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**BEHAVIORAL ANALYSIS OF CONDITIONS AND TREATMENTS AFFECTING
MOVEMENT AND NOCICEPTION**

by

Indu Mithra Madhuranthakam

A Thesis

Submitted to the
Department of Chemistry and Biochemistry
College of Science and Mathematics
In partial fulfillment of the requirement
For the degree of
Master of Science in Pharmaceutical Sciences
at
Rowan University
March 26, 2021

Thesis Advisor: Thomas M Keck, Ph.D.

Committee Members:
Kandalam Ramanujachary, Ph.D.
Subash Jonnalagadda, Ph.D.

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Abstract

Indu Mithra Madhuranthakam
BEHAVIORAL ANALYSIS OF CONDITIONS AND TREATMENTS AFFECTING
MOVEMENT AND NOCICEPTION

2019-2021

Thomas M Keck, Ph.D.
Master of Sciences in Pharmaceutical Sciences

This thesis work includes three projects. First part deals with the investigation of novel D2 receptor ligands in Prepulse inhibition. The goal of this study is to establish a relationship between dopamine receptor antagonists and agonists and prepulse inhibition which can then serve as a working model for an in-vivo efficacy of novel dopamine D2 drugs. The second part of the thesis work deals with Niemann pick disease type C. Niemann pick disease type C is a progressive genetic disorder that is characterized by the lysosomal accumulation of lipids which causes neurodegeneration, dementia, ataxia, and death. NPC1^{nmf164} mutant mice (knockout) have a delayed acquisition of motor skills during development. The aim of our study was to identify cellular and molecular pathways involved in causing and progressing NPC in young patients from which effective therapeutic interventions can be designed and tested to prevent or delay the onset of the disease and prolong and improve the quality of life of young NPC patients. Finally, the last part of this thesis work includes studies on synergistic anithyperalgesic and antinociceptive effects of morphine and MP-III-024, a positive allosteric modulator at $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors. The purpose of this study was to examine the interactive effects of the μ -opioid agonist morphine and the $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptor positive allosteric modulator MP-III-024 in preclinical models of mechanical hyperalgesia and thermal nociception.

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Chapter 1

Investigating Novel Dopamine D2 Receptor Ligands in Prepulse Inhibition

Introduction

Prepulse inhibition is the reduction in startle amplitude to an acoustic startle stimulus when preceded by a weaker pre-stimulus within a certain time frame (fig.1). The information processing begins during the initial 100ms following the onset of a stimuli with a set of preattentive automatic processes in organisms with complex nervous systems that trigger different response mechanisms, one among which is the startle response, a neuronal reflex mechanism that prepares an organism to face a potentially relevant stimulus. Therefore, acoustic startle response is a protective mechanism that is elicited by a sudden and intense acoustic stimulus¹. PPI is normal in healthy individuals but impaired in patients with schizophrenia and Alzheimer's disease due to abnormalities in sensorimotor gating. Commercially available drugs used in the treatment of psychotic disorders, such as the dopamine D2 receptor antagonist haloperidol, increase PPI. The goal of this study is to establish a relationship between dopamine receptor antagonists and agonists and prepulse inhibition which can then serve as a working model for an in-vivo efficacy of novel dopamine D2 drugs.

Reflex and Its Importance

Reflex is an involuntary movement that arises in response to a peripheral stimulus and gets conducted to the nerve center in the brain or spinal cord. Spinal cord is an abode of the centers that govern reflex movements in addition to the ones that control certain

groups of muscles. The reflex begins in an impulse upon the stimulation of a sensory or afferent nerve. It is conducted to the center in the spinal cord, from the sensory cell bodies to the neurons of the corresponding motor center. As a result, a motor impulse originates in the motor nerve which then is transmitted to the muscle fibers that are supplied by the nerve. This complete path is called as a reflex arc. A reflex arc is complicated when sensory impulses are conducted to the higher or lower-level centers and excite several motor impulses. If the arc, either in its sensory or motor limb or in the center, is interrupted, the reflex is lost. Spinal reflexes are very important for survival and include the stretch reflex, the Golgi tendon reflex, the crossed extensor reflex, and the withdrawal reflex³.

The stretch reflex, also called a myotatic reflex is a monosynaptic reflex that causes muscle contraction in response to stretch within the muscle. These are the shortest latency reflexes as they travel the shortest distance between the muscle and spinal cord and from the same segment of the spinal cord that it entered to the muscle. The knee jerk reflex is an example of a stretch reflex³.

Golgi Tendon reflex is a negative feedback mechanism that prevents the tendon from breaking due to muscle force by causing muscle relaxation². As tension builds in the tendon, the Golgi tendon organ gets stimulated and propagates nerve impulses along the sensory neurons into the spinal cord where it synapses with and activates an inhibitory interneuron that releases the neurotransmitter glycine and inhibits the α motor neuron, a neuron that innervates extrafusal muscle fibers of skeletal muscle and initiate contraction³.

Crossed extensor reflex is a contralateral stimulus that occurs on the opposite side of the body from the stimulus. Upon receiving the stimulus, sensory nerve fibers cross to the

contralateral side of the spinal cord from the stimulated side of the body and synapse with and inhibit the alpha motor neurons that innervate the muscles of the contralateral limb. An example of this reflex action is when a person steps on a nail. The painful stimulus propagates and causes the flexors of the ipsilateral leg (the one that experiences the stimulus) to contract and extensors to relax. On the contralateral leg (the other leg that bears the weight of the body) the flexors relax and the extensors contract to support the weight of the body³.

Withdrawal reflex is a protective reflex that protects the body from damaging stimuli. It is polysynaptic and an example is when a person withdraws his hand from a hot object even without consciously wanting to do so. The sensory neurons get stimulated by the receptors that receive heat and synapse with the interneurons that relay message to the motor neurons. The motor neurons that innervate flexors allow them to withdraw from the stimulus³.

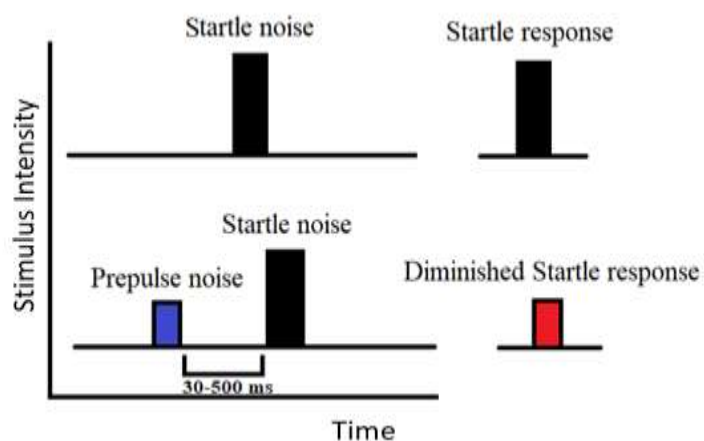
A reflex need to be sufficiently constant and noticeable to give importance to its alterations, particularly in case of its absence, in normal subjects. Reflexes of the lower extremity are more vital than those of the upper extremity and knee-jerk is the prominent among them. Babinski reflex, ankle clonus, Achilles jerk, abdominal reflex, the cremasteric or inguinal reflex, epigastric reflex, and planter reflex etc., are some of the other reflexes that are important. Absent or diminished or exaggerated reflexes are indicative of diseases of the central nervous system and muscles that are involved in that reflex⁴.

Startle Reflex

The startle reflex response is induced by stimuli of adequate intensity reflecting the activation of motor paths in the bulbopontine reticular formation of the brainstem. Within a few milliseconds, facial and skeletal muscles are activated, leading the whole body to flinch in rodents due to changes in the electrical activity. In rats, the startle response can be observed within 5msec in the neck and 8msec in the hindleg. Neural pathways mediate this acoustic startle response attributing to its very short latency. According to Davis *et al.*, the acoustic startle response in the rat is mediated by three synapses:- 1) cochlear root neurons; -, 2) neurons in the nucleus reticularis pontis caudalis and 3) motoneurons in the facial motor nucleus or spinal cord⁵.

Figure 1

Difference Between Startle Response with and Without Prepulse Stimulus in Healthy Individuals.

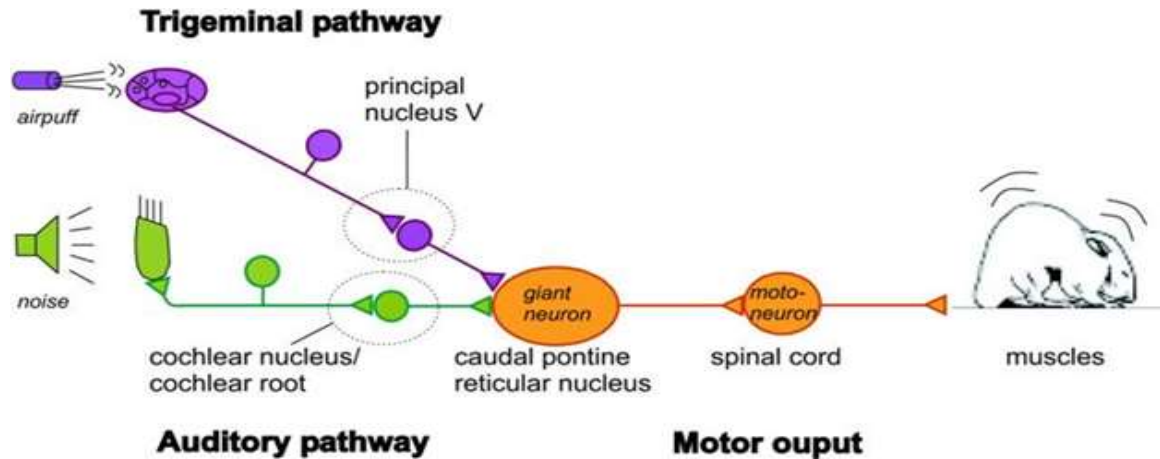


Neural Pathway for Acoustic Startle Response

The neural pathway for Acoustic Startle Response begins in the cochlear nucleus upon receiving the stimulus. This signal is transferred to the cells in the nucleus reticularis pontis caudalis (PnC) located in the pons of the brainstem which then reaches the reticular formation neurons that form circuits with the motor neurons of spinal cord or facial motor neurons which control the muscle movements (fig.2)^{2,3}. This causes the startle response⁶. The startle response is adaptive in nature in terms of its plasticity in response that it can be either be high or low depending on the intensity of the stimulus produced and the circumstances. For instance, when the organism is in a state of emotional activation the startle response is augmented because of a non-associative learning process called sensitization or attenuated due to a process called habituation, which is because of repeated presentations of stimulus. The other circumstance in which the startle reflex is decreased is when presented with a low intensity stimulus before a startle stimulus, a process known as prepulse inhibition¹.

