ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

# INDIRECT TARGETING OF nNOS THROUGH NMDA RECEPTORS TO RELIEF PAIN IN CHRONIC PANCREATITIS PATIENTS

Nedim Can ÇEVİK
M. Sc. THESIS

DEPARTMENT OF HEALTH SCIENCES

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| Department: | Health Sciences |
| Program: | Medical Biotechnology |
| Thesis Title: | Indirect Targeting of nNOS Through NMDA Receptors To Relief Pain in CP |
| Student's name and Surname: | Nedim Can ÇEVİK |
| Date of Defence: | / / |

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# DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethicel rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

03.06.2022
Nedim Can ÇEVİK

# PREFACE AND ACKNOWLEDGEMENT

All my research and work about this thesis was done in Acıbadem University's Pancreatic Cancer Research Center. Throughout my master degree, as a researcher I was a proud member of Pancreatic Cancer Research Center for 3 years. This facility has given me a great opportunity to learn, develop and experience in vitro and in vivo skills. In this great journey of enthusiastic research, Prof. Dr. Güralp Onur CEYHAN and Prof. Dr. İhsan Ekin DEMİR demonstrated a great influence of leadership and provided great mental excellence in my research and under their guidance I felt always responsible, eager and dedicated to my research. In addition to that, my laboratory manager Dr. Elif SEVER has given me a great mentorship, support and belief in my work that has given me confidence in my research. Lastly, I would like to give a special thanks to my laboratory partners Utku ERTETİK and Deniz BAYBAĞ for their friendship, support and ideas during my research.

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# ÖZET

**Kronik Pankreatit Hastalarındaki Ağrının Giderilmesi İçin nNOS'un NMDA Reseptör İlişkisi Kullanılarak Dolaylı Olarak Hedeflenmesi**

Ciddi nöropatik ağrı oluşumu Kronik Pankreatitte (KP) açığa çıkan ciddi bir semptomdur. KP seyir olarak da hasta açısından birçok ciddi soruna yol açmaktadır; yeme isteğinde azalış, kilo kaybı, mental sorunlar, ve sürekli şiddetli ağrı hissi yaşamı ciddi anlamda etkilemektedir. Şu zamana kadar kullanılmış olan en ciddi opioidler bile bu ağrıyı engellemekte istenilen düzeyde sonuçlar verememiştir. Günümüzde bu soruna analjezik olarak etkin bir çare bulunması ciddi ve acil bir önem kazanmıştır. Grubumuz tarafından yakın zamanda gerçekleştirilen bir çalışmada (Demir vd, 2019), bir nNOS antagonisti olan N(ω)-propyl-L-arginine (NPLA)'in abdominal ağrıyı ciddi anlamda azaltabileceğini göstermiştir. Nöronlarda, nNOS mekanizmasının aktivasyonu, bir voltaj-kanalı prensibinde çalışan NMDA reseptörlerinin aktivasyonu ile dolaylı olarak değiştirilebileceği bilinmektedir. Bu tezde, KP ağrıyı azaltabilmek için araştırmanın ağırlığı ve yoğunluğu ise NMDA antagonist ilişkisi kullanılarak hem nNOS aktivasyonuna etki ederken, hem de bu mekanizmanın içerisinde ve alt yolağında bulunan diğer mekanizmaları da anlamlandırmakla ilişkilidir. qPCR, WB ve IHC gibi in-vitro olarak yapılan öncül sonuçlarda, NMDAR1A'in ifadesinin KP'te normal pankreas (NP) dokuları ile karşılaştırıldığında ciddi anlamda artış gösterdiği gözlemlenmiştir. Buna ek olarak, öncül çalışmalarda NMDAR1A antagonisti TCN-201'in in-vivo çalışmalarda caerulein ile indüklenen KP fare modellerinde kullanıldığında abdominal ağrıda ciddi anlamda azalmaya yol açtığı gösterilmiştir. Bu bulguları desteklemek adına, transkripsiyonel ve translasyonel olarak $Ca^{+2}$ seviyesinin değişimine bağlu olan PKA, PKC, PKs, $Ca^{+2}$/CM ve CREB gibi yolaklar TCN-201 tedavisi ile birlikte in-vitro ve in-vivo olarak da incelenecek ve bu hücre içi mekanizma daha çok ortaya çıkarılacaktır. TCN-201 antagonisti dışında diğer NMDA antagonistleri de denenecek olup NPLA gibi daha önce kullanılan antagonistlerle de etkinlik olarak karşılaştırılacaktır.

**Anahtar Sözcükler:** Kronik pankreatit, nöropatik ağrı mekanizması, NMDA, nNOS

## ABSTRACT

### Indirect Targeting of nNOS Through NMDA Receptors to Relief Pain in Chronic Pancreatitis Patients

Severe neuropathic pain is one of the indicator symptoms of Chronic Pancreatitis (CP). It may leads into significant problems such as low appetite, weight loss, mental problems, disability and decline in the patient's life quality. So far, even the most potent painkillers such as opiates were not effective enough to cause a sufficient results in ameliorating pain in CP. Today, development of an analgesic treatment for CP pain is and urgent necessity. Recent study of our group, demonstrated that one of the nNOS antagonist such as N(ω)-propyl-L-arginine (NPLA) was capable in decreasing the severe abdominal pain in caerulein induced CP mice model (Demir et al., 2019). In neurons, one mechanism that leads to nNOS activation is the opening of the voltage-gated NMDA receptors. In this thesis, therefore investigation focused relieving pain in CP via NMDA receptor antagonist relationship since nNOS activation is one of the downstream effectors. In preliminary qPCR, WB and IHC results, NMDAR1A has been found to be significantly increased in CP patients when compared with normal pancreas (NP) tissues. In addition, preliminary NMDAR1A antagonist (TCN-201) treatment demonstrated a significant relief in caerulein induced CP mouse models when it compared with the control group. To elaborate these findings and decipher neuropathic intracellular mechanism of NMDA in CP. Further investigation should be done in both transcriptional and translational levels of $Ca^{+2}$ mediated downstream factors such as PKA, PKC, PKs, Ca+2/CM and CREB upon treatment of TCN-201 in both in-vivo and in-vitro experiments. Further studies will be performed preclinical trials in caerulein induced CP mouse models to investigate role of NMDAR1A with TCN-201 and comparing these results with other antagonist such as NPLA to evaluate neuropathic effects of treatment and understanding the role of NMDA in CP.

**Keywords :** Chronic Pancreatitis, neuropathic pain mechanisms, NMDA receptors, nNOS

# 1 INTRODUCTION AND AIM

## 1.1 Definition, Epidemiology, Pathogenesis and Etiology of Chronic Pancreatitis

Pancreas is a retroperitoneal organ located behind the stomach connecting to the duodenum and small intestine through pancreatic duct that contacts with the liver. In 1986, Chronic Pancreatitis (CP) first identified by H. Chiari as 'the pancreas succumbs to its own digestive properties', and it was elaborating that CP has autodigestive capability [1]. In general perspective, CP is characterized by three divergent sub-problems as; chronic obstructive pancreatitis, chronic calcifying pancreatitis and inflammatory related pancreatitis which revealed by pathological changes in CP. Due to these differences in pathology, surgical approaches also evolved to give response through this phenomenon [3]. One of the main contributors of CP considered to be use of long-term excessive alcohol consumption. This repetitive process leads production of excessive amount of enzymes and proteins within pancreatic juice causing protein plugs, gallstones especially in the ampulla of Vater and calcification within the pancreatic ducts. Accumulation of these events mediates the disruption of acinar cells located within ducts. Enzymes eventually could not be directly go through duct to duodenum and autodigestion of the pancreas occurs with proteases, amylases and lipases. Following autodigestion, most patients experience an unbearable abdominal pain. Over time, pancreas loses endocrine and exocrine functions due to culminated tissue necrosis and fibrosis [2].

### 1.1.1 Preliminary treatment approaches in chronic pancreatitis

Over four decades of research, especially for opioid related therapeutic pain management approaches in chronic pancreatitis could not yield an expected outcome. Variety of experimental designs have been administrated to overcome pain management in CP, such as drug usage (mostly analgesics), surgical and endoscopic interventions. In general, first approaches solely focused on regaining the endocrine and exocrine functional mechanism of the pancreas. To achieve that purpose, Claude Bernard injected mutton fat into the canine pancreatic duct which found to be supportive in regaining protein and carbohydrate content as a secretion model of pancreas. Later on, this treatment model applied in different cases and became a valuable tool in aiding exocrine replacement therapies [4]. Following these results, porcine pancreas treatment (amylase, lipase, and trypsin) was used on patients with the exocrine deficiency problems. This application was also widely accepted over the past decades, however, thanks to modernization of the techniques to date, better results were expected and, this technique coupled with the addition of a variety of highly concentrated enzymes. On the other hand, surgical techniques were mainly divided into two different subcategories as: pancreatectomy and decompression of drainage. In 1935, Whipple et al. began their work via successful resection of pancreas. However, he improved his technique in 1940 by shortening the procedure from two-step to one-step stage by employing bile duct jejunum anastomosis, instead of gallbladder. To this date, stomach anastomosis is a widely applicable technique and considered a landmark procedure in pancreatic surgery [5]. In 1954, hypertension problems in CP patients relieved by a successful pancreatic duct decompression [6]. On the other hand, Alfred Pearce Gould managed to remove pancreatic stones to alleviate pressure on the pancreatic duct and, thanks to this successful procedure, disease progression and pain ameliorated in CP patients [7]. To date, most effective pain relief mechanism for CP is knows as resection of pancreatic head (duodenum-preserving pancreatic head resection and pancreaticoduodenectomy) was proven itself to be an asset in surgical approaches [30-36]. Over the past years, technology, and techniques evolved and emerging endoscopic techniques developed itself to become promised interventional therapy. With this technique, drainage of pseudocysts, extraction of pancreatic duct stones, and dilation of pancreatic duct became more applicable in a simplified manner without affecting patients with minimal adverse conditions. In time, coupling endoscopy technology and surgical techniques become one of the hot topics, to yield better treatment and prognosis in pre and post interventions.

CP and unbearable abdominal pain is clinically challenging due to the 90% prevalence rate in patients, and it requires a better understanding since multiple levels of mechanisms in CP can be mediated by different factors. To date, there is still no viable opioid based therapy option to ameliorate abdominal pain in CP which can be a struggle in prognosis of CP and directly affecting quality of life of patients. When it is considered in general perspective, pain emerges due to ductal and parenchymal obstruction, and lead to inflammation, autodigestion, pressure and pain management, via removal of gallstones only complies a limited response to overcome pain relation in CP [13]. On the other hand, inflammation-related irreversible changes within the ducts and loss of the endocrine and exocrine functions in the pancreas, could not be fully alleviated with present perspectives. Hence, directing these problems has high demands of approaches, and recently emerged studies points out that consideration of CP pain relation to neurobiology and neuro-pathophysiology. To comprehend these changes in molecular levels, consensus should be shifted into action potential related reprogramming capability of central and peripheral neuronal levels, susceptible nociceptors, and neurotransmitters.

# 2 BACKGROUND

## 2.1 Molecular Driving Factors and Pain Mechanisms of Chronic Pancreatitis

Pathogenesis of CP is poorly understood with only common etiology was heavy alcohol use [43], Bordero et al., suggested that ethanol may lead to fatty degeneration in acinar tissues which was a similar manner to the hepatocytes observed in alcohol-related liver diseases. In his experiments, morphologic relevance of CP sustained with direct or indirect use ethanol led to disruption of the integrity of pancreatic acinar cells [43-44]. On the other hand, Braganza et al., proposed to use of oxygen-free radicals to produce a toxic effect on pancreatic acinar cells. Introduction of excessive free oxygen radicals were used in different clinical trials, and as a response to that, different antioxidants were administered for the treatment of chronic pancreatitis which yielded promising results (NCT00142233) [45-47]. Another pancreatitis hypothesis was proposed by Sarles and Sahel which was pinpointing destruction of pancreatic acini due to ductal hypertension. This process was dependent on aggregating protein precipitates with the due to primary intraductal obstruction. With the aid of alcohol consumption, precipitation of calcium crystals and proteins within the duct lumen caused duct obstruction. In normal conditions, acinar cells require production of lithostatin to avoid such stone formation, where lithostatin release increases fluidity of pancreatic juice and prevents the formation of such stones via preventing precipitation of calcite crystals and protein plaques. The validity of this hypothesis was questioned since researchers could not prove lithostatic function in pancreatic juice but however, the role of duct plugging cystic fibrosis was unquestioned by authorities [48-49]. Comfort et al., previously suggested that chronic pancreatitis occurs due to recurrent episodes of acute pancreatitis [50]. When Klöppel and Maillet revisited Comfort's idea, they revealed that focal fat and necrosis in pancreatic parenchyma led to the infiltration of fibroblasts, macrophages and lymphocytes, while fibrosis assumed to be the consequence of necrosis. They have also valued the idea, that underlying cause of recurrent bouts of acute pancreatitis, which occurs due to activation of zymogen in pancreatic acini subsequently led to the development of chronic pancreatitis [51]. Fibrosis is defined as the accumulation of an excessive amount of extracellular matrix proteins within tissue, and it is one of the constant hallmark feature of chronic pancreatitis. In recent researches, fibrosis does not to be considered as an end product occurring upon chronic injury, rather this phenomenon believed to be active in active dynamic process and it can be reversible in the early stages of the injury. This phenomenon encoruaged research field through understanding of pancreatic fibrogenesis and significant methods have been established to isolate and culture pancreatic stellate cells (PSCs). Klöpell and Maillet have successfully demonstrated that PSCs activation can lead to the activation of fibrosis. Normally PSCs resides within the periacinar region of the pancreas in the quiescent rest state and function as keeping maintenance of synthesis and degradation of the extracellular matrix (ECM) [52-55]. Activation of PSCs can be triggered via different mechanisms but one of the prominent and potent activator of PSCs is known as ethanol and its metabolites causing oxidative stress via reactive oxygen species (ROS) within the gland. In addition to that, different cytokines can also initiate activation of PSCs, such; as tumor necrosis factor α (TNF α), transforming growth factor β (TGF β) and interleukins like IL-1-6-10 [56,57]. On the other hand, lipopolysaccharides (LPSs) can activate PSCs via their interaction with Toll-like receptor 4 (TLR4), leading activation of endotoxins to induce pancreatic fibrosis [58].

Upon activation, PSCs transform into a myofibroblast-shaped cell type that is capable of secreting upregulated amounts of extracellular matrix proteins such as collagen type-I, fibronectin, and laminin. Due to the activation, PSCs act on phagocytic cells limiting the extent of inflammation via ingesting neutrophiles and antigens while it is also possible to observe activated PSCs via the use of smooth muscle actin (α-SMA) [59,60]. General process of fibrosis and destruction cycle results in endocrine and exocrine malfunction insufficiency in pancreatitis patients. Exocrine insufficiency occurs in the later stages of the CP, and leads to release of amylase, lipase and proteases into the pancreatic juice. To demonstrate clinical symptoms of CP, 10% less lipase secretion than normal is sufficient in most of the cases [61]. In a clinical manner, assessment of the exocrine function capability of pancreas either performed by the help of direct and indirect tests. In a direct function test, measurement is done via collection of pancreatic juice from the duodenal tube considered to be the gold standard in exocrine function measurement of the pancreas. On the other hand, indirect assessment is done with the use of serum. The decreased amounts of pancreatic enzymes along with elevated levels of amylase/lipase are considered to be a sign for CP [62,63]. Whereas, endocrine dysfunction reveals itself as pancreatic diabetes with abnormal glucose tolerance levels, and gradually it evolves into an overt diabetes mellitus in CP via loss of insulin secretion. This case can also be triggered due to insulin resistance that contributes to diabetes in the event of episodes of hypoglycemia which can be a lethal problem due to the lack of absence of glucagon as parallel counter control mechanism [63,64].

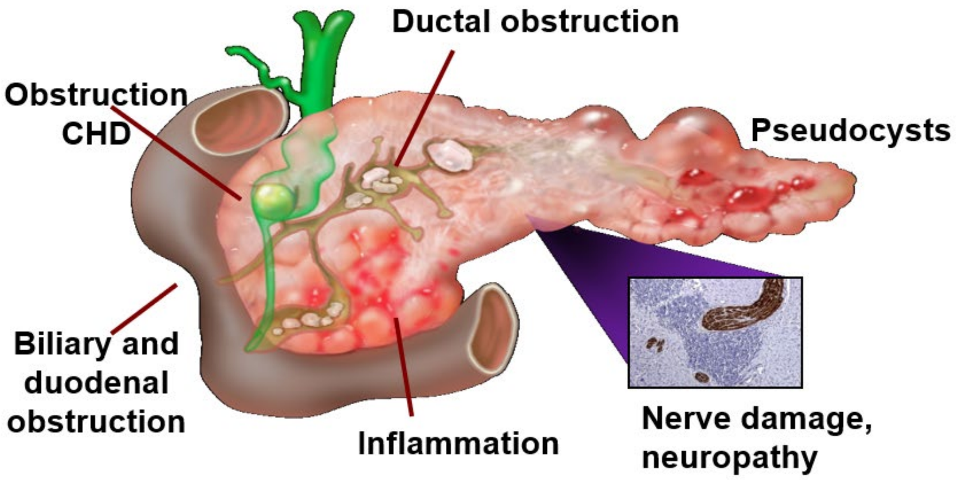



Figure 1 – General Changes in Chronic Pancreatitis [2,3,16,17]
Accumulation of proteins plugs and gallstones, leads inflammation of pancreas and calcification within the pancreatic ducts. Consequent of this repetitive problematic behavior leads disruption of acinar cells located within ducts and eventually enzymes could not be directed through duct to duodenum, instead autodigestion of pancreas occurs with proteases, amylases, and lipases.

Collection of researches revealed that inflammation and fibrotic tissue in CP leads morphological and functional changes in intrapancreatic nerves. Accumulation of these changes results in wiring problems related with the wide spectrum of alterations of central pain and peripheral nociception [14]. Ultrastructural analysis aided

Bochman et al., to investigate CP tissues and in his observations nerves had dramatic changes in their hypertrophy and density. They have also revealed that CP tissues have severe neural damage which could be a consequence of disrupted edematous neural contents, perineurium and penetration of inflammatory cells into the interior nerves [29]. Unfortunately, these findings did not go further investigation until today. Following larger studies correlated to CP with extent of neural damage, neural hypertrophy, neural sprouting, inflammation around nerves (called as pancreatic neuritis) and neuropathic pain intensity [17,26]. Ceyhan et al., correlated pain in CP with neuropathic alterations, where neural remodeling occurs via increased neural density and significantly enlarged intrapancreatic nerves found to be related CP patients with severe pain scores. Ceyhan and colleagues also identified these nerves were infiltrated or surrounded by inflammatory cells and this infiltration within the nerves causing damaged perineurium called as 'pancreatic neuritis'. Patients with neural and perineural alterations have correlated with a long-lasting abdominal pain with 95% prevalence. This observation illuminated research on the neural remodeling in which altering pancreatic innervation in CP, became one of the most investigation area managing pain in CP [16]. Parallel to this study, Ceyhan et al., also demonstrated the relation of neural alteration and pain relation in a cohort including 546 CP patients. [17]. Consequently, Demir et al., attempted to explain pain mechanisms in chronic pancreatitis since this problem become a true challenge over the past decades. They have shifted their focus on peripheral nociception, peripheral/pancreatic neuropathy-neural plasticity and central neuropathy-neuroplasticity. Their research revealed, pain related factors in CP is not only dependent on nociceptive agents but also dependent on neuropathic agents. In this mixed type of pain relation, nociceptive mechanisms characterized by direct stimulation of intact nerve fibers due to responsible agents while neurotrophic ones occur only in the presence of nerve damage [18].

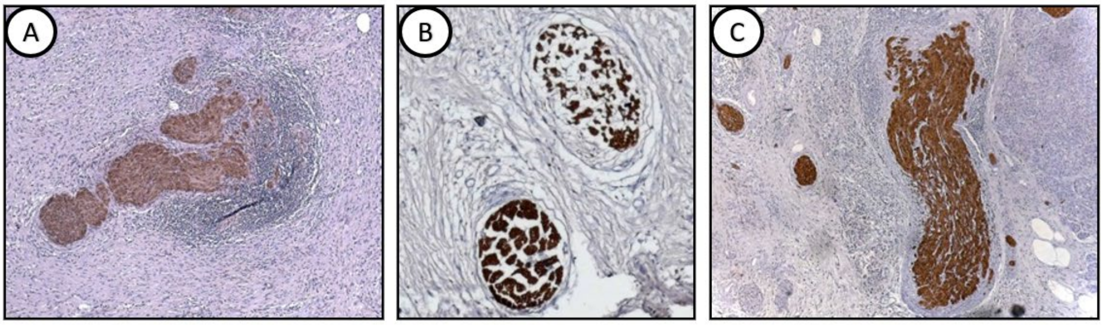



Figure 2 – Neural Remodeling in CP [16,17,362-364]

(A) Myxoid appearance and neural edema (B) Extraordinarily enlarged intrapancreatic nerves (C) Growth-Associated-Protein-43 expression correlates with active neural growth and enlargement of intrapancreatic nerves in CP

CP is referred as sum of recurrent attacks of acute pancreatitis (AP), when action potential reaches a required threshold, whole process could trigger and activate pain in CP. Activation of nociceptive fibers related with the release of agents from damaged acinar cells such as calcium, hydrogen sulfide, bradykinin, and serotonin but so far, these molecules were never demonstrated in any animal model of CP or human CP [19]. Since it is difficult to track down activators of nociceptive fibers in CP, research trend was shifted to elaborate activation markers in CP. Following activation, nociceptive nerve fibers secrete elevated amounts of neurotransmitters like substance P, calcitonin gene-related peptide (CGRP) and glutamate into the pancreas. In these events peripheral nociceptive fibers orchestrated chemotactic and vasodilatory effects of these mediators which results in neurogenic inflammation in AP [20,21]. On the contrary, short-lived action of neurotransmitters in CP makes things more complicated while revealing neurogenic involvement of CP. Büchler et al. and his colleagues overpower this phenomenon via semi-quantitative analysis, and they have demonstrated that SP and CGRP were upregulated in immunoreactive nerve fibers of human CP specimens [22]. In addition to that, still majority of the neurotrophic factors under investigation to elaborate their relationship with CP. Especially nerve growth factors (NGFs) considered to be one of the important suspects in terms of nociception. NGF is known to activate nociceptive nerve fibers while modulation of SP expression via its high-affinity receptor tyrosine kinase A (TrkA) and its low-affinity receptor p75

[23]. NGF previously demonstrated to be overexpressed and localized in hypertrophic nerves, metaplastic ductal cells, intrapancreatic ganglia and degenerating acinar cells. Within this study, results were demonstrated upregulation of TrkA related with degree of pain in CP, but NGF expression and pain correlation could not be proven [24]. In another study, pain correlation and upregulation of brain-derived neurotropic factor (BDNF) was revealed to act on the p75 receptor [25]. In a follow up study, glial cell-line derived (GDNF) family artemin and its receptor GFRα3 was also investigated. Due to conjunction with NGF, artemin was able to induce hyperalgesia. While both artemin/GFRα3 found to be located within Schwann cells, smooth muscle arteries and intrapancreatic ganglia and both artemin/GFRα3 were significantly overexpressed in CP with a strong correlation in pain severity [26,27]. Keith et al. was the first researcher reporting the prominent infiltration of inflammatory cells (especially eosinophiles) into the intrapancreatic nerves in CP. Eosinophilic infiltration around the nerves and pain severity in patients reported as in major correlation with his study [28]. Interleukin-8 (IL-8) was found to be the only inflammation marker that overexpressed in CP tissues when it was compared with the normal pancreas (NP) tissue. In addition, different inflammatory cells were found to be in hypertrophic nerves [37]. Chemo-attractants were investigated to enlighten which factor mostly contributing attraction of inflammatory cells into the nerve mediated neural damage and neuropathic pain. Along with nerve immunoreactivity, upregulation of fractalkine and CXCR1 was found in patients with severe pancreatic neuritis and neuropathic pain [38]. Fractalkine was also reported as responsible in neuropathic alteration of in CP via playing vital role as chemo-attractants for immune cells, inducing neuropathic pain via glial activation and contribution of tissue fibrosis [39-41]. In a recent study, Demir et al., aimed to decipher specific mediators of CP to discover a novel therapy option to prevent that unbearable pain in patients. To achieve that aim, his group tried to elaborate neurotransmitter and neuro-enzyme profile of the intrapancreatic nerves of CP and pancreatic cancer (PCa) patients. Their investigation focused on SP, CGRP, VIP and nNOS on human normal, CP and PCa tissues. They have found out nNOS expression was significantly increased in CP tissues, and was also correlated with pain and neuritis score of CP patients. Following these findings, they have used caerulein induced CP mouse model, and nNOS specific inhibitor NPLA to assess the abdominal

pain in mouse. When it is compared with no treatment group, results of their experiments were satisfactory in pain management and this pathway looked promising for an applicable therapy option [42]. In overall picture, syndrome in CP requires elaboration of neuropathic pain mechanisms with the definition of activated numerous cellular and molecular activators. Generation of neuropathic pain requires understanding of different factors such as immune cells, damaged nerves, activated glia, chemokines, neurogenesis, cytokines, and possible unidentified mediators. Due to the interdependence of neuropathy and nociception, clinicians may consider pain in CP as a predominantly neuropathic, mixed-type pain and a convincing treatment option may need a consideration of this special mixed mechanism [18].

## 2.2 Deciphering the Glutamate Mediated Pain Management and Therapeutic Options in CP

Throughout the history, pain relief considered to be one of the major focus of medical science. In some special cases pain could not be ameliorated with the natural defense mechanism. Normally upon a tissue injury, it follows up with a withdrawal response of perceived pain or CNS aids to prevent further injury. However, in chronic pain or extreme pain conditions, persisted pain occurs even after complete healing process, and it may become inconvenient to the patients. Unfortunately in those cases, use of relieving analgesics and repeated usage of those opioids often associates with the analgesic tolerance or the use of low-affinity-target drugs leads cytotoxicity problems over time. In order to reach a maximized affect for a drug with a minimal adverse effect, understanding of the disease and deciphering molecular mechanisms of pain became requisite for CP To overt this expectation, recent researches involving pain relief, focused on the glutamate and glutamate related pathways.

### 2.2.1 Glutamate maintenance

Glutamate is the one of the most principal mediators of the fast excitatory neurotransmitter in central nervous system (CNS) that controls the cognition, memory learning, sensory information, emotions, memory retrieval and motor coordination. Approximately 80-90% of the synapses in the brain are excited with the glutamatergic pathway [65]. Therefore, all of the cells in the brain, whether glial or neuronal, contain glutamate in their process, which is found in both mitochondria and cytosol cell bodies. Formation of glutamate occurs with the metabolization of amino acids and glucose, through glycolysis and tricarboxylic acid (TCA) cycle as it is converted into α-ketoglutarate. Due to transamination process (α-ketoglutarate receives amino group), glutamate is produced. In the quiescent form, glutamate continuously converted into α-ketoglutarate and metabolized with the aid of TCA cycle [66]. All the glucose that passes through the blood-brain barrier (BBB), gives rise to two molecules of acetyl-CoA that activates TCA cycle to become α-ketoglutarate and it eventually metabolized as glutamate. Current studies demonstrated amino acids such as valine, leucine and isoleucine are important amino donors for glutamate synthesis since these amino acids has no problems passing through the BBB. When the uptake volume of amino acids are less than glucose uptake, the amino group uptake requires glutamate recycle in the brain where carbon backbone broken down into $H_2O$ and $CO_2$. In addition to that, highly active aspartate is also important in glutamate synthesis since it shuttles intermediate molecule oxaloacetate by the aminotransferase [67]. In the TCA cycle, α-ketoglutarate has been continuously produced with the aid of glutamate release from neurons. If this continuous cycle does not countered with other contributors, the downstream product oxaloacetate could not produce citrate. Therefore, energy metabolism stops with the halt of TCA cycle. As complimentary system, the return of glutamine for transmitter glutamate reduces the effect of lost α-ketoglutarate but in the end, it cannot prevent that loss completely. To maintain the level of glutamate, two additional mechanisms emerge; one is reuptake of glutamate from extracellular fluid and directing it to nerve terminals, and other reaction occurs with the aid of pyruvate to form malate which is a prerequisite for oxaloacetate termed as 'pyruvate carboxylation'[68]. Particularly, glutamate found to be released in response to nerve, tissue or nociceptive stimulation upon an injury [69-76]. In different studies, glutamates role has been shown in CNS where it induces neural depolarization. On the other hand, glutamate in the spinal cord after chemical or electrical stimuli found to be released to counteract through this process [77-84]. In addition to that, in vitro experiments demonstrated that glutamate has a role in noxious chemical stimulation such as nerve injury, chronic inflammation, hind-paw retrieval cases where it released into the CSF [85-87]**.** In an experiment, the inhibition of glutamate release after an injury or inflammation on rodents presented the attenuation of hyperalgesia. On the other hand, glutamate injection into the knee joint cavity of rats found to be induced hyperalgesia [88]. Upon electrical stimulation of the primary afferent fibers, aspartate and glutamate release also observed in rat spinal cord [89]**.** All in all, there is a strong relation and evidence about glutamates' role in pain on nociceptive and excitatory neurotransmitters whether in periphery and spinal cord. However, up to this point, due to the complex function of glutamate, it may be triggered in different paths and complex mechanism which required to be deciphered on event and disease basis.

### 2.2.2 Ionotropic and metabotropic glutamate receptors

Learning is a key anatomical process, and it requires CNS to undergo some form of changes termed as 'synaptic plasticity' which enables storage of data and response through information. Electrophysiologically, two different phenomenon occurs at the synaptic level; while long term-potentiation (LTP) occurs where synapses has been strengthened and long-term depression (LTD) occurs when a synapse is weakened through response. In this phenomenon, high-frequency glutamatergic (100 Hz) stimulation may relay impulses more efficiently and therefore leads those responses to be kept for months or longer. On the other hand, prolonged low-frequency stimulation (1 Hz) leads a relay in the impulses in the long-term basis where responses vanish over time [90]. In theory, glutamatergic synapse communication relies on presynaptic and postsynaptic (axo-axonal) terminals. In fact, even microglia, oligodendrocytes and astrocytes express different types of glutamate receptor that may lead into the release of glutamate through nerve terminals. Glutamatergic receptors can be identified under two major categories; ionotropic receptors are cation channels shifts into open state via glutamate binding while metabotropic receptors do not conduct any ion fluxes but instead these receptors activate intracellular enzymes through G coupled proteins when they interact with glutamate.

Ionotropic glutamate receptors forced into a conformational change due to agonist binding that increases the chance of channel opening. Ionotropic receptors have three different classes: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainite (KA). In general, ionotropic receptors relies on fast responses and they have different affinity against their agonist glutamate. These ligand-gated ion channel receptors are in tetrameric assemblies of the individual subunits and each subunit can produce functionally different response leading to the heterogeneity of glutamate receptors throughout the CNS. While $EC_{50}$ for glutamate at NMDA receptors approximately 1 µmol/L, it increases approximately 400 µmol/L for AMPA receptors. Aside from glutamate receptors, several endogenous molecules may play role in the activation of NMDA such as aspartate and homocysteate [91].

#### 2.2.2.1 NMDA receptors

The NMDA receptor has been one of the major interest points in both human and animal nociceptive studies. Three NMDA receptor subunit families have been identified as single gene GluN1, GluN2A to GluN2D and GluN3A to GluN3B. As like AMPA and Kainate receptors, most of the NMDA receptors in the brain are known to be heteromeric and mRNAs that encoding most of the NMDA receptors are differentially distributed within different regions. While GluN1 expression in the CNS nearly ubiquitous, the four GluN2 subunits demonstrates differential patterns of expression. Specifically, GluN2A is mostly present within the cerebellum and forebrain while GluN2B highly expressed in the forebrain and GluN2C found mostly in cerebellum. On the other hand, GluN2D expression seems to be complementary to the GluN2A expression within the hindbrain and midbrain region but has low expression in the forebrain. GluN3A has mostly identified in the cortex and spinal cord, whereas GluN3B mainly found within motor neurons of the spinal cord, medulla and pons.

Unlike any other glutamatergic receptor, NMDA receptors are most tightly regulated, and it has at least six distinct endogenous ligand binding sites that may influence the probability of ion channel opening. NMDA receptors have C terminus linked to an ion channel permeable to sodium ($Na^{+}$), calcium ($Ca^{2+}$) and potassium $K^{+}$ [92-94]**.** NMDA has two unique agonist sites for the glutamate and glycine and polyamine regulatory sites, all of which promote the receptor activation with distinct recognition sites for $H^{+}$, $Mg^{2+}$, and $Zn^{+2}$ that acts to inhibit ion flux against bound agonist through a voltage-gated manner. NMDA receptors have short-chain dicarboxylic amino acids as agonists, such as aspartate and glutamate. Moreover, NMDA receptor has a unique requirement since it can be activated by binding of two different agonist, glycine, and glutamate. While glycine binding site on the GluN1 subunits, neither glutamate nor glycine is not sufficient to activate and open the ion channel and due to that fact, they referred as coagonists for NMDA receptor activation [95]**.** In addition to that, the glycine binding site within NMDA receptor is distinct since it could not be blocked by strychnine or not activated by β-alanine as like other glycine receptors. Interestingly, most of the glycine site agonists of NMDA also competitively block AMPA receptors, suggesting possible structural similarities between two ligand-recognition sites [96]. While glutamate and glycine act in open NMDA receptor ion channel, extracellular $Mg^{2+}$, can exert a voltage-dependent block on the open ion channel [97]. Other voltage-dependent blockers of NMDA receptor channels are phencyclidine (PCP), MK-801 and ketamine which interferes with the channel opening against glutamate and glycine coaginsts. Another important endogenous allosteric inhibitor occurs with $H^{+}$ activation leading to a reduction in channel opening frequency especially for GluN2B-containing receptors over a physiological pH range, such that, at 6.0 pH receptor activation seems to completely to be suppressed. This situation proves that the ionizable cysteine and histidine may play a key role in NMDA receptor activation [98]. NMDA receptors mediated with the excitatory postsynaptic activation and potentials. Their stimulation affects PKC, PKA activation and nitric oxide (NO) production via $Ca^{2+}$/Cd mediated nitric oxide synthase (NOS) [99-100]**.** Upon activation, NMDA receptors leads accumulation of intracellular $Ca^{+2}$ levels that is not only effecting activation of the downstream processes but also contributes a positive feedback loop which eventually leads NMDA activation enormously. Under these circumstances the inhibition of overexpressed NMDA activation and downstream processes such as PKA, PKC and $Ca^{+2}$/Calmodulin pathway become an important issue in challenging the pain mechanism. To demonstrate this kind of approach, $Ca^{2+}$ dependent phosphatase, calcineurin was used to inactivate NMDA receptors that which suggesting dephosphorylation of the NMDA receptor can also lead dampening NO production [101-102].

Post-synaptic density (PSD) is a multi-protein complex that play role of signal transduction via converting the extracellular glutamate signal into various intracellular signals. Apart from glutamate receptors, PSD also contain ion channels, transporters, glycolytic enzymes, cell adhesion molecules, intracellular signaling pathway proteins and scaffolding proteins that links PSD as a functioning complex. In addition to that, PSD made from lipophilic proteins which requires binding of the two palmitic acid residues leads lipid rafts and small areas of the plasma membrane rich with sphingomyelin and cholesterol. These rafts all together aids to concentrate proteins within the spines of PSD [103]. NMDA and AMPA receptors spread through PSD, while metabotropic receptors generally located within the periphery of PSD. Between NMDA and AMPA, NMDA seems to appear in all or most of the glutamatergic synapses when it is compared with AMPA receptors and some of these synapses does not elicit AMPA receptor currents since they are 'silent' containing only NMDA receptors [104]. In glutamatergic pathway, PSD95 seen as one the major scaffolding protein, it contains several domains: PDZ, guanylate kinase (GK) and Src homology (SH3) domain. PSD95 anchors NMDA receptors to the cytoskeleton of the dentritic spine and couples them to the intracellular signaling system via affecting NMDA intracellular activation. Prior to calcium intake, postsynaptic NMDA receptors bind to the calmodulin at all glutamatergic synapses where $Ca^{2+}$ binding activates $Ca^{2+}$ / calmodulin-dependent kinase II within that downstream. Activation of CaM kinase II leads to phosphorylation of other proteins such as GluA1 (from serine 831 residue) increases conductance of the AMPA receptor which contributes to potentiation of the glutamatergic synapses [105]. The $Ca^{2+}$-calmodulin (Ca/Cd) complex also activates nitric oxide synthase (NOS), which binds to a PDZ domain of the PSD-95. Activated NOS leads to the production of NO from arginine; and in turn NO activates guanylate cyclase enzyme that catalyzes the conversion of GTP to the intracellular cGMP, leading to the activation of protein kinase G (PKG). On the other hand, A-kinase anchor protein (AKAP) is another scaffolding protein of PSD, binds to protein kinase A (PKA), protein kinase C (PKC) and protein phosphatase calcineurin (PP2B) as well as PSD95. These processes ensures proximity of NMDA receptors to PKA. AMPA recruitment within the postsynaptic membrane strengthen the synapse during LTP, while PKC plays an important role in phosphorylation of other GluA1 serines (S816 and S818), where phosphorylation means GluA1 can bind onto an actin-binding protein and can travel along actin filaments to the plasma membrane [106]**.** LTD of the glutamatergic synapses can also lead to the activation of the NMDA receptors with the aid of $Ca^{2+}$-dependent activation, while calcineurin dephosphorylates GluA1 on serine 845 residue and plays role in relocation of the AMPA receptors from the plasma membrane through to the cytosol [107]**.** Unlike the relationship of NMDA and PSD95, AMPA receptors do not directly binds to the PSD95, but they appear to bind it indirectly through other proteins such as trans-membrane AMPA-R regulatory proteins (TARPs) [108]. On the other hand, C-termini of the AMPA receptors directly binds to the other PSD proteins via the aid of PDZ domains, such as protein interacting with C kinase 1 (PICK1), glutamate receptor interacting protein (GRIP), AMPA receptor-binding protein (ABP) and synapse-associated protein of 97kDA (SAP97) [103]. PSD95, PICK1 and GRIP also known to interact with GluK1 and GluK2 kainate receptor subunits. PKC binding onto the PICK1 may aid to phosphorylate their subunits while leading anchoring of the more stable synapse and strengthen the kainate receptor-mediated transmission. On the other hand, metabotropic glutamate receptors use homer and shank scaffolding proteins to be anchored to the periphery of the PSD. Homer is a cytoplasmic protein which aids the link and multimerize proteins that have homer-binding proteins of mGlu receptors while leading connection of 3,4,5-triphophate receptors ($IP_3$) that causing release of $Ca^{+2}$ from smooth endoplasmic reticulum. On the other hand, shank binds onto PSD95 through the GK domain and links mGlu receptor to the PSD95 [106]**.**

All the changes occur in LTD or LTP relies on synaptic plasticity and with this process, formation of new proteins requires specific transition of certain genes. Upon activation, NMDA receptors may lead transcription of more than 100 genes during LTP stage. That process results in formation of mRNAs and immediate or late activation of those certain genes. Genes that encoding transcription factors, intracellular signaling molecules, enzymes of energy metabolism and other receptor subunits (such as AMPA) contributes to these synaptic changes [109]**.** Upon activation by glutamate, intracellular mechanism relies on monomeric GTPases that have important role in activation or silencing of different signaling pathways that lead to changes in gene transcription. Ras is known as a small GTPase that plays a central role in mediating alteration of the gene transcription within NMDA receptor activation. Raf-MEK-ERK and PI3 kinase intracellular pathways can be stimulated via Ras, and those pathways latter leads activation of MEKK-HNKK-JNK and protein kinase B (Akt/PKB) pathway. Consequent to these events, phosphorylated ERK translocate from the cytosol to the nucleus and contributes to the activation of transcription factors such as CREB and Elk. On the other hand, Akt/PKB activation affects the nuclear translocation and activation of NF□B and CREB while activation of JNK pathway leads activation of c-Fos, c-Jun and ATF2. In principle small GTPases cycle between an inactive GDP-bound and active GTP-bound state and regulated by activating guanyl nucleotide exchange factors (GEFs) and inhibitory GTPase-activating proteins (GAPs). In short, GEFs stimulate exchanges of GDP for GTP leading small GTPases such as Ras bound to their effector proteins, while GAPs lead to hydrolyzation of GTPases and causes them to lose their signaling activity. In addition to that fact, $Ca^{2+}$/Cd or diacylglycerol can also activate GEFs and GAPs through binding of $Ca^{2+}$ ions. In that case, a Ras-specific GAP (synaptic GAP) binds to the PSD95 complex and Ras GEFs could be identified with NMDA receptor subunit leading ERK activation. Through this glutamate receptor activation both GEFs and GAPs can be modulating the activity of Ras and its downstream effector pathways [110].

Electrophysiological data represents that NMDA has a role in the primary afferent neurons in rat hind-paw retrieval experiments [111]**.** Moreover, ionotropic NMDA application within the spinal cord induces enhanced dorsal horn neuronal response in noxious and innocuous mechanical stimulation [112-115]**.** To counter that, use of NMDA antagonists led to attenuation of the dorsal horn neuronal responses to noxious mechanical stimulation [116], and intrathecal supplementation of NMDA antagonists revealed inhibition of dorsal horn neuronal firing rate [117]. In addition to that, NMDA antagonists also demonstrated effects on dorsal root ganglia (DRG) [118,119]. Upon tissue injury or inflammation, use of antagonist also demonstrated to be preventing the development of wind-up induced repetitive C-fiber stimulation, sensitization and dorsal horn firing [120-122]. Behavioral data also suggest that NMDA has significant role in nociceptive processing where NMDA administrated and spontaneous nociceptive behaviors (SNBs) were observed along with mechanical and thermal hyperalgesia [123-130]. In another experiment, NMDA agonist has been infused with a low dose for a long time, and this infusion has been shown to elicit a chronic 'central' experimental pain disorder in rats, which can be prevented with co-infusion of $Mg^{2+}$ sulphate that aids blocking NMDA receptor channel [131]. Following a formalin test on rats, both competitive or non-competitive NMDA antagonists affected in reducing nociceptive scores whether they have been given as an injection or orally [132-145]. On the other hand, when Complete Freund's Adjuvant (CFA) induced to observe chronic inflammation in sensory axons revealed that correlation in increased NMDA receptor activation [146]. Moreover, NMDA antagonist found to be reducing heat/cold hyperalgesia and mechanical allodynia in rats accompanied with nerve injury [147-162]. It has been also demonstrated in spinal cord ischemia or peripheral nerve injury situations [163-165]. These accumulation of data may suggest that NMDA is a valuable target and some of the antagonists can be useful in treatment of chronic pain syndromes.

#### 2.2.2.2 AMPA & kainate Receptors

AMPA receptor subunits GluA1-GluA4 serve as fast excitatory synaptic transmission and these receptors are widespread throughout in the CNS. Subunits of AMPA receptors co-assemble with one another to form tetramers with a specific pharmacological profile. AMPA and Kainate receptors are permeable to the $K^+$ and $Na^+$ and they have been mediated through fast excitatory postsynaptic potentials [166-169]. While subunits of GluR1 to GluR4 has higher AMPA affinity, GluR5 to GluR7 subunits are found to be specifically Kainate receptor selective. While expression of

GluR2 within the neurons evident, $Ca^{2+}$ currents seem to abolish signals. Conversely, in the absence of GluR2 subunit, it may contribute to $Ca^{2+}$ influx triggered by glutamate [170,171]**.** Kainate receptor subunits constituted with GluK1-GluK3 family but it requires co-assemblance with GluK4 or GluK5 to produce a functional receptor profile. In general, GluK4 and GluK5 are known as modulatory subunits, when these receptors found to be expressed alone, they become virtually inactive ion channels [172-175].

As like the NMDA receptors, there are also different hints about AMPA and KA receptors involvement in nociceptive processing. Administration of AMPA/KA antagonist into the spinal cord demonstrated diminishing dorsal horn neuronal responses against thermal and mechanical noxious/non-noxious stimulation [176,177]**.** It should be also noted that, GluR5 specific antagonist can also reduce spinal noniceptive responses as in vitro studies but fail to administer an analgesic effect in acute thermal nociception experiments [178]**.** Application of AMPA/KA antagonist also revealed inhibition in dorsal neuronal responses in periphery and in afferent neurons [179-181]**.** Behavioral evidence also reveals the role of AMPA/KA receptors in pain mechanism where administration of agonist produces SNBs as well as mechanical and thermal hyperalgesia in the allodynia [182-188]**.** In chronic pain conditions, such as injury of sciatic nerve, or chronic inflammation AMPA/KA receptors also found to be alleviated within the spinal cord and peripheral areas [189-192]**.**

#### 2.2.2.3 Metabotropic receptors

Metabotropic glutamate receptors are linked to the G-coupled proteins and these receptors are consisted of mGlu1-mGlu8 proteins possessing unusually large N-terminal extracellular domain. While glutamate itself activates all recombinant mGlu receptors, Group I mGlu receptors stimulates phospholipase C activity to produce $IP_3$ that leading release of $Ca^{2+}$ from cytoplasmic stores. Activation of phospholipase C leads not only formation of $IP_3$ but also diacylglycerol where it activates protein kinase C. On the other hand, group II and group III mGlu receptors results in the inhibition of adenyl cyclase (Conn, 2003). In general, mGlu receptors responsible in modulation of variety of voltage and ligand gated ion channel expression in central neurons and their coupled effector enzymes. Activation of mGLU leads inhibition of L-typed voltage dependent $Ca^{+2}$ channels which decreases high-threshold $Ca^{+2}$ current in spiking neurons. Through this process, activation of mGlu receptors leads depolarization and consequent neuronal excitation via closing voltage-dependent, $Ca^{2+}$-dependent $K^{+}$ channels in cortical and hippocampal neurons. Although exact mechanism still unclear, general understanding relies on activation of CaMKII. On the other hand, mGlu activation also affects activation of ligand-gated channels such as NMDA and kainate receptors via inhibition or potentiation. Upon activation of mGlu I, inhibitors of protein kinase C (PKC) or Src kinase leads inhibition of NMDA-receptor-induced uptake of intracellular calcium [193]. On those cases, intracellular $Ca^{2+}$ level increase may contribute to trigger production of NO via $Ca^{2+}$/Cd activation of NOS. On the other hand, Group II (mGlur2-3) and group III (mGlur4,6,7,8) are negatively coupled with the adenylate cyclase enzyme, and activation of these receptors leads inhibition of production of cAMP [194,195]. Electrophysiological data reveals that use of mGluR agonists may induce neural depolarization on the brain, spinal cord and periphery [196-202]. Intrathecal administration of selective mGluR agonists, such as (RS)3,5-dihydroxy-phenylglycine (DHPG) affects group I and induces SNBs in rats [203,204]. On the other hand, while group I mGluRs related to be involved in acute or tonic pain situations, group II/III mGluRs found to be pronociceptive [205]. Another group of investigators shown that rat models with postoperative pain reduction could not be alleviated in hyperalgesia or allodynia even with the use of several mGLuR antagonist [206]. As mentioned before, group I mGluRs are directly coupled with the PI hydrolysis that leads into activation of NMDA receptors and PKC. There are also accumulating evidence about the role of PKC and pain relation since inhibition of PKC also revealed a pain relief against mechanical and thermal injury [207-210]. In addition to that, PKC found to be translocated within the membrane of the spinal cord in the cases of nerve injury or inflammation and PKC thought to be increased in activity on those areas [211-215]. Moreover, PKC knockout mice also did not develop any neuropathic pain function following nerve constriction [216]. All in all, activation or inactivation of mGluR plays are role in modulation of

NMDAR receptor activity. Activation of group I mGluRs enhances NMDA receptor activity via PKC phosphorylation of the NMDA-associated ion channel [217-224], so group I agonists enhances the effect of NMDA-induced neuronal degeneration [225]. On the other hand, group II and III mGluRs contributes NMDA receptor activity and it also preserves neurons against NMDA or KA induced excitotoxicity [226].

### 2.2.3 Suggested pain related mechanistic model

Given all the data, it could be possible to suggest a mechanistical model about relationship of glutamate and glutamate receptors' collaborative work against a stimuli such as pain. With this mechanism, it is evident that there is a co-localisation of different class of glutamate receptors. These receptors can contribute into feedback responses, direct and silent activations. Under simultaneous events or consequent orders it is an inevitable that these actions effects each underlying mechanism. In the previous researches, these connections have been demonstrated where NMDA receptors and opioid receptors found to be co-localized [227,228] while NMDA, AMPA and mGluRs can be an overlapping sequence due to the glutamate activation [229]. Whether in acute or chronic pain states, glutamate release is enhanced as a primary response, thus all other connected glutamatergic responses become overstimulated with the aid of this event. Postsynaptically, enhanced activity of iGluRs leads intracellular accumulation and influx of $Ca^{2+}$ ions that latter leads activation of different intracellular pathways. Enhanced activity of group I mGluRs increases PI hydrolysis, ultimately leading increased release of $Ca^{2+}$ from the stores of ER via activation of $PI_3$. Further accumulation of promoted $Ca^{2+}$ influx promotes opening of voltage-gated calcium channels that assisting intake of further $Ca^{2+}$ ion intake [230-233]. Accumulation of intracellular $Ca^{2+}$ levels triggers the activation of $Ca^{2+}$/Cd that leads the activation of $Ca^{2+}$/Cd dependent kinases and neuronal nitric oxide synthase (nNOS). $Ca^{2+}$ stimulation also activates PKC which can be also promoted due to PI hydrolysis following stimulation of group I mGluRs. As previously described, PKC involved in different acute and chronic pain states, as well as opioid tolerance, PKA and PKC together further phosphorylates NMDA receptor-associated ion channels [234-240], which also helps relieving the receptor from $Mg^{2+}$ block and further promotes intake of $Ca^{2+}$ ions, thus creating a one of the feedback loops. On the other hand, through $Ca^{2+}$/Cd pathway and activation of nitric oxide synthetase (NOS), leads enhanced nitric oxide (NO) production. As another feedback loop, accumulation of NO production could diffuse out of the cell and elicit more glutamate release presynaptically [241]. In addition to that, PKC phosphorylates G protein coupled opioid receptors thereby desensitizing these receptors and rendering opioid agonist analgesic effects [242-246]. This process within the cycle seem to be important since neuropathic pain analgesic therapy is considerably take a hit due to less effective opioid response. On the other hand, group II/III mGluRs effects on pain mechanism is less clear, but they have assumed to be co-localized with group I mGluRs and/or NMDARs which leads phosphorylation of the coupled opioid receptors. This whole process induces desensitization and reduction by inhibition of cAMP production, while on the contrary increased intracellular cAMP levels means enhanced PKA-mediated phosphorylation of NMDA that contributing feed-back loop and chronic pain sensation with neuronal excitation [247-249]. Further research on this mechanism still needs to be enlightened to provide new possibilities in the chronic pain.

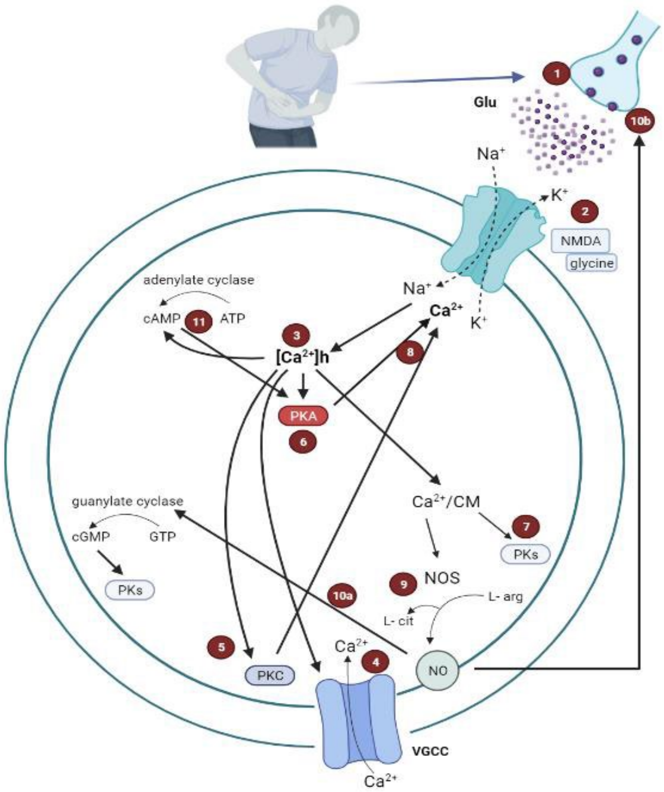



Figure 3 – NMDA Receptors Related Pain Mechanism

(1) Upon highly active chronic pain stimulation, presynaptic glutamate is released into ionotropic receptors, in this case NMDA receptors. (2) Activated NMDA receptors provides intracellular $Ca^{2+}$ influx (3) and contributes elevation of $Ca^{2+}$ levels dramatically. (4) Increased intracellular $Ca^{2+}$ influx leads activation of Voltage-Gated Calcium Channels (VGCC). These channels provide more intracellular $Ca^{2+}$ influx which activates (5) protein-kinase C (PKC), (6) protein-kinase A and (7) $Ca^{2+}$/Cd dependent protein kinases (PKs). (8) Upon stimulation, PKA and PKC phosphorylates NMDA receptors which enables intracellular intake of more $Ca^{2+}$. (9) Accumulation of intracellular $Ca^{2+}$ levels also leads stimulation of $Ca^{2+}$/Cd pathway and activation of nitric oxide synthetase (NOS) leading increased production nitric oxide (NO). (10a) Accumulation of intracellular NO, activates PKs through cyclic guanosine monophosphate (cGMP) and (10b) NO also diffuses out of the cell, reactivates presynaptic area triggering release of further glutamate release. (11) In addition to that, accumulation of intracellular $Ca^{2+}$ also activates cAMP which later leads activation of PKA. This activated PKA also phosphorylates NMDA receptors and enables intracellular $Ca^{2+}$ influx.

### 2.2.4 NMDA antagonists

There are several studies that are involving the use of NMDA antagonists to counter different problems such as pain related syndromes, therapeutic studies against cancer or therapeutic trials to cure any memory related disease. In one of these approaches, healthy volunteers were evaluated for pain sensation after the use of NMDA antagonists such as dextromethorphan and ketamine. This research has yielded decrease in pain sensation due to repeated chemical or electrical stimulation on those individuals [250,251]**.** In another study ketamine was also used as an analgesic against acute pain conditions in human volunteers, and this experiment was proven that ketamine can be a useful agent to alleviate acute pain conditions [252,253]**.** Another experiment with NMDA antagonists was done on rats, and this experiment also proven that targeting NMDA could be valuable in preventing spreading hyperalgesia [253-258]**.** On the contrary, some of the researches could not yield expected outcome from NMDA antagonists but these antagonists also did not induce any adverse effects on patients [259-261]**.** Although, there were some suggestions about use of NMDA antagonists with common opioids may induce a synergetic effect, in the end it did not attain any synergetic effect to induce a meaningful analgesic effect [262]**.**

In general, NMDA antagonists was seen to capable of relief pain in chronic recurrent pain conditions where commonly used opioids could not attain an expected outcome. Antagonists such as amantadine, ketamine and memantine are non-competitively act on blocking NMDA receptor associated ion channels. Upon non-competitive blocking on, voltage-gated channels affects various types of neuropathic pain in cancer, surgery-induced nerve injury and limb pain after traumatic sciatic nerve injury [263-266]. Administration of ketamine in cancer patients especially with mixed or neuropathic pain often observed as to be useful managing pain conditions [267]. On the other hand, efficacy of non-competitive NMDA antagonist treatment in chronic pain conditions remain as controversial. Up to now, there are no conclusive scientific data whether use of non-competitive NMDA antagonist with analgesic regimens is still a debatable area, and it is generally hands of the experienced clinician's decision. Several groups were investigated effects of non-competitive NMDA antagonist's efficacy on postoperative pain conditions, and they have given these antagonists as pretreatment, during or after surgery conditions. These studies also observed some controversial results, some researches found out that NMDA antagonists may induce reduction in post-operative pain [268-270], while others reported that pretreatment could not yield any analgesic benefits [271,273]. Likewise to post treatment (after surgery) conditions, reduction in pain has been reported in several studies [272-275]. In another study, $Mg^{2+}$ was used to block NMDA ion channel and when it supplemented with anesthesia it could give and postoperative analgesic benefits and while exclusively it also reported in one trial patients had more episodes of unbearable pain [276,277]. With this outcome $Mg^{2+}$ found to be not a candidate for analgesic therapy. In different cases including animals and humans, some of the non-competitive NMDA antagonist also found to have some adverse effects in motor levels, such as extensor paralysis in rat hindlimbs, balance loss and weaving, righting, and grasping reflexes and poor placing of hands and legs [278,279]. In humans, these non-competitive antagonists have produced perceptual disturbance, neural neurotoxicity cases and psychotomimetic effects [280-287]. All in all, up to now non-competitive NMDA antagonists found to be effective in pain management where commonly used opioids could not yield an expected analgesic outcome. Although, some of these NMDA antagonists found to have some adverse effects, studies should be implemented to better understanding benefits or risks of this class of antagonists and in further studies these should be improved to have more effective antagonists with less adverse effects but more stability and affinity.

## 2.3 Animal Models of CP

Chronic Pancreatitis (CP) characterized as occurrence of continuous or recurrent inflammation that leads irreversible morphological changes such as acinar cell atrophy and interstitial fibrosis. Although excessive alcohol use seen as one of the key reasons behind chronic pancreatitis, in the experimental aspect clinically relevant models require more delicate approaches to establish a desired pathological model. In detail, perilobular and intralobular fibrosis occurrence within the parenchyma leads stone formation within the pancreatic duct, eventually leads into pain and permanent impairment in endocrine and exocrine functions in the advanced stages of pathogenesis. Since there is no viable opiod related treatment option for chronic pancreatitis, animal models needed to recapitulate the original disease to understand and evaluate the viability therapeutical approaches and source of the disease itself. On that aspect, when special focus shifted to induce acute and chronic pancreatitis, this attention led researchers to different approaches such as pancreatic duct ligation, repetitive overstimulation with caerulein and chronic alcohol feeding. In addition to those chemical stimulations, genetic models were also generated to mimic the features of chronic pancreatitis with distinct background and limitations. So far, certain differences underlined between human and experimental animals needs to be resolved for critical understanding of the results. Pathological features of chronic pancreatitis include different aspects such as the loss of endocrine and exocrine cell mass, formation of intraductal protein plugs, infiltration of chronic inflammatory cells, calcification and interstitial fibrosis required to be mimicked to develop a fully functional chronic pancreatitis model. On the other hand, there are some natural physiological differences in humans; pancreatic and bile ducts separate ducts normally form a very short channel in the duodenal wall while rat has common long channel, and the bile duct serves as a conduit for the pancreatic juice. Another difference is the absence of gallbladder in rats while their pancreas composed of multiple segments in the shape of an omentum while on the other hand, human pancreas is a single solid organ abutting the duodenal wall. Despite these differences rat pancreas also shares similarities; pancreatic duct drains into the duodenum, similarity of cellular components such as ductal, acinar, endocrine and stellate cells presented. In addition to that rat pancreas also serves for both endocrine and exocrine functions [360].

To recapitulate acute and chronic pancreatitis models, Lieber and DeCarli performed a series of studies to evaluate the effects of alcohol administration on the liver and their research led development of different animal models focusing different pathophysiological configurations to stimulate pancreas [288,289]**.** To produce a genuine model, several models combined with two or more stimuli to enhance effects of fibrogenic factors on pancreas. Up to this date, most popular models include surgical pancreatic duct ligation, repetitive caerulein injections and ethanol feeding which complement with different stimulatants. Also, genetic models has emerged to demonstrate pathogenesis of chronic pancreatitis and these models can be manipulated with different chemicals, agents and immune cells to enhance the mildness of chronic pancreatitis.

In 1856, Claude Bernard demonstrated acute pancreatitis can be mimicked through oil olive injection into the pancreatic duct of dogs and this observation led intensive research into the mechanism to determine the onset of biliary pancreatitis. In 1901, Eugene Lindsay Opie published two different autopsy reports suggesting in both cases of pancreatitis was connected with gallstone where in the first report gallstone occluded the orifice of pancreatic duct while patient lost his life due to acute pancreatitis. Opie also tested pancreatic duct ligation in cats and he noted that the development of pancreatic tissue leaded fat necrosis. Along with this knowledge, he was proposed that there was an obstruction event which leading “impaired outflow obstruction hypothesis” triggering cause of acute pancreatitis. In another post-mortem research he also found out that impacted stone within the papilla of Vater region created a different communication between the main pancreatic ducts and common bile duct, allowing the patient’s bile enter the pancreatic duct [290]**.** One of the earliest study for bile duct ligation was implemented by Churg et al., where this procedure was applied on dogs. After common bile duct closed to the duodenum of the pancreatic tissue, examinations done for 1 week of time. Results of that experiment demonstrated that pancreas morphology was significantly altered where distinctive architectural changes for chronic pancreatitis were seen; as acinar cells exhibited atrophy, loss of zymogen granules and fragmentation of endoplasmic reticulum. In addition to that, infiltration of leukocytes, macrophages and fibroblasts identified within the interstitial space [291]**.** Similar results were observed when Watanabe et al., published his research while performing duct ligation on mice including a 16 weeks of observation after the procedure. On the other hand, anatomy of mouse pancreas was different where three lobes; duodenal, splenic and gastric and most the cases splenic duct joins the gastric duct, which eventually leads into the common bile duct. To overcome this problem, splenic lobe separated from rest of the pancreas and with this manipulation it could demonstrate the different parts of the organ can contribute mechanical obstruction of pancreatic duct whereas rest of the organ can be used as control without any effect and animals did not suffer from any complete loss of the organ functionality. However, this technique was challenging to implement since it required precise surgical skills to minimize trauma and pancreatic duct which was only compensating 150 uM diameter of working area. This area was also ligated proximal to pancreas near the liver and a cannula which is inserted abote the ligature to obtain pure bile while another cannula inserted into biliopancreatic duct through the ampulla of Vater to collect pancreatic juice with an additional cannula inserted into the duodenum to return biliopancreatic juice. Nonetheless, this method was useful to practice later effects in the course of chronic pancreatitis while it could sustain remarkable observations in gradual replacement of parenchymal and fatty fibrotic tissue after 2 weeks which was also the same with the clinical observations of humans [292]**.** Interestingly, after this procedure Langerhans islets were found to be preserved during chronic pancreatitis and exocrine functionality kept until the advanced stages while exocrine insufficiency occurred since more than 90% of the parenchyma was lost. Watanabe also observed similar outcomes with mice when ligation mediated pancreatic juice out-flow is limited with 60% and mice maintained their weight [292,293]**.** In different studies, time point of the impairment in the loss of exocrine and endocrine functions drastically changed. In general, glucose intolerance with decrease of insulin levels was observed between 28 days and up to 6 to 12 months [294-298]. On the other hand, as pancreatic stellate cells (PSCs) were considered to be responsible in generation of fibrosis, with proinflammatory stimulus of duct ligation which leads changes that can be observed via α – SMA expression test after 7 -10 days [299,300]. While it demonstrates that PSCs activation dependent on multifactorial factors, the biggest activator seem to be elevated intraductal pressure due to pancreatic duct obstruction [301,302].

Morphological changes during pancreatitis occurs due to digestion of the gland by enzymes. These enzymes normally secreted and synthesized by pancreatic acinar cells due to clogging, presence of these enzymes leads to necrosis of the gland. Probably most potentially harmful digestive enzymes are proteases, normally synthesized and secreted as inactive zymogens where they have activated only with the duodenum via brush-border enzymes [303-307]. Moure et al, reported that excessive cholinergic stimulation is correlated with progression of pancreatic injury that mediated with the acinar cell necrosis and vacuolization [308]. Moure and Hans Chiari also demonstrated that trypsin might be involved in development of pancreatitis with proposed autodigestion mechanism in 1896 [309]. In the light of these events, experimental animal models designed to employ cholinergic agonists such as cholecystokinin (CCK), carbamylcholine, carbachol and its analogs as well as scorpion venom to induce pancreatic injury to administer both dose and time dependent approach [310-314]. In humans, pancreatic acinar cells do not directly give a response against CCK stimulation rather, they generally manipulated due to cholinergic pathways involving neurogenic CCK stimulation. On the other hand, in rodents CCK plays a direct role in maintenance of exocrine pancreatic functions after stimulation via food ingestion [315]. In 1977 Lampel and Kern was given caerulein (a CCK analog derived from the Australian tree frog *Litoria caerulae*) to the rats and they have observed characteristics development of excessive edema even after 1 hour onset of disease [316,317]. The primary physiological effect of CCK has been observed as stimulation of protein-rich secretion while doses of CCK lead continuum of maximal stimulation of enzyme secretion. Following stimulation of maximal secretory doses of caerulein, pancreas loses storage of exocrine enzyme by 75% within several hours due to supraoptimal stimulation with the aid of increasing concentration of CCK and maximal secretion, hyperstimulation occurs and leads the accumulation of secretory proteins within the pancreas and pancreatic injury [318-321]. In rats caerulein injection can be continuously infused intravenously either through jugular vein with the help of a polyethylene catheter or into the tail while in mice it is administered through peritoneal cavity as repeated injections. General concentration of caerulein was given to the animals between 50 to 100 ug/kg/h and thereby it has reaches maximal secretory concentrations after 12 h. Changes within the pancreas such as edema, inflammation and necrosis can be obtained after 48 hours via continuous infusions. Within the first hours of caerulein hyperstimulation, it leads in increased hydrostatic pressure, vascular permeability and release of pancreatic enzymes which can track down through blood serum. Calcium upregulation and breakdown of actin cytoskeleton can be observed with premature zymogen activation and abolishment of secretion of zymogens into the pancreatic duct. All in all, these events lead to occurrence of systematic inflammatory response syndrome and extra-pancreatic damage such as pancreatitis related lung injury [322]. In different studies of acute pancreatitis, repeated caerulein induction reported to be comparable with humans since these models can induce hyperglycemia and diverse histopathological findings such as infiltration of inflammatory cells within the pancreas, acinar cell vacuolization, pancreatic edema, and the presence of activated pancreatic enzymes [361]. To induce chronic fibrotic changes in the pancreas, repeated stimuli required with variable intervals. These models can also mimic clinical observations such as repeated attacks of acute pancreatitis, eventually results in fibrosis and atrophy [323]. At the beginning of the injections, pancreas produces components for recovery that temporarily exceeds the degradation of extracellular proteins [324]. Following this process, in favor of fibrosis cytokines released into the environment and TGFβ1, one of the most potent fibrogenic modulator, overexpressed in stromal cells and pancreatic acinar due to continuous caerulein administrations [325,326]. Due to repeated episodes of acute pancreatitis organ becomes vulnerable and unable to degrade ECM components which leads fibrosis after a series of injuries in the pancreas. Neuschwander-Tetri et al., has performed these injections twice a week for 10 weeks to observe fibrotic changes in the mice [327]. In the rats, repetitive caerulein injections alone could not attain fully developed chronic pancreatitis while causing only minor effects on endocrine cells. To provoke sufficient endocrine disfunction, toxins and different triggering factors needs to be used to increase severity of chronic pancreatitis. On the other hand, Ohashi et al., demonstrated that exocrine functions of rats can decrease significantly after 6 weeks, while Wistar Bonn/Kobori (WBN/Kob) rats with chronic pancreatitis also demonstrated with severely impaired exocrine function [328]. All in all, exocrine functions do not require more triggering factors such as endocrine functions. L-arginine is one of the essential amino acid that has been used to induce severe necrotizing pancreatitis and this also implemented in acute pancreatitis studies with murine and rat models [329-331]. After 24 h of intraperitoneal injection, L arginine (500 mg/100 g body weight) leads necrosis in acinar cells up to 100% with a dose dependent manner and after 48 h necrotic cells are replaced by interstitial tissue composed of fibroblasts and leukocytes [332,333].

There are also several reports demonstrating that combination of caerulein with a proinflammatory agents whic can cause synergetic affect and enhances pancreatic fibrogenesis. In general lipopolysaccharides (LPS), Ethanol, Cyclosporin A (CsA) and dibutyltin dichloride (DBTC) was used along with caerulein injections. In addition to that, intraperitoneal caerulein injections can also be given to genetically engineered mouse models to enhance the effect of pancreatic fibrosis. As an endotoxin, LPS induces strong immune response in animals when it is attached into LPS binding protein (LBP). This complex binds into CD14, further activates the TLR4. Recently it was demonstrated not only immune cells but also PSCs expresses CD14 and TLR4 and aggravates pancreatitis [334,335]. In a study, mice were treated with (50 ug/kg body weight) and LPS (3,125 mg/animal) twice a week for 10 weeks. Mice were killed following the procedure and morphology of the pancreas of treated mice demonstrated severe signs of chronic pancreatitis [336]. Cyclosporin A (CsA) is another used as immunosuppressant in organ transplantations, and it can induce TGFβ expression on pancreatic stellate cells. Combination of CsA with cearulein injections leads increased glandular atrophy, reduced pancreatic blood flow and collagen deposition resulting more potent glandular histological damage [337]. Dibutylin dichloride (DBTC) is another compound that is used as heat stabilizer of polyvinylchloride plastics, