yield new insights into the cortical GABAergic interneuron-related pathogenesis of schizophrenia. This in turn may lead to the development of new treatments targeted to particular cellular pathways for human psychiatric illnesses.

RESEARCH STRATEGY:

A. SIGNIFICANCE

The pathophysiology of schizophrenia has long remained a mystery, but a range of underlying structural and functional abnormalities have been suggested to underlie the disease process. One leading theory, NMDAR hypofunction (Coyle, 1996; Javitt and Zukin, 1991), has gained considerable support. The impetus for this hypothesis is driven by the reaction in healthy subjects to NMDAR antagonists, including phencyclidine, ketamine and MK-801, that resembles many schizophrenia symptoms. These reagents reproduce both positive and negative symptoms of the disease, as well as cognitive deficits, including memory and attention deficits. Genetic studies have provided further evidence supporting this theory. For example, a NR1 (Grin1) hypomorph mouse, in which NR1 expression is reduced to 5-10% of control levels, displays deficits in social interaction and impairment in prepulse inhibition (PPI) of auditory startle reflex (Gainetdinov et al., 2001). However, a fundamental question remains: Is NMDAR hypofunction at *specific* cell-types crucial for these phenotypes to emerge? Converging evidence has suggested that cortical GABAergic interneurons are a prime target for NMDAR hypofunction (Olney and Farber, 1995). For example, despite the abundant expression of NMDARs in excitatory neurons acute systemic administration of NMDAR antagonists results in hyperactivity of cortical pyramidal neurons (Jackson et al., 2004), and spillover of cortical glutamate (Moghaddam et al., 1997). These findings suggest that fast-spiking interneurons are preferentially affected by NMDAR hypofunction and that their subsequent impaired functioning disinhibits cortical excitatory neurons. Our initial study explicitly tested this idea and demonstrated that a postnatal deletion of NMDARs specifically in corticolimbic interneurons confers several behavioral and pathophysiological features in mice that resemble human schizophrenia (Belforte et al., 2010). However, the boundary conditions (e.g., sensitive period or responsible cell-types) for NMDAR hypofunction are still not fully understood. Our contribution in this application will derive from the assessment of the time and cell-types in which NMDAR hypofunction occurs in order to induce the emergence of schizophrenia-like phenotypes and delineate the subsequent cellular and network events that occur in those cell-types. *This contribution is significant because it is the first step in a continuum of research that is expected to*; (1) clarify the cellular events following NMDAR hypofunction elicited in the sensitive period; (2) determine the crucial cellular events that contribute to latent development of the disease; (3) identify the novel roles played by NMDAR function during cortical maturation; and (4) pave the way for the discovery of potential drug targets related to maturation of cortical circuitry, which could significantly impact the treatment of schizophrenia and related mental illnesses.

B. INNOVATION

Until recently, schizophrenia NMDAR hypofunction research was conducted by pharmacological intervention using NMDAR antagonists or global (conventional) NMDAR KO mutant mice. However, due to drug off-target effects it is extremely difficult to delineate the downstream pathways of NMDAR hypofunction by relying solely on this experimental approach. Similarly, while conventional NMDAR genetic manipulations in mice avoid these off-target effects, they preclude a direct investigation into the involvement of specific cell-types and developmental time periods due to the global nature of the mutation. To overcome these limitations, a cell-type specific transgenic approach is required. *Innovation in this application is evident in the concept of the research, the approaches to be used, and even the research material itself.* The concept of a sensitive period for NMDAR hypofunction is itself unique since it was clearly formulated based upon the findings of our recent study (Belforte et al., 2010). Our experimental approach is highly innovative in several ways. Cell-type restricted KO approach will allow us to identify the cell-types responsible for the manifestation of schizophrenia-like phenotypes. The combinatory use of particular Cre lines and the floxed lines may also allow us to control the onset of the knockout (see Aim #1/Study #1.2). We also use a variety of advanced methods and multidisciplinary experimental approaches in this project. For example, we employ a mouse conditional transgenic approach, mouse basic behavioral battery tests and neuropsychiatry disorder-related behavioral tests, slice patch-clamp recordings in interneurons from *in vitro* slices, *in vivo* tetrode recordings for units and local field potentials (LFPs). Since no biomarker or biological measure is available to diagnose schizophrenia, it is essential to employ a combinatory use of these techniques to identify the mouse phenotypes that mimic human endophenotypes and to delineate the mechanisms involved. Finally, we are uniquely positioned to conduct this innovative research as few other research groups have transgenic models that are confined to GABAergic neurons during that crucial sensitive period. Our preliminary studies strongly suggest that our approach will be effective in identifying the molecular, cellular and network events during the sensitive period of NMDAR hypofunction, which may open a window of new therapeutic potential.

C. APPROACH

In this application, we propose two related and overlapping specific aims are directed at understanding: (1) the spatial and temporal boundary of NMDAR hypofunction-mediating pathophysiology; and (2) the critical cellular and network events that follow NMDAR hypofunction. The Aim #1 is relevant because NMDAR hypofunction at, for example, cortical excitatory neurons, besides interneurons may contribute to the subsequent emergence of schizophrenia-like phenotypes. Focusing specifically on interneurons in Aim#2 is justified by our previous study showing that schizophrenia-like phenotypes emerge following the induction of NMDAR hypofunction in corticolimbic interneurons (Belforte et al., 2010).