Figure 2

Trigeminal Pathway and Startle Response



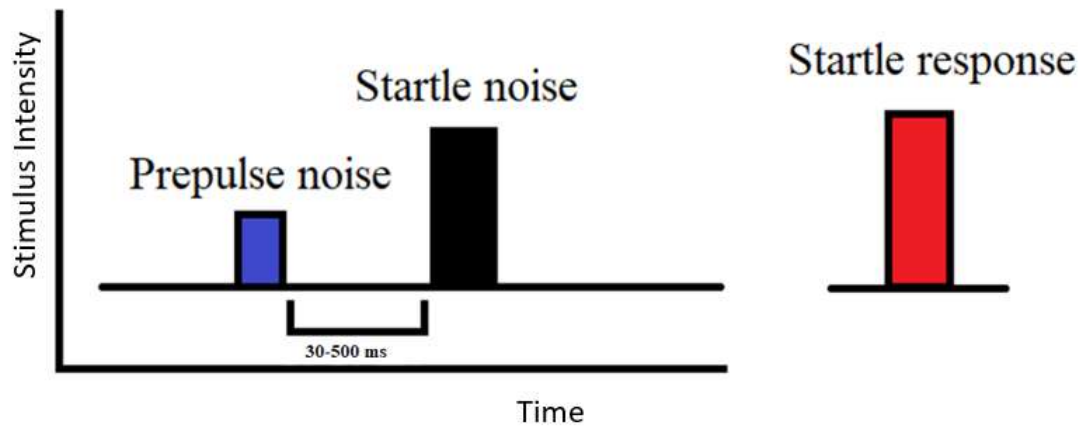
Prepulse Inhibition, Sensorimotor Gating, and Schizophrenia

Prepulse inhibition plays a key role in filtering relevant and irrelevant sensory information, alteration of which causes an overload of sensory information and can be used as a measure of sensorimotor signaling which is impaired in patients with Schizophrenia¹(fig.3). Several studies confirmed the presence of PPI disruption in schizophrenia spectrum patients and in other disorders with disrupted gating mechanisms which in healthy people involves an integrated symphonic array of time-coordinated events across multiple sites that are brought about by a cascade of neurons in multiple loci. Thus, PPI is considered as an operational measure of sensorimotor gating which gates out of awareness the excess or insignificant stimuli, so that an individual can focus on the most important aspects of the stimulus-loaded environment. We use the term sensorimotor

gating to describe the construct calculated by PPI because it is involved in the weak sensory event or stimulus (the prepulse) inhibiting (or gating) the startle motor response to a startling event or stimulus⁷.

Figure 3

Impaired Prepulse Inhibition in Schizophrenia Patients.



Animal studies suggest that PPI can be because of the descending forebrain circuitry of cortico-striato-pallido-pontine (CSPP) interconnected substrates. The regulatory tone is transmitted by the pedunculo-pontine nucleus from the forebrain via descending ventral projections from ventral pallidum to the primary startle circuitry thus altering the inhibitory effect of the prepulse stimulus on startle reflex. The psychopathological manifestations observed in schizophrenia patients is because of this circuitry overlapping with the regions

associated with the disease symptoms. Sensorimotor signaling is further influenced by any structural abnormalities in the brain regions related directly or indirectly to PPI regulation⁸.

Literature has constantly demonstrated the reduction in prepulse inhibition in patients with schizophrenia compared to healthy subjects and has thus proposed PPI as a biomarker of the disease¹.

Schizophrenia

It is a serious psychotic illness characterized by an array of symptoms that can be categorized into positive, negative and cognitive symptoms, which when left untreated can be persistent and disabling. Positive symptoms include hallucinations, delusions and thought disorder. Negative symptoms include avolition, anhedonia, flat affect and reduced speaking. Cognitive symptoms of schizophrenia are subtle in some patients while in others they are more prominent and include difficulty in decision making, paying attention, and learning new things⁹. There are several theories as to what causes schizophrenia that center on either an excess or a deficiency of neurotransmitters such as dopamine, serotonin, or glutamate.

Dopamine Hypothesis of Schizophrenia

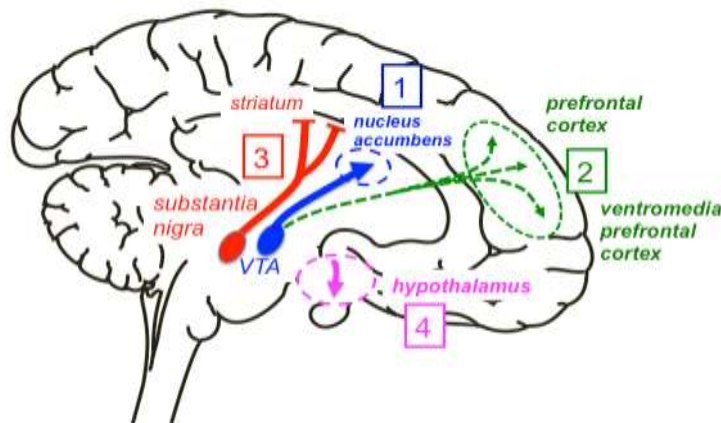
It is the widely accepted theory, and it involves four dopaminergic pathways (fig.4). The low dopamine levels within the nigrostriatal pathway that extends from the substantia nigra to caudate nucleus is responsible for motor symptoms. Excess dopamine levels in the mesolimbic pathway that originates from the ventral tegmental area (VTA) and ends in the limbic areas cause positive symptoms of schizophrenia. Negative symptoms

and cognitive deficits in schizophrenia are caused by dopamine levels in the mesocortical pathway that extends from the VTA to the cortex. The decrease or blockade dopamine levels in tuberoinfundibular pathway extending from the hypothalamus to the pituitary gland results in elevated prolactin levels and causes galactorrhea, amenorrhea, and reduced libido¹⁰.

Figure 4

Dopamine Pathways Involved in Schizophrenia

Four Dopamine Pathways & Schizophrenia



- 1) Mesolimbic (SCZ - increase in DA causes positive symptoms)
- 2) Mesocortical (SCZ - DA hypoactivity; negative & cognitive & affective symptoms)
- 3) Nigrostriatal (Drugs - EPS & TD drug side effects)
- 4) Tuberohypophyseal (Drugs - hyperprolactinemia side effects)

Role of Dopamine Receptors. Heightened D2 receptor activation due to increased subcortical release of dopamine is responsible for the positive symptoms and reduced D1 receptor activation in the prefrontal cortex for the negative symptoms of schizophrenia¹¹.

Serotonin Hypothesis of Schizophrenia

This hypothesis was developed when a structural similarity between serotonin (5-hydroxytryptamine; 5HT) and lysergic acid diethylamide (LSD), a hallucinogenic drug, was established 40 years ago¹². Drugs that blocked both dopamine and serotonin instead of just dopamine were found to be more effective in alleviating both positive and negative symptoms of schizophrenia¹¹.

Glutamate Hypothesis of Schizophrenia

This theory is centered on the lack of glutamate, an excitatory neurotransmitter, activity at the NMDA receptor. Increased co-agonists at NMDA receptor showed some efficacy in clinical trials in schizophrenic subjects but not yet firmly established¹³.

Prepulse Inhibition and Other Psychiatric Disorders

Prepulse inhibition is deficient in patients with Huntington's disease, obsessive compulsive disorder, nocturnal enuresis, bipolar disorder, Asperger's syndrome, fragile X syndrome, 22q11 syndrome, Alzheimer's disease, blepharospasm, and Tourette syndrome¹⁴. Studies in OCD patients revealed dysfunctional PPI which was hypothesized to be due to abnormalities in striatal regions and sensorimotor gating. In another study, it was found that OCD patients with a history of a tic disorder were more likely to have PPI deficiency¹⁵.

Huntington's disease is a genetic neurodegenerative disorder that causes progressive breakdown of nerve cells in the brain. Patients with HD have movement abnormalities, cognitive impairments, and emotional disturbances¹⁶. Corpus striatum plays an important role in inhibiting involuntary and intrusive movements. Degeneration of striatum in Huntington's disease causes a loss of motor inhibition which is manifested as abnormal involuntary choreiform movements. Sensorimotor abnormalities or inhibition in such patients can be measured using startle reflex¹⁷.

Bipolar disorder shows similar psychotic symptoms as schizophrenia such as hallucinations and delusions. Deficits of PPI in bipolar disorder patients were like the deficits in schizophrenia. Studies suggested that active manic symptoms and acute psychosis might be responsible for PPI dysfunction in bipolar patients¹⁸.

Patients with major depressive disorder showed lower PPI values compared to the control subjects in research studies but the impairment was not as profound as was observed in patients with psychotic symptoms¹⁹.

Alzheimer's disease is a progressive neurodegenerative disease that is characterized by cognitive and memory deficits due to the structural and functional abnormalities including neurofibrillary tangles, amyloid- β accumulation and neuron loss. Decreased prepulse inhibition and increased latency to startle was observed in the transgenic mouse model Tg4-42²⁰.

Post-traumatic stress disorder is a condition that develops in people who have experienced an injury or a traumatic event. In such patients, strong evidence from previous studies show

an increased baseline startle amplitude. PPI in PTSD patients isn't quite conclusive yet due to controversial results. Also, the causes of altered PPI values must be assessed with further caution as baseline startle amplitude is elevated in the patients²¹.

Niemann Pick Disease Type C is a progressive genetic disorder characterized by the lysosomal accumulation of lipids which causes neurodegeneration, dementia, ataxia, and death. In some cases of adult-onset NPC, psychiatric disorders such as schizophrenia, attention deficit hyperactivity disorder, and depression were pre-diagnosed suggesting neural circuitry dysfunction precede the neurological symptoms. So, PPI can be used as a diagnostic tool in NPC disease.

PPI in Previous Studies

PPI has been effectively studied in various neurological disorders, drug-challenge studies and in studies of antipsychotic drug efficacy²². Studies were conducted to determine the role of dopamine in prepulse inhibition of acoustic startle response and behavioral variations among different genetic strains of mice²³. Studies were also conducted to determine a relationship between dopamine drugs and prepulse inhibition. Amphetamine, an indirect dopamine agonist, was shown to decrease prepulse inhibition by increasing dopamine overflow in nucleus accumbens markedly in one study while in the other experiment, a combined treatment with subthreshold doses of SKF38393 and quinpirole, selective dopamine D1 agonist and a selective dopamine D2 agonist, respectively, decrease prepulse inhibition. Lastly, raclopride, a selective dopamine D2/D3 antagonist was shown to increase prepulse inhibition²⁴.

Parametric Effects on PPI

Prepulse

For the prepulse stimulus to inhibit startle response, it must precede the startle stimulus by 30-500ms²⁵. The stronger the prepulse stimuli the greater the Prepulse Inhibition. In spite of this general rule, prepulse inhibition is not as straightforward as it might appear at first. There is more than one factor on which the prepulse inhibition depends, one among them being the background noise. The background noise is the constant source of sound that is neither the prepulse nor the startle stimulus. Prepulse stimulus arising from an uncontrolled ambient background does not elicit a reliable relationship between prepulse and startle stimuli and sensorimotor gating as the exact same brain processes are not involved in regulating prepulse inhibition arising from different background noise levels²⁵. The other factor on which the prepulse inhibition depends is the prepulse duration. Bloch's law stipulates that the stimulus salience reflects an interaction of intensity and duration.

Prepulse-to-Pulse Intervals

Interstimulus interval (ISI), the onset of prepulse to the onset of pulse or startle stimuli, for eliciting PPI normally ranges from 30 to 240 ms. ISI of 1000ms or more can cause facilitation of the startle response magnitude in humans. Thus, in order to assess both PPI and facilitation in a single experiment or a session, short and long ISIs can be used respectively. In addition to the magnitude of the startle response, the pre-stimulus effects on latency of the startle reflex response are quite important as the latency-to-startle onset

and latency-to-startle peak are measures of the rapidity with which the startling stimulus evokes the startling response²⁵.

Inter-Trial Intervals

Inter-trial intervals (ITI) lie within a range of 8-30 or more seconds with a range centered around 15-20 seconds. Compared to fixed ITIs, variable ITIs causes less habituation of startle reflex responses habituation and are thus more favorable for testing²⁵.

Prepulse Type

Discrete white noise prepulses were observed to have maximal PPI effects in both normal comparison subjects and schizophrenics in various studies²⁵.

Aim

The main aim of the present study was to establish a working model that helps in the discovery of effective novel dopamine drugs that can treat various psychoses. Tests were conducted on two different strains of mice, all male, using haloperidol, a dopamine receptor antagonist to demonstrate its effects on prepulse inhibition¹³.

Materials and Methods

Two different strains of mice, C57BL/6J and CD1, weighing 23–30 g at the start of experiments were used. The C57BL/6J mice were obtained from Charles River Laboratories (Wilmington, MA) and were housed in a room that was maintained at a constant temperature at 21-23°C and humidity at 45-50% at a vivarium located in Cooper Medical School of Rowan University at Camden. The vivarium has a barrier facility and

animals were kept under a 12h light/dark cycle (lights on at 0700 and off at 1900) in groups of four per cage in polycarbonate cages with ad libitum food and water and enrichment provided by paper Bio-Huts and/or nestlets. The mice were weighed and marked almost every morning over the period of the research and monitored for general health and behavioral parameters.

Animals

C57BL/6J

These are widely used in several different research studies because of their availability and robustness. C57BL/6J are inbred mice strain developed by C. C. Little in 1921 which were handed over to Charles River in 1974 from NIH²⁶. Also known as B6, B6/J mice, C57BL/6J mice are used mainly in research areas like cardiovascular biology, developmental biology, diabetes and obesity, immunology, genetics, and sensorimotor research. Their preference for alcohol and morphine and sensitivity to noise and odors make them the best fit for addiction and prepulse inhibition studies²⁷. C57BL/6J's are deep brown to black in color with a tendency to bite. The dominant mice tend to remove hair and whiskers of cage mates²⁸.

CD1

These are the most popular strain of the outbred mice that are used in toxicology and pharmacology research. The CD1 IGS mice are albino (have completely white hair) with large genetic diversity similar to that found within and between human populations.

They are docile and easy to handle compared to other types and display minimal evasive, struggling, and aggressive behavior when caught and held²⁹.

Drugs

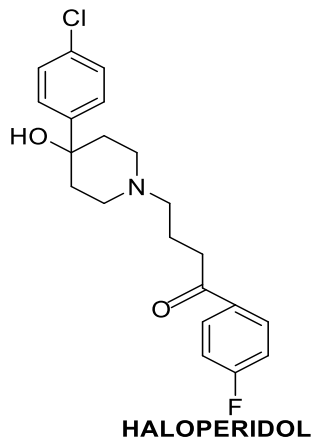
Haloperidol was obtained from Sigma Aldrich, (St. Louis, MO, USA). It is a white to faintly yellowish amorphous or micro-crystalline powder. It is poorly soluble in water but freely soluble in chloroform, methanol, acetone, benzene, and dilute acids³⁰.

Haloperidol is a first-generation butyrophenone antipsychotic drug active at postsynaptic dopamine (D2, D3, and D4) receptors in the mesolimbic system of the brain. It is effective in the management of the positive symptoms of Schizophrenia such as hallucinations, delusions, hearing voices, aggression/hostility, disorganized speech, and psychomotor agitation. Haloperidol along with other first-generation antipsychotics has side effects of tardive dyskinesia, drug induced Parkinsonism, sedation, and weight gain³¹.

Three different doses, 0.1mg/kg, 0.3mg/kg, and 1mg/kg, and a vehicle dose of haloperidol were used in this study. The drug, 0.1N HCl and DMSO were vortexed until the drug completely dissolved in the solution and finally saline was added to make up the volume.

Figure 5

Structure of Haloperidol



Apparatus

Prepulse Inhibition

A Med-associates apparatus was used. The mouse was held in a restraining tube attached to a movement-sensitive plate and screwed onto a platform table in a sound attenuating cubicle. To ensure proper functioning of the system, both the sound attenuating chambers were calibrated using the “calibrate audio” utility and the ANL-929A-PC USB microphone package¹⁷. Acoustic startle and prepulse stimuli were produced by a speaker that fit snugly inside the chamber. The analog-to-digital converter converts the analog voltage signal from the startle sensor to a digital unit.

Procedure

Two strains of mice, C57 and CD1, each with a different protocol were tested in PPI. Two different software were used; Startle reflex, which is a manual setup software and Startle pro, which is a highly automated software.

Startle Reflex Software

The pilot testing in C57 mice was divided into an initial acclimation period and Block I startle only period with 6 trials, Block II prepulse plus startle period with 30 trials, and Block III startle only period with 6 trials. Six different startle intensities between 95dBs and 120dBs and five different prepulse intensities between 70-100 dB with rise/fall time of 1 ms and 0 ms respectively and a 100 ms prepulse/startle delay were tested. In the next stage, the same procedure with 0.1mg/kg, 0.3mg/kg, and 1mg/kg, doses of haloperidol or vehicle (95% saline, 5% DMSO, 1N HCl) was tested. This protocol had two main drawbacks. Firstly, prepulse noise levels itself caused startle in the animals. Secondly, the drug solution caused ulcers at the site of injection and severe catalepsy which led to the death of many mice. The testing was later carried out in cd1 mice with a modified protocol. Initially, all the animals were acclimated to 70 dBs of white background noise for 15 minutes for three consecutive days. The next three days animals were subjected to varying startle levels of 70 dB, 80 dB, 90 dB, and 100 dB. In the next stage, experiments were conducted at four different startle intensities as in the previous step along with a prepulse level of 70 dBs at three different frequencies of 4000 Hz, 12000 Hz, and 20000 Hz with rise/fall time of 0ms and 3ms respectively and prepulse/startle delay of 100ms⁹. The testing period was divided into 3 blocks with an initial 1-minute acclimation period at 70dBs white

noise. All the blocks had 18 trials with both prepulse and startle stimuli. Prepulse frequencies for block I, II, and III were set to 4000 Hz, 12,000 Hz, and 20,000 Hz, respectively. In the next step, the same procedure was followed with 0.1 mg/kg, 0.3 mg/kg, 1 mg/kg doses of haloperidol or vehicle. Open field tests were conducted simultaneously to study the effect of drug on locomotor properties of mice.

Startle PPI Software

8 C57 mice were used for testing in this procedure. Animals were initially acclimated to the chambers for three days with constant white background noise at 60 dB for 5 mins. The animals were then tested without any injections in the startle pro software for 3 days. The test is highly automated, meaning all parameters are preset by the software. The parameters include: 60 seconds acclimation, 10 msec prepulse duration, 3 msec prepulse rise, 70 dB prepulse intensity at 3 different frequencies of 4, 12, 20 kHz, 100 msec prepulse delay and, 10 msec startle duration, 0 msec rise, startle noise bursts of 70, 80, 90, 100 dBs, fixed sequence 3->8 seconds inter-trial-interval all summing up to 55 trials (24 with prepulse stimuli).

Statistical Analysis

Acoustic Startle responses evoked by 100-dB startle only (S2-only) intensities without prepulses (S1s) establishes a baseline startle amplitude for each mouse. PPI for each of the prepulse frequencies was calculated by dividing startle amplitudes with prepulse amplitudes by baseline amplitude (S1+S2 trials / S2 only trials). Therefore, a smaller ratio indicates stronger Prepulse inhibition.

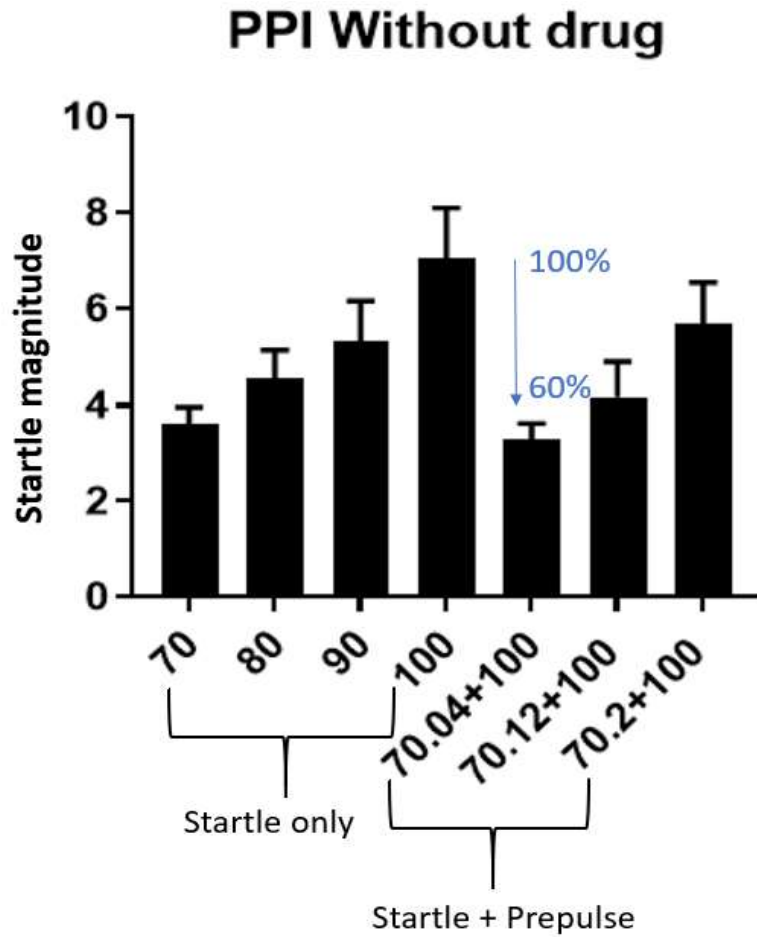
Results

8 male CD1 mice were tested using the Med-PC's Startle PPI software. Animals were acclimated to the chambers with 65dB white background noise the first three days followed by the three-week testing period, first week without any injections, second week with vehicle, 1 mg/kg, and 0.1 mg/kg doses of Haloperidol, and third week with the same doses of 7-OH-DPAT.

Sixteen startle-only noise bursts, eight 70.04+100, eight 70.12+100, eight 70.20+100, five 70dB noise bursts, five 80dB noise bursts, and five 90dB noise bursts were produced. All the data were trimmed prior to plotting on the graph to eliminate outliers.

Figure 6

PPI Without Drug

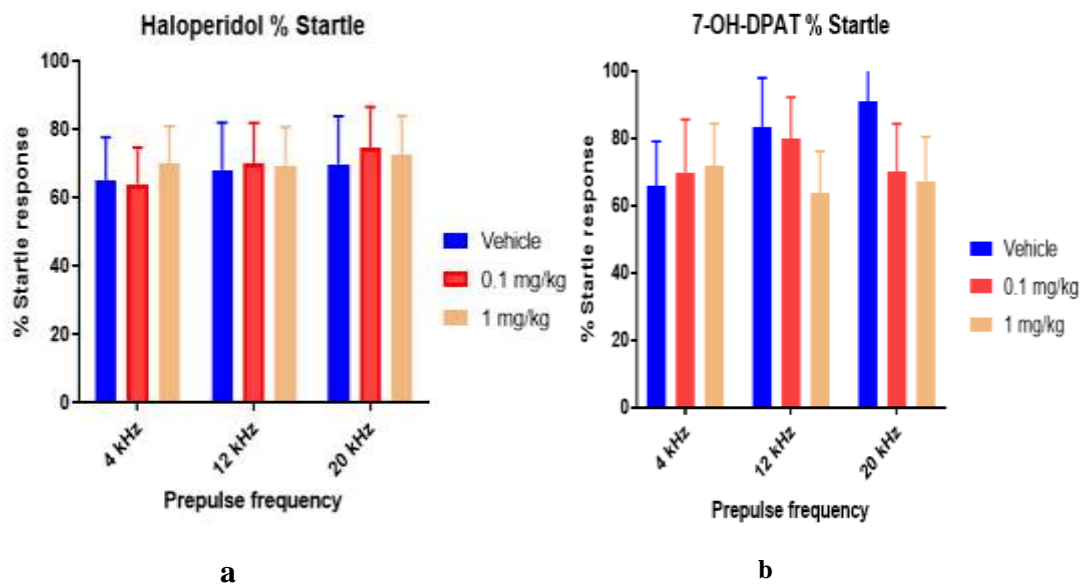


Animals were tested at four different startle-only intensities, 70 dB, 80 dB, 90 dB, and 100 dB, and at three different prepulse + startle intensities (70 dB 4 kHz frequency, 70 dB 12 kHz frequency, and 70 dB 20 kHz frequency followed by 100dB startle noise). The data shows an increase in startle response to the increase in startle noise. When preceded by a

prepulse stimuli, startle response to startle stimuli reduced significantly. These results are presented as means \pm SEM.

Figure 7

Effect of Dopamine Drugs on Prepulse Inhibition



Note. Effect of two different doses of haloperidol(a) and 7-OH-DPAT(b) in startle response at three different prepulse frequencies. No significant effects were produced by both the drugs. Startle response increased with the increase in prepulse frequency in both the cases, but the drugs have no consistent effect on the prepulse inhibition.

Discussion

Prepulse inhibition is a measure of sensorimotor signaling that is impaired in patients with Schizophrenia and thus can be used as a biomarker of the disease. When a weaker pre-stimulus precedes the startle stimulus, PPI filters out the irrelevant stimulus and decreases the sensory overload. Haloperidol, an antagonist at D2 receptor is used in the treatment of positive symptoms of Schizophrenia. In the present study, Prepulse inhibition of startle stimuli was measured at various sound intensities and frequencies. Haloperidol and 7-OH-DPAT were injected to in the male CD1 mice to observe the effects of Dopamine agonists and antagonists on prepulse inhibition. We expected to observe an increase in prepulse inhibition with haloperidol and a decrease in prepulse inhibition with 7-OH-DPAT, but the data showed no such effects. However, in both the studies, a decrease in startle response to startle stimuli when preceded by prepulse stimuli was observed indicating that all the mice have intact prepulse inhibition and thereby the sensorimotor gating.

There are limitations to this study. Firstly, the major limitation is the ineffectiveness of haloperidol and 7-OH-DPAT on prepulse inhibition. In previous studies, haloperidol prevented the disruption of PPI induced by dopamine agonists. Though 7-OH-DPAT showed a significant disruption of PPI in other studies, in the present study it had no effect on Prepulse inhibition. The effect of haloperidol on the disruption of 7-OH-DPAT (if any) has not been assessed. Secondly, there are some sex mediated differenced in dopamine receptor expression and signaling in humans and rodents. Since all the tests were conducted

in male mice, there could be a significant difference in results if female were used for the same studies³².

Chapter 2

Niemann Pick Disease Type C

Introduction

Niemann Pick Disease Type C is a progressive genetic disorder characterized by the lysosomal accumulation of lipids which causes neurodegeneration, dementia, ataxia, and death. Cognitive impairments, severe motor deficits, and primary behavioral disturbances are some of the other symptoms observed as the disease progresses. In some cases of adult onset, psychiatric disorders such as schizophrenia, attention deficit hyperactivity disorder, and depression were pre-diagnosed suggesting neural circuitry dysfunction precede the neurological symptoms.

Pathophysiology of NPC

NPC is caused by a mutation in NPC1 gene that encodes for a protein that is in the limiting membrane of endosomes and lysosomes. The protein binds cholesterol to its N-terminal domain and mediates cholesterol trafficking. The mutations in NPC1 gene includes small intragenic deletions/insertions, and missense, nonsense, and splice site variants that cause the dysfunction of autophagic-lysosomal pathway in cells. This leads to the formation of structural abnormalities and ectopic dendrites in mature neurons which suggests that lysosomal dysfunction in NPC modifies neuronal structure and function. During the developmental ages and after injury, neuronal autophagy and glial phagocytosis are important for the structural and degenerative changes in dendrites and axons.

In addition, autophagic-lysosomal activity inside the axons and in surrounding phagocytes is important for the degeneration and removal of axon terminals during developmental pruning but in case of NPC and other Lysosomal Storage Diseases (LSDS), lysosomal dysfunction alters the process of synaptic pruning and removal of unwanted synaptic structures during development. Purkinje cells are hypersensitive to the loss of NPC1 protein and degenerate earliest and to a greater severity than other neurons in the brain which contribute to the development of early neurological symptoms such as clumsiness, gait defects, and ataxia. Nevertheless, it is still unclear as to how the NPC1 deficiency effects synaptic development at postnatal stages. Presumably, this susceptibility of Purkinje cells to early neurodegeneration could possibly be because of intrinsic and/or extrinsic pathological events during neurodevelopment caused by NPC1 deficiency. In order to understand the underlying mechanisms involved in the early dysfunction and degeneration of PCs, it is important to detect the timeline of pathological changes in the cerebellum caused by NPC1 deficiency. In a study with mice with complete NPC1 deletion, microglial cells of neuroinflammatory phenotype were recognized as early as two weeks of age and at 3 weeks genes associated with immune responses in the cerebellum showed a change in their expression. A knockout mouse showed neurological symptoms as early as 4-5 weeks of age which made it difficult to find out if the neuroinflammation was caused in response to the absence of NPC1 gene function in microglial cells or a result of PCs degeneration or both.

NPC and Neurological Disorders

Recent studies presented evidence to the contribution of microglial cells to the developmental synaptic pruning defects in several neurological disorders like epilepsy, schizophrenia, autism spectrum and fragile X syndrome. Interestingly, NPC in humans cause neurological diseases that rapidly develops and progresses during childhood but shows no apparent neurological symptoms during early infantile age. In addition to these neurological diseases, some cases of juvenile and adult-onset NPC in humans were pre-diagnosed with psychiatric disorders like ADHD and schizophrenia due to cerebellar dysfunction prior to the manifestation of the NPC neurological symptoms. This provides the evidence that neural circuitry instabilities precede the loss of neurons in NPC and the neurological symptoms which may alter psychomotor behaviors.

Current Research at Rowan

Microglial cells have the highest expression of NPC1 gene in brain which is why it is important to understand how the NPC1 deficiency influences the proinflammatory responses in the microglial cells and the disease progression. The late-onset NPC mouse model at our laboratory showed neuroinflammatory responses in the synaptic region of the cerebellum (molecular layer) of the brain such as microglial reactivity and monocyte infiltration preceding the PCs degeneration. Moreover, PCs were degenerated by active engulfing of calbindin and dendrites of PCs by IBA1 and microglia/macrophages which suggest that preceding neuronal death, synaptic structures of PCs were targeted and removed by phagocytes. Interestingly, by the end of postnatal development and before any sign of PCs loss, the number of microglia at the synaptic region of the cerebellum at the

molecular layer increased which lead to the conclusion that the abnormal development at the molecular layer of the cerebellar synaptic structures along with microglial reactivity due to NPC1 deficiency synergistically lead to synaptic defects that augment neuroinflammatory responses in the cerebellum which precipitate the onset of PCs degeneration.

Recent studies showed that cerebellar microglia have a gene expression that is different from the microglia in other regions of the brain. Higher immune defense and immunoregulatory genes were observed in healthy microglia obtained from cerebellum than in the ones obtained from cortex or hippocampus. This shows that the microglia in cerebellum are more immune-vigilant and deficiency of NPC1 in microglia is more detrimental in cerebellar microglia than in the microglia from other parts of the brain.

Prepulse Inhibition in NPC

Previous studies showed a decreased prepulse inhibition in mice with NPC1^{nmf164} mutation compared to the wild type mice due to impaired sensorimotor gating.

Aim

The aim of this proposed research is to identify cellular and molecular pathways involved in causing and progressing NPC in young patients from which effective therapeutic interventions can be designed and tested to prevent or delay the onset of the disease and prolong and improve the quality of life of young NPC patients. The first part of the research plan was aimed at dissecting the effects of Npc1^{nmf164} mutation on the microglial phenotype during cerebellar postnatal development. The second aim was to

determine whether the $Npc1^{nmf164}$ mutation affects developmental axon pruning in the cerebellum. Finally, the third aim was to examine whether the mutation causes cerebellar synaptic defects and psychomotor behavioral abnormalities before the degeneration of the PCs.

The $Npc1^{nmf164}$ mouse model is based on the C57BL/6J mouse strain with a point mutation in NPC1. This mouse model has a single amino acid change from aspartate to glycine in the cysteine-rich luminal loop of the NPC1 protein at position 1005 or bp 3163 and is very similar to usually occurring human mutation. The factors that make $Npc1^{nmf164}$ mouse model a valuable model for understanding this disease and its progression are the location of this mutation which is like that found in humans, late-onset and slower disease progression. Breeding colonies were established for the knockout mice and tests were conducted on knockout, heterozygous and wild type offspring on 21/22 day. Typically, in humans, for a couple who are both carriers the chances of having an affected child is 25%, a child who is a carrier is 50%, and a child who is unaffected is 25%. In NPC like recessive genetic disorders, the risk is the same for both male and female offspring.

Animals

Heterozygous $NPC1^{nmf164}$ and wild-type C57BL/6J mice, weighing 23-30g at the start of experiment were used. Upon arrival to the laboratory, the mice were given free access to food and water in a room that was maintained at a constant temperature (21-23°C) and humidity (45-50%) on a 12h light-dark cycle. Breeding colonies were thus established, and the litter were genotyped on P18 by tail clipping and weaned on P20/21. Gestation period for mice is typically 21 days so care was taken to wean the older pups before the

arrival of the new litter. Behavioral studies were conducted from P21/22 depending on the day of weaning.

Equipment

Open-Field Locomotor

This test was conducted in an apparatus obtained from med associates. It has four chambers with cameras mounted on top of each chamber to monitor the movement of the mice. All the chambers and the cameras were connected to the computer and run by Any-maze software. The field was divided into center region and outside regions, and the time spent in the two regions was recorded.

Rotarod

This equipment has a horizontal rod that can be set to rotate at different speeds. Mice were placed on the rod for 300seconds and the speed and time at which the mice tripped off the rod was recorded.

Procedure

21/22-day old pups were tested in open field locomotor and rotarod equipment. The pups were placed inside the boxes of locomotor equipment and tested for their locomotor activity for 30 minutes to find out if there was a difference in movement among various genotypes of mutant mice⁵. NPC1^{nmf164} mutant mice (knockout) have a delayed acquisition of motor skills during development. All the animals in the colony were tested in rotarod equipment for their motor skills. The animals were placed on a rod that rotates at a speed of 4-40rpm and the time at which they fall onto the sensor plate before reaching the constant

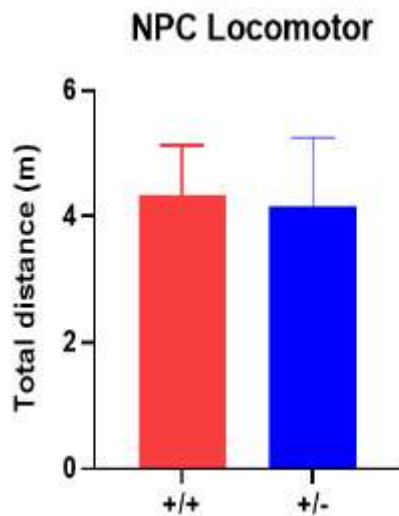
speed of 40rpm was noted down. At the end of 4 weeks, the animals were euthanized and perfused.

Results

All the +/- (het), and +/+ (WT) were tested first on rotarod followed by open-field locomotor for a span of 10 days to observe for any diminished motor activity by time.

Figure 8

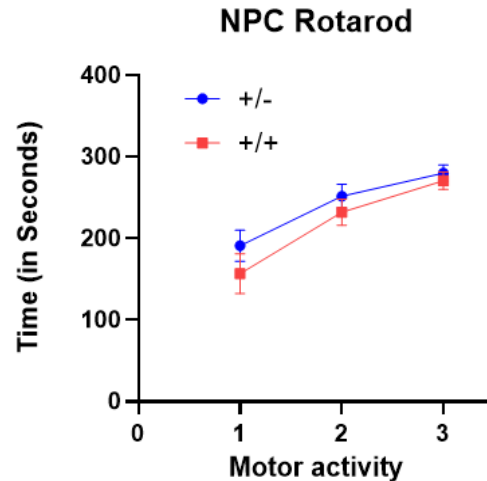
Locomotor Activity of NPC Mice



Note. The Total Distance Travelled in Meters in the Open-Field Locomotor Chambers by 17 +/+, and 22 +/- C57 Mice. +/+ Mice Show Slightly but not Significantly More Locomotor Movement Than +/- Mice.

Figure 9

Rotarod Test in NPC Mice



Note. In Rotarod test, +/- and +/+ show an increase in motor activity day-1 through day-3 indicating no diminished motor activity. +/- shows a slightly better motor activity than +/+.

Discussion

NPC is a progressive genetic disorder that causes neurodegeneration leading to severe motor and behavioral disturbances. It is caused by a mutation in NPC1 gene that codes for a protein that is involved in cholesterol trafficking. NPC1^{nmf164} mutant mice (knockout) have a delayed acquisition of motor skills during development. We established breeding colonies to obtain knock-out mice and test them in rotarod, open-field locomotor and PPI. The breeding colonies produced abundant +/+ and +/- pups. No knock-out mice were detected in the colony, hence tests were only conducted in the +/+ and +/- mice which produced no significant differences in their motor activity.

The study was called to a halt due to a non-compliance issue that raised due to the weaning of mice pups in the colonies prior to the protocol mentioned weaning date (P21).

Chapter 3

Synergistic Antihyperalgesic and Antinociceptive Effects of Morphine and MP-III-024, a Positive Allosteric Modulator at $\alpha 2$ GABA_A and $\alpha 3$ GABA_A Receptors

Introduction

Opioids are drugs that relieve pain. Prescription opioids are used to treat moderate to severe pain, but they are also associated with serious side effects like constipation, euphoria, and respiratory depression. They are often misused for their euphoric effect. Opioids interact with the opioid receptors in the central nervous system and the gastrointestinal tract and cause pain relief and constipation, respectively³³.

Classification

Based on Origin

Opioids are classified into either endogenous or exogenous opioids. Endogenous opioids are the opioids that are produced by the body. There are three endogenous opioids:

- Beta-endorphin
- Enkephalin
- Dynorphin

Exogenous opioids are not produced within the body. They are further classified into 3 types of based on their origin³³.

Table 1³³*Classification of Opioids Based on their Origin*

Natural opioids	Morphine, Codeine, Thebaine, and Papaverine
Synthetic opioids	Methadone, Fentanyl, Pethidine, Alfentanil, Remifentanil, and Tapentadol
Semi-synthetic opioids	Heroin, Buprenorphine, Dihydromorphine, and oxycodone

Based on Opioid Receptors

There are three receptors at which opioids usually show their effects. They are mu (MOP) receptors, delta receptors, and kappa receptors. Opioid receptors are G-protein-coupled receptors. Only after the discovery of these receptors was a series of endogenous opioid ligands discovered in brain extracts. A fourth receptor with opioid-like receptor activity called as the nociception receptor (NOP) was subsequently discovered.

Table 2³³*Clinical Effects of Opioid Receptors*

MOP	DOP	KOP	NOP
Analgesia	Analgesia	Analgesia	Analgesia
Euphoria	Modulation of MOP	Dysphoria	Sedation
Respiratory depression	Inhibit dopamine release	Diuresis	
Physical dependence			

Table 3³³*Opioid Receptors and their Respective Endogenous Ligands*

Receptor	Endogenous ligand
DOP	[Met]-enkephalin [Leu]-enkephalin
KOP	Dynorphin-A Dynorphin-B
MOP	B-Endorphin Endomorphin-1 Endomorphin-2
NOP	Nociceptin/orphanin (N/OFQ)

Table 4³³*Classification of Opioids Based on their Receptor Binding and Affinity*

Full agonist	Partial agonist	Mixed agonist	Antagonist
Morphine Hydromorphone Oxymorphone Heroin Codeine Hydrocodone Oxycodone Meperidine Methadone Fentanyl	Tramadol Butorphanol Pentazocine Buprenorphine	Nalbuphine Butorphanol Buprenorphine Pentazocine	Naloxone Naltrexone

Mechanism of Action

Opioid receptors are coupled to inhibitory G-protein coupled receptors $G_{\alpha i}$ or $G_{\alpha o}$.

Opioids cause two types of inhibitions; Presynaptic or Postsynaptic.

Presynaptic inhibition: G protein is made of 3 subunits, α , β , and γ subunits. When an opioid bind to the receptor, a number of structural and molecular changes occur at the G protein and the α subunit dissociates from $\beta\gamma$ subunit. $\beta\gamma$ subunit interacts with the voltage gated calcium channels on the presynaptic neuron that help in releasing the neurotransmitter and prevents them from opening. This inhibits the action potential from propagating from presynaptic neuron to postsynaptic neuron.

Postsynaptic inhibition: When opioids bind to the opioid receptors on the postsynaptic neuron the α subunit dissociates from $\beta\gamma$ subunit. $\beta\gamma$ subunit interacts with the G protein

coupled potassium channels, opens them and the potassium moves out of the cell. When a neurotransmitter binds to the receptors on postsynaptic neuron and opens Na^+ channels (depolarization), the positive charge that is brought about by the Na^+ ions is negated by the K^+ ions moving out thus causing hyperpolarization and inhibiting the action potential from further propagation.

$G_{\alpha i}$ role: The α subunit of the G protein is involved in inhibiting the cAMP production. Adenylyl cyclase is an enzyme, present in the plasma membrane, converts ATP to cAMP which activates cAMP dependent protein kinases. Protein kinases are made of regulatory subunit and catalytic subunit. cAMP binds to the regulatory subunit and gets dissociated from the catalytic subunit. The catalytic subunit then phosphorylates the neuronal proteins and channels which either activates or inhibits them. This in turn starts some signaling pathways and stops some. $G_{\alpha i/o}$ interacts with the adenylyl cyclase and inhibits it converting ATP to cAMP.

β -Arrestin signaling: β -Arrestin proteins play a significant role in signal transduction at G protein-coupled receptors. β -Arrestins regulate especially Mu opioid receptor signaling by receptor desensitization. Desensitization occurs by phosphorylating the GPCRs by serine/threonine kinases called G protein coupled receptor kinases. The phosphorylated GPCRs recruit β -Arrestin which blocks G-protein mediated signaling either by desensitizing the receptors or internalizing the receptors³⁴. Regulation of GPCRs by β -Arrestin is multifaceted and the other way in which they regulate is by activating the mitogen-activated protein kinase (MAPK) downstream signaling cascade which inhibit G protein signaling at these receptors for long periods³⁵.

Analgesic Mechanism of Action

There are two pain pathways, ascending and descending. Ascending pathway sends pain signals to the brain while descending pathway blocks the ascending pathway.

Ascending pathway: the pain stimulus gets propagated from the site of pain through the primary sensory neuron which meets secondary sensory neuron in the spinal cord. The secondary sensory neuron passes through the brain stem into the thalamus which processes the sensory information and synapses with tertiary neurons that activate brain cortex that is involved in giving meaning to the pain.

Descending pathway: The neurons in the descending pathway are normally inhibited by the GABA neurotransmitter that are released from the inhibitory interneurons in the brain stem. In response to pain certain neurons release endogenous opioids that bind to the inhibitory interneurons in the descending pathway and prevents GABA neurotransmitter release. Without GABA, the neurons in the descending pathway are no longer inhibited and the endogenous opioid releasing interneurons in the spinal cord get activated and inhibit the pain signal propagation between primary and secondary neurons of the ascending pathway.

Exogenous opioids act the same way endogenous ligands do in activating the descending and inactivating ascending pathway.

Opioid receptors are also present in the ventral tegmental area, and respiratory center which upon binding with opioids causes addiction and respiratory depression, the two main side effects associated with long-term opioid use.

Addiction Mechanism

Mesolimbic pathway, also called the reward pathway connects ventral tegmental area of the midbrain to the ventral striatum of the basal ganglia of the forebrain. Nucleus accumbens and olfactory tubercle make up the ventral striatum. Nucleus accumbens plays a pivotal role in the reward pathway. Dopaminergic neurons that extend from the ventral tegmental area to the nucleus accumbens are regulated by GABAergic interneurons that have opioid receptors. When opioids bind to the opioid receptors on the GABAergic interneurons, GABA inhibition of dopamine release is stopped as a result of which excessive dopamine is released which activates reward pathway inducing pleasure. After repeated use of opioids, a direct association is developed between opioid and pleasure which is called as drug liking.

Tolerance Mechanism

It is a condition when body requires more drug to produce same effects after repeated use of the drug. Repeated Opioid use deplete the cAMP reserves in the body. To achieve homeostasis and compensate for the anticipated loss of cAMP, neurons preemptively produce more cAMP which makes the neurons hyperactive. Opioid intake followed by this increase, decreases the cAMP as expected but to a level that is within the normal range. For the opioids to produce effects similar to those in the early stages, the dose needs to be increased. Other factors that contribute drug tolerance are receptor phosphorylation that changes the receptor shape, receptor internalization, decrease in number of receptors available for ligand binding, and receptor-effector uncoupling which stops the downstream signaling pathways.

Withdrawal Mechanism

Withdrawal symptoms are due to the increased cAMP levels in the body that overfires the neurons. cAMP levels are spiked in order to compensate for the loss of it upon opioid intake. In case of no drug present in the body, the cAMP that is produced by the body preemptively (to achieve homeostasis like discussed previously) overfires the neurons causing withdrawal symptoms. Ventral tegmental area, locus coeruleus, and intestines are the most affected areas of the body.

Locus coeruleus is the site for norepinephrine synthesis in the brain. Locus coeruleus is responsible for alertness or wakefulness in humans or causing psychological stress. It also activates the sympathetic nervous system or fight or flight response of the body. Activated sympathetic nervous system produces sweating, pupil dilation, increased heart and breathing rates. Opioid use produces the opposite effects, while withdrawal symptoms exaggerate the normal functions of locus coeruleus. When opioids bind to the opioid receptors in locus coeruleus, common side effects of opioid use like drowsiness, sedation, dry skin, pinpoint pupil, and decreased heart and breathing rate are observed. During withdrawal, the over firing neurons cause jitteriness and insomnia, anxiety, excessive sweating, wide dilated pupils, and rapid heart and breathing rate.

Opioid receptors on the neurons that innervate and control gut movement, when bound by opioids cause constipation, a common side effect observed with opioid use. During withdrawal, gut movement becomes rapid and expels feces even before enough water is absorbed resulting in diarrhea.

In the ventral tegmental area, the GABAergic interneurons that keep the dopaminergic neurons in check become tolerant to opioids and regain their ability to inhibit dopaminergic reward pathway even in the presence of opioids which causes the feeling of discomfort. To avoid these consequences, patients continue to use opioids and fall into the category of opioid use disorder.

Overdose Mechanism

Breathing is controlled by Pre-Botzinger complex in the brain stem. This complex sends signals rhythmically to diaphragm and other respiratory muscles that enables breathing. When breathing is stopped by the person for whatever reasons, the feedback loop that has chemoreceptors detect increased carbon dioxide levels and decreased oxygen levels in the blood and activates Pre-Botzinger complex that forces breathing. These chemoreceptors and Pre-Botzinger neurons have opioid receptors and at high opioid doses, they get shut down and the complex no longer forces the person to breath and is followed by death.

Naloxone is an opioid receptor antagonist that binds and prevents morphine from binding to the receptors thus preventing respiratory depression.

Uses of Opioids

Analgesia

Opioids are generally prescribed in the management of severe acute or chronic pain. More than 100 million people in the United States suffer from acute and chronic pain and around 6-8 million undergo long-term opioid treatment³⁶. Opioid analgesics work

effectively against both cancer and non-cancer pain. Intravenous opioid analgesics are used to heal neuropathic pain like central pain, postherpetic neuralgia, and neuropathic pain while oral opioid analgesics are effective against neuropathic, musculoskeletal, and other non-cancer pain³⁷. Morphine alone can manage severe cancer pain in almost 85% of the patients. Combination therapy in the treatment of pain seems promising.

Edema

Morphine is a vasodilator and can be used in the treatment of pulmonary edema³⁸. Cardiogenic pulmonary edema is a condition in which the left ventricle cannot pump blood efficiently out of the heart and builds up pressure. Morphine helps by acting as a vasodilator and reducing hydrostatic pressure by lowering preload and afterload.

Diarrhea

Loperamide is an opioid agonist that acts like morphine and decreases the tone of the longitudinal and circular smooth muscles thereby decreasing the peristaltic movement. This increases the duration the material stays in the intestine and the water absorption from the feces³⁹. Opioids can also be used in the treatment of Irritable Bowel syndrome with Diarrhea (IBS-D). Although there is no effective method available to treat IBS-D, opioids can help in alleviating the symptoms. Eluxadoline is a Schedule IV drug approved by FDA to manage IBS-D associated symptoms. The drug has a mixed pharmacology. It is an agonist at MOR, and KOR, and antagonist at DOR. Since Eluxadoline acts locally by targeting gut opioid receptors, CNS side effects are not common⁴⁰.

Cough Suppressant

Opioids act centrally by suppressing the cough center that is in the medulla oblongata in the brain stem. Opioids are the only effective centrally acting anti-tussive drugs that act through MOR and KOR agonism. Codeine is effective against acute and chronic cough. Dextromethorphan is the most used anti-tussive that is as effective as codeine. Hydrocodone is another opioid drug that is used as an anti-tussive, but it is only available as a combination drug in the USA. The use of this drug with antihistamine drugs often enhances the sedative effects of the drug due to which there is a limit to the maximum dose a patient can take. Hydrocodone has fewer gastrointestinal side effects and is as effective as codeine making it the drug of choice for cough⁴¹. Prescribing opioids as an anti-tussive to patients below 18 years of age is not recommended by FDA⁴².

Anesthetic

Opioids are widely used as anesthetics in major surgeries, especially those involving cardiovascular disease patients. Opioids do not cause cardiac depression making them the drug of choice for inducing anesthesia in patients with cardiovascular diseases⁴³.

Adverse Effects

The major adverse effects associated with opioids use is respiratory depression, sedation, constipation, and bradycardia. Tolerance, hyperalgesia, dependence, and immunologic effects, hormonal change, sleep disturbances, abuse, and addiction are other side effects associated with opioid use⁴⁴.

Respiratory Depression

Opioids bind to the receptors located in the respiratory center or Pre-Botzinger complex in the brain stem and induce respiratory depression. This opioid induced respiratory depression initiates cardiorespiratory arrest with subsequent hypoxia and hypercapnia resulting in fatalities. Mortality in opioid addicts that overdose on morphine and heroin usually die of respiratory depression and respiratory failure.

Sedation

It is the second most observed adverse effect associated with opioid use. Opioids are the primary choice of drugs used in the management of pain in hospitalized patients. Sedation is a common adverse effect during first 24 hours of opioid therapy. To combat this effect, opioids are often prescribed with other drugs like methylphenidate, which is a first-line therapy for reducing sedation⁴⁵. 10-15mg of methylphenidate has been observed to reduce drowsiness significantly in cancer patients. Simultaneously, reducing opioid doses without reducing analgesic effect may also be possible. Other drugs like dextroamphetamine, donepezil, modafinil, and caffeine can be used to reduce opioid induced sedation⁴⁶.

Constipation

40-60% of the non-cancer patients receiving opioids experience opioid induced constipation. Patients also develop other GI side effects like nausea, vomiting, bloating in the stomach, abdominal pain, and straining. Stimulants like Senna or Bisacodyl are administered with or without a stool softener and laxatives like magnesium citrate are used

to prevent constipation. Lubiprostone increases fluid secretion in the GI tract by activating type 2 chloride channel increasing the tone and peristalsis, accelerating the small bowel and colonic transit times. Methylnaltrexone bromide, a peripherally acting opiate antagonist is used in the treatment of opioid induced constipation. It does not cross the Blood Brain Barrier to induce symptoms of opioid withdrawal making it the most superior agent among all the available agents. Naloxegol, alvimopan, and naldemedine are approved for the treatment of OIC in non-cancer pain patients⁴⁷.

Cardiac Effects

Opioids cause bradycardia and vasodilation. Opioids when administered with other medications like benzodiazepines, may decrease cardiac function. Methadone and Buprenorphine are known prolong QTc, especially in patients at high risk for QTc prolongation. Morphine, hydromorphone, hydrocodone, and meperidine decrease systemic vascular resistance and blood pressure by releasing histamine⁴⁸.

Tolerance

Opioid tolerance occurs when the same amount of drug shows reduced responsiveness and is manifested as the need to use an increasing dose to achieve a desired effect⁴⁹. Increased doses of opioids given to counter tolerance can result increased opioid associated adverse effects and opioid induced hyperalgesia. Factors that dictate the development and extent of tolerance are the dose of drug and frequency of drug administration.

Hyperalgesia

Opioids induce hyperalgesia, a state of increased pain sensitivity following long-term use of high-dose opioids. It occurs when neoplastic modifications occur in both peripheral and central nervous system. There are no well-established molecular mechanisms as to how Opioid induced hyperalgesia occurs, but it could be due to the molecular adaptations in MOR-expressing neurons that change the interaction between cells and activate the independent oppositional system. In addition, opioid induced apoptosis could also contribute to the development of hyperalgesia. This sensitization is pain is a paradoxical response and the pain experienced in OIH can be very close to the patient's original pain⁵⁰.

Immunologic Effect

Morphine has been observed to decrease the effectiveness of both natural and acquired immunity in animal and human studies by interfering with the intracellular pathways involved in immune regulation. Not all the opioids have same immunosuppressive effects⁵¹. Morphine, codeine, methadone, fentanyl, sufentanil, and remifentanil produce strong immunomodulating effect while oxycodone, tramadol, buprenorphine, and hydromorphone produce weak immunomodulating effect. In selective vulnerable populations like elderly or immunocompromised patients, opioid mediated immune effects could be particularly risky. The least immunosuppressive opioid is buprenorphine which is used as a first-line analgesic⁵².

Hormonal Changes

Endocrinopathy (OE) is a term used to describe hormone problems. Opioids induce endocrinopathy by affecting different hormones like testosterone, estrogen, luteinizing hormone, gonadotropin releasing hormone, dehydroepiandrosterone, and adrenocorticotropin and corticotropin-releasing hormone, and cortisol. Opioids affect men and women differently. In men, sexual disorders like erectile dysfunction, decreased libido, depression and decreased energy levels are common side effects. Testosterone levels drop significantly one to four hours after acute administration of opioids and takes around 24 hours to return to normal levels. Chronic administration of opioids results in tonic decrease in both total and free testosterone levels⁵³. In women, opioids cause depression, dysmenorrhea, sexual dysfunction, and potentially reduced bone mineral density⁵⁴.

Sleep Disturbances

Disorders of sleep-wake cycles, disorders of initiating and maintaining sleep. Dysfunction associated with sleep stages and partial arousals are some of the sleep disturbances that are associated with long-term opioid use such as in cancer patients^{55,56}. Studies showed that opioids can increase the number of sleep-wake transitions and reduce the sleep time and efficacy⁵⁷. Opioids alter the balance of neurotransmitters that regulate sleep like serotonin, acetylcholine, noradrenaline, histamine, dopamine, GABA, melatonin (sleep hormone), other pituitary hormones and affect sleep⁵⁸.

Opioid Addiction

Opioids have high abuse potential due as they induce euphoria and reduce negative dysphoric moods⁵⁹. Addiction is a chronic, relapsing disorder that is characterized by strong desire to take the drugs even when medically unnecessary. Prescription opioids can also cause addiction in people⁶⁰.

The Opioid Epidemic

The Opioid epidemic is a major health concern occurring from the over-prescription of opioids to relieve pain, increase in opioids use/abuse/overdose significantly impacting patient health and economy. According to the center for disease control and prevention, there are three different waves in the modern American opioid epidemic that raised the mortality due to opioid overdose. The first wave was in 1990s, when opioids were prescribed increasingly. The second wave was in 2010, when heroin caused large number of overdose deaths. The third wave began in 2013 due to significant deaths due to synthetic opioid overdosing, mainly those that were illicitly manufactured like fentanyl (IMF)^{61,62}.

In 2016, 11.5 million Americans were misusing opioid prescriptions, more than 2.1 million people had a diagnosable opioid use disorder and more than 42,000 died from opioid overdosing. In 2017, US department of Health and Human Services declared a public health emergency for this opioid crisis following 70,000 overdose deaths⁶³. Over the last two decades, hundreds of thousands of lives have been lost and millions of people and their families have been affected by opioid epidemic. Opioid misuse, abuse, and overdose have been constantly surging in USA as well as in the whole world.

Addiction Treatments

Opioid addiction has limited treatment options. Recovery requires Behavioral therapy and Pharmacotherapy either individually or in combination along with counseling and support^{64,65}. Treatment is generally started with counseling, opioid replacement therapy, and gradual discontinuation of the drug. Sudden discontinuation of the drug causes severe withdrawal symptoms in which case drug detoxification is the only choice for the physicians⁶⁶.

New Analgesia and Strategy

NSAIDs and opioids are used in the treatment of pain. NSAIDs show weak analgesic activity than opioids and act peripherally. Opioids act both centrally and peripherally owing to their both central and peripheral side effects³³. High abuse potential of opioids raises the need for new treatment regimens and strategies in pain management.

Methods to Determine Analgesic Activity

To test analgesic agents, animals are subjected to nociceptive/painful stimuli prior to administering an analgesic. The painful stimuli generates a response like jumping, licking, shaking of the paws, skin twitch, tail flick, or flight which is indicative of painful sensations. Popular methods for determining analgesic activity are

Writhing Test

Irritants like Phenylquinone or acetic acid are injected into the mice or rats in their abdominal regions to induce pain and cause writhing. This is followed by the injection of the synthesized analgesic compound which should decrease the frequency of writhing

significantly^{67,68}. This test is suitable to test for the analgesic profile of the peripherally acting painkillers like chlorpromazine and meprobamate. The major drawback with this test is that it is difficult to evaluate the analgesic duration as the frequency of writhing decreases over time^{69,70}.

Hot Plate Test

It is a thermal pain sensitivity test in which the rodents are placed on a hot surface that is maintained at a specific temperature for a specific time frame and observed for nocifensive activity like paw licking, fluttering, and jumping⁷¹. Upon injecting the animal with analgesic drug, the latency to respond on hot plate increases indicating that the drug is showing analgesic activity. Hot plate is a tricky test comparatively to other thermal assays as rodents show very complex and subtle behavioral response⁷².

Von Frey Test

This is a mechanical pain sensitivity test. Animal is subjected to noxious stimulus using a 50mm long filament of varying diameters. The animal is placed on an elevated mesh screen and poked with filaments to different diameters to induce pain⁷³. If the animal withdraws the paw or starts licking or shaking the paws, it means the animal is feeling pain. The exact force of the fiber is determined by its thickness. After injecting the analgesic drug, the latency to respond to this painful stimulus increases indicating the analgesic activity of the drug⁷⁴.

Tail Flick Test

This is one of the most common nociceptive assays. In this technique, heat is applied to the animal's tail and the pain sensitivity is determined by measuring the tail flick latency⁷⁵. If this latency is prolonged upon administration of the test drug, it indicates that the drug has analgesic properties. The major drawback with this technique is that spinal transection above the lumbar fails to block the tail flick response, thus it may not be measuring the pain but the spinal nociceptive reflex⁷⁶.

Formalin Test

This test is used to measure clinical pain due to injury. Dilute solution of formalin is injected onto the planter surface of the rodent's hind paw and is observed for rodent's stereotypical behavior such as flinching, licking, and biting of the affected hind paw which are used as the measurements of inflammatory pain. These effects last for 15-60minutes⁷⁷. This method is preferred over other techniques because this model is useful to measure both acute and tonic pain⁷⁸ that affects inflammatory, neurogenic, and central mechanisms of nociception^{79,80}.

GABA Receptors

γ -aminobutyric acid is a neurotransmitter that has inhibitory activity in the CNS. Inotropic GABA_A receptors are one of the two major classes of GABA receptors that include various $\alpha\beta\gamma 2$ isoforms assembled from varying α , β , and γ subunits with differential signaling and expression patterns. Benzodiazepine-like drugs act as positive allosteric modulators (PAMs) on $\alpha 1$ GABA_A, $\alpha 2$ GABA_A, $\alpha 3$ GABA_A, and $\alpha 5$ GABA_A

receptors⁸¹. These receptors are distributed within the CNS with $\alpha 2$ GABA_A and $\alpha 3$ GABA_A especially expressed in the Dorsal Horn Spinal pathway that is important to nociceptive transmission⁸². Previous studies have demonstrated that intrathecally administered benzodiazepines mediate antihyperalgesic effects via $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors at the level of the spinal cord^{83,84,85}. Moreover, systemically administered compounds with PAM activity at $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors showed reduced activity at supraspinally located $\alpha 1$ GABA_A receptors which mediate side-effects of clinically used, commercially available benzodiazepines like diazepam^{83,86,82,87,88}.

μ -opioid receptors are co-expressed with $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors in nociceptive pain pathways which raises the possibility for interactive or combination effects⁸⁸. If supra-additive or synergistic effects are detected on endpoints related to pain reduction, combination therapy may be useful to treat pain. A recent benzodiazepine-type compound methyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (MP-III-024), with GABA_A PAM effects was synthesized that displayed functional selectivity for $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors over other GABA_A receptors like $\alpha 1$ GABA_A and $\alpha 5$ GABA_A. This selectivity produced antihyperalgesic effects with no off-target effects as observed with operant responding and locomotor activity⁸⁸. These data are along the same lines with other structurally related $\alpha 2$ GABA_A and $\alpha 3$ GABA_A preferring ligands, HZ-166⁸⁶ and KRM-II-81⁸⁹. MP-III-024 is a close analog of HZ-166 which showed non-sedating anxiolytic activity in rhesus monkey and antinociceptive activity in rodents^{82,86}. In addition, MP-III-024 showed a similar time

course and duration of action compared to morphine, a prototypical μ -opioid receptor agonist making it ideal for combination studies⁸⁸. In the present study, dose addition analysis was used to assess $\alpha 2$ GABA_A / $\alpha 3$ GABA_A receptor-opioid interactions.

Materials and Methods

CD-1 mice 10 weeks of age were obtained from Charles River Laboratories. Animals were housed in a vivarium that is maintained at a constant temperature and humidity with constant access to air and water, under a 12h light/dark cycle (lights on at 7:00 AM). Mice were housed in groups of four in polycarbonate cages with ad libitum food and water and enrichment provided by paper Bio-Huts and/or nestlets. No experiments were conducted for 2 weeks upon their arrival to habituate them to the colony room environment. Mice were handled for 2 days prior to the initiation of experiments. Typical testing period was between 11:00 AM and 3:00 PM. Care of animals was taken in accordance with the guidelines of the Institutional Animal Care and Use Committee of Rowan University and all testing adhered to the “Guide for the Care and Use of Laboratory Animals” (National Research Council, National Academy of Sciences, Washington, D.C., USA, 2011).

Animals

CD-1 laboratory mice are used in biomedical and pharmaceutical research. They are the progeny of nine Swiss mice, two male and seven female albino mice that were imported to the USA by Dr. Clara Lynch of the Rockefeller Institute for Medical Research, now named Rockefeller University in 1926.

Experiments were started when the animals were 35 days old and were weighing approximately 22-25 g. The animals were aggressive the first few days of handling and gradually got accustomed to human handling.

Drugs

Methyl 8-ethynyl-6-(pyridin-2-yl)-4Hbenzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (MP-III-024), the novel benzodiazepine analog was synthesized at the Department of Chemistry and Biochemistry at the University of Wisconsin-Milwaukee. Morphine was purchased from Sigma-Aldrich. 0.5% methyl cellulose dissolved in 0.9% NaCl was used as a vehicle to suspend the drugs and was administered intraperitoneally in a total volume of 10 ml/kg body weight.

Hot Plate Testing

A 25.3 × 25.3 cm measuring hot plate analgesia meter was used to assess antinociception. The plate was maintained at $56 \pm 0.1^\circ\text{C}$. The antinociceptive response was measured by assessing the latency to lick or shuffle or flutter the hind paw(s) and/or jump from the hot plate surface. To prevent tissue damage, a predetermined cutoff time of 20 s was defined as a maximal response. The antinociceptive response was measured at 30 and 15 min prior to the beginning of the drug administration and was used to yield a baseline value.

Apparatus

The hot plate analgesia meter (Columbus Instruments, OH) for small laboratory animals were used for this analgesic test. (Figure 9) The hot plate could continuously provide 55 °C temperature on an aluminum surface, with a digital built-in thermometer to maintain surface temperature to 0.1 °C precision and a timer with a 0.1 sec precision. The square shaped surface plate was enclosed by a clear acrylic cage to confine animals during testing. Pushes on start/stop button related to the timer, which displayed the time on the screen which was recorded manually⁹⁰.

Procedure

The hot plate was maintained at 56 °C. Animals were placed on the surface of the hot plate and observed for certain behavioral changes like paw licking, flutter, and jumping, which indicates animal's pain feeling. Latency time to respond to the stimuli after placing the mice on the hot plate provided a threshold level. Two baseline studies were conducted prior to injecting the animals with the drug. After weighing the animals, testing drug was administered and animals were placed on hot plate in 15, 30, 45, 60 and/or 75/90 minutes time intervals to measure the latency time. The mouse was immediately taken off the hot plate if any behavioral changes were observed and the time was recorded. Animals were removed from the hot plate after 20 seconds even though no considerable behavioral change was observed to avoid tissue damage. Animals that showed a latency time more than 20 seconds were excluded from further investigation.

Statistical Analysis

The anti-nociceptive effect for each dose was calculated as the % of the Maximal Possible Effect (% MPE) using the following formula:

$$\%MPE = \left(\frac{\text{Latency time after drug administration} - \text{Mean latency time of baseline studies}}{\{\text{Maximal latency time (20)} - \text{Mean latency time of baseline studies}\}} \right) \times 100$$

Von Frey Test

A subcutaneous injection of 0.06 mg Zymosan A suspended in 20 µl 0.9% NaCl was given into the plantar surface of the right hind paw to induce inflammation and study antihyperalgesia and the non-injected left hind paw was used as control. 24 hours after the Zymosan A injection, mechanical sensitivity was measured by applying von Frey filaments of increasing stiffness (5-26 g) to mid plantar surface of the hind paws until the filament bends. Paw withdrawal response is indicative of positive response and the mechanical threshold is defined as the minimum force necessary to elicit a positive withdrawal response.

Apparatus

The apparatus consists of a mesh screen that is mounted on a stand at eye level. Animals are placed on top the mesh and poked from below with von Frey filaments of increasing stiffness of 0.008-300 g to determine the threshold that produces a hind paw withdrawal response. The minimum gauge von Frey filament that causes a withdrawal reflex is marked as the mechanical withdrawal threshold.

Procedure

Each mouse was weighed, and baseline studies were conducted prior to injecting drugs. Testing drug was injected, and the animals were placed on the wide gauge, wire mesh surface in 15, 30, 45, 60, and/or 75/90 minutes time intervals and von Frey filaments of increasing stiffness were applied until the filaments were seen to bend. If the same filament made the mouse respond by flicking its paw away 3 times, the diameter of the filament was recorded, and the process repeated with increasing gauges of von Frey filaments that have different stiffness until stimulation forced a hind paw withdrawal.

Statistical Analysis

The mechanical threshold following drug administration was normalized to the baseline measurement of the non-injected left hind paw and expressed as a percentage of the maximal possible effect (%MPE) from the following formula:

$$\%MPE = \left(\frac{\text{post drug right paw threshold (g)} - \text{baseline right paw threshold (g)}}{\text{baseline left paw threshold (g)} - \text{baseline right paw threshold (g)}} \right) \times 100$$

Isobolographic and Dose-Addition Analysis

Graphical and statistical approaches were used to assess the interaction between MP-III-024 and morphine. With the use of isobolograms, graphical approach made a distinction between sub additive, additive or synergistic interactions. In the current study, isobolograms were constructed by plotting and connecting the ED₅₀ of MP-III-024 on abscissa with the ED₅₀ of morphine on ordinate to obtain an additivity line. The additivity line had the loci of dose pairs that produce an ED₅₀ equal to the ED₅₀ of morphine or MP-

III-024 alone. Dose pairs that fell below the additivity line suggested ED₅₀ was reached with lesser quantities of the drugs which was suggestive of synergism and the experimental points representing dose pairs that fell above the line suggested subadditivity.

Drug interaction can be analyzed in different ways. Among them, a comparison of the ED₅₀ values for each mixture with the predicted additive ED₅₀ values is a good way to determine the potency of the prospective combination drug. ED₅₀ values for each mixture can be represented by Z_{mix} and predicted additive ED₅₀ values can be represented by Z_{add}⁹¹. The total drug dose of combined MP-III-024 and morphine that produces a 50% maximum possible effect is called Z_{mix}. If two drugs, e.g., morphine and MP-III-024 added together in a specific ratio have no sub- or supra-additive effects, the predicted additive response can be measured by a predicted ED₅₀ (Z_{add}). In this mechanical sensitivity assay, if both drugs were effective equally, an equation $Z_{add} = fA + (1 - f)B$ can be used to calculate Z_{add} values individually. In that equation, A is the ED₅₀ for MP-III-024 alone, B is the ED₅₀ for the morphine alone, and f is the fraction. For determining the proportion of MP-III-024 in each mixture equation $fA/[fA + (1 - f)B]$ can be used. This study examined effects produced by mixtures in which f = 0.25, 0.5, and 0.75. When f = 0.25, the mixture contains a proportion of $[A/(A + 3B)]$ MP-III-024 and a mixture ratio of $[(A/B)/3]$ parts MP-III-024 to one-part morphine; f = 0.50 leads to a proportion of $[A/(A + B)]$ MP-III-024 in the mixture and a mixture ratio of (A/B) parts MP-III-024 to one-part morphine; and f = 0.75 leads to a proportion of $[A/(A + B/3)]$ MP-III-024 in the mixture and a mixture ratio of $[(A/B) \times 3]$ parts MP-III-024 to one-part morphine.

Isobolograms were constructed to determine analgesic properties of morphine alone or in combination with MP-III-024 in hot plate and von Fret test. In combination study, upon plotting the ED₅₀ on isobologram, three different cases were observed. Points representing dose pairs can be on the additive line or lower-left side or upper-right side. If the points fall on the additive line, no significant effects are possible of that combination. If the points fall on the upper-right side, subadditive or counterproductive effects. If the points fall on the lower-left hand side, supra-additive or synergistic effects are possible of that combination. Upon testing with different ratios of the mixture in von Frey and hot plate, a slight leftward shift of the ED₅₀ values on isobologram were noticed.

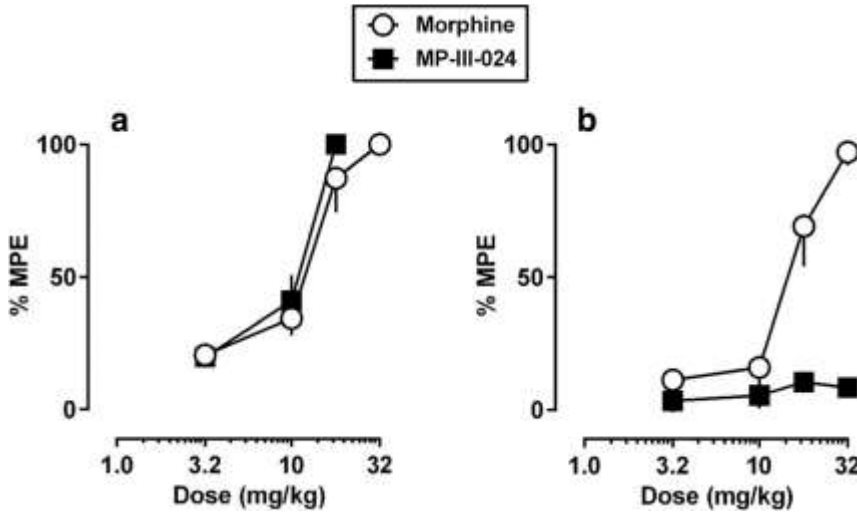
Open-Field Locomotor Test

To test the effect of MP-III-024 on locomotion, an open field locomotor test was conducted in drug naïve CD-1 mice. A cumulative dosing strategy was used in this experiment. 3.2 mg/kg, 6.8 mg/kg, 8.0 mg/kg, and 14 mg/kg of morphine alone and MP-III-024 alone tests were conducted followed by 1:0.94 ratio of morphine to MP-III-024 drug mix.

Results

Figure 10

Morphine and MP-III-024 in Von Frey and Hot Plate Technique



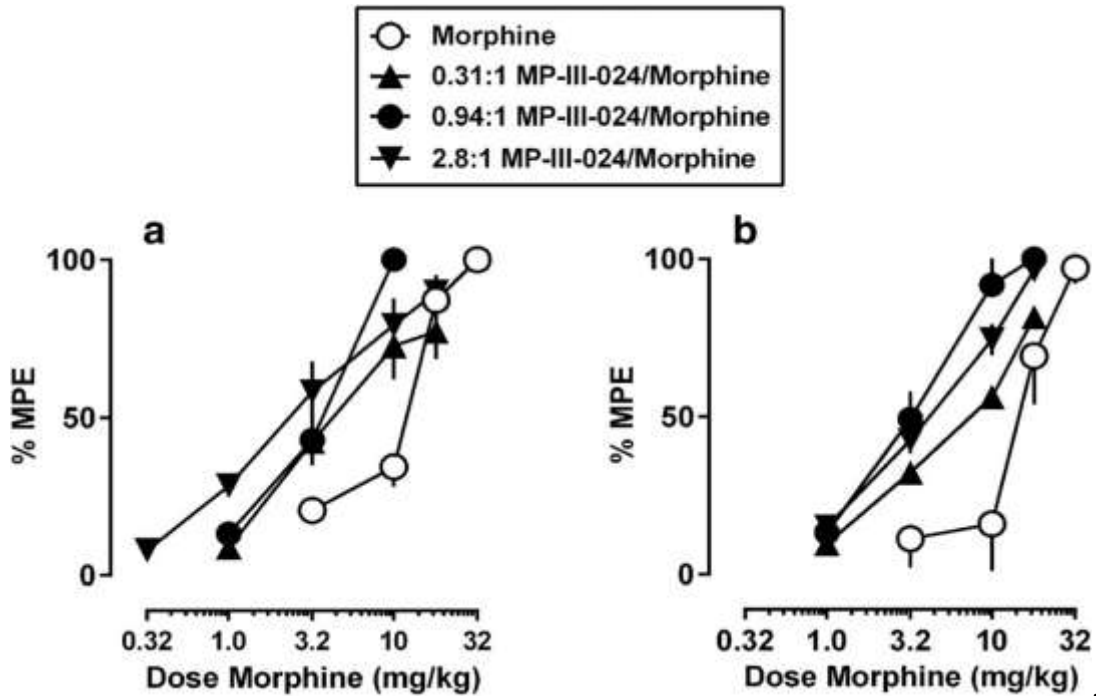
Note. Dose- effect curves of analgesic properties of morphine and MP-III-024 in von Frey technique (left) and Hot plate technique(right). Morphine and MP-III-024 showed effectiveness in the von Frey test. MP-III-024 induced antinociception comparable to morphine. In the hot plate technique, Morphine showed a dose dependent effect, while MP-III-024 was with no effect.

Figure 10 demonstrates the dose response curves for morphine alone and MP-III-024 alone in both the hot plate and von Frey procedures. In the von Frey test, the right hind paw that was injected with Zymosan A showed reduced mechanical sensitivity relative to the non-injected left hind paw whose withdrawal threshold was unaffected (data not shown). In this assay (right panel) each compound produced dose- and time-dependent increases in

mechanical sensitivity as expressed as %MPE. A statistical test for parallelism revealed that the morphine and MP-III-024 dose-effect curves were parallel ($p < 0.05$). These relative potency values were used to determine relative proportions of the compounds used in subsequent studies assessing MP-III-024/morphine mixtures. In the hot plate test, Morphine produced a dose-dependent increase in the latency to respond to the thermal stimulus while MP-III-024 was without effect in this assay. Thus, relative potencies determined in the von Frey test were used to determine the relative proportions of the compounds in each mixture⁹⁰.

Figure 11

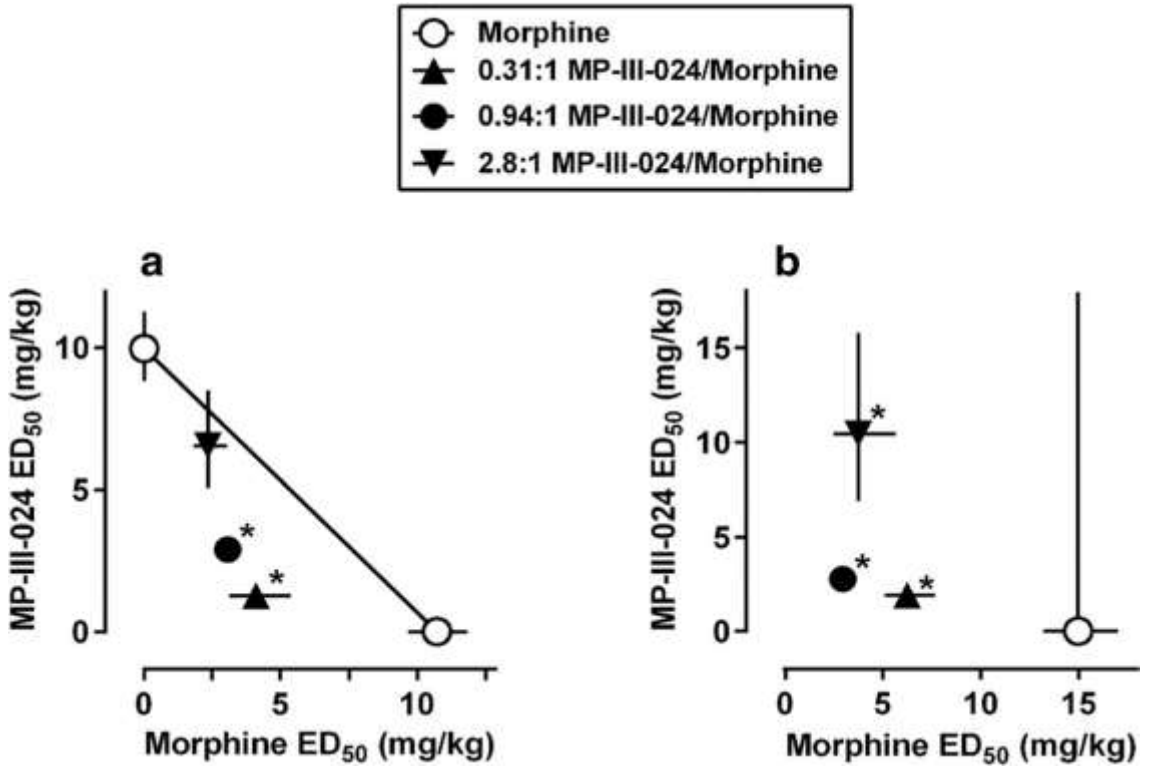
Morphine Alone and Morphine+MP-III-024 Mixtures in Von Frey and Hot Plate Technique



Note. Dose-effect curves of morphine alone and in combination with MP-III-024 in the assay of mechanical hyperalgesia and in the assay of thermal nociception b. Abscissae, cumulative dose of morphine in each mixture in mg/kg. Ordinate, antihyperalgesic (left) or antinociceptive (right) effects of each drug expressed as percent maximum possible effect. Each point shows the mean (\pm S.E.M.) from 8 mice⁹⁰

Figure 12

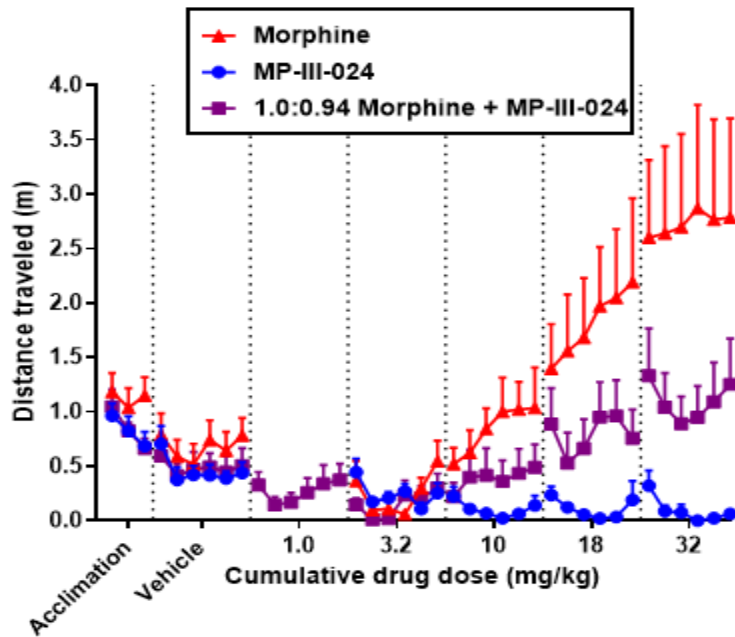
Isobolograms for MP-III- 024/Morphine Mixtures in the Assay of Mechanical and Thermal Hyperalgesia



Note. Isobolograms for MP-III- 024/morphine mixtures in the assay of mechanical hyperalgesia (a) and in the assay of thermal nociception (b). Abscissae, ED₅₀ value for morphine in mg/kg. Ordinate, ED₅₀ value for MP-III- 024 in mg/kg. Each point shows the mean (\pm S.E.M.) from 8 mice. *Significantly different from additivity⁹⁰

Figure 13

The Comparison of Locomotor Activity in Mice Injected with Morphine, MP-III-024, and Morphine+MP-III-024.



Note. Figure 13 demonstrates the locomotor activity in mice when injected with morphine alone, 0.94:1 ratio of MP-III-024 and morphine, and MP-III-024 alone. The curves indicate that MP-III-024 can be as potent as morphine as an analgesic but with fewer side effects than morphine.

Discussion

Morphine and MP-III-024 showed a dose and time-dependent reduction in mechanical sensitivity in von Frey procedure while in the hot plate procedure only morphine was observed to be effective.

1:0.94 combination mixture of morphine and MP-III-024 demonstrates an interaction that is synergistic in activity in both hot plate and von Frey procedures.

Variations in withdrawal response is an important tool used to assess changes in the tactile sensitivity in mouse models of pain and inflammation. Nociceptive behaviors like paw licking, flutter, and jumping and increased response latency following drug administration was observed in hot plate technique and interpreted as an antinociceptive response. In von Frey test, mechanical sensitivity induced by Zymosan A injection was reduced by each compound and dose indicating the antinociceptive response. The two techniques showed different effects for both the drugs but in combination, a synergistic effect raised the possibility for a new combination of analgesics that could be used in lower doses and obtain higher efficacy. Locomotor studies reveal the potential use of MP-III-024 as an analgesic that has similar potency, time course and duration of action as morphine but with significantly fewer adverse effects.

There are some limitations to this study. Firstly, all the tests were conducted in male mice. Since there are sex-mediated differences in opioid signaling and expression, if the tests were conducted in female mice the effect of combination study might have turned out to be different. Secondly, all the animals were housed in light/dark cycle, but all the

experiments were conducted during the day. The inactive part or the night part of the mouse diurnal cycle may provide variable opioid receptor expression and opioid receptor activation itself can change the circadian rhythms. If these tests were performed during the dark cycle, there is a possibility that it could have yielded different results.

We aim to test GABA_A PAMs such as HZ-166, or MP-III-080, and MP-III-024, in combination with other opioids in the future.

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