Regulation of Biochemical and Electrical Signaling in Mouse Prefrontal Cortex by Clozapine, a Therapeutic Agent for Schizophrenia

by

Baishali Kanjilal

A dissertation submitted to the Graduate
faculty in Biochemistry in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

The City University of New York

2008

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_____ _____

Date Chairperson of Examining Committee
Dr. Probal Banerjee

_____ _____

Date Executive Officer
Dr. Lesley Davenport

Dr. Andrzej Wieraszko, The College of Staten Island, CUNY

Dr. Abdeslem El Idrissi, The College of Staten Island, CUNY

Dr. Eitan Friedman, City College, CUNY

Dr. Yu-Wen Hwang, New York State Institute for Basic Research in Developmental Disabilities

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ABSTRACT

Regulation of Biochemical and Electrical Signaling in Mouse Prefrontal Cortex by Clozapine, a Therapeutic Agent for Schizophrenia

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Baishali Kanjilal

Advisor: Professor Probal Banerjee

Atypical antipsychotic-induced cortical dopamine release, which is believed to improve the negative and cognitive symptoms of Schizophrenia, is reportedly mediated by $5HT_{2A}/D_2$ receptor blockade and activation of $5HT_{1A}$ receptor ($5\text{-}HT_{1A}$-R) in rat prefrontal cortex (PFC). In the present study we have investigated the involvement of the $5\text{-}HT_{1A}$ receptor in clozapine-evoked regulation of electrical signals in PFC slices. Clozapine (15 μM) showed a dramatic increase in population spike in PFC slices isolated from postnatal (Day-20-30) mouse brain. This increase in neuronal activity was eliminated in the presence of the $5\text{-}HT_{1A}$ receptor antagonist WAY100635, the phospholipase C (PLC) inhibitor U73122, and a membrane permeable Ca^{2+}/CaM Kinase IIα (CaMKIIα) inhibitor. In

contrast, the MAP kinase kinase (MEK) inhibitor PD98059 failed to block the effect. Clozapine therefore functions as a 5-HT_{1A}-receptor agonist, and PLC and CaMKIIα are also involved in the clozapine-evoked signaling that results in an increase in electrical activity in the PFC. As revealed by Western blot analysis for CaMKIIα phosphorylation at T^{286}, 15 min of clozapine treatment of PFC slices evokes maximal activation of CaMKIIα and this induction is completely blocked by WAY100635. Thus, our observations from electrical as well as biochemical analyses suggest that clozapine elicits induced electrical activity in the PFC through a 5-HT_{1A}-R-mediated signaling pathway that involves CaMKIIα. The involvement of the 5-HT_{1A}-R was further corroborated by a dramatic increase in electrical activity and CaMKIIα activation following treatment with the 5-HT_{1A}-R agonist 8-OH-DPAT. Clozapine also showed a significant increase in population spike in 5-HT_{1A}-R(-/-) mice. However, a CaMKII inhibitor failed to block the effect of clozapine in knockout mice, indicating the involvement of other receptors that do not require CaMKII activation. The clozapine-induced electrical activity in the PFC also involved the NMDA receptor because it was completely eliminated upon pretreatment of the slices with the NMDA antagonist APV. Collectively, our findings indicate a synergism between the NMDA receptor and 5-HT_{1A}-R-mediated signaling via

CaMKIIα, which is responsible for PFC activity elicited by the antipsychotic agent clozapine.

I dedicate this thesis to my loving family:

To my parents for all your unconditional support with my studies.

To my husband Ricky and my son Aarush, for believing in me and helping me further my studies.

Without all of you I am not complete.

ACKNOWLEDGEMENTS

This work could not be completed without the efforts of hardworking and dedicated people. I would like to express deep gratitude to all the members of the Chemistry and Biology department of the College of Staten Island and Biochemistry doctoral program at the Graduate School of the City University of New York for all the moral and financial support. Special thanks and appreciation goes to the ever so patient and encouraging mentor of this project Dr. Probal Banerjee for his scientific wisdom, efforts in helping direct the project, and the endless advise both personal and academic. I would like to extend a heartfelt gratitude to the members of the supervisory committee. Also the members of Dr. Banerjee's lab: A special thanks to Sudarshana and Farah for your insights and guidance with the project. Sincere thanks to Zhaglool for passing his wisdom and knowledge on. I would also like to thank my dearest friends and lab partners Shawn, Kelly, Buddhi, Phyllis and last but not least Priya and Amit for all your support and the shoulders provided to lean on – you all are true friends indeed.

Finally, I would like to thank my immediate family members, especially my grandparents who were very supportive, understanding and

patient in helping finish my thesis. A special thank you to my husband and my son Aarush for all your help, support and patience during my long days in lab and the time spent writing my thesis. Last but not least, thank you to my mother for coming to my rescue in my final days of graduate school, you made it easy for me to get to the finish line.

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ABBREVIATIONS

AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APD	Atypical antipsychotic drug
APV	(±-2-Amino-5-phosphonovaleric acid)
BSA	bovine serum albumin
Ca^{2+}	Calcium
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CLZ/CLOZ	Clozapine
DA	Dopamine
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tertraline
EPS	Extrapyramidal symptom
EPSP	Excitatory postsynaptic potential
ERK 1/2	Extracellular signal regulated kinase 1/2 , MAPK
FBS	Fetal bovine serum
GABA	γ-aminobutyric acid
5-HT	Serotonin
HRP	Horse-reddish peroxydase
IgG	Immunoglobulin G
LTP	Long term potentiation
MAPK	Mitogen activated protein kinase
NMDA	N-methyl-D-aspartic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline

PKC	Protein kinase C
PLCβ	Phospholipase Cβ
PMSF	Phenylmethylsulfonyl fluoride
PCP	phencyclidine
PFC	Prefrontal cortex
RIPA	Radio immuno precipitation assay
SDS	Sodium dodecyl sulfate
VTA	Ventral tegmental area

Chapter 1

Introduction and Background:

Schizophrenia:

Schizophrenia is a highly complex and heterogeneous disorder involving perceptual, behavioral and cognitive abnormalities. The disease which is equally prevalent in men and women affects approximately 1% of the population worldwide (Lang 2007). In addition to the 1% with schizophrenia, another 2% to 3% of the general population suffer from *schizotypal personality disorder*, a condition considered to be a milder form of the disease (Kandel 2000). The first psychotic episodes of schizophrenia is very often preceded by *predormal signs* which include social isolation and withdrawal, impairment in fulfilling expected roles, odd behavior and ideas, neglect of personal hygiene and blunted affect. This period is then followed by one or more psychotic episodes that may include loss of reality testing, memory disturbances, delusions and hallucination. These episodes are sometimes separated by long non-psychotic period in which the patient is not overly psychotic, socially isolated and shows a flattened affect (a low level of emotional arousal) (Kandel 2000). All these symptoms are categorized into three categories:

Positive symptoms, which include delusions, prominent hallucinations, usually auditory hallucinations, disordered thoughts, loss of normal association between ideas; *Negative symptoms***,** which is usually a long term outcome of positive symptoms, mainly include alogia, loss of emotional expression (Flattening of affect), social withdrawal; *Cognitive-attentional symptoms***,** such as impairment of sensory-gating, working and verbal memory (Millan 2000). The symptoms of the non-psychotic period are called *negative symptoms* because they reflect the absence of certain social and interpersonal behavior. In contrast the abnormalities of psychotic episodes are called *positive symptoms* because they reflect the presence of distinctively abnormal behaviors (Kandel 2000).

The area of the brain affected in schizophrenia:

The major areas of the brain implicated in the schizophrenia are the fore brain, hindbrain and the limbic system. The popular belief is that schizophrenia may be caused by disruption of different functional circuits in the brain, rather than a single abnormality in one part of the brain. Although the brain areas involved are not well-defined, the frontal lobe, temporal lobe, limbic system (specifically the cingulate gyrus, amygdala, hippocampus and the thalamus) are believed to be involved. Functional neuroimaging studies with schizophrenia subjects have shown dysfunction in the areas of the brain

critical for decision-making, which are prefrontal cortex, anterior cingulate and parietal cortex. fMRI studies have shown that both chronic and first-episode never-treated schizophrenia patients showed deficits in working memory performance associated with an increased or decreased activation of dorsolateral prefrontal cortex. The dorsolateral prefrontal cortex dysfunction in schizophrenia subject during a working memory task has been associated with disorganization symptoms (Paulus 2003). Postmortem studies have also revealed enlargement of the lateral ventricle, reduced size of temporal lobe structures, decreased thalamic volume and enlarged basal ganglia among schizophrenia patients (Harrison 1999). A number of functional studies in both humans and animals reveal physiological abnormalities of limbic-cortical structures in schizophrenia. Neuro-anatomical studies have revealed decrease in the density of hippocampal pyramidal cells. Reduced gray matter volume as well as abnormal cytoarchitecture has also been reported within parahippocampal gyrus. There are many interrelationships between limbic-cortical structures within medial temporal lobe, cingulated gyrus and frontal lobe with regard to both anatomy and function, and anatomical abnormalities in any one of these regions are likely to affect other functional regions of the brain. Therefore it is unlikely that abnormalities in any one brain structure would be sufficient to explain the symptoms of schizophrenia patients.

Rather, the abnormalities in the neuronal circuits within different functional regions of the brain may attribute to the pathophysiology of schizophrenia (Csermansky 1998).

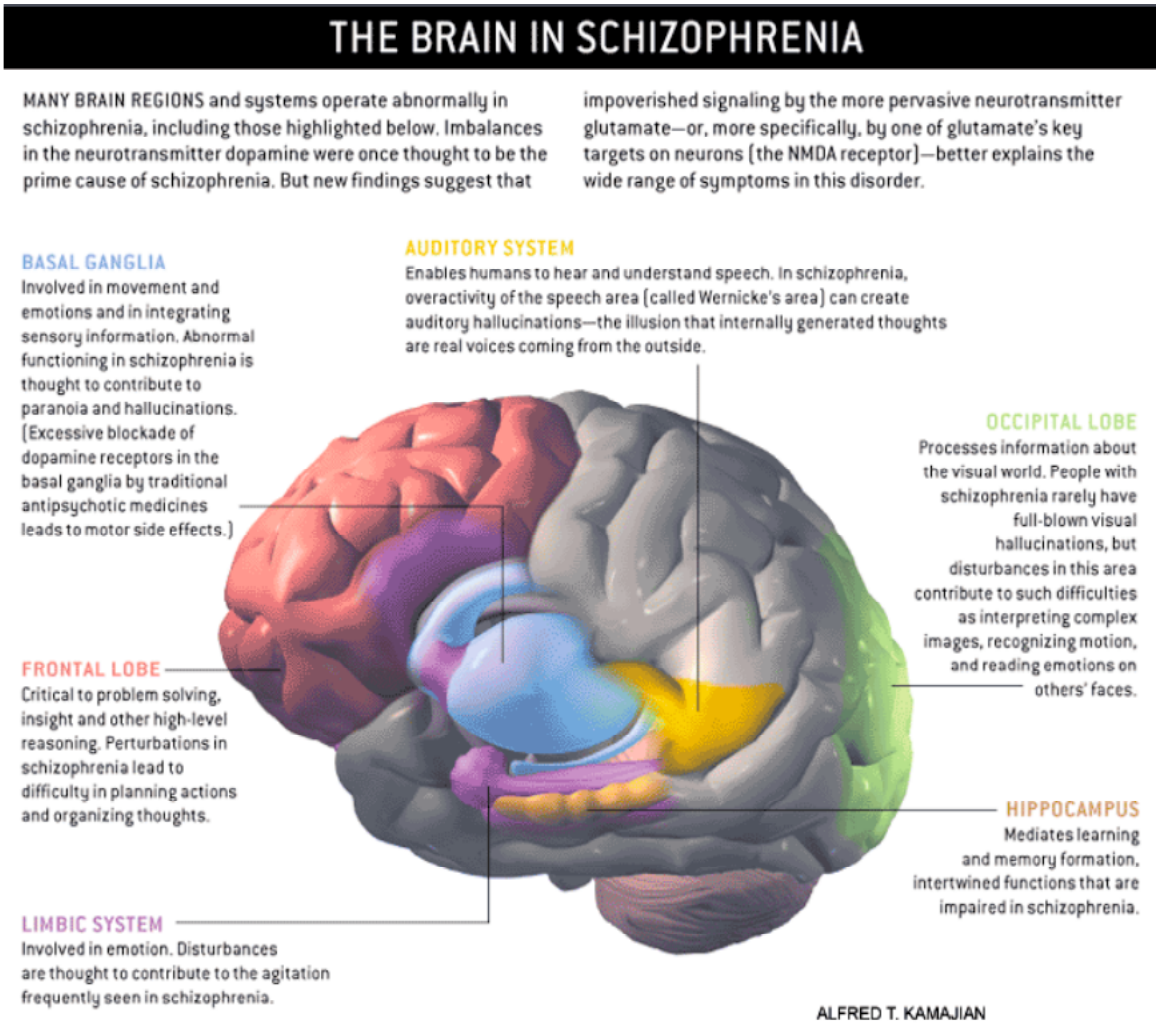


Figure 1: The regions of the brain affected in schizophrenia. Frontal lobe, basal ganglia, limbic system, hippocampus, occipital lobe, auditory system are the major regions of the brain affected in schizophrenia.

Ref: http://www.schizophrenia.com/images/schizophrenia_brain_large.gif

Antipsychotic drug:

Treatment with antipsychotic (or neuroleptic) drugs currently represents the most common therapy for schizophrenia. Introduction of the first antipsychotic drug chlorpromazine in the 1950s shifted the focus of schizophrenia pathophysiology to the dopamine D_2 receptor. Chlorpromazine was both an effective treatment for schizophrenia and a dopamine receptor antagonist. Since then a large number of typical or conventional antipsychotic drugs (e.g. haloperidol, thiothixene, loxapine) have been developed for the treatment of schizophrenia (Roth 2003). Dopamine receptors, particularly the D_2 –like receptor family are known to be involved in the pathophysiology of psychotic disorder and more directly in the pharmacological basis of the beneficial effects of neuroleptic-antipsychotic drugs (Millan 2000). Although the typical antipsychotic drugs are quite effective at reducing positive symptoms in schizophrenia in many patients they are not without serious side effects. One major limitation in the use of conventional antipsychotic drug is that their prolonged administration induces acute and chronic movement disorders (e.g. drug-induced Parkinsonism, acute and tardive dystonia, acute and chronic akathisia, tardive dyskinesia), elevation of serum prolactin levels (hyperprolactinemia) and neuroleptic malignant syndrome (Roth 2003). All these symptoms that

normally include involuntary movements, tremors, rigidity, body restlessness, muscle contraction and change in breathing and heart rate are collectively named as *extra pyramidal symptoms* (EPS) (Millan 2000).

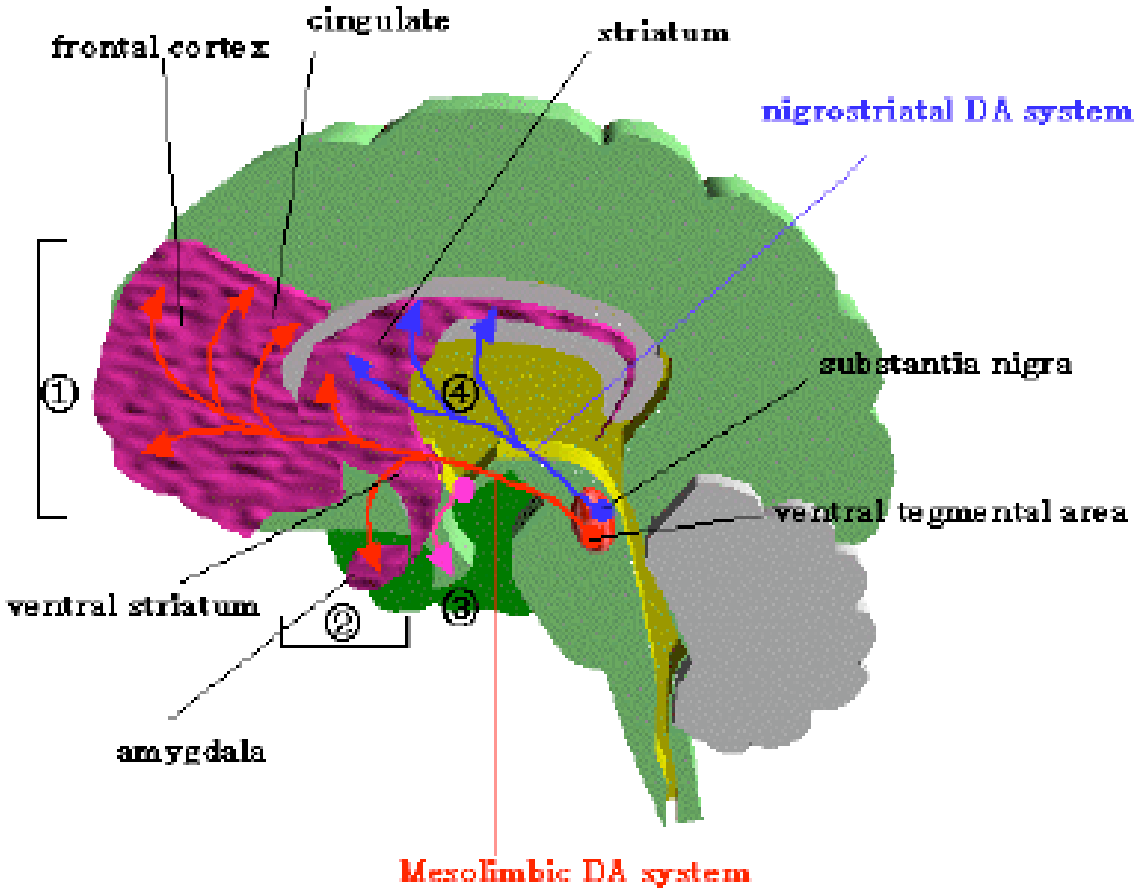


Figure 2: Dopaminergic system. The antipsychotic drugs work on (1) mesocortical DA system and (2) mesolimbic DA system, and shows antipsychotic and sedative actions. Extrapyramidal effects are caused by the inhibition of D2 receptor of (4) the nigrostriatal system. Secretory inhibition such as prolactin (Hyperprolactinemia) is caused by DA block in the hypothalamus and hypophysis system (3).