Aim 1: Define the cell-types and sensitive period for NMDAR hypofunction critical for the manifestation of schizophrenia-like phenotypes

Introduction

Our initial study (Belforte et al., 2010) demonstrated that a postnatal deletion of NR1 in a subset (40-50%) of cortical and hippocampal interneurons, a majority of which were parvalbumin (PV)-positive, was sufficient to trigger several behavioral and pathophysiological features in mice that resemble human schizophrenia. Therefore, it provided strong experimental support for the long-standing hypothesis (Olney and Farber, 1995) that NMDAR hypofunction in cortical interneurons is a primary site of schizophrenia pathogenesis (Nakazawa et al., 2011). Notably, we did not observe schizophrenia-like behaviors when NR1 ablation was introduced in the same GABAergic neurons after adolescence, suggesting that there is a sensitive period of NMDAR hypofunction and the NR1 deletion impairs the postnatal maturation of GABAergic neurons. This idea of abnormal maturation of cortical circuits is consistent with the neurodevelopmental hypothesis of schizophrenia (Weinberger, 1987). These results also provided the concept of a 'sensitive or critical period' of NMDAR hypofunction. To narrow the boundary conditions of the sensitive period of NMDAR hypofunction in interneurons, we will focus on two key questions. The first question (**Study #1**) is whether NMDAR hypofunction in the cortical and hippocampal *excitatory* neurons also contributes to the emergence of schizophrenia. Many genes encoding the NMDAR complex proteins, such as neuregulins, are expressed in both excitatory and inhibitory neurons in the cortex. If the mutation was introduced in these genes, NMDAR hypofunction and subsequent schizophrenia-like phenotypes could occur in excitatory neurons as well. We will assess the impact of cortical excitatory neuron NMDAR hypofunction on the disease process. The second question to be addressed (**Study #2**) is precisely when during the postnatal period is NMDAR hypofunction necessary to induce neurodevelopmental disturbances and the subsequent emergence of schizophrenia-like phenotypes after adolescence. Firing properties of PV-positive fast-spiking interneurons mature rapidly by postnatal day 25 (P25) in primary somatosensory (S1) cortex (Goldberg et al., 2011; Okaty et al., 2009). It is plausible that the impaired maturation of PV neurons by P28, but not simply NMDAR deletion from PV neurons, is crucial for development of schizophrenia-like phenotypes. To test this hypothesis, we will analyze a novel mutant mouse line in which NR1 is deleted at around P28 from cortical PV neurons, to examine whether the mutant displays robust schizophrenia-like phenotypes. If this is the case, we could estimate that the sensitive period for PV neuron NMDAR hypofunction would be closed by P28.

Study #1.1: *Evaluate the consequence of corticolimbic excitatory neuron-restricted NR1 knockout:*

Justification and Feasibility

To introduce the NR1 deletion into excitatory neurons for behavioral and physiological analysis, cell-type specific transgenic approach is essential, because global or conventional NR1 knockout mice are perinatally lethal (Li et al., 1994). In the past, at least 3 different types of excitatory cells' NR1 KO mutants have been generated. The first is the αCaMKII-Cre/NR1 KO mutants (Tsien et al., 1996), in which NR1 was deleted from postnatal 3-4 weeks in principal neurons in the entire forebrain, including the striatum and the thalamus. These mice displayed hyperlocomotion and spatial memory deficits. However, the excessive hyperlocomotion precluded an in depth analysis of the psychiatric-like behavioral phenotypes. The second line was the Emx1-Cre/NR1 KO mutants (Iwasato et al., 2000), in which NR1 was deleted from embryonic day 11 (E11) in excitatory neurons in the cortex and hippocampus. These mice showed impairments in the whisker-barrel formation in S1 cortex. However, the mutants' body size was much smaller (~70 %) at P7 and later, which also precluded a behavioral analysis. The third line, which we previously published, was the so-called G35-3 Cre/NR1 KO mutants (Sawtell et al., 2003), in which NR1 deletion is confined to excitatory neurons in the cortex and hippocampus during the postnatal period. The mutants demonstrated impaired ocular dominance plasticity in V1 during adulthood. Behavioral analyses of this line have not yet been published. However, this line appears to be the most appropriate for behavioral tests, because these mutants are viable, fertile, normal in size and do not display any gross physical or anatomical abnormalities until adulthood. To explore whether NMDAR

hypofunction in cortical excitatory neurons triggers schizophrenia-like phenotypes later in adulthood, we analyzed the G35-3 Cre/NR1 KO mutants. **Figure 1** shows the pattern of Cre-recombination in the G35-3 Cre

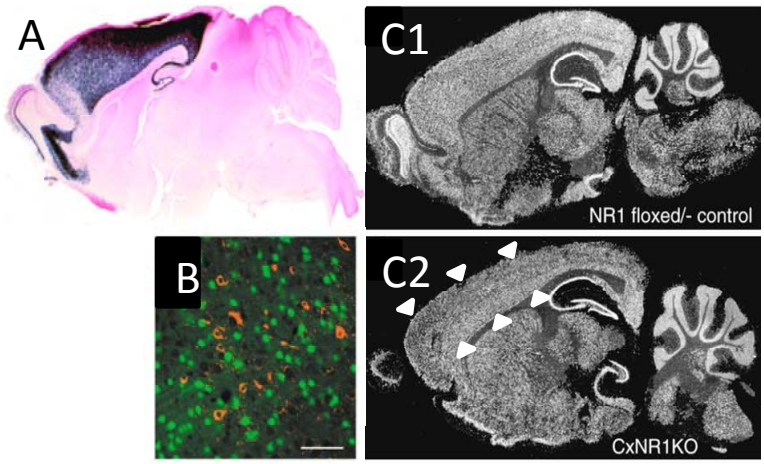

Figure 1. G35-3Cre/fNR1 KO mutant. (**A**) Cre recombination pattern at P17 in G35-3 Cre /R262RLacZ mice, visualized by X-Gal staining. (**B**) Double immunofluorescent staining with antibodies against β-galactosidase (green) and GAD-67 (red), showing no cre recombination in GABAergic neurons. Scale bar, 200 μm. (**C**) NR1 mRNA signals are reduced selectively in the cortex (shown by arrowheads). Dark-field images of *in situ* hybridization performed on parasagittal sections from fNR1 controls (C1) and its mutant littermate (C2) using a ^{33}P-labeled NR1 cRNA probe.

line at P17, which is exclusively confined to excitatory neurons at the cellular level, and NR1 deletion from the cortex in G35-3 Cre/fNR1 KO mutants at 8-week-old. Furthermore, preliminary data suggested that onset of the Cre recombination in the G35-3 Cre/R26RLacZ line is at late embryonic stage to postnatal Day 0, and the regional pattern of the recombination was confined to cortical and hippocampal excitatory neurons throughout life. Thus, we decided to use the animals older than 11 week-old for behavioral study. To assess the behavior

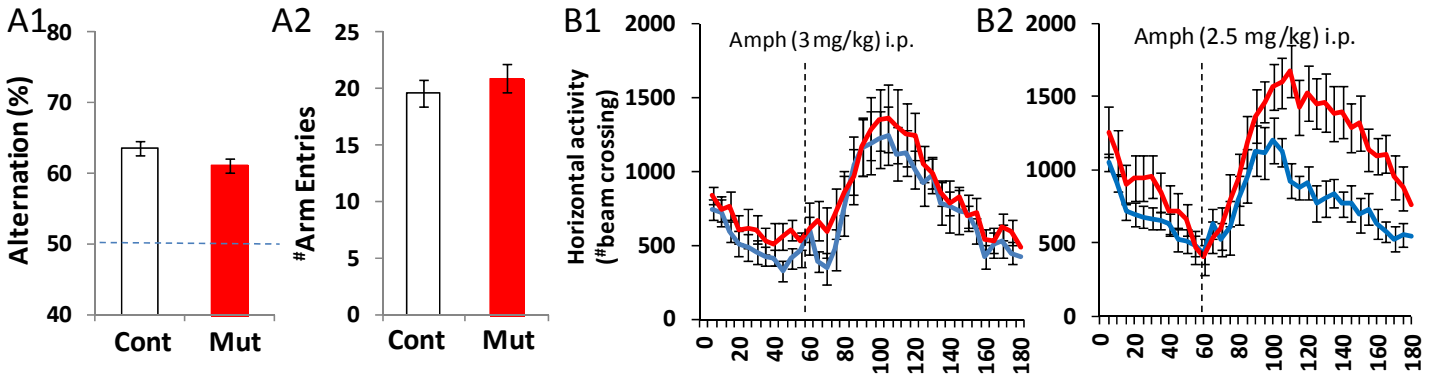

Figure 2. G35-3 Cre/NR1 KO mutants were normal in spatial working memory in Y-maze alternation task and d-amphetamine (Amph)-induced hyperlocomotion test. (**A**) Both genotypes showed higher alternation index than chance level (50%, A1) with equal number of arm entry (A2). (**B**) No difference was observed in Amph-induced locomotion change (Red, mutant; Blue, fNR1 control (B1) although Ppp1r2-Cre/NR1 KO mutants (Red) were supersensitive to Amph (B2).

of the mutants, we subjected the mutants and floxed-control littermates to a battery of tests. Pilot data showed that the mutants are normal in novelty-induced locomotor activity, and anxiety level on elevated-plus maze (data not shown). Furthermore, there was no difference in spatial working memory in the Y-maze alternation task and amphetamine-induced hyperlocomotion test (**Fig. 2**). These preliminary results were in stark contrast to the interneuron knockout mutants of NMDARs. For example, Ppp1r2 (protein phosphatase 1, regulatory subunit 2)-Cre/NR1 KO mutants, in which NR1 ablation is introduced during the early postnatal period selectively into cortical and hippocampal GABAergic neurons, a majority of which were PV-containing, exhibited severe impairments in novelty-induced locomotor activity, anxiety, spatial working memory, and amphetamine-induced hyperlocomotion (Belforte et al., 2010), (**Fig. 2**). These preliminary data suggested that schizophrenia-like phenotypes are not prominent in the G35-3 Cre/NR1 KO mutants, compared to the interneuron knockout mutants, thereby emphasizing the importance of NMDAR hypofunction in the cortical interneurons. We will continue to characterize the involvement of excitatory neurons' NMDARs in the neuropsychiatric disorder-like phenotypes.

Research Design

1. *Histological verification of knockout onset and regional expression pattern*. We will first examine by non-radioactive double *in situ* hybridization whether NR1 mRNA ablation takes place in the cortical neurons by the 1st postnatal week and is completed in cortical deep layers by the 10th week. We use an anti-sense cRNA

probe for αCaMKII or Emx1 as an excitatory neuron marker. Disruption of the barrel structure in Emx1-Cre/NR1 KO mutants at P7 is a signature of functional NMDAR deletion, which can be visualized by anti-5HTT immunostaining (Datwani et al., 2002). Therefore, we will also determine whether NR1 is already eliminated by P7 by assessing the integrity of barrel cortex using anti-5HTT immunocytochemistry.

2. *Basic behavioral battery tests and psychiatric disorder-like behavioral tests.* We will subject the mutants and control littermates to basic behavioral battery tests (home-cage locomotion, exploration and motor coordination), schizophrenia-related cognitive tasks (fear conditioning, Y-maze spontaneous alternation test for working memory, PPI of acoustic startle reflex, (Belforte et al., 2010), attention set-shifting test, negative symptom-related tasks (sociability test, saccharine preference test, female urine sniffing test, home cage-running wheel test (Malkesman et al., 2011), and mood disorder-like tests (forced swim test, learned helplessness test, elevated plus maze (Malkesman et al., 2011).

3. *Schizophrenia pathophysiology-related assessment*. Ppp1r2-Cre/NR1 KO mutants did not elicit MK-801-induced hyperlocomotion perhaps because the brains are already sensitized by NMDAR hypofunction (Belforte et al., 2010). We will administer MK-801 to determine whether G35-3Cre/NR1 KO line is also non-responsive to NMDAR antagonists. Exacerbation of amphetamine-induced hyperlocomotion is a reliable measure mimicking human positive symptoms of the disease (Lipska, 2004), and Ppp1r2-Cre/NR1 mutants showed this exacerbation. We will, therefore, subject the mutants and their littermate controls to amphetamine-induced hyperlocomotion task.

4. *Synchronized activity and LFP gamma oscillations*. Impaired synchronized unit firing among cortical principal neurons in S1 cortex and a deficit in auditory tone-evoked LFP gamma oscillations are excellent markers for schizophrenia pathophysiology (see Aim 2). We will conduct *in vivo* tetrode recording from the cortices of G35-3Cre/NR1 KO mutants and their littermate controls to assess these two measures.

Expected Outcomes

Preliminary behavioral tests using G35-3Cre/NR1 KO mutants showed normal behavior in a spatial working memory task and sociability task, suggesting the mutants do not show robust schizophrenia-related behavior. However, considering extinction learning deficit was induced by AP5 infusion to the mPFC (Hsu and Packard, 2008), we expect to see some cognitive deficits, such as in long-term memory and attention.

Potential Problems & Alternative Strategies

Here we focus on the cortex and hippocampus as a putative site of NMDAR hypofunction, and use the G35-3 Cre line in which Cre-recombination pattern is confined to cortical and hippocampal excitatory neurons, as opposed to the Ppp1r2-Cre line, in which Cre-recombination pattern is confined to cortical and hippocampal inhibitory neurons. However, we do not exclude the possibility that NMDAR hypofunction outside the cortex and hippocampus also contributes to the schizophrenia-like phenotypes. Yet, this will be beyond the scope of this application.

Study #1.2: *Evaluate the consequence of PV neuron-specific NR1 knockout:*

Justification and Feasibility

Regarding the onset of the NMDAR hypofunction in the interneurons, we have evaluated the consequence of NR1 deletion starting from the mid-embryonic stage using a Dlx6a-Cre line (Monory et al., 2006), which was crossed to a floxed-NR1 line. However, very few Cre/homozygously floxed-NR1 mutants survived to the postnatal period, suggesting that the sensitive period of NMDAR hypofunction for schizophrenia begins after birth (unpublished data). On the other hand, it is unclear at which postnatal stage NMDAR hypofunction is requisite to elicit schizophrenia-like phenotypes. The interneuron cell-types responsible for NMDAR hypofunction also remains to be identified, because only ~70% of Cre-targeted interneurons are PV-positive neurons in our Ppp1r2-Cre/fNR1 KO mutants. Focusing on PV neurons, two recent reports (Korotkova et al., 2010)(Carlen et al., 2011) have addressed the cell-type issue. These groups recently generated and characterized PV-Cre/fNR1 mutant mice, in which NMDARs are deleted selectively from entire PV neuron populations in the brain. The first mutants (Korotkova et al., 2010), in which lack of NMDA currents were confirmed in two thirds of PV neurons during the postnatal week 7 to 8, displayed spatial working and reference memory deficits, but no other schizophrenia-related phenotypes were reported. The second mutants (Carlen et al., 2011), in which Cre-mediated LacZ expression was detected in a majority of PV neurons by P29 (Carlen et al., 2011; Hippenmeyer et al., 2005), exhibits several behavioral deficits, including habituation, working memory and fear conditioning. However, Carlen et al. did not observe other, more characteristic schizophrenia-like phenotypes, such as PPI deficits, and concluded that this mutant may manifest only cognitive-like deficits. While this report did not mention the onset of NMDAR deletion, it is suspected that NMDAR deletion in PV neurons takes place much later than P29, because they used the floxed-NR1 line (so-called 'fNR1 line-B' in this application), in which Cre recombination efficiency is considerably low. Indeed, we previously differentiated the

onset of NR1 gene ablation at the postnatal 2nd week versus after adolescence (Belforte et al., 2010) by crossing the same Ppp1r2-Cre line to two distinct *loxP*-flanked or "floxed" NR1 (fNR1) lines; one was a fNR1 line-A (obtained from Dr. Yuqing Li)(Dang et al., 2006) that elicits knockout from P10-14, and another was a fNR1-line-B (obtained from Dr. Susumu Tonegawa)(Tsien et al., 1996) that causes knockout after 8-week-old. It is known that the distance between two loxPs (*e.g.,* 2.3 kb in fNR1 line-A vs over 12 kb in fNR1 line-B) has a huge impact on the Cre recombination efficiency (Wang et al., 2009; Zheng et al., 2000). Therefore, when fNR1-line-B was bred to the PV-Cre line by Carlen et al (2011), it is conceivable that onset of NR1 deletion was delayed into much later postnatal stage, by which the sensitive period ended. In this application we hypothesize that a novel PV-Cre/fNR1-line-A KO mutant, in which NR1 deletion is expected to be accomplished in a majority of cortical PV neurons by P28, would display the disease phenotypes. This study may enable us to confirm that PV neuron is a site of NMDAR hypofunction and further estimate the sensitive period of NMDAR hypofunction up to P28. We will also characterize the onset of NR1 deletion in PV-Cre/fNR1-line-B mutants (Carlen et al., 2011) to identify the closing time of the sensitive period. Preliminary data indicated that the PV-Cre/fNR1-line-A KO mutant is viable, fertile, normal in size and does not display any gross physical or anatomical abnormalities until adulthood. To visualize the PV-neurons, the mutant or PV-Cre mice will be further bred to a floxed-YFP reporter line (Srinivas et al., 2001).

Research Design

1. *Characterize the onset of NR1 mRNA deletion*. We will examine the time course of NR1 mRNA ablation in cortical and other area PV neurons (e.g., P14, P21, P28 and P56) by non-radioactive *in situ* hybridization. We will next examine the basic firing properties of PV neurons in cortical slices to assess if the passive membrane properties, excitatory and inhibitory synaptic inputs and sEPSC/sIPSC, and firing properties are altered by NR1 deletion at P28.
2. *Basic behavioral battery tests and psychiatric disorder-like behavioral tests.* We will subject the mutants and control littermates to basic behavioral battery tests, schizophrenia-related cognitive tasks, negative symptom-related tasks, and mood disorder-like tests, as described in ***Study #1.1***.
3. *Schizophrenia pathophysiology-related assessment*. We will administer MK-801 to examine whether these mutants are also less responsive to a NMDAR antagonist. We will also employ the amphetamine-induced hyperlocomotion to assess the positive symptom-like sign.
4. *Synchronized activity and LFP gamma oscillations*. We will conduct *in vivo* tetrode recording from the cortices to assess these two measures.

Expected Outcomes

It is our expectation that NMDA receptor ablation in PV-interneurons, if completed by P28, may develop similar but more robust neuropsychiatric phenotypes to those observed in our Ppp1r2-Cre/NR1 KO mutants. These potential findings have the possibility of strengthening the developmental PV-interneuron origin of NMDAR hypofunction for schizophrenia pathogenesis.

Potential Problems & Alternative Strategies

In case we do not observe the robust phenotypes predicted even if NR1 deletion was almost completed in PV neurons by P28, it is plausible that PV-*negative* neurons may play a major role in the emergence of NMDAR hypofunction-induced psychiatric phenotypes in the adulthood. In this case, we would explore the cell-type specific NR1 knockout in other interneuron cell-types, such as Somatostatin (SS) neurons, as described in *Future Directions.*

Aim 2: Determine the cellular events that follow as a consequence of NMDAR hypofunction during the sensitive period

Introduction

Correlated activity of cortical principal neurons (Salinas and Sejnowski, 2001) and concomitant LFP synchronization, in particular, at the gamma frequency range (30-100 Hz)(Fries, 2009) play a fundamental role in normal cortical network functioning. It is widely accepted that inhibition from PV-positive basket cells projecting to perisomatic areas of excitatory neurons is essential for synchronization of neural activity (Bartos et al., 2007);(Mann and Paulsen, 2007). However, the exact mechanisms for synchronous oscillations involving PV neurons are still unclear. Two principal mechanisms have theoretically been proposed to underlie gamma oscillations: (1) feedback loops from principal neurons onto PV neurons; and/or (2) oscillations in mutually connected PV neuronal networks, via chemical and/or electrical transmissions. The literature thus far suggests that the former circuitry is more dominant, because the gamma power is reduced when excitatory drive is selectively reduced onto PV neurons (Fuchs et al., 2007) but not when synaptic inhibition is ablated in PV neurons (Wulff et al., 2009). Regardless of the generation mechanisms, involvement of PV-positive basket cells for the generation of synchronous gamma oscillations appears to be critical due to their unique characteristics.

Accumulating evidence implicates disturbances in gamma frequency neuronal synchrony as a major pathophysiological feature of schizophrenia (Uhlhaas and Singer, 2010). Consistent with the early sensory impairments in schizophrenia, several studies have recently demonstrated reductions in the amplitude and phase locking of evoked gamma oscillations during the sensory processing or following application of transcranial magnetic stimulation. However, a major question that still exists in this field is whether impairments in the gamma oscillations are causally linked to the disease pathophysiology or represent an epiphenomenon. To answer the question, it is imperative to delineate the underlying mechanisms of synchronous oscillation impairment in schizophrenia.

Justification and Feasibility

The synchronized firing of cortical principal neurons is largely diminished in our mouse model, Ppp1r2-Cre/fNR1-lineA, in which NMDAR hypofunction is induced in cortical GABAergic interneurons in early postnatal development. (Belforte et al., 2010). These mutants also display defective tone-evoked cortical gamma oscillations after adolescence (**Fig. 3**). Interestingly, the synchronized firing of cortical principal neurons was normal when NMDARs were ablated from interneurons during adulthood in the Ppp1r2-Cre/fNR1-line-B mutants (Belforte et al., 2010). Thus, the loss of NMDARs in PV neurons may not cause a functional disruption directly but rather impair the postnatal maturation of the mechanisms underlying synchronized oscillations. How does

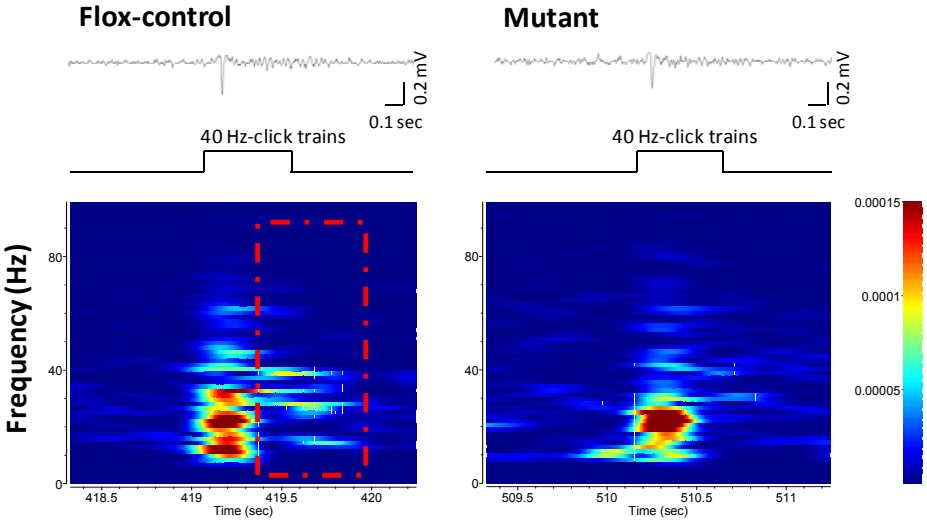

Figure 3. Ppp1r2-Cre/fNR1-line-A mutants, in which NR1 was deleted from P10-14, were impaired in auditory tone-evoked gamma oscillations. Power spectrum showed that 40 Hz-click tones elicits tone-evoked LFP responses in A1 cortex under head-restrained awake condition, which were followed by the persistent evoked oscillatory activity in the controls (red enclosure), but not in the mutants.

postnatal NMDAR ablation in the FS interneurons lead to the impaired synchronized activity of principal neurons and furthermore induce LFP gamma oscillation deficits? Unfortunately, it is a technical challenge to record in vivo synchronized activity of principal neurons or LFP gamma oscillation during the maturation process of PV neurons in awake mice. To overcome this limitation, we generated another mutant line (Ppp1r2-Cre/ErbB4 KO mutant) with equivalent deficits in PV signaling, but with normal synchronized firing of postsynaptic principal neurons in the S1 cortex. The reason why we chose ErbB4 was: (1) a receptor of EGF family proteins named neuregulins, is selectively expressed in the PV-positive interneurons in the cortex in the postnatal rodent brains and appears to facilitate GABA release (Fazzari et al., 2010); and (2) Variations of the genes that encode the Neuregulin 1 (NRG1)–ErbB4 receptor complex are reported to be associated with schizophrenia illness (Banerjee et al., 2010). Indeed, we found an equivalent reduction of mini-IPSC frequency from mPFC principal neurons of Ppp1r2-Cre/ErbB4 KO mutants compared to that of postnatal NR1 KO mutants (**Fig. 4E**), suggesting an impairment in presynaptic GABA release. Consistent with this reduced inhibition, multi-electrode unit recording from awake mutants also showed robust increase in the mean firing of S1 cortex principal neurons (**Fig. 4F**), suggesting cortical disinhibition. However, we unexpectedly observed fairly normal behavior of the mutants: anxiety, locomotion, spatial working memory, PPI, and mating/nest building were all normal (**Fig. 4A-D**). In addition, we found that synchronized activity was normal in the ErbB4 mutants as measured by cross-correlation analysis (**Fig. 4G & H**), which is in sharp contrast to the data showing an impaired synchronized activity in the NR1-line-A mutants. Taking into account the correlations between the behavioral data and the synchronous firing data among Ppp1r2-Cre/NR1-line-A KO mutant, Ppp1r2-Cre/NR1-line-B KO mutant, and Ppp1r2-Cre/ErbB4 KO mutant, it is plausible that impaired cortical synchronized activity may be requisite for schizophrenia-like pathophysiology. Therefore, we predict that in the NR1-lineA mutants, but not in ErbB4 or NR1-lineB mutants, behavioral deficits result from impaired synchronized cortical activity rather than from impaired GABAergic inhibition per se. Why is the synchronized firing of postsynaptic excitatory neurons diminished selectively in NR1-line-A KO mutants? It is unlikely that any intrinsic properties of excitatory neurons are defective because the Ppp1r2-Cre-mediated genetic manipulation is confined to interneurons. Thus, we

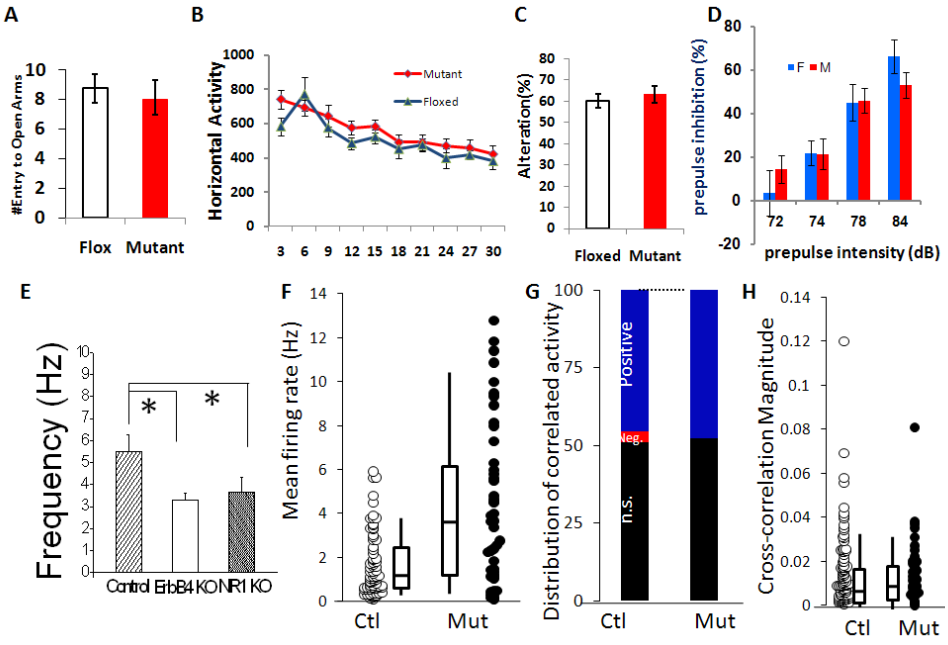

Figure 4. *Ppp1r2-Cre*/ErbB4 KO mutants display normal behaviors and neuronal synchrony despite impaired GABAergic inhibition. There were no differences in anxiety-like behavior (**A**), open field activity (**B**), Spontaneous Y-maze alteration (**C**), and prepulse inhibition (**D**) between genotypes. Ablation of ErbB4 from interneurons was confirmed by a decreased GABA release, as measured by mini-IPSCs from mPFC principal neurons (**E**). Correspondingly, the *in vivo* mean firing rates of S1 cortical principal neurons were robustly increased (**F**). Nonetheless, the synchronized activity of these S1 principal neurons appeared to be normal, as measured by cross-correlations of the pairs of neurons from the same tetrodes (**G** and **H**).

postulated that the synchrony of GABAergic inputs to excitatory neurons is defective in the NR1-line-A mutants. We assessed this prediction by double patch recording of sIPSCs from nearby pyramidal cells in tissue slices. If the synchronous GABAergic inputs were disturbed, the IPSC events between two nearby pyramidal neurons would be desynchronized. Results of a pilot study suggest this is the case in mPFC slices of the NR1-line-A mutants at P28-P35 (**Fig. 5**), implicating that the precision of spike transmission, by which single interneuron spikes precisely translate into postsynaptic response, is disturbed in the mutants. Then, what actual cellular mechanisms are impaired to explain these mutant phenotypes?

One plausible explanation may be that action potential impulses arriving at the presynaptic terminals fail to properly evoke GABA release. This may be caused by altered expression of proteins whose functions are responsible for the evoked GABA release, such as K^+ and Ca^{2+} channels. A second possibility is that electrical couplings, which are thought to contribute to spike synchronization among PV neurons (Tamas et al., 2000) may be diminished in the mutants. However, synchrony may not necessarily depend on gap junctions, rather on synaptic connections (Hu et al., 2011). Thus, it is less clear how impairments in the electrical couplings would impact on the mutant phenotypes. A third possibility is that impaired fast-spiking property diminishes synchronized oscillations (Cardin et al., 2009). However, our preliminary results suggest that the mutant fasts-spiking property at P28-P35 is fairly normal (Zsiros et al., unpublished). Therefore, we hypothesize that this is caused by an impaired evoked GABA release at presynaptic terminals of NMDAR-deleted interneurons. For this purpose, it would be beneficial to compare the three mutants, NR1-line-A KO mutant, NR1-line-B KO mutant, and ErbB4 KO mutant.

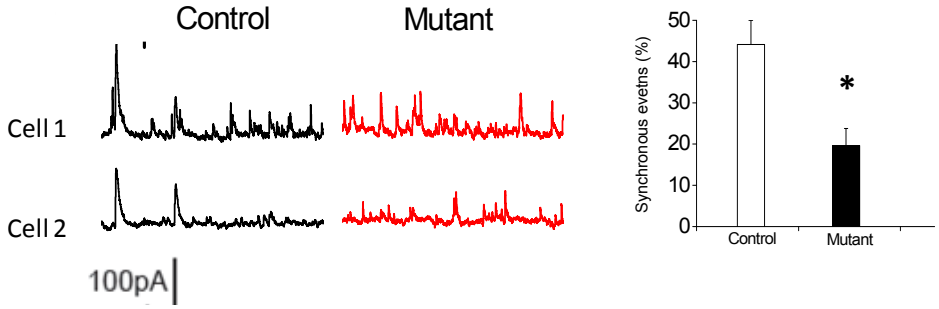

Figure 5. Synchronous GABAergic inputs to pyramidal neurons were significantly lower in Ppp1r2-Cre/fNR1 postnatal mutants, compared to fNR1 control mice. Synchrony of spontaneous IPSC events was measured from pairs of pyramidal neurons in mPFC at P28-P40. The recorded cells were 25-30 μm apart.

The *objective* of this aim is to identify the elements downstream of NMDAR hypofunction that impair the synchronized activity in the cortical excitatory neurons and induce the gamma oscillation deficits. To achieve this goal, we will test our *working hypothesis* that NR ablation in the PV-positive neurons results in an impaired evoked GABA release to postsynaptic excitatory neurons, which results in impaired synchronized firings between these neurons. *The experimental approach* to be employed includes electrophysiological techniques (slice physiology and in vivo physiology) and genomic approaches (microarray/qPCR) to compare Ppp1r2-

Cre/NR1-line-A KO mutants, Ppp1r2-Cre/NR1-line-B KO mutants, and Ppp1r2-Cre/ErbB4 mutants. It is our expectation that that we will be able to identify the molecular targets responsible for the impaired coordinated spiking of cortical pyramidal neurons by pinpointing the mechanisms by which PV neuron maturation is impaired by NMDAR deletion.

Research Design

1. *Auditory tone-evoked gamma oscillations in vivo.* We will first examine whether auditory tone-evoked gamma oscillation is defective in Ppp1r2-Cre/ErbB4 KO mutants and adult NR1 KO mutants (Ppp1r2-Cre/fNR1-line-B KO). Toward this end, we will chronically implant a nichrome wire array to the primary auditory (A1) cortex and after a 10 day recovery period we will apply 500-ms click trains of tones at 40-Hz to elicit auditory-evoked potentials followed by emergent gamma oscillations (Franowicz and Barth, 1995)(**Fig. 3**). If there is no impairment in both mutants, this could strengthen the idea that synchronized firing of the principal neurons is correlated with the LFP synchronous oscillations because both are impaired in postnatal NR1 KO mutants, while both would be normal in either ErbB4 mutants or adult NR1 KO mutants.

2. *Synchronous GABAergic inputs to excitatory neurons.* We will continue the experiments showing impairments in synchronous sIPSC events from nearby pyramidal neurons in mPFC of NR1 mutants at P28, (**Fig. 5**). In addition, we will test whether the same measure is normal in ErbB4 KO mutants at P28. If this is normal, it is likely that the synchronous GABAergic input to pyramidal neurons contributes to their synchronous firing *in vivo*. Therefore, the measure of double electrode-sIPSC recording may possibly be used as a neuronal marker for the impaired synchronous firing, which is highly challenging to measure during the postnatal period in mice.

3. *Assess the maturation of NR1-deleted PV neurons.* We will explore the possibility of impaired fast-spiking properties in fluorescent labeled PV-positive, NR1-deleted interneurons. The fluorescent-labeling will be attained by further crossing postnatal Ppp1r2-Cre/NR1-line A homozygous KO mutants or Ppp1r2-Cre/ErbB4 KO mutants to a floxed R26R-YFP line. Through current clamp recording, we will assess the action potential firing patterns and other electrophysiological properties in the mPFC and/or S1 cortex slices. Since ~30% of Cre-targeted neurons in the Ppp1r2-Cre line are PV-negative, *post hoc* immunostaining or single-cell PCR will be conducted to determine the cell shapes and whether the biocytin-loaded cells are PV-positive. We will collect action potentials using 1-s long pulses of current injection with low-chloride internal solution. To classify the neurons, we will measure electrophysiological parameters, including resting membrane potential, input resistance, capacitance, subthreshold oscillations, spike threshold, spike frequency adaptation, and firing frequency distribution.

4, *Determine the mechanisms of impaired synchronous GABAergic inputs to excitatory neurons in postnatal NR1 mutants.* How does NMDA deletion in PV neurons impair their precise spike transmission to excitatory neurons? It is plausible that the impaired maturation of PV neurons, but not simply NMDAR deletion from PV neurons, is crucial for this phenotype. As mentioned above, we will evaluate three major possibilities: (1) a reduction of fidelity of evoked GABA release at the presynaptic terminals of NR1–deleted PV neurons; (2) a deficit in interneuronal electrical coupling among PV neurons, which may lead to unsynchronized firing of PV neurons; and (3) an impaired fast-spiking property diminishing spike synchronization among PV neurons. To address possibility #1, we will measure the sIPSC events from postsynaptic pyramidal neurons upon repetitive stimulation of NR1-deleted PV neurons from a patch pipette. To address possibility #2, we will conduct simultaneous double whole-cell patch clamp recordings to measure electrical coupling to assess the potential deficits in the mutant mPFC slices at 3-4 weeks of age. To address possibility #3, we will evaluate the electrophysiological parameters of the fast-spiking neurons at P14, P28, and P42.

5. *Cell-type specific microarray.* To identify the molecular targets of impaired synchronous GABAergic inputs to excitatory neurons in postnatal NR1 mutants, we will employ GABAergic neuron-selective qPCR and/or a gene-expression microarray to identify candidate genes whose expression is dysregulated during the maturation process without NMDARs. This genomics approach may also provide us a hint as the identity of other potential impairments in NR1-deleted interneurons. To selectively enrich the Cre-targeted neurons (labeled by fluorescence), we will treat fresh cortex slices with protease for ~45 min. After trituration the fluorescent cells will be collected and analyzed by microarray and/or qPCR (Sugino et al., 2006). Since PV-positive interneurons are known to be dynamically altered in postnatal development (Okaty et al., 2009), the timing of harvesting the cells will be critical. This part of the project will be conducted in collaboration with Dr. Abdel Elkahloun at NHGRI/NIH. Upon identification of candidate molecular targets, we will first focus on the molecular changes, which may explain the cellular phenotypes determined by slice physiology. For example, if the evoked GABA release deficit would be a major cause of the impaired coordinated firing, we will focus on the genomic changes that may account for the evoked GABA release deficit. Furthermore, we will try to rescue the evoked GABA release deficit in the mutant slices if the pharmacological approach is feasible, such as using agonist/antagonist.

Otherwise, identification of causal relations between the candidate molecular changes and the cellular and behavioral phenotypes will await the generation of new transgenic mutants in the next round of grant funding.

Expected Outcomes

It is our expectation that NR1-deletion in PV neurons during early postnatal development will lead to impaired precise spike transmission from PV neurons to pyramidal neurons. This may result in impaired synchronized firing of cortical pyramidal neurons, thereby leading to the synchronous oscillation deficits. Being able to delineate the actual cellular phenotypes will be beneficial in the search for the target molecular changes responsible for the cellular change. This could significantly impact on the discovery of potential drug targets.

Potential Problems & Alternative Strategies

One of the final goals of this project is to examine whether the molecular targets are responsible for the impaired the coordinated firing. As mentioned before, this evaluation could depend on which molecules are identified. Although one potential method would be to rescue the phenotypes by pharmacological manipulation if the specific agonists/antagonists exist, it may not always be feasible. Alternatively, we could generate the interneuron-specific KO mutant or transgenic mice of the gene encoding the particular molecule. For example, if the coupling via interneuronal gap junctions was reduced upon NR1 deletion in PV neurons, we would then test whether gap junction proteins are implicated in the impaired synchronized firing or impaired gamma oscillations. In this case, we would generate Ppp1r2-Cre/connexin 36(Cx36) KO mutants, because Cx36 is a major interneuronal gap junction protein (Ma et al., 2011). However, this sort of genetic approach would require additional time and resources, so we propose this type of approach in *Future Directions*.

D. Timeline

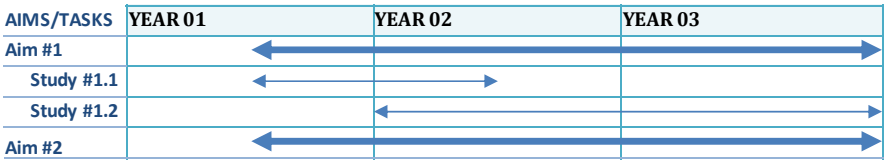

Table. All the studies in this application require double transgenic lines (the cross of Cre line and floxed-line) or triple transgenic lines (the cross of Cre line, floxed-line, and floxed-YFP line). Therefore, when we move to a new institution, we will need to consider extra-time of expanding the mouse colony to re-start the behavioral and physiology experiments.

E. Future Directions

At the conclusion of this project, we expect to understand; (1) the degree to which NMDAR hypofunction at cortical excitatory neurons contributes to the development of schizophrenia-like phenotypes; (2) the precise time window of the NMDAR hypofunction-sensitive period in PV neurons, and (3) the cellular mechanisms that are associated with the impaired synchronized activity of the cortical principal neurons following NMDAR hypofunction in cortical interneurons. There are at least two future goals that follow from this application; first, to further delineate the cell-type-specific roles of NMDARs in the psychiatric disorder-like phenotypes and second to clarify whether the molecular targets in Aim #2 are directly responsible for triggering schizophrenia-like phenotypes following adolescence. To further delineate the cell-type specific roles of NMDAR hypofunction, we would investigate, for instance, somatostatin (SS)-positive GABAergic neurons which are another major class of cortical interneurons. Interestingly, the deficits in GAD67 expression in schizophrenia are not only restricted to PV neurons but also included in SS cell-type (Lewis et al., 2005). The ratio of NMDAR components relative to non-NMDARs is also much higher at SS cell-synapses than that at PV cell synapses in rat S1 cortex (Lu et al., 2007), and SS neurons also directly inhibit the PV-expressing FS neurons (Gibson et al., 1999). It will be interesting to analyze the NMDAR KO mutants selectively in SS neurons, in case PV-Cre/NR1 KO mutants do not display robust schizophrenia-like phenotypes in Aim#1/Study#1.2.

To clarify whether the identified downstream molecular targets are truly responsible for triggering the schizophrenia-like phenotypes, as mentioned previously, we will monitor synchronous IPSC events between nearby pyramidal neurons in slices to predict whether the synchronized activity is impaired in vivo. However, it is imperative to also introduce such mutation or deletion in animals to determine the causal relations between molecular deficits and functional deficits, such as in vivo synchronized firing and gamma oscillations, and behavior in mice. Therefore, first, we will generate cell-type specific overexpression mice or knockout mice to explore if the dysregulation of the candidate gene itself is sufficient to induce the phenotypes observed in Ppp1r2-NR1 KO mutants. e will use a floxed-line of the candidate gene for the knockout. If the candidate gene is upregulated, we will generate Cre-dependent over-expression transgenic line(s) using a loxP-STOP-loxP cassette for cell-type-specific over-expression. We will subsequently set-up genetic rescue experiments by crossing the floxed line with the Ppp1r2-Cre/NR1 KO mutant background. In this manner the functions of the identified genes will be normalized in the NR1 KO mutants, allowing us to assess whether the genetic rescue in PV neurons restores the synchronized activity, gamma oscillations, and, furthermore, cognition. The detailed plan of this future experiment would depend on which cellular and molecular changes are identified.