Ref: http://park12.wakwak.com/~pharma1/textbook/Antipsychotic/Antipsychotic-e.html

The introduction of the antipsychotic agent Clozapine in 1958 was a major step in developing drugs with less risk of neurological side effects (Roth 2003). Clozapine is not only superior to typical antipsychotic drugs in treating both positive and negative symptoms but also in not inducing the extra pyramidal side effects commonly caused by the conventional antipsychotic drugs. Because of all these properties clozapine is termed as *"atypical"* and represents the prototype drug of this class (Duncan 1999). Since then a number of atypical antipsychotic agents have been developed (Risperidone, Olanzapine ,Ziprasidone etc) and most of them have proved to be useful in the treatment of schizophrenia.

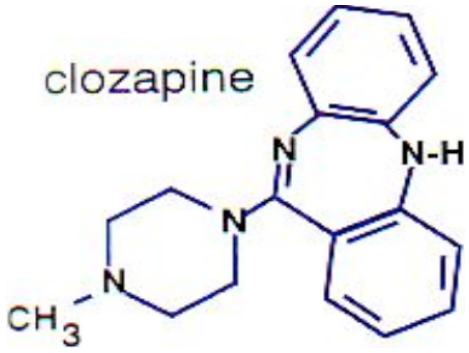
Pharmacological basis of the function of atypical APD Clozapine:

It is not clear how such atypical antipsychotic drugs work to decrease the extra pyramidal side effects i.e. the pharmacological basis of their clinical properties. Clozapine, an antipsychotic drug commercially available as clozaril is a dibenzodiazepine derivative. It is particularly effective in a subpopulation of patients not responsive to typical neuroleptics and exerts antipsychotic activity in the virtual absence of any extra-pyramidal motor syndrome. In addition, clozapine might be effective in ameliorating negative symptoms of schizophrenia, which are generally resistant to neuroleptic treatment (Josselyn 1997). Clozapine is not, however a panacea because it is

associated with serious hemotoxicity in a small group of patients, while its blockade of muscarinic and histaminergic receptors provokes significant cardiovascular-autonomic side-effects (Millan 2000).

Table 1

Structure of clozapine



Pharmacokinetics

Time of peak plasma level: 1-4 hours
Half life: 9-30 hours
Optimal plasma level: 200 - 350 ng/mL
Volume of Distribution: Vary with patients
Usual daily dose (mg): 300
Daily dose range (mg): 300-900

(Ref:http://www.chemsoc.org/ExemplarChem/entries/2004/Nottingham_chong/Bpharmacology.htm)

Table 2 **In-vitro binding profile of clozapine:**

Receptor	Clozapine K_i(nM)	Receptor	Clozapine K_i(nM)
D_2	83.0	5-HT_{2A}	7.8
D_4	1.6	5-HT_{1A}	120.0
Alpha1	5.6	H_1	23.0

(Spouse 1999).

The significance of the antagonist property of Clozapine at mesolimbic D_2 receptors in the control of positive symptoms cannot be ruled out. A clinical dose of clozapine is known to result in lower D_2 receptor occupancy than haloperidol (Millan 2000). However, such observations linking only one receptor fail to provide a satisfactory explanation for the very unique clinical

profile of clozapine, which cannot just be mediated by a single receptor. The atypical property of clozapine is not limited to its clinical profile but also extends to its pharmacological profile. Clozapine interacts with higher potency at serotonergic (5-HT_{2A}, 5-HT_{2C} and others), acetylcholinergic (muscarinic), adrenergic (α_1, α_2, β_2) and histamine receptors. In contrast, it has only moderate affinity for both D_1 and D_2 receptor [Table 2](Tarazi 2001). Clozapine also displays relatively greater affinity for D_4 than other dopamine receptors (Schoots 1995; Tarazi 2001). However, repeated treatment with clozapine did not result in any increase in D_4 mRNA expression or the receptor protein in rat cerebral cortex, although haloperidol elicited a significant increase in D_4 mRNA levels in both brain areas (Moghaddam 1990; Schoots 1995). The atypical antipsychotic drugs (APDs) clozapine and olanzapine at high doses, but not typical APDS like haloperidol, are known to produce greater increase in dopamine release in rat prefrontal cortex compared to the nuclear accumbens or striatum (Kuroki 1999; Ichikawa 2000; Ichikawa 2001). The increased dopamine release in the medial prefrontal cortex has been hypothesized to contribute to the ability of atypical APDs, to improve the negative symptoms and some domains of cognition in schizophrenia (Bantick 2001; Ichikawa 2001). Although atypical APDs have been shown to have higher affinity for 5-HT_{2A}

than D_2 receptors *in vitro* and *in vivo*, often they have strong affinity for other monoamine receptors (Ichikawa 2001).

Involvement of the serotonin 1A receptor in the action of atypical antipsychotics:

In patients with schizophrenia, the majority of post-mortem studies have reported an increase in 5-HT_{1A} receptor densities in the prefrontal cortex in the approximate range of 15%-80% (Bantick 2004).The mechanism underlying this increase in 5-HT_{1A} receptor density remains uncertain but, because 5-HT_{2A} sites are decreased in parallel it is unlikely to reflect up-regulation due to deficient serotonergic transmission. Furthermore, levels of mRNA encoding 5-HT_{1A} receptors are not modified implying that post-transcriptional modifications are not involved (Millan 2000). Alterations in levels of 5-HT_{1A} receptors in schizophrenia is not the only evidence, functional activation of the 5-HT_{1A} receptor and partial agonist properties of clozapine at the 5-HT_{1A} receptor also indicate the implication of the receptor in the pathogenesis and management of schizophrenia (Millan 2000). Previous studies have revealed that treatment with 5-HT_{1A} receptor agonist causes reduction in D_2 antagonist-induced catalepsy and elicits an increase in prefrontal dopamine release (Goff 1991). In preliminary studies in patients with schizophrenia, 5-HT_{1A} partial agonists caused reduction in extra

pyramidal side effects and negative symptoms (Goff 1991; Sumiyoshi 2001; Bantick 2004) while significantly improving executive functions and verbal memory ratings (Goff 1991; Bantick 2004; Newman-Tancredi 1996). Some atypical antipsychotic agents show greater affinity to the 5-HT_{1A} receptors. Particularly, clozapine functions as a 5-HT_{1A} partial agonist with submicromolar affinity for the receptor (Goff 1991; Ichikawa 1999; Bantick 2004).

Meltzer and coworkers showed that atypical antipsychotic-induced cortical dopamine release was mediated by 5-HT_{2A} and D_2 receptor blockade which occurred as a sequel to 5-HT_{1A} receptor activation in the rat medial prefrontal cortex (Ichikawa 2001). It was hypothesized that the mechanism by which atypical APDs increase dopamine release in the medial prefrontal cortex might be due to direct 5-HT_{1A} receptor activation, 5-HT_{1A} receptor activation secondary to combined blockade of 5-HT_{2A} and D_2 receptors or both.

However, 5-HT_{1A}-R agonists could modify neither conditioned avoidance responses in rats nor apomorphine-induced climbing in mice or the “classic” models predictive of the control of positive symptoms (Cassaday 1993; Millan 2000). Although, it is very unlikely that the activation of 5-HT_{1A} receptors can improve positive symptoms, 5-HT_{1A} autoreceptors may

facilitate their control by neuroleptics (Millan 2000). The 5-HT_{1A}-R agonists have been shown to enhance the activity of mesocortical dopaminergic pathways in sequence to the actions expressed proximally to ventrotegmental dopaminergic cell bodies, which may improve the negative and cognitive symptoms of schizophrenia. The current hypothesis is that the engagement of the 5-HT_{1A} autoreceptors relieves a tonic, inhibitory influence of 5-HT_{2C} receptors exerted via excitation of inhibitory GABAergic interneurons in the VTA. In addition, GABAergic interneurons may be directly inhibited by postsynaptic 5-HT_{1A}-R.

The atypical antipsychotic drug clozapine particularly functions as a partial agonist of the 5-HT_{1A} receptor. Many properties of the 5-HT_{1A} auto receptor agonists have been observed with clozapine, which similarly activates and blocks pre-and postsynaptic 5-HT_{1A} receptors, respectively (Millan 2000). Previous studies have shown that, WAY100635 attenuated dopamine levels in frontal cortex elicited by clozapine and its ability to moderate the cataleptogenic actions of haloperidol (Kuroki 1999; Millan 2000). In a separate study, clozapine showed a significant increase in dopamine release in rat prefrontal cortex, which was partly due to its 5-HT_{1A}-R agonist properties as the effect could be blocked by WAY-100635, a 5-HT_{1A}-R antagonist (Rollema 1997; Meltzer 2002). The 5-HT_{1A}-R agonist

BAY increased the firing rate and burst firing of dopamine neurons in the ventral tegmental area (VTA) and dopamine release in both VTA and mPFC, both the effects were reversed by the 5-HT_{1A}-R antagonist WAY-100635. Furthermore, atypical antipsychotics like clozapine, olanzapine enhanced dopamine release in the mPFC of wild-type but not 5-HT_{1A}-R knockout mice after systemic and local administration of the drugs (Mataix 2005). Taken together these studies indicate that 5-HT_{1A}-R might play an important role in the mechanism of action of clozapine.

NMDA receptor and its implication in the mechanism of atypical antipsychotic drug:

The dopaminergic dysfunction theory has been critically linked to the etiology of schizophrenia for last two decades. However, the pharmacological and clinical profiles of several drugs raise serious questions about the fundamental importance of D_2-R blockade in the potency of antipsychotic drugs (Lidsky 1997). Ineffectiveness of dopamine antagonists to treat some of the symptoms of schizophrenia led investigators to postulate the involvement of other signaling systems like glutamatergic system in the pathophysiology of schizophrenia. Antagonism of glutamatergic NMDA receptor complex has produced behavioral and cognitive deficits in otherwise normal subjects that closely mimic schizophrenia and in

therapeutic trials, in which agents that enhance NMDA receptor activity have selectively improved certain symptoms of schizophrenia (Goff 2001). The psychotomimetic properties of phencyclidine (PCP) were recognized immediately after its introduction as a general anesthetic. The remarkable similarity of PCP-induced psychosis to schizophrenia led, in 1962, to the formulation of an hypothesis alternative to dopamine hypothesis (Lidsky 1996) Glutamate hypothesis of schizophrenia proposes a possible link between hypoactive glutamate receptor neurotransmission (particularly NMDA receptor hypo function) with this disease (Arvanov 1997). In addition postmortem studies have revealed abnormalities of glutamate receptor density and subunit composition in the prefrontal cortex, thalamus and temporal lobe (Goff 2001).

Carlsson and Carlsson suggested that the development of a novel glutamatergic agonist without side effects could be a promising antipsychotic agent. In fact several antipsychotic drugs have shown diverse affect in both pre and postsynaptic glutamatergic transmission. Chronic haloperidol treatment up-regulates NMDA receptors in the cortex and striatum and along with clozapine, affects the expression of mRNA of both NMDA and non-NMDA receptors in a region-specific fashion. There are two plausible mechanisms by which antipsychotic drugs can achieve

glutamatergic effects: The first involves influencing dopaminergic processes and the second mechanism involves direct effect on glutamate receptors (Lidsky 1997). Previous studies on the brain slices from rat prefrontal Cortex have shown that the atypical antipsychotic drug Clozapine but not Haloperidol produced bursts of excitatory postsynaptic potentials (EPSPs), which were specifically blocked by glutamate receptor antagonist. Clozapine also showed a significant increase in the amplitude of these EPSPs compared to Haloperidol treatment (Arvanov 1997). Additional mechanisms are thought to be involved in potentiation of NMDA receptor by clozapine. Previous studies have shown that clozapine potentiated the late NMDA receptor mediated component of evoked rEPSPs which were augmented further when dopamine transporter inhibitor Buproprion blocked dopamine re-uptake (Chen 2002). On the other hand pre-treatment with the D1 antagonist SCH23390 blocked clozapine-mediated potentiation of the late rEPSP. All these results suggested that the dopamine released by clozapine caused an activation of D1 receptor to achieve a sustained enhancement of the NMDA receptor-mediated synaptic responses in pyramidal neurons (Chen 2002). Based on all theses findings, the involvement of NMDA receptor and the possible interaction of NMDA receptor with other receptors

cannot be ruled out in the mechanism of action of atypical antipsychotic drug like clozapine.

Antipsychotic drug and other components of an intracellular signaling pathway:

Most mechanistic studies of antipsychotic drugs have focused mainly on dopamine release from neurons. In our present study we are asking more fundamental questions like if clozapine is causing any change in the excitability of neurons in the prefrontal cortex and if it does, what signaling pathway it may follow to cause neuronal excitation. Previous studies have shown that typical and atypical antipsychotics have opposing effects on MEK/ERK pathway in mouse dorsal striatum (Pozzi 2003). Atypical antipsychotic treatment caused an inhibition of ERK1/2 in mouse brain, while typical antipsychotics like haloperidol showed opposite effect. In contrast, clozapine administration selectively increased phosphorylation of MEK1/2 and its downstream substrate ERK1/2 in rat prefrontal cortex (Browning 2005). Chronic administration of clozapine decreased protein kinase C activity and mRNA and protein levels of PKC isozymes in membrane and cytosol fraction of cortex, hippocampus and cerebellum (Dwivedi 1999). In a separate study phosphopholipaseC-beta-1 knock out

mice exhibited schizophrenia–like symptoms, which were rescued by clozapine administration (McOmish 2007). Antipsychotic drug treatment showed induction of differential gene expression in different animals. For example, acute administration of the typical antipsychotic drug haloperidol induced the expression of c-fos transcription factor, mainly, in the striatum and nuclear accumbens. Previous studies have shown that the atypical antipsychotic drug clozapine also acutely induced c-fos expression in the prefrontal cortex (Kontkanen 2002). Fos family of genes encodes certain transcription factors, which regulate the expression of other genes. Microarrray analysis of gene expression to search for genes regulated in the PFC by acute clozapine treatment identified several genes involved in the presynaptic function (e.g. ChromograninA, SynaptogaminV, CalcineurinA) to be regulated by antipsychotics.

Chronic clozapine treatment also induced differential cortical expression of Chromogranin A, Son of sevenless (sos) and Sec1 (Kontkanen 2002). These data suggest that the administration of antipsychotic drug triggers expression of genes involved in synaptic function and regulation of intracellular Ca^{2+}.

Involvement of CaMKII signaling pathway in the mechanism of action of atypical antipsychotic drug:

Calcium/calmodulin dependent protein kinase II (CaM kinase II), an enzyme which plays pivotal role in synaptic plasticity and cognitive functions has been implicated in the actions of anticonvulsants and antidepressants, but little is known about the enzyme's role in the action of different antipsychotic drugs. Previous studies have shown that antidepressants like fluvoxamine and despramine which specifically block monoamine reuptake caused an up-regulation of the enzyme in the prefrontal cortex, where as typical antipsychotic drug haloperidol did not show any significant change in the kinase protein level (Celano 2003). On the other hand in a separate study both the typical antipsychotic drug haloperidol and the atypical antipsychotic drug clozapine caused an increase in mRNA expression of many Ca^{2+}-activated kinases including CaMKII in the rat frontal cortex (MacDonald 2005).

Prolonged ingestion of phencyclidine (PCP) in humans produces long-lasting schizophrenia like cognitive dysfunctions. Long-term PCP treatment has been used as a behavioral model of schizophrenia. Mouri and coworkers have shown a direct involvement of NMDA receptor and CaMKII pathway in the impairment of latent leaning in the PCP model of schizophrenia. In the

same study impairment of learning-associated CaMKII activation was observed in the prefrontal cortex of PCP-treated mice. PFC slices from PCP-treated mice also failed to exhibit exogeneous-NMDA induced activation of CaMKII compared to the control mice. All these findings strongly indicate prefrontal cortical dysfunction of NMDA-CaMKII signaling in the pathophysiology of schizophrenia (Mouri 2007). Intracellular recording from PFC slices showed direct involvement of CaMKII in the facilitating action produced by clozapine on NMDA induced inward currents and excitatory postsynaptic currents in pyramidal cells. The CaMKII inhibitor, KN-93, but not the inactive isomer, KN-92, blocked clozapine's augmenting effect on NMDA-evoked responses in pyramidal cells of the rat mPFC. In contrast to results in pyramidal cells from rats or wild-type mice, clozapine was not able to potentiate NMDA-induced currents in the mPFC pyramidal cells from the CaMKII mutant mouse. Taken together, these results suggest that the facilitating action of clozapine on the NMDA receptor and electrically evoked responses in pyramidal cells of the mPFC requires activation of CaMKII enzyme (Ninan 2003).

Objective:

Most mechanistic studies of antipsychotic drugs have focused mainly on dopamine release from neurons. Behavioral abnormalities observed in schizophrenia and other studies of brain activity implicate abnormal function of the prefrontal cortex in schizophrenia. Therefore, in our study, we have asked a more fundamental question, "Does clozapine cause a change in neuronal excitability of neurons in the PFC?"

In the present study we are investigating the effect of clozapine, an atypical antipsychotic drug on the neuronal excitability in mouse prefrontal cortex and the underlying pathways, which can give a mechanistic insight into the physiological action of this life-saving psychotropic drug.

Chapter 2

Material and Methods:

Animals:

Mice (age: Postnatal day 20-day 30) of Swiss Webster wild type and 5-HT_{1A} receptor knock out were used for the experiments. Animals were kept in a 12h light/dark cycle with *ad libitum* access to food and water.

Materials:

The antibodies to CaMKinaseII and phosphoCaMKinaseII and the horse radish peroxidase-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Clozapine, D_2 receptor antagonist sulpiride, 5-HT_{1A} receptor agonist 8-OH-DPAT, 5-HT_{1A} receptor antagonist WAY100635, MAPKKinase inhibitor PD98059, NMDA receptor agonist NMDA and NMDA receptor antagonist APV (±-2-Amino-5-phosphonovaleric acid) were obtained from Sigma Chemicals (St. Louis, MO, USA). CaMKII inhibitor CaMKIINtide (Myristoylated) and Phospholipase Cβ inhibitor U73122 were purchased from Calbiochem (La Jolla, CA, USA).

Preparation of acute slices from mouse prefrontal cortex:

Prefrontal cortex slice were prepared from P20 to P30 Swiss Webster (+/+) and (-/-) mouse brain (Stoppini 1991; Xiang 2000; Franke 2003). Mouse pups of specific ages were subjected to spinal cord dislocation and decapitated. The brain was removed under sterile conditions. The brain was cut at 60^{o} angle along the longitudinal fissure. Each of the two hemispheres was then cut saggitally into two parts and coronal sections of prefrontal cortex were (300µm thick) obtained from each of the two saggital sections previously cut from each hemisphere using a manual tissue slicer. The slices were then placed in pre-oxygenated Ringer buffer and structures were inspected using a dissection microscope for the presence of uninterrupted bright transparent multiple neuronal layers characteristic of the cortical structure. The slices were incubated for 1 hour and then the slices were treated with respective drugs. After drug treatment, the slices were lysed with RIPA buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM Na_3VO_4, with freshly added protease inhibitor cocktail) and the lysate was used for Western blot analysis.

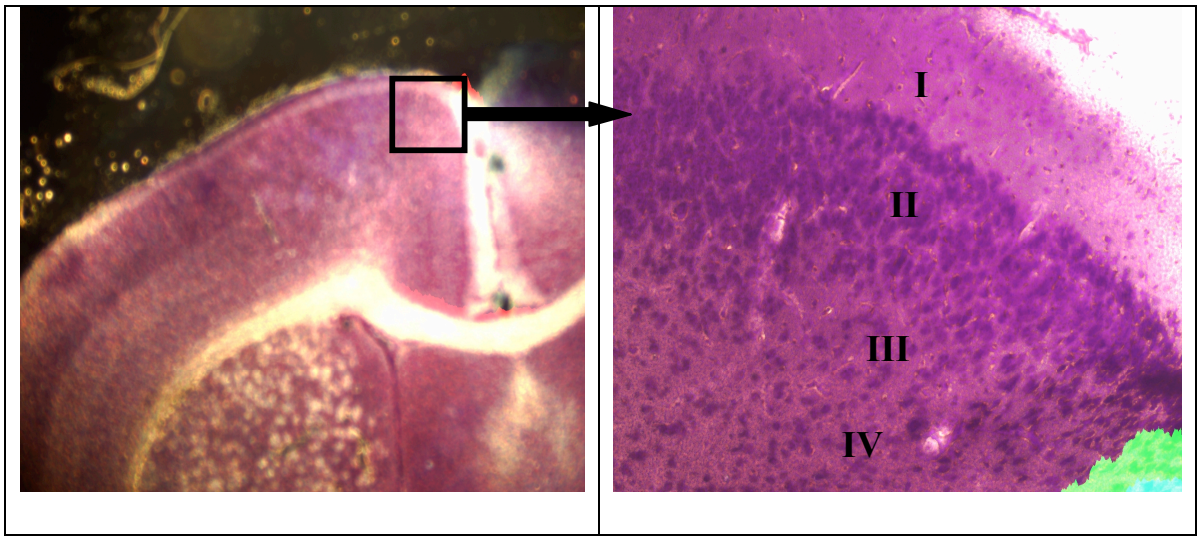


Figure 3: Pictures above show different cortical cell layers.

Concentration of drugs used:

The acutely isolated slices were treated with different drugs. The inhibitors were added 20 minutes prior to clozapine (15 µM) treatment.

The concentrations of the antagonists and inhibitors were as follows:

5-HT_{1A}-R antagonist WAY100635 (4 µM), phospholipse Cβ inhibitor U73122 (1 µM), CaMKII inhibitor (1 µM), MAP kinase kinase inhibitor PD98059 (25 µM), Dopamine D_2 receptor antagonist sulpiride (1 µM), 5-HT_{1A}-R agonist 8-OH DPAT (100 nM), NMDA-R antagonist APV (±-2-Amino-5-phosphonovaleric acid) (15 µM) , NMDA-R agonist NMDA (30 µM).

Electrophysiology experiments:

The experiments were done on prefrontal cortex slices of Swiss Webster wild type and knock out P20 to P30 young adult mice. Following decapitation, brains were removed and each of the two hemispheres was then cut saggitally into two parts and placed into ice cold Ringer solution consisting of (in mM): NaCl 124, KCl 3.1, KH_2PO_4 1.3, $MgSO_4$ 1.3, $CaCl_2$ 3.1, $NaHCO_3$ 25.5 and glucose 10.0.

Coronal sections of prefrontal cortex were (300 μm thick) obtained from each of the two saggital sections previously cut from each hemispheres using a manual tissue slicer and placed in an incubation chamber (33°C) constantly oxygenated with CO_2 / O_2 (5% /95%) mixture. Following 1-hour pre-incubation slices were transferred to the recording chamber, which was maintained at 33°C. A bipolar concentric (stimulating) electrode was placed on the white matter area (layer 5 or 6) of prefrontal cortex slices and the recording electrodes were guided into layer 2 or 3 of prefrontal cortex to record population spike .

The magnitude of each population spike was measured by a computer program as the average distance between the highest negativity and the following positivity. The strength of the stimulus in all tested slices were

adjusted at the beginning of each experiment to approximately 75% of the maximum response.

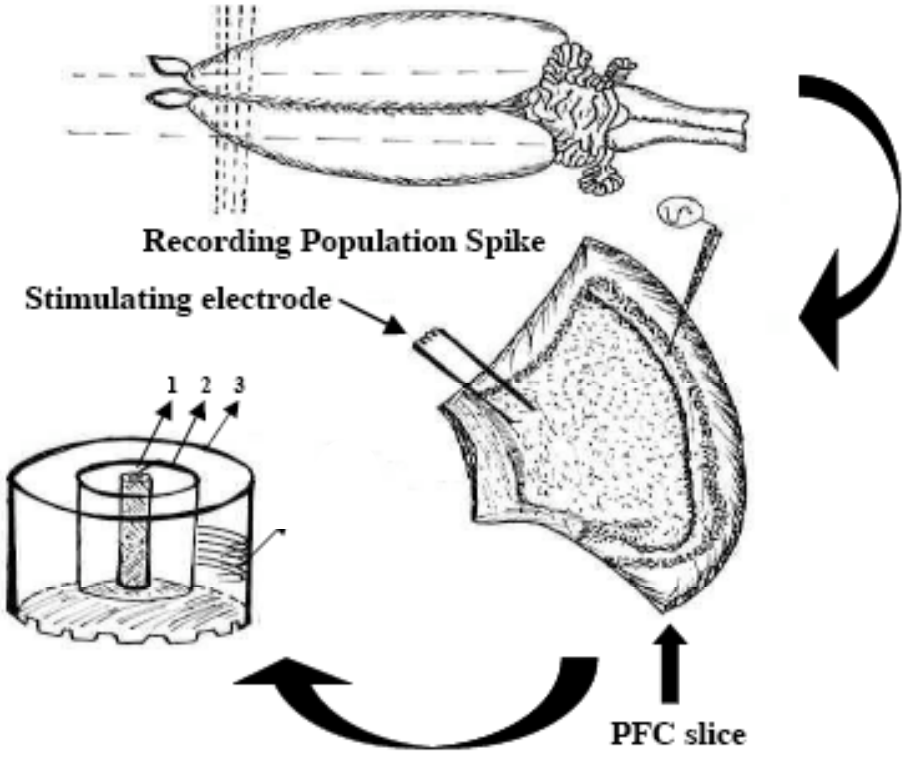


Fig. 4. 1. The slice containing chamber 2. Water bath 3. Acrylic frame

The potential was monitored at this level for 10-15 minutes and experiments were performed only on slices demonstrating stable response to low frequency (0.03 Hz) stimulation. Each experiment was performed on a separate slice. The recording was continued for the 50 to 60 minutes. Data was presented as mean $\pm$ S.D. (Standard Deviation). Statistical analysis was carried out using "Paired student-t test"[Figure 4].

Statistical analysis:

Statistical analysis of sets containing more than two groups was carried out using One Way ANOVA with Bonferroni Post Hoc Test (a = 0.05). Paired t-test was used to compare between two sets (e.g. with and without clozapine treatment) from triplicate or multiple repeats of the same experiment.

Western blotting:

The drug-treated slices were lysed in 1ml RIPA buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM Na_3VO_4 and freshly added protease inhibitor cocktail; Boeringer), the lysate (10 µg-15 µg protein) was resolved using a 7% to 16% gradient acrylamide gel , protein bands transferred to a nitrocellulose membrane, in a blocking solution containing either 5% Milk or 2.5% BSA in 0.1% tween in TBS (20 mM Tris-HCl, pH 7.4, 0.8% NaCl)(t-TBS) and then the membrane probed with phospho-CaMKIIα antibody (1:1000) followed by treatment with horseradish peroxidase (HRP)-linked goat anti-rabbit IgG (1:100000). Both antibodies were dissolved in the blocking solution. After probing with phospho-CaMKIIα antibody, the blot was stripped by incubating for 1 h at room temperature in 0.2 M glycine (pH 2.5), and then blocked in 5%

defatted milk and re-probed using a monoclonal, CaMKIIα antibody at 1:1000 dilution and then with HRP-conjugated antimouse IgG (1:5000). The immunoreactive bands were visualized using the Supersignal luminol kit (Pierce). The CaMKIIα bands were used to confirm that the observed increase in P- CaMKIIα was not due to an increase in the amount of CaMKIIα protein.

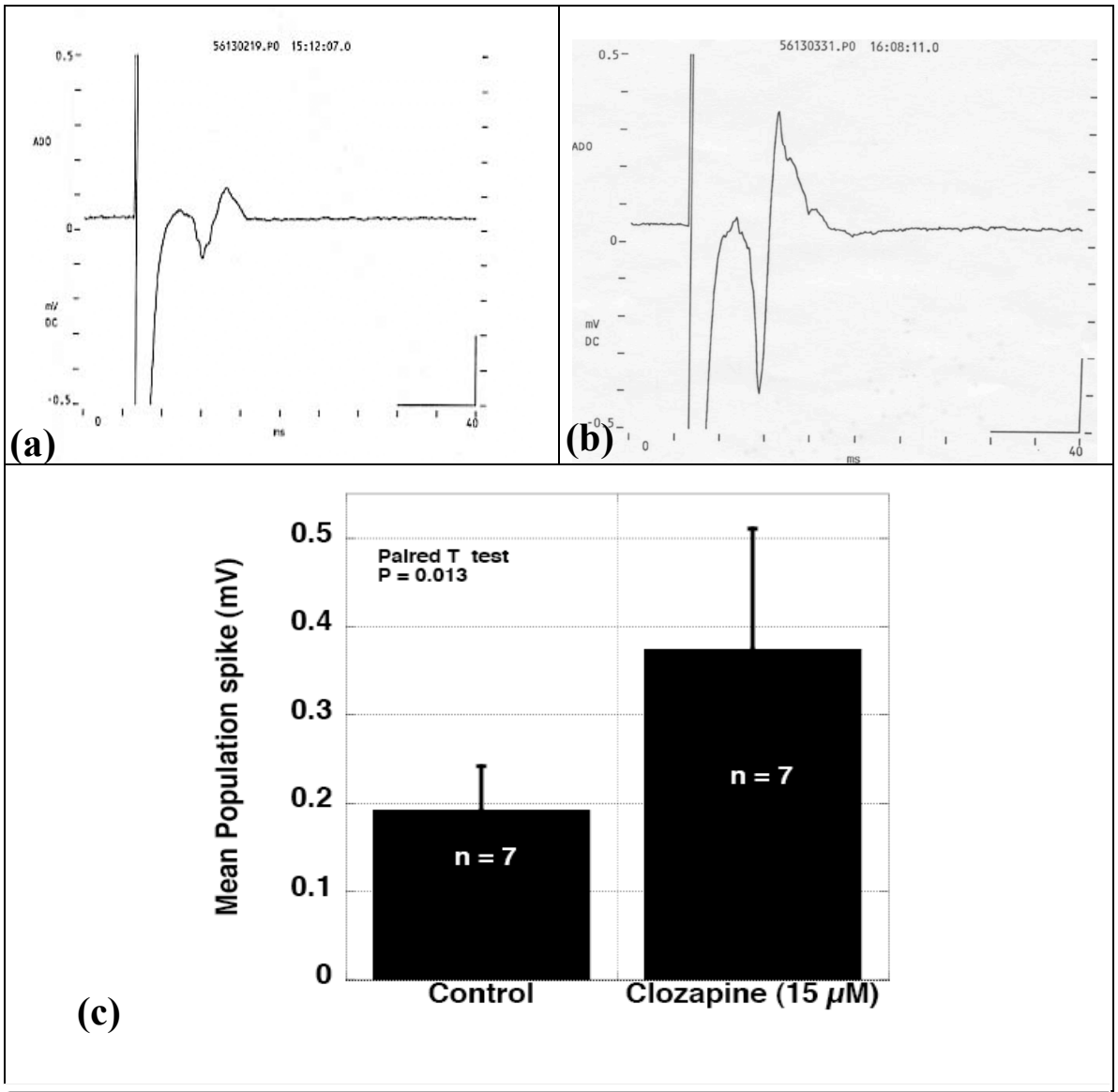
Chapter 3

Results:

Clozapine treatment results in a significant increase in Population Spike in Mouse Prefrontal Cortex:

Previous studies have shown that atypical antipsychotic drug treatment induced cortical dopamine release in rat prefrontal cortex compared to nucleus accumbens or striatum. To further understand how atypical antipsychotic drug treatment affect synaptic transmission and neuronal firing, electrophysiology recording was performed on acute Prefrontal cortex slices from postnatal day 20 to day 30 Swiss-Webster mice. Coronal sections (300 μm) from Prefrontal cortex were used for the electrical recording and population spikes were measured from layer (iii) and layer (iv) after stimulating from layer (v). Low frequency (0.03 Hz) repeated stimulation was given at every 30-seconds. Once the population spike reached the basal level after about 10 minutes, the slice was treated with Clozapine (15 μM) and the recording was continued for 50 to 60 minutes. The experiment was repeated seven times (n=7). Overall clozapine resulted in a significant increase in the population spike. Within each set of experiments, "Paired student t-test" was performed for statistical analysis. A highly significant

95% increase in Mean population spike was observed after clozapine treatment [Figure 5].



	Base Line (mV)	Clozapine (15 μM)	Treatment	Mean Population spike (mV)	Std.Dev.
0	0.14000	0.33000	Control	0.19143	0.050143
1	0.14000	0.32000	Clozapine (15 μM)	0.37429	0.13636
2	0.23000	0.28000			
3	0.16000	0.22000			
4	0.25000	0.42000			
5	0.17000	0.64000			
6	0.25000	0.41000			

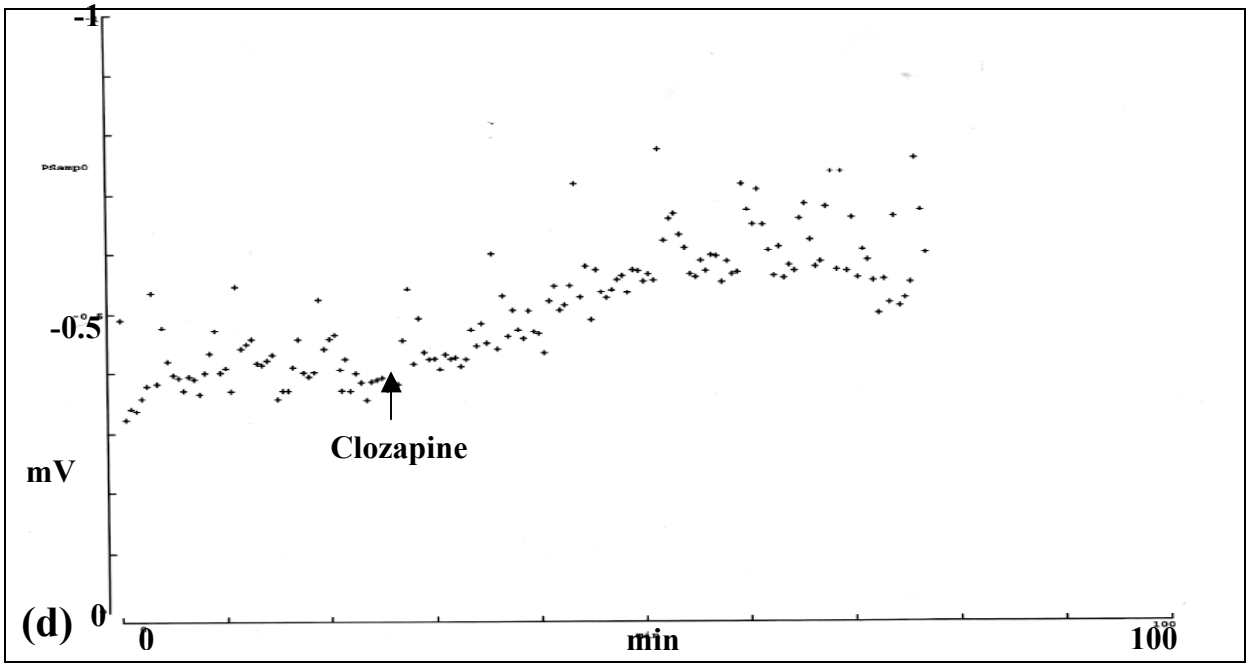
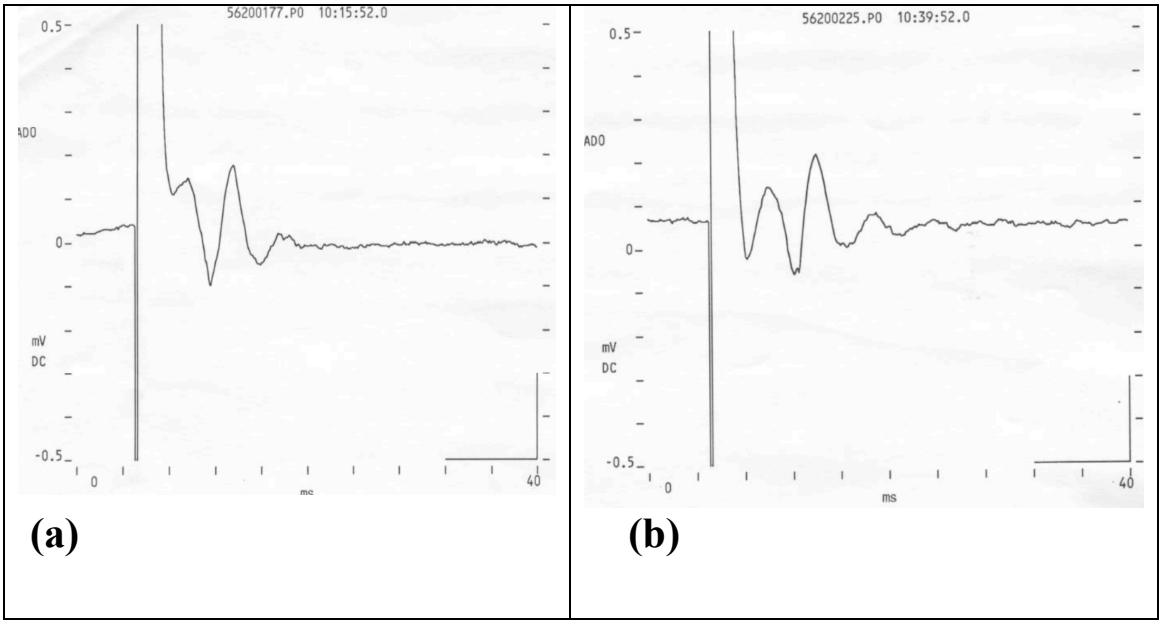


Figure 5. Increase in population spike after Clozapine (15 µM) treatment in mouse prefrontal cortex. (a) Population spike before clozapine treatment (b) Population spike after clozapine treatment for 60 minutes (c) Clozapine treatment resulted in a 95% increase in Mean population spike (d) Repeat stimulation, once in every 30 seconds over 50 to 60 minutes time period showed a significant increase in population spike.

The 5-HT$_{1A}$-receptor antagonist WAY-100635 blocks clozapine-evoked increase in population spike:

Isolated PFC slices were treated with the selective 5-HT$_{1A}$-R antagonist WAY-100635 (4 μM) 20 minutes prior to clozapine treatment. As shown in Figure 6, administration of WAY prior to clozapine treatment resulted in a complete blockage of the clozapine-evoked increase in population spike observed earlier [Figure 5]. PFC slices were treated with WAY for 20 minutes followed by clozapine treatment. After the drug treatment no significant increase in population spike was observed.



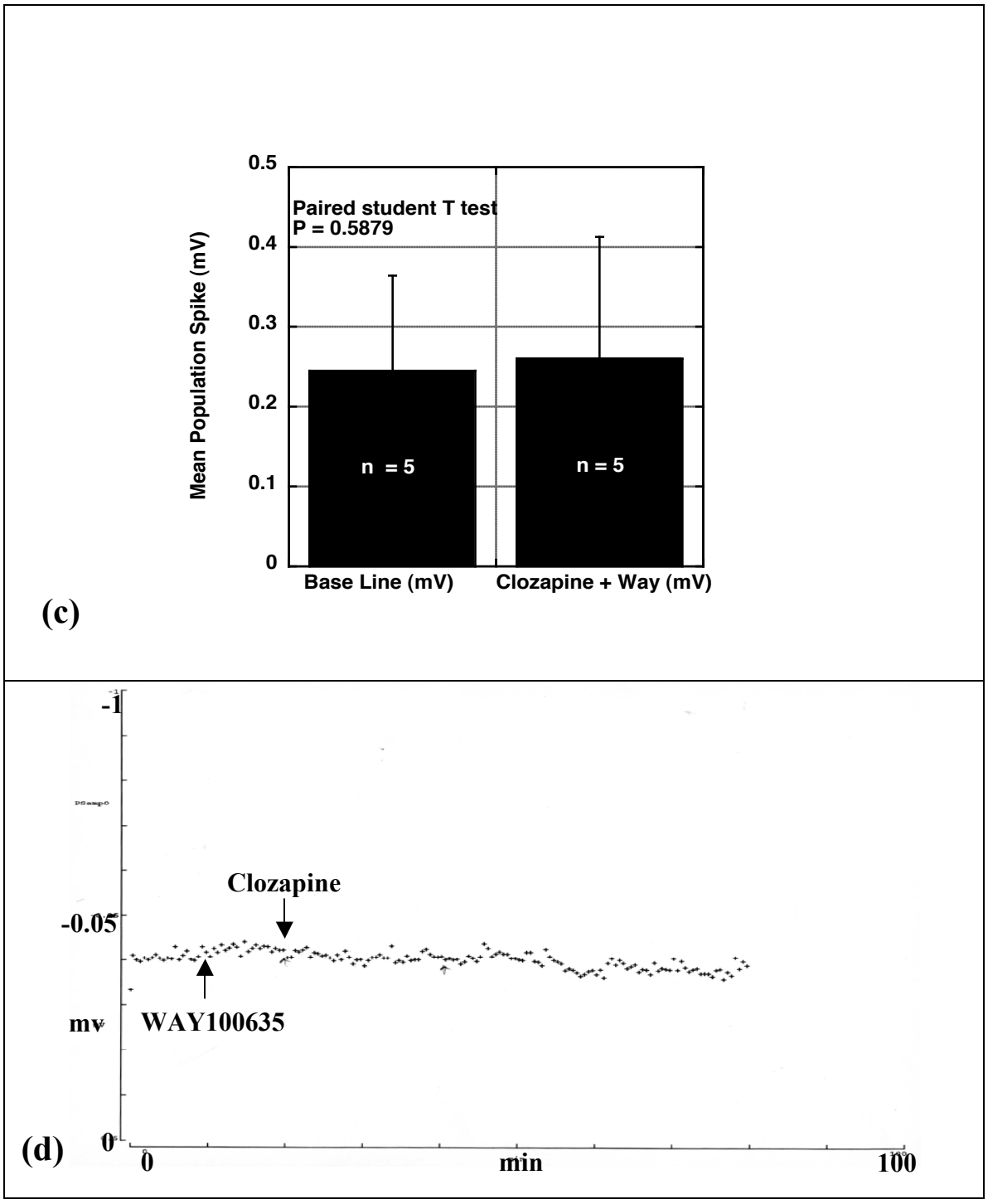
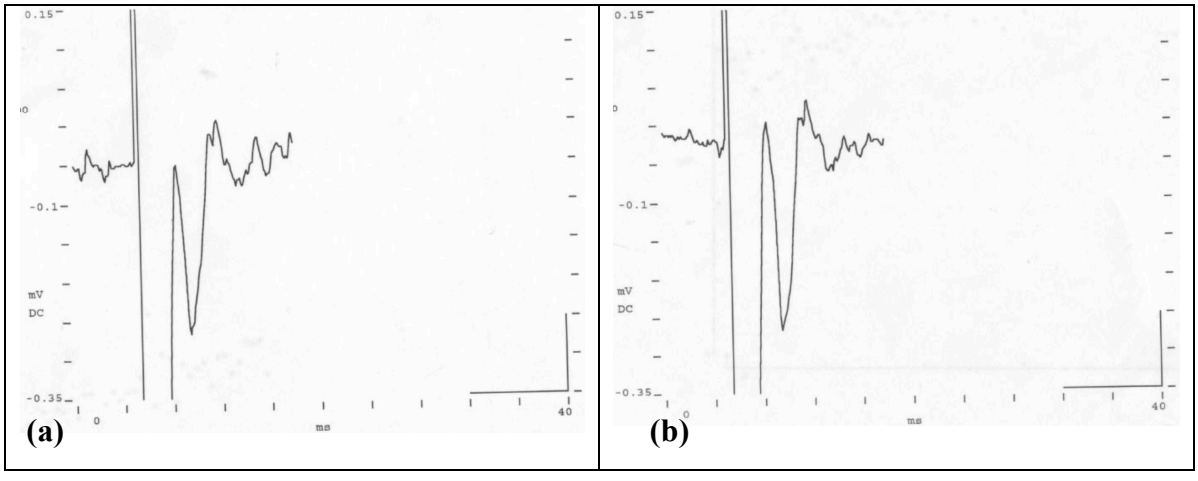


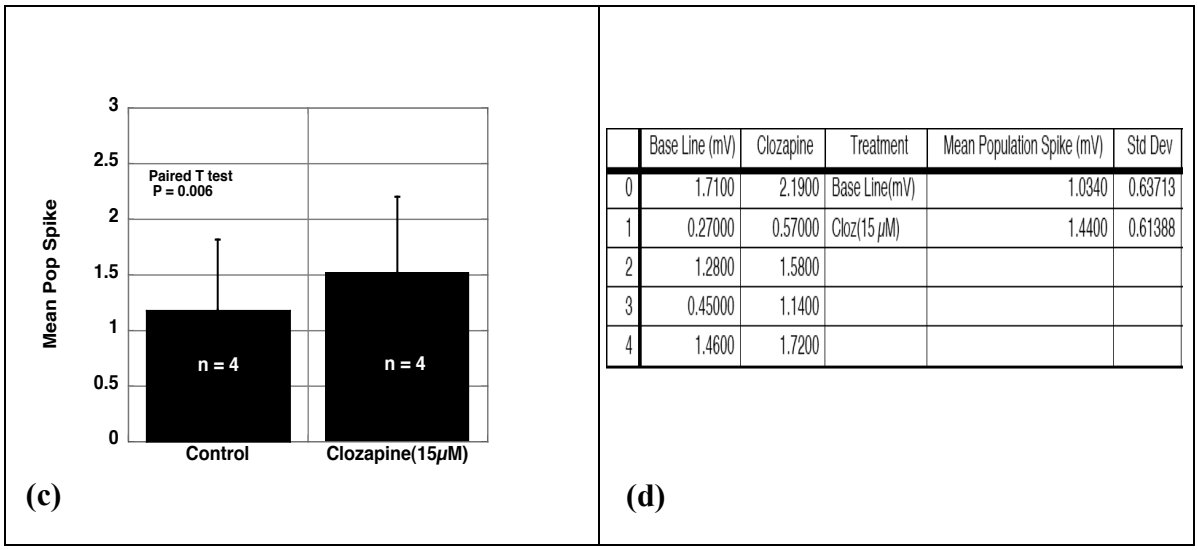
Figure 6: Clozapine-evoked increase in population spike in mouse prefrontal cortex is mediated by 5-HT$_{1A}$-R activation. (a) Population spike before drug treatment (b) No significant change in Population spike when the PFC slice was treated with the 5-HT$_{1A}$-R antagonist WAY-100635 for 20 minutes prior to Clozapine treatment (15μM) (c) The 5-

HT_{1A}-R antagonist WAY-100635 caused a complete blockage of clozapine-evoked increase in population spike in wild type mouse slices.

Clozapine treatment also shows an increase in population spike in 5-HT_{1A}-R (-/-) mouse prefrontal cortex:

Electrophysiology studies on Day 20 to 30 postnatal mouse prefrontal cortex slices in our laboratory provided more supporting evidence to the involvement of the 5-HT_{1A} receptor in the mechanism of action of clozapine. Electrical recording from layer 3 or 4 of prefrontal cortex slices of P20 to P30 5-HT_{1A}-R (-/-) mouse brain also showed an increase in population spike in the presence of clozapine [Figure 7]. Treatment of clozapine on the PFC slices from 5-HT_{1A}-R knockout mice also resulted in an increase in population spike, indicating the involvement of a different receptor in the clozapine-evoked population spike in the absence of the 5-HT_{1A} receptor.





	Base Line (mV)	Clozapine	Treatment	Mean Population Spike (mV)	Std Dev
0	1.7100	2.1900	Base Line(mV)	1.0340	0.63713
1	0.27000	0.57000	Cloz(15 μM)	1.4400	0.61388
2	1.2800	1.5800			
3	0.45000	1.1400			
4	1.4600	1.7200			

(d)

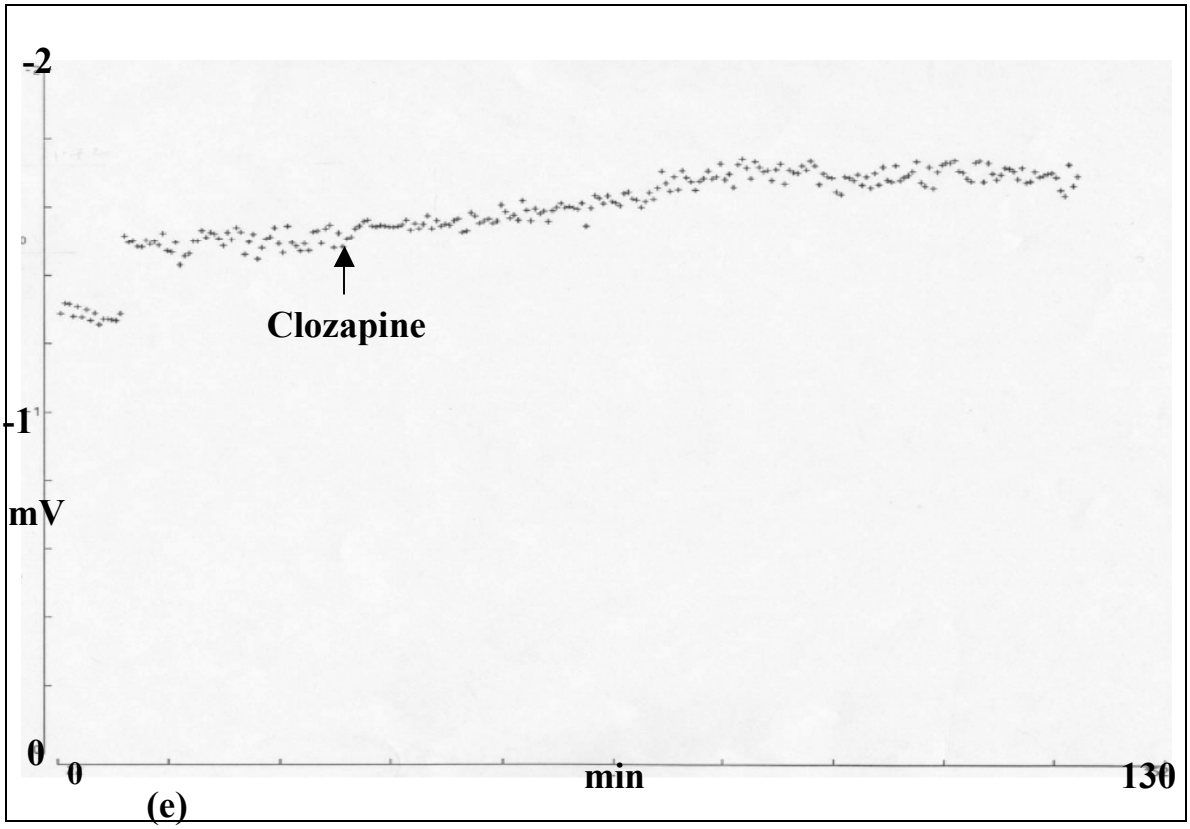


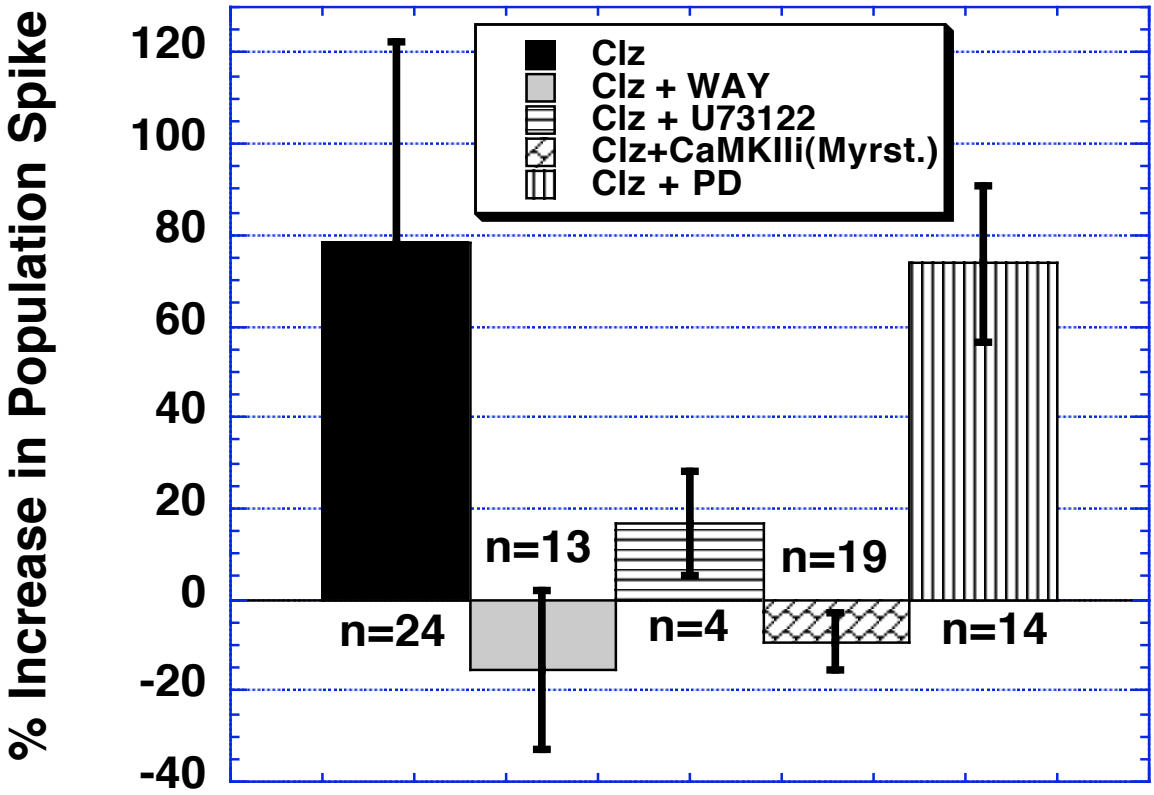
Figure 7: Treatment of clozapine on the PFC slices from 5-HT_{1A}-R knockout mice also resulted in an increase in population spike (a) & (e)Population spike before drug treatment (b) Population spike after drug treatment (c) Clozapine (15 μM) resulted in a

slower increase in Population Spike in Prefrontal Cortex from $5HT_{1A}$ knockout mice (P20-P30).

Clozapine-evoked increase in population spike requires subsequent activation of Phospholipase C and Ca^{2+}dependent CaMKII but does not show any involvement of the MAPKinase pathway:

In separate experiments PFC slices from wild type mice were treated with the Phospholipase C inhibitor U73122 (1 μM) and a CaMKII inhibitor (1 μM) respectively, followed by clozapine (15 μM) treatment. This caused elimination of the clozapine-mediated increase in population spike in mouse prefrontal cortex. Here, the PLC inhibitor U73122 exhibited a clear trend of inhibition, where as a CaMKII inhibitor caused a complete block of the clozapine-evoked increase in population spike. In sharp contrast, the MEK inhibitor PD98059 showed no significant inhibition ($P = 0.37$). On the other hand the effect of WAY-100635, U73122 and a CaMKII inhibitor on clozapine-evoked increase in population spike was statistically significant as revealed by ANOVA ($p < 0.001$). These results clearly delineate the mechanistic role of Phospholipase C and CaMKII in clozapine-mediated augmentation of population spike [Figure 8]. Therefore, it is likely that, clozapine functions as a 5-HT_{1A}-R agonist, which subsequently triggers activation of PLC and CaMKII to finally cause an increase in population

spike. Apparently this pathway does not show any significant involvement of MAPKinase.

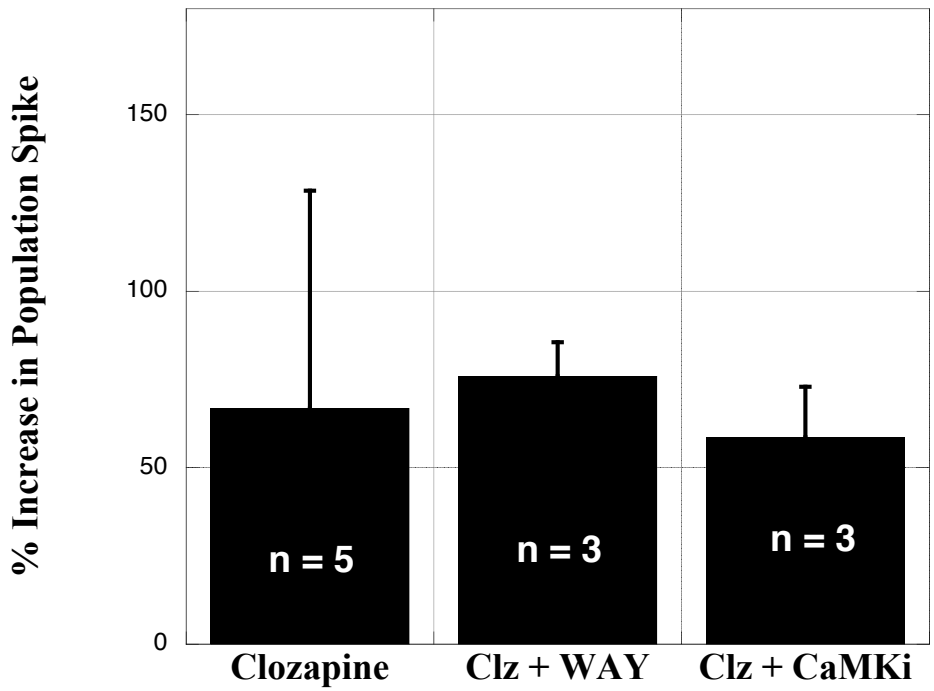


Analysis by ANOVA using Bonferroni's all pair's comparison

Comparison	P value
Clozapine vs WAY+Clozapine	<.0001
Clozapine vs U73122+Clozapine	0.0032
Clozapine vs CaMKIIi+Clozapine	<.0001
Clozapine vs PD98059+Clozapine	1.000

Figure 8: Clozapine-evoked augmentation of population spike involves activation of Phospholipase C and Ca^{2+}-dependent CaMKIIα.

Clozapine-mediated increase in population spike is independent of CaMKII activation in 5-HT_{1A}-R knockout mice:



Increase in population spike observed after clozapine + WAY and clozapine + CaMKi treatment shows statistically insignificant differences in the value compared to clozapine alone. P value =1

Figure 9: Clozapine induced elevation of population spike is not dependent on CaMKII activation in 5-HT_{1A}-R knock out mice.

Clozapine-mediated rise in population spike observed in wild type mice was completely blocked upon treatment with the CaMKII inhibitor and WAY100635.The result demonstrated the involvement of both CaMKII as

well as 5-HT_{1A}-R activation. However, it is not clear that, clozapine-evoked activation of CaMKII, which eventually elicits a significant increase in population spike, is mediated *via* 5-HT_{1A}-R activation. To understand the connection between 5-HT_{1A}-R and CaMKII activation in the clozapine-mediated signaling pathway, we performed electrophysiology experiments with 5-HT_{1A}-R (-/-) mice in the presence of the CaMKII inhibitor and WAY100635.

PFC slices from 5-HT_{1A}-R (-/-) mice were treated with clozapine in the presence of CaMKII inhibitor. As we have seen before, clozapine also shows an increase in population spike in knockout mice. A CaMKII inhibitor failed to inhibit clozapine- elicited increase in population spike in the knockout mice (Figure 9). This piece of data indicates that the increase in populations spike observed in 5-HT_{1A}-R knockout mice does not involve CaMKII activation. This is in sharp contrast to the CaMKII-dependence of the clozapine-evoked increase in population spike observed in wild type mice.

As WAY100635 also fails to reverse the clozapine-mediated change in electrical activity in knockout mice, it excludes the possibility of WAY100635 functioning through a different receptor causing activation of CaMKII in the wild type mice.

Clozapine regulates CaMKinase phosphorylation in a time-dependent manner:

Treatment of acute prefrontal cortex slices with 15μM clozapine produced a time-dependent change in CaMKII phosphorylation [Figure 10] with a maximal effect reaching around 15 minutes after the drug treatment. The levels of phospho-CaMKII after clozapine administration reached a maximum activation in about 15 minutes.

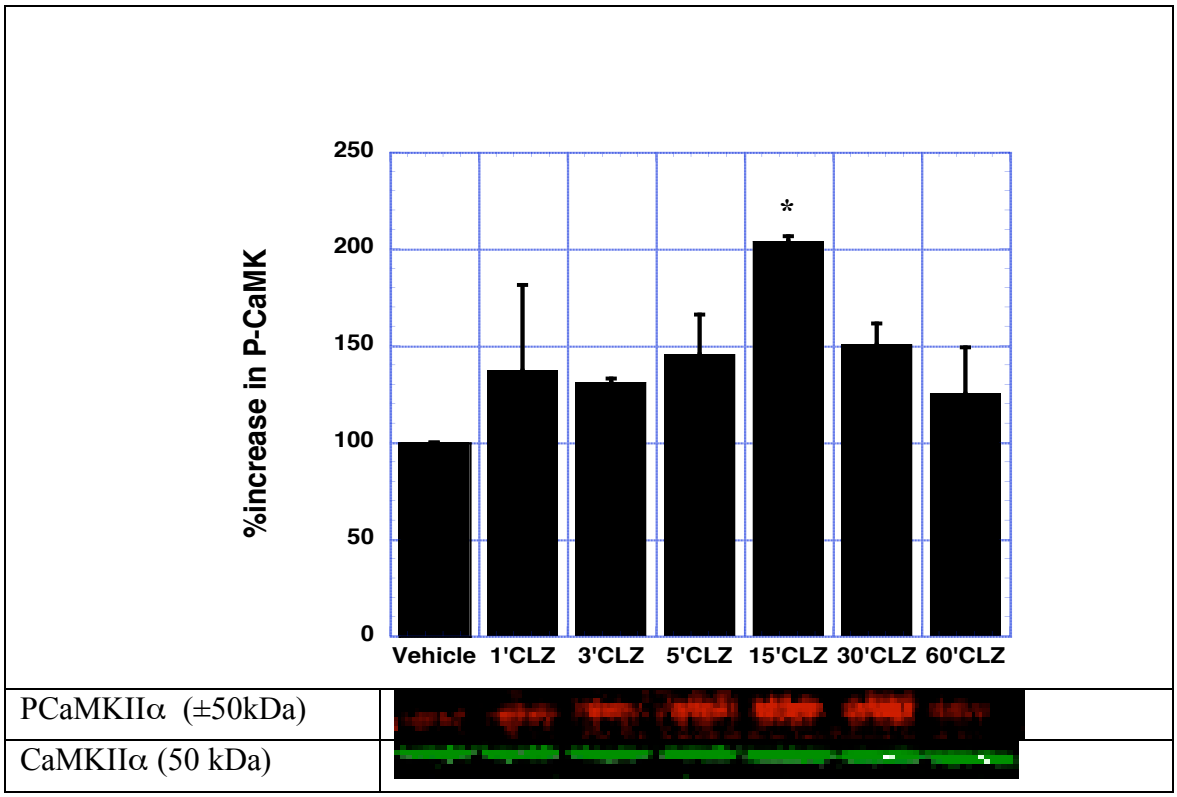


Figure 10: Clozapine triggers phosphorylation of CaMKII. *P Value (0.0346) is statistically significant at 15 minutes of Clozapine treatment as revealed by One Way ANOVA.

This finding further supported the electrophysiology data, where treatment of the CaMKII inhibitor prior to the addition of clozapine caused a complete blockage of clozapine-evoked increase in population spike.

The 5-HT$_{1A}$-R antagonist WAY-100635 blocks clozapine-mediated regulation of CaMKinaseII phosphorylation :

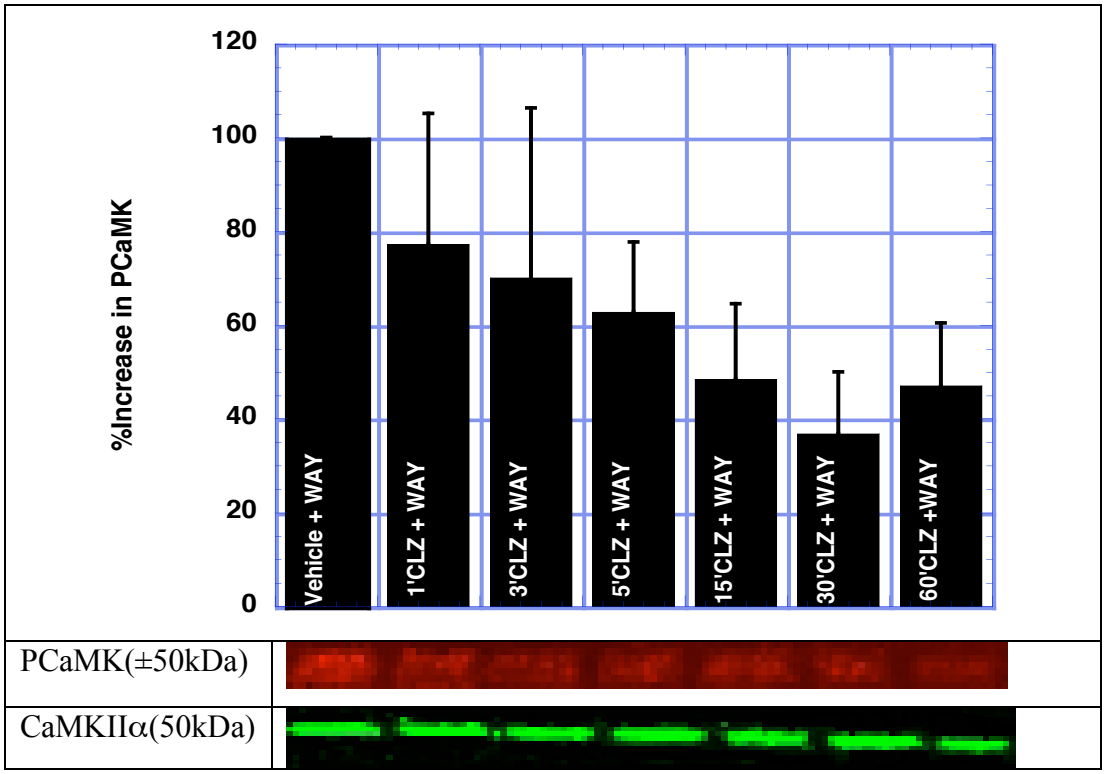
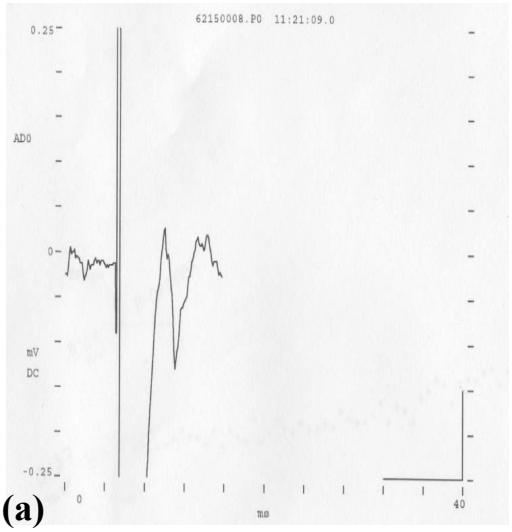


Figure 11: The 5-HT$_{1A}$-R antagonist WAY-100635 blocks the clozapine-evoked activation of CaMKII.

When cortical slices were treated with clozapine in the presence of the 5-HT_{1A}-R antagonist WAY-100635 (4 μM), it showed complete inhibition of Phospho-CaMKII [Figure 11]. This piece of data further supports the fact that clozapine mediated activation of CaMKII may be mediated through 5-HT_{1A} receptor. This result provides additional evidence for the pathway where clozapine functions as an agonist to 5-HT_{1A}-R, which eventually triggers an activation of CaMKII and finally an increase in population spike in the prefrontal cortex.

The 5-HT_{1A}-R agonist 8-OH-DPAT-treatment results in an increase in population spike in mouse prefrontal cortex:

PFC slices were treated with the selective 5-HT_{1A}-R agonist 8-OH-DPAT (100 nM) and the population spike was measured for 30 minutes. 8-OH-DPAT treatments resulted in more than two folds of increase in population spike over 30 minutes of drug treatment. This result further supports the fact, that 5-HT_{1A}-R agonism plays an important role in increasing population spike observed after clozapine treatment in prefrontal cortex [Figure 12].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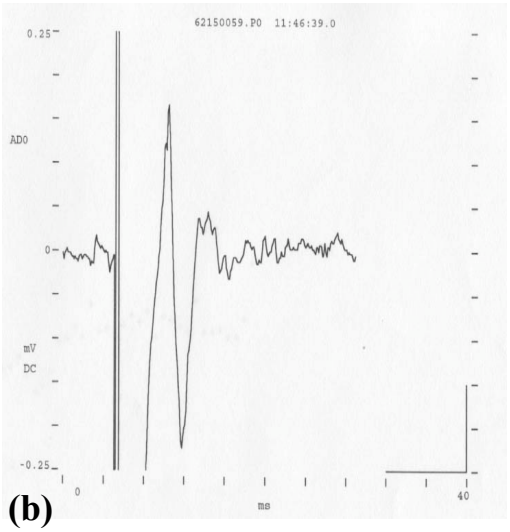
0.25
AD0
0
mV
DC
-0.25
0
ms
40

(a)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mV
DC
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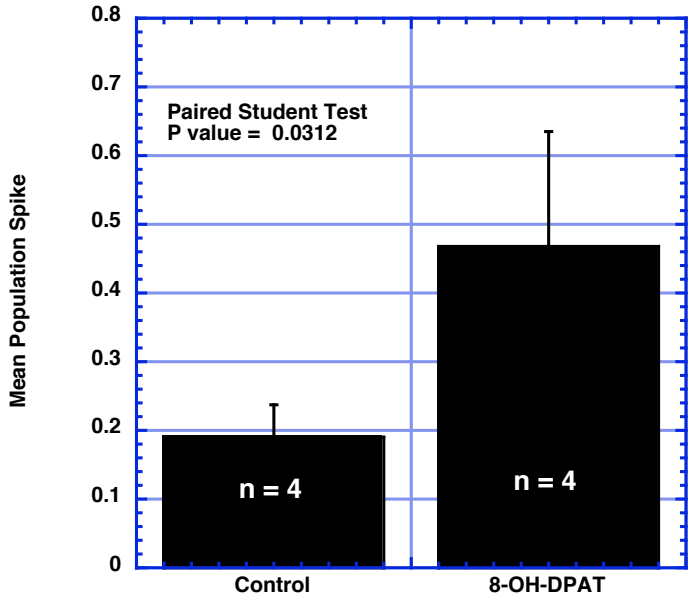
0
ms
40

(b)


Mean Population Spike
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Paired Student Test
P value = 0.0312
n = 4
n = 4
Control
8-OH-DPAT

(c)

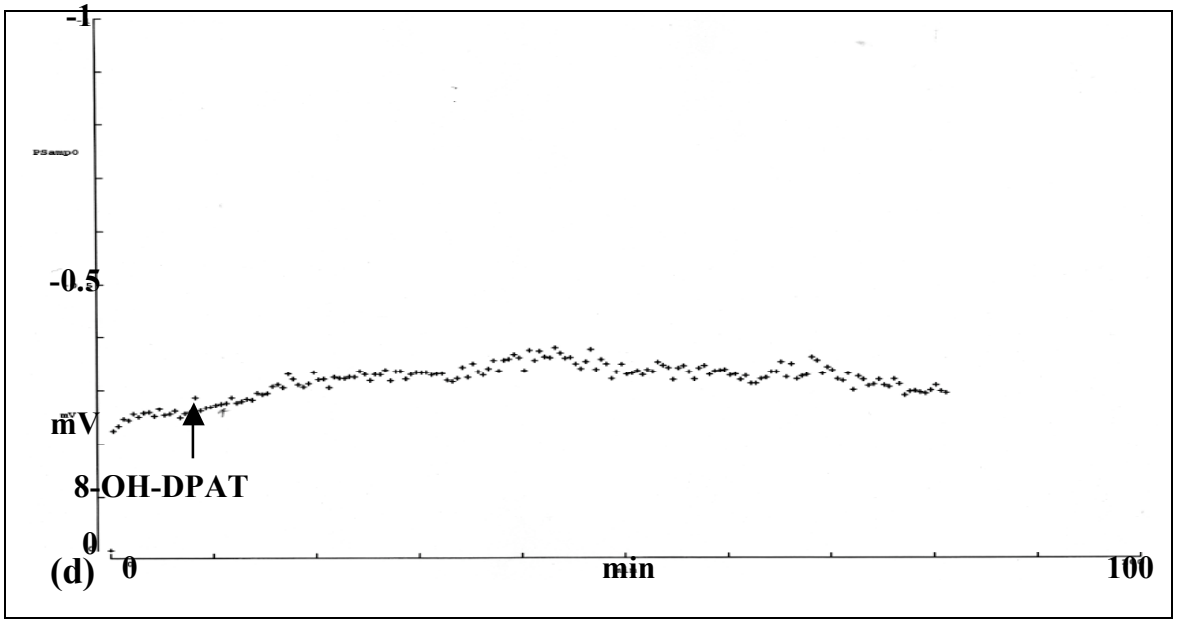


Figure 12: The 5-HT_{1A}-R agonist 8-OH-DPAT treatment results in an increase in population spike in mouse prefrontal cortex. (a) Population spike before drug treatment (b) Population spike after drug treatment (c) The 5-HT_{1A}-R agonist (8-OH-DPAT) treatment of PFC slices elicited a greater than two-fold increase in population spike for 30 minutes of drug treatment

The 5-HT$_{1A}$-R agonist 8-OH-DPAT treatments on PFC slice results in phosphorylation of CaMKinaseII :

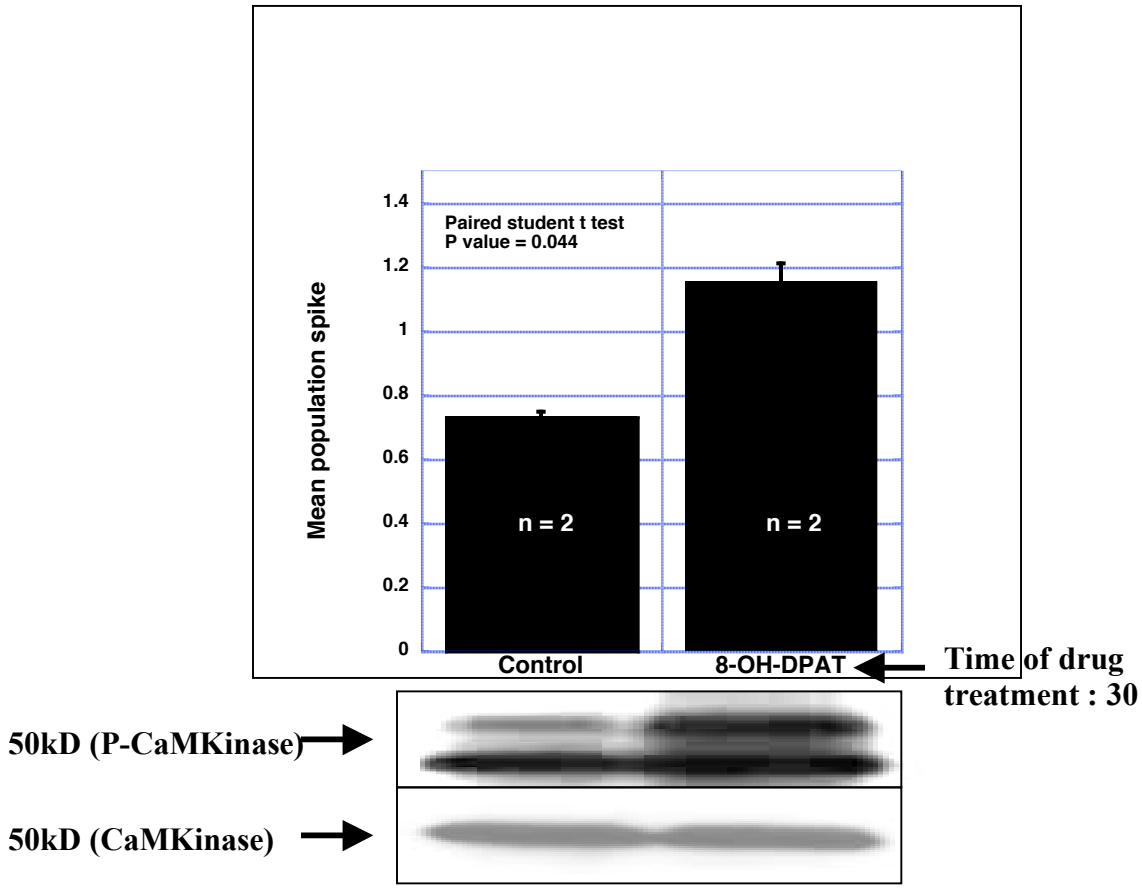
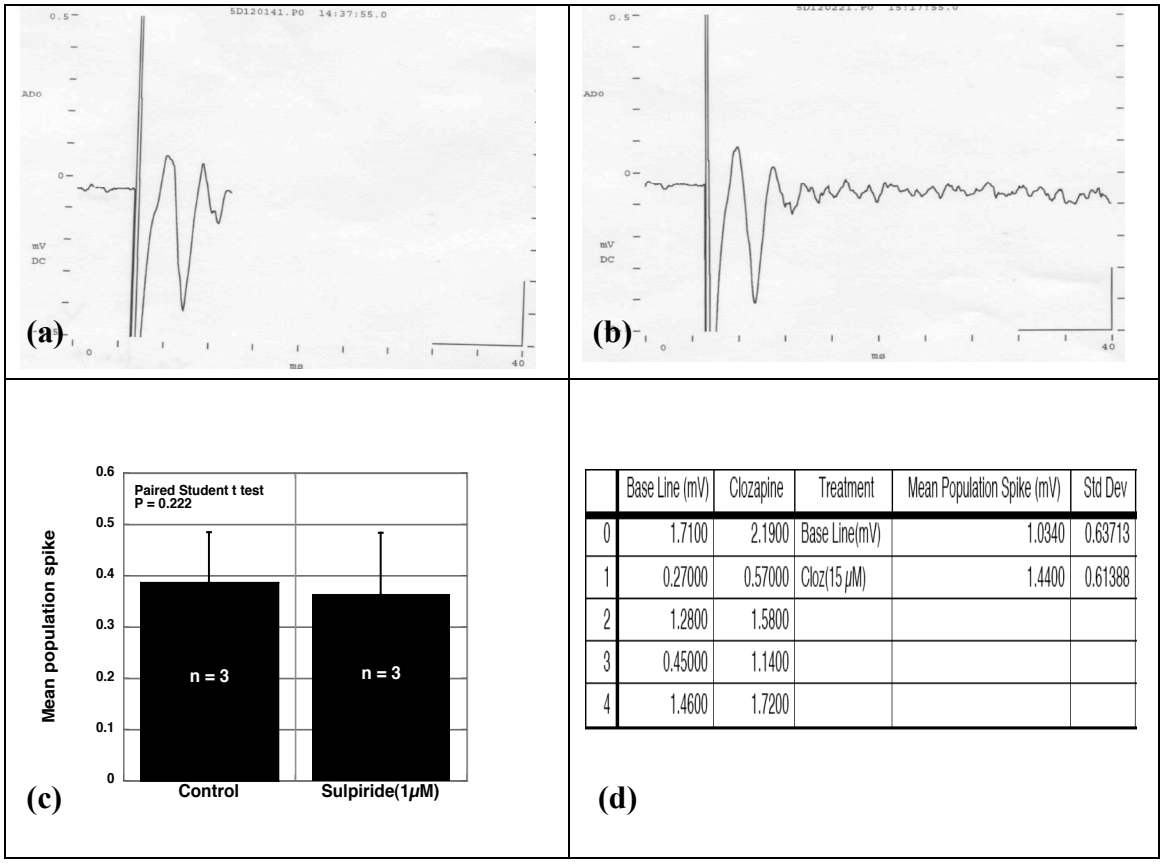


Figure 13: The 5-HT$_{1A}$-R agonist 8-OH-DPAT triggers phosphorylation of CaMKinaseII. P value (0.044) is statistically significant as determined by Paired student t test.

PFC slices from postnatal day 20 to 30 mouse were isolated and treated with the selective 5-HT$_{1A}$-R agonist 8-OH-DPAT for 30 minutes and the increase in population spike was measured by electrical recording. The same slice was then lysed with RIPA buffer and subjected to Western blot

analysis. It showed a significant increase in CaMKinase Phosphorylation. This result further supports the fact that activation of 5-HT_{1A}-R by a specific agonist triggers an activation of CaMKinaseII, which eventually results in an increase in Population spike.

The D_2 receptor antagonist sulpiride did not cause any change in population spike.



	Base Line (mV)	Clozapine	Treatment	Mean Population Spike (mV)	Std Dev
0	1.7100	2.1900	Base Line(mV)	1.0340	0.63713
1	0.27000	0.57000	Cloz(15 μM)	1.4400	0.61388
2	1.2800	1.5800			
3	0.45000	1.1400			
4	1.4600	1.7200			

Figure 14: D_2 receptor antagonist sulpiride failed to bring any change in population spike. (a) Population spike before drug treatment (b) Population spike after drug treatment (c) & (d) The D2-R antagonist sulpiride treatment on PFC slices failed to show any increase in population spike.

Clozapine is known to display a limited antagonism at the dopamine D_2 receptor, the receptor commonly thought to mediate the antipsychotic activity of neuroleptics (Josselyn 1997). To investigate the involvement of D_2-R in the mechanism of action of antipsychotic drug, we treated the PFC slices with specific D_2-R antagonist sulpiride (1μM). Sulpiride was expected to show an increase in population spike the same way clozapine did as both were known to function as D_2-R antagonists. However, sulpiride treatment on PFC slices failed to show any increase in population spike [Figure 14]. Therefore, the involvement of D_2-R in the clozapine-evoked increase in population spike can be ruled out.

The NMDA receptor antagonist APV blocked clozapine-evoked increase in population spike:

In the present study, we have measured the changes in neuronal activity upon clozapine administration by recording population spikes from prefrontal cortex slices. Population spikes are the synchronous action potentials of the field of neurons, triggered by the excitatory post-synaptic potential (EPSP). Usually EPSP is generated when a bundle of axon fibers that send synapses to a group of neurons are electrically stimulated. It causes

the synchronized synaptic release of transmitter (usually glutamate). This activates excitatory receptors (e.g. NMDA receptor) on all of the target neurons, giving rise to a "population excitatory postsynaptic potential (pEPSP or field EPSP, also termed fEPSP when measured outside)". Therefore, the involvement of NMDA receptors in clozapine-evoked increase in population spike can be predicted. The NMDA receptor has been known to play an important role in the pathophysisology of schizophrenia as well as in the mechanism of action of antipsychotic drugs. In order to study the functional involvement of the NMDA receptor in clozapine-mediated increase in neuronal activity, cortical slices were treated with the specific NMDA antagonist APV (2-amino-5-phosphonovaleric acid) (15 μM), 15 minutes prior to clozapine treatment. The experiment showed no significant increase in population spike in the presence of clozapine and APV (P values were greater than 1 as revealed by One Way ANOVA) [Figure 15]. The result strongly indicated the involvement of NMDA receptor in the clozapine-evoked increase in population spike.

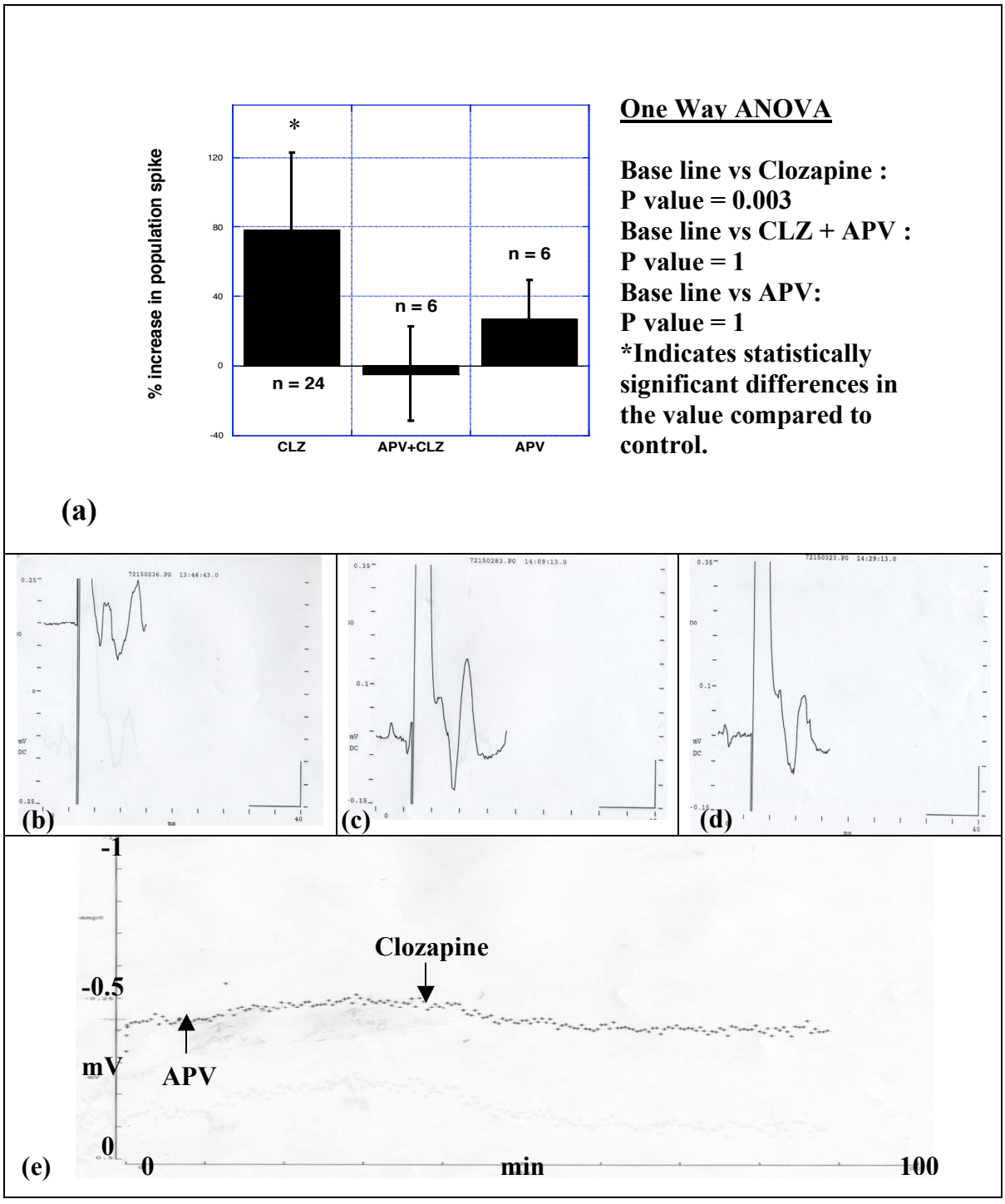


Figure 15: Clozapine-evoked increase in population spike requires the involvement of NMDA-R. (a) & (e) The NMDA-R antagonist APV treatment of PFC slices caused complete blockage of clozapine-evoked increase in population spike (b) Population spike before drug treatment (c) Population spike after treatment with the NMDA antagonist APV (d) Population spike after treatment with clozapine in the presence of APV.

Clozapine potentiates NMDA mediated fEPSP in mouse prefrontal cortex, which requires 5-HT$_{1A}$ receptor activation but does not show involvement of the CaMKII pathway:

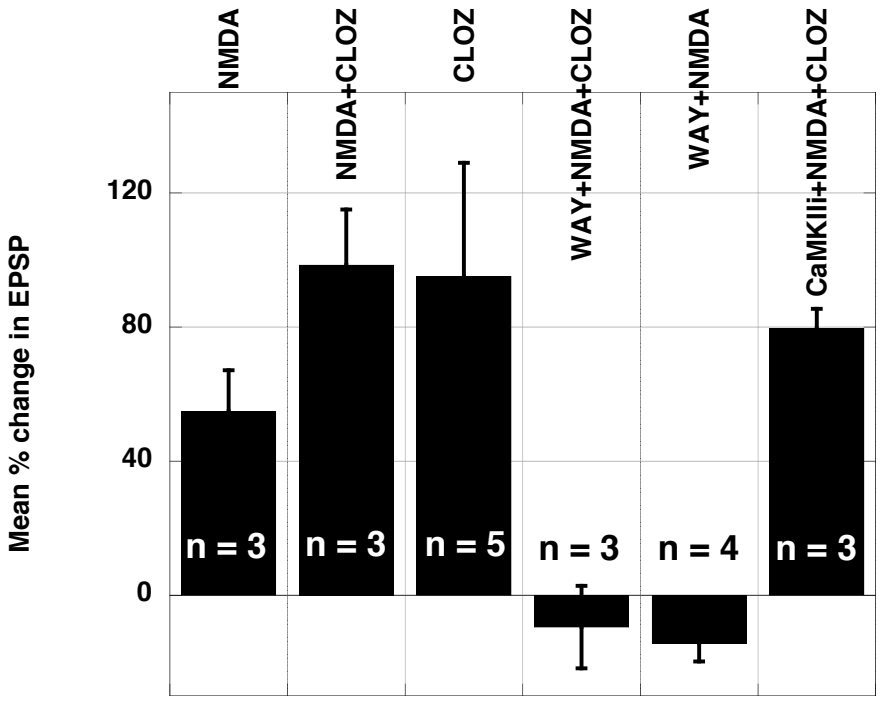


Figure 16: Clozapine potentiates NMDA (N-Methyl-D-aspartate)-evoked increase in fEPSP. The change in fEPSP is completely blocked upon treatment with the 5-HT$_{1A}$-R antagonist WAY100635, whereas a CaMKII inhibitor is unable to eliminate the effect.

Analysis by ANOVA using Bonferroni's all pair's comparison

One Way ANOVA	P value
NMDA vs NMDA+CLOZ :	0.0216
NMDA vs CLOZ :	0.1
NMDA vs WAY+NMDA,CLOZ :	0.0036
NMDA vs CaMKIIi +NMDA,CLOZ :	0.0393
NMDA vs WAY+CLOZ :	0.0001
NMDA+CLOZ vs CLOZ :	0.878
NMDA+CLOZ vs WAY+NMDA,CLOZ :	0.001
NMDA+CLOZ vs CaMKIIi +NMDA,CLOZ :	0.122
NMDA+CLOZ vs WAY+CLOZ :	< 0.0001
WAY+NMDA,CLOZ vs CaMKIIi+NMDA,CLOZ :	0.0005
WAY+NMDA,CLOZ vs WAY+CLOZ :	0.5396

In the previous experiment clozapine-evoked increase in population spike showed an involvement of the NMDA receptor. The understanding of the connection between the 5-HT_{1A}-R signaling pathway and NMDA-R activation is necessary as both the components are involved in the clozapine-mediated increase in population spike and plays an important role in the signaling pathway.

In the present set of experiments the NMDA receptor agonist NMDA triggers an increase in fEPSP recorded from acute PFC slices. Treatment with clozapine potentiates NMDA-evoked fEPSP [Figure 16]. However, the increase in fEPSP observed after clozapine and NMDA treatment never exceeded the fEPSP isolated from the slices treated with clozapine alone.

Pretreatment with the 5-HT_{1A}-R antagonist WAY100635 not only blocks the excitatory effect of clozapine on the NMDA-evoked fEPSP, but it also suppresses the fEPSP below the basal level. One possible explanation may be the direct inhibitory influence of 5-HT_{1A}-R on GABAergic interneurons, which may contribute to a state of depolarization. This depolarization is essential for the removal of Mg^{2+}ion from the ligand-gated NMDA receptor resulting in an increase in EPSP. Overall this effect may be eliminated in the presence of WAY100635. Taken together, the result demonstrates the involvement of 5-HT_{1A}-R in clozapine-evoked potentiation of NMDA current. A CaMKII inhibitor failed to block the clozapine-evoked potentiation of NMDA-mediated fEPSP. This result strongly suggests that the activation CaMKII is not upstream but downstream of NMDA receptor activation in clozapine-evoked signaling pathway.

Chapter 4

Discussion:

A number of atypical antipsychotic drugs have been developed over the last few years. Although, these new drugs have been classified as “atypical”, their widely varying chemical structures and pharmacological actions indicate their heterogeneity. Understanding the mechanism of action of these atypical antipsychotic drugs is not only useful in exploring the pathophysiology of schizophrenia but it also helps in establishing realistic goals for treating schizophrenia (Ananth 2001).

A major breakthrough in schizophrenia research came with the advent of clozapine in early 1950s. Clozapine showed diminished extra-pyramidal side effects (EPS) and clinical superiority in the treatment of refractory psychosis and the possibility of a broader spectrum of clinical efficacy, such as improvement in negative as well as positive symptoms (Remington 2003).

The very unique clinical features of clozapine demand a review of our thinking regarding the pharmacological mechanisms underlying schizophrenia. The superiority of clozapine over already existing highly selective D_2 antagonists (typical or conventional antipsychotic drugs) shifts the focus from Dopamine, in particular the D_2 receptor, to other receptors. clozapine shows different affinities towards various receptors and its

complex pharmacological profile [Table 3] suggests the possibility that the drug works through many receptors.

Table 3: Receptor binding profile for clozapine

Receptor	Relative affinity
Dopamine D_1	++
Dopamine D_2	+
Dopamine D_3	—
Dopamine D_4	++
Serotonin 5-HT_1	++
Serotonin 5-HT_{2A}	+++
Serotonin 5-HT_{2C}	++
Serotonin 5-HT_6	++
Serotonin 5-HT_7	++
α_1-Adrenergic	++
α_2-Adrenergic	++
Muscarinic M_1	+++
Histamine H_1	++
+ = low ; ++ = intermediate ; +++ = high	

(Remington 2003)

Relatively higher affinity of clozapine and other similar atypical antipsychotic drugs towards different serotonin receptors evoked a

considerable interest in the role of serotonin receptors in the action of clozapine (Meltzer 2002).

Previous studies showed that the ability of clozapine to reverse olanzapine-induced catalepsy was blocked by the selective 5-HT_{1A}-R antagonist WAY-100635, suggesting that the effect of clozapine was mediated by the stimulation of the 5-HT_{1A}-R (Meltzer 2002). Systemic administration of atypical antipsychotics also showed an increased extracellular dopamine in the mPFC by a 5-HT_{1A}-R-dependent mechanism (Rollema 1997; Kuroki 1999; Ichikawa 2001). In a separate study, systemic and local administration of clozapine resulted in elevated dopamine release in mPFC of wild type mice, but failed to show the same effect in the 5-HT_{1A}-R knockout mice (Mataix 2005). All these results strongly indicate the involvement of 5-HT_{1A}-R in the mechanism of action of this atypical antipsychotic drug. However, most of the mechanistic studies have focused mainly on dopamine release from neurons. In the present study, we have asked a more fundamental question to find out if clozapine brings about any changes in neuronal excitability in the prefrontal cortex, which is expected to be prime cause of dopamine release.

Electrical recording from layer II, III (and/or other layers) of prefrontal cortex slices of postnatal day 20 to 30 mouse brain have shown that the

treatment with atypical antipsychotic drug clozapine causes a dramatic increase in PFC neurons as measured by population spikes [Figure 5]. Although atypical APDs show relatively lower affinity towards D_2-like receptors, importance of this receptor as a common target cannot be ignored. However, the possibility of involvement of the D_2-R antagonism in clozapine–evoked increase in population spike was excluded, because the selective D_2 receptor antagonist sulpiride did not show any change in electrical activity [Figure 14]. Therefore, the involvement of other receptors, like the 5-HT_{1A} and NMDA receptors was taken into consideration.

Previous studies showed that systemic administration of atypical antipsychotics increased extra cellular dopamine release in the mPFC by a 5-HT_{1A}–R-dependent mechanism. Although the in-vitro affinity of clozapine for the 5-HT_{1A}-R is lower than that of the high affinity antipsychotic drugs like ziprasidone, clozapine shares a common pattern of *in vivo* action to modulate prefrontal dopamine release (Diaz-Mataix 2005). Our study gives a more direct evidence of the involvement of 5-HT_{1A}–R in the mode of action of clozapine in regulating electrical activity in the prefrontal cortex. While administration of clozapine induced a marked increase in population spike in mouse prefrontal cortex, prior bath administration of the selective 5-HT_{1A}–R antagonist WAY100635 completely blocked this response [Figure

6]. These results clearly indicate that the activation of 5-HT_{1A}–R accounts for the observed clozapine-induced increase in electrical activity in mouse prefrontal cortex. Similar studies on 5-HT_{1A}-R knockout mice also showed an increase in population spike in the presence of clozapine [Figure 7]. Collectively these results show that the 5-HT_{1A}-R is possibly involved in the clozapine-evoked increase in population spike in wild type mice, but it also indicates the involvement of a different receptor in 5-HT_{1A}-R (-/-) mice.

To further understand the signaling mechanisms by which clozapine increased the electrical activity, PFC slices were treated with selective antagonists to multiple signaling components and the change in electrical activity was measured in the presence of clozapine.

Studies with Phospholipase C beta-1 knock out mice showed schizophrenia-like symptoms, which were rescued by clozapine administration (McOmish 2007). Signaling studies have revealed that 5-HT_{1A}-R, which is a G protein- coupled receptor, is further linked to different effector molecules like Phospholipase C (stimulation), Adenylate cyclase (inhibition), N-type calcium channel (inhibition), Hyperpolarizing K^+ channel (stimulation), MAPKinase cascade (stimulation) (Mehta 2007).

To identify the effector molecules involved in the mechanism of action of clozapine *via* 5-HT_{1A}-R activation, slices were treated with a phospholipase

C inhibitor U73122, a CaMKII inhibitor CaMKIINtide (Myristoylated) and a MAPK kinase inhibitor PD98059 prior to the administration of clozapine. Both phospholipase C inhibitor U73122 and CaMKII inhibitors suppressed the Clozapine-mediated increase in Population Spike. In contrast, the MAPkinase kinase inhibitor PD98059 failed to block the effect. These results clearly demonstrate the functional roles of Phospholipase C and CaMKII in clozapine-mediated increase in population spike. Taken together, it is likely that clozapine functions as a 5-HT_{1A}-R agonist to activate PLC and CaMKII that finally cause an increase in population spike [Figure 8].

Meltzer and co-workers showed that atypical antipsychotic drug-induced cortical dopamine release was caused by $5HT_{2A}$ and D_2 receptor blockade via $5HT_{1A}$ receptor activation in the rat medial prefrontal cortex (Ichikawa 2001). But the D_2 receptor antagonist sulpiride did not cause an increase in population spike. This result eliminates the possibility of D_2 receptor blockade as a mechanism of clozapine-evoked increase in population spike. Western blot analysis revealed that clozapine treatment of PFC slices causes a time-dependent activation of CaMKII, which peaked after 15 min of drug treatment. However, similar drug treatment in the presence of 5-HT_{1A}-R antagonist WAY100635 showed complete inhibition of CaMKII during the course of drug treatment. In this particular experiment we have made an

interesting observation that WAY100635 not only blocked CaMKII activation, but also reduced the activity of the enzyme even below the basal level (control + WAY) with increased time of exposure to the drug (clozapine + WAY100635). This phenomenon can further be explained by the inverse agonist property of WAY100635, which reverses constitutive activity of the receptor and exerts the opposite pharmacological effect of the receptor agonist. There is experimental evidence that the so-called silent 5-HT_{1A}-R antagonist WAY100635, shows inverse agonist properties to cloned human 5-HT_{1A}-R and also the receptors in rat hippocampus and cortex (Cosi 2000; Abbas 2007).

As revealed by previous reports, like atypical antipsychotic drugs, R (+) 8-OH-DPAT-mediated stimulation of 5-HT_{1A} receptors caused an increase in dopamine release in the mPFC, and this increase was completely blocked by WAY100635 (Rollema 1997; Kuroki 1999; Ichikawa 2001). In our study, 5-HT_{1A}-R agonism, which is thought to be responsible for clozapine-evoked increase in population spike, is further supported by the experimental results where the selective 5-HT_{1A}-R agonist 8-OH-DPAT also elicited in an increase in synaptic activity in a population of neurons [Figure 12]. The same slice, which showed an increase in electrical activity, also exhibited an increased level of CaMKII phosphorylation after DPAT treatment [Figure

13]. All these findings suggest that the 5-HT_{1A}-R agonism may trigger CaMKII phosphorylation and the atypical antipsychotic drug clozapine stimulates such a pathway.

If the activation of 5-HT_{1A}-R and CaMKII are the identifying mechanisms involved in clozapine-mediated increase in synaptic activity, it is most likely that these two components are within the same signaling pathway. To understand the connection between 5-HT_{1A}-R and CaMKII activation in clozapine-mediated signaling, we performed electrophysiology experiments with 5-HT_{1A}-R (-/-) mice in the presence of a CaMKII inhibitor and WAY100635. PFC slices from 5-HT_{1A}-R (-/-) mice were treated with clozapine in the presence of a CaMKII inhibitor. The CaMKII inhibitor failed to inhibit the clozapine-elicited increase in population spike in the knockout mice [Figure 9]. This piece of data further establishes the fact that the clozapine-evoked increase we have seen in wild type mice may be due to 5-HT_{1A}-R-mediated activation of CaMKII but the clozapine-evoked increase in electrical activity observed in 5-HT_{1A}-R knockout mice does not require CaMKII activation.

While the results above point to a mechanism involving clozapine-mediated activation of the signaling pathway 5-HT_{1A}-R→PLC→CaMKII, which eventually results in an increase in population spike, they cannot

exclude an additional contribution of other receptors like the NMDA receptors.

Antagonism of the glutamatergic NMDA receptor complex has been reported to produce schizophrenia-like behavioral and cognitive deficits in normal subjects (Goff 2001). Electrical recording from PFC slices have shown that the atypical antipsychotic drug clozapine but not the typical antipsychotic drug haloperidol produces bursts of excitatory postsynaptic potentials (EPSPs) and the effect is inhibited by a glutamate receptor antagonist (Arvanov 1997). In the present study, we have examined the involvement of the NMDA receptor in clozapine-mediated modulation of electrical activity in prefrontal cortex. Bath application of the NMDA antagonist APV prior to clozapine administration showed a complete inhibition of clozapine-triggered increase in population spike.

5-HT_{1A}-R being an inhibitory receptor, the activation of this receptor is expected to cause hyperpolarization and therefore inhibition of action potential. However, in the present study, we have seen that clozapine – evoked activation of 5-HT_{1A}-R triggered an increase in neuronal activity in PFC. 5-HT_{1A} receptors have been reported to be expressed by GABAergic interneurons in the mPFC. Previous studies have also shown that the effect of different antipsychotic drugs and 5-HT_{1A}-R agonist BAY3702 on

extracellular dopamine concentration was cancelled by GABA-R antagonist bicuculline (Diaz-Mataix, 2005). Results from this study as well as ours indicate that stimulation of the 5-HT_{1A} receptors on the inhibitory GABAergic interneurons results in the suppression of this inhibitory effect, thereby causing depolarization of the post-synaptic neurons in layers II and III. This depolarization could then cause an increase in neuronal excitability by activation of excitatory NMDA receptor.

Therefore, the summary of the experimental results is as shown in Figure 17.

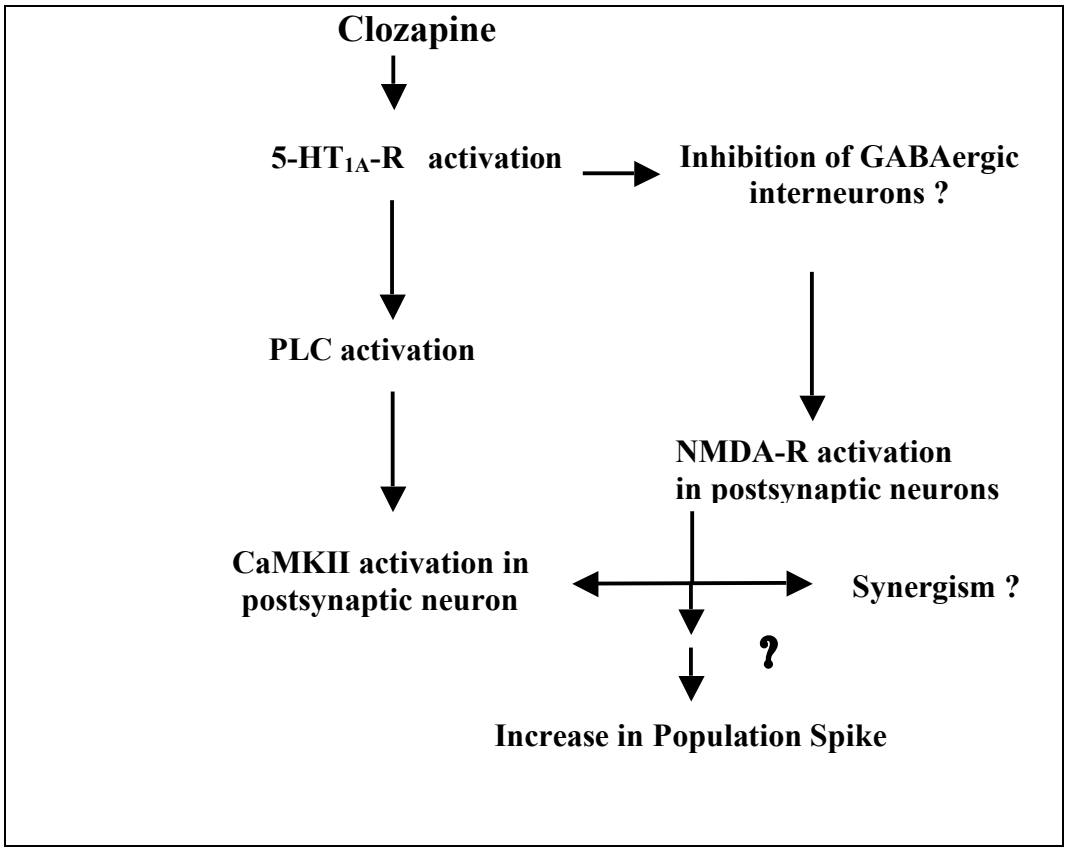


Figure 17: Summary of the experimental results.

Since treatment with both NMDA-R and 5-HT_{1A}-R antagonists results in a complete blockage of the clozapine-triggered increase in population spike, it is possible that both the receptors are in some way linked within the signaling pathway. However, we do not yet have any enough information to answer the following question:

- How do 5-HT_{1A}-R and NMDA-R connect in the proposed signaling pathway?

One possible hypothesis is that clozapine elicits activation of both the receptors, thereby stimulating two parallel pathways, which may eventually trigger an increase in population spike. The alternative hypothesis is both the receptors are someway connected in the mode of action of clozapine. To address this question, we next examined the effect of clozapine on NMDA-evoked fEPSPs recorded in the absence and presence of the selective 5-HT_{1A}-R antagonist WAY100635. Since bath administration of the NMDA receptor agonist NMDA showed an increase in amplitude of fEPSP, we conducted electrical recording in the presence of clozapine and NMDA to examine if clozapine would elicit any change in the NMDA-evoked EPSP. Interestingly, treatment with clozapine further potentiated the NMDA-evoked current [Figure 16]. However, the increase in EPSP observed after

clozapine and NMDA treatment never exceeded the effect of clozapine alone. This result indicates that clozapine alone can occlude the facilitating effect it has on NMDA-evoked EPSP.

Administration of the 5-HT_{1A}-R antagonist WAY100635 not only blocked the excitatory effect of clozapine on NMDA-evoked fEPSP, but also elicited a sub-basal level of fEPSP. One possible explanation may be the direct inhibitory influence of 5-HT_{1A}-R on GABAergic interneurons, which may contribute to a state of depolarization. This depolarization may be essential for the removal of Mg^{2+}ion from the ligand-gated NMDA receptor resulting in an increase in EPSP. Overall this effect may be eliminated in the presence of WAY100635. The result further demonstrates the involvement of 5-HT_{1A}-R in clozapine-mediated potentiation of NMDA current [Figure 16].

If CaMKII functions upstream of NMDA receptor activation by clozapine, we may expect to see the inhibition of clozapine-potentiated NMDA current in the presence of CaMKII inhibitor. However, a CaMKII inhibitor failed to block the effect of clozapine on NMDA-mediated EPSP [Figure 16]. This result strongly indicates that the activation of CaMKII is not upstream but downstream of NMDA receptor activation in clozapine-evoked signaling pathway.

This study further opens the opportunity in several areas to investigate:

- What is the downstream pathway following CaMKII activation that eventually results in an increase in population spike?

One way to address these questions is by looking for a possible link between Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and the NMDA receptor, two molecules that have been known to play major roles in synaptic plasticity. CaMKII is a multifunctional protein abundant in brain cytosol and in postsynaptic densities. Postsynaptic Ca^{2+} influx is known to trigger autophosphorylation at Thr^{286} residue in the autoinhibitory domain of the enzyme, which makes the kinase persistently active and causes translocation of soluble CaMKII to the postsynaptic densities (Strack 1998). Multiple lines of evidence indicate that autophosphorylation of CaMKII is necessary for NMDA receptor–dependent LTP. On the other hand, LTP induction in both the hippocampal and neocortical pyramidal neurons depends on the activation of NMDA-type glutamate receptors and Ca^{2+} influx through these receptors play a crucial role in the induction of LTP (Schiller 1998). Postsynaptic density-associated CaMKII phosphorylates ionotropic glutamate receptors, thereby providing a route to increased synaptic strength (Strack 1998). Recent studies have revealed a fascinating fact regarding CaMKII–NMDA interaction. Ca^{2+}/calmodulin binding causes

the autoinhibitory domain to move away, thereby activating the kinase. Moreover, the interaction of the NR2B subunit of NMDAR with the active enzyme prevents the return of the autoinhibitory domain, thereby keeping the kinase persistently active. This results in hyper-phosphorylation of the enzyme, a condition that causes the enzyme to remain active even in the absence of Ca^{2+} (Lisman 2001; Fink 2002). This hyperactivation of CaMKII may have multiple consequences:

- Active CaMKII may phosphorylate AMPA receptors (at $Ser^{831}$of the GluR1 subunit) eliciting a significant increase in EPSP, which may further be potentiated during LTP (Lisman 2001; Semyanov 2001).
- The active kinase may phosphorylate local phosphatase molecules, thereby preventing dephosphorylation of CaMKII (Lisman 2001).
- Hyperphosphorylation of CaMKII may trigger the assembly of molecular linkage in which different structural proteins along with actin-binding protein serve as linking elements to connect the NMDAR with the GluR1 subunit of AMPAR. This process is believed to be involved in the delivery of additional AMPAR into synaptic sites which may contribute to LTP (Lisman 2001; Fink 2002).

On the basis of our data and literature findings we would like to postulate a scheme, in which clozapine functions as an agonist at the 5-HT_{1A}-R, which triggers activation of Phospholipase C, thereby resulting in a rapid release of Ca^{2+} in the cytosol of a postsynaptic neuron. Elevated levels of Ca^{2+} result in an activation of cytosolic CaMKII. On the other hand clozapine treatment may stimulate the excitatory NMDAR via 5-HT_{1A}-R activation (by inhibition of GABAergic interneurons) causing a transient influx of Ca^{2+} in the postsynaptic neurons, which may trigger accumulation of CaMKII in the postsynaptic densities. In the postsynaptic region, interaction between NMDAR and CaMKII creates a persistently active state for the enzyme, which eventually triggers phosphorylation of AMPAR, thereby increasing single channel conductance through the receptor (Fink 2002). Both Ca^{2+} -triggered excitation of NMDA and AMPA receptor may result in an increase in population spike.

In the present study, we have made an interesting observation that clozapine-evoked increase in population spike requires activation of CaMKII via 5-HT_{1A}-R activation. However, the facilitating effect of clozapine on NMDA-evoked EPSP is not dependent on CaMKII activation. From this observation we can predict that clozapine-evoked

increase in population spike requires simultaneous activation of CaMKII as well as NMDA receptor in postsynaptic neuron via 5-HT_{1A}-R activation. Synergistic actions of these two pathways allow the postsynaptic neurons to reach a threshold potential and eventually result in an increase in population spike. On the other hand, facilitating effect of clozapine on NMDA-evoked fEPSP does not require synergistic actions of these two parallel pathways. Clozapine-mediated activation of 5-HT_{1A} R alone can trigger activation of excitatory NMDA receptor (possibly through inhibition of GABAergic interneurons) without any activation of CaMKII thus causing an increase in EPSP. Finally we can conclude that, the possible clozapine-mediated pathway resulting in an increase in electrical activity in prefrontal cortex may be the following:

Clozapine evoked 5-HT_{1A}-R activation causes phospholipase C activation, which finally results in an activation of CaMKII. Furthermore, 5-HT_{1A}-R activation may trigger NMDAR activation (Possibly by inhibiting inhibitory GABAergic interneurons) and can result in an increase in Ca^{2+} influx in the postsynaptic neurons, which may trigger activation of CaMKII. Synergistic interaction between NMDAR and CaMKII may create a hyperphosphorylated active state of the kinase, finally resulting in an increase in the population spike by AMPA receptor

phosphorylation. Clozapine also triggers an increase in NMDA-evoked EPSP via 5-HT_{1A}-R-mediated activation of excitatory NMDA receptor. However, this increase in fEPSP does not require synergistic activation of CaMKII.

Hypothesis 1:

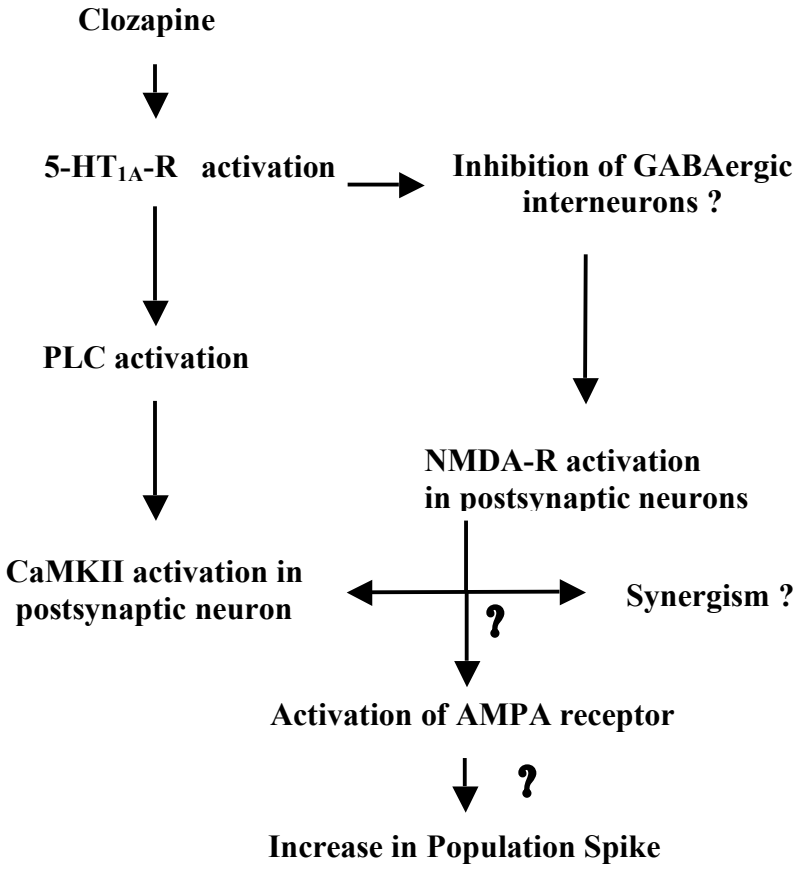


Figure 18: Proposed Clozapine-mediated signaling pathway which results an increase in population spike.

Hypothesis 2:

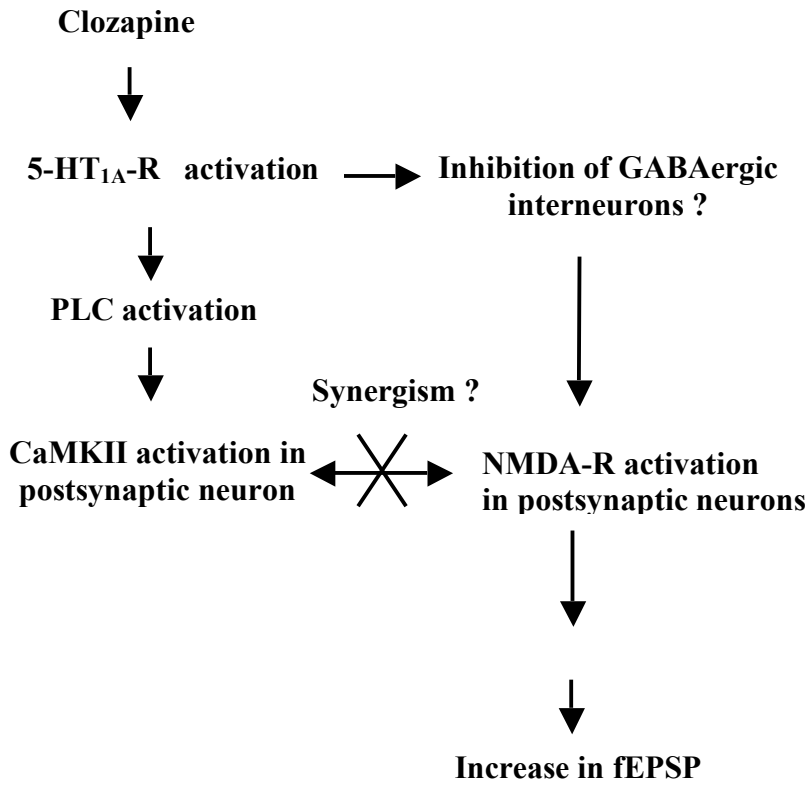


Figure 19: Postulated clozapine-mediated signaling pathway, which facilitates NMDA-evoked EPSP.

- We have also made an interesting obersevation that the 5-HT_{1A}-R antagonist WAY100635 inhibits NMDA-evoked fEPSP even in the absence of clozapine [Figure 16]. This interesting finding indicates a possible interaction between the antagonist and the NMDA receptor, which requires further investigation.

- Clozapine-mediated activation of the NMDA receptor is dependent on 5-HT_{1A}-R activation, which may possibly involve inhibition of inhibitory GABAergic interneurons (Millan 2000). The nature of interaction between these two receptors and possible interaction with GABAergic interneurons opens a new area of research.

Final thought:

The major purpose of this project is to elucidate mechanistic events triggered by atypical antipsychotic drug clozapine, which eventually lead to an increase in electrical activity in mouse prefrontal cortex. The overall study will not only help in developing new therapeutic approaches to treat schizophrenia, but also shed a new light on the putative "Atypicality" of this new generation antipsychotic drug.

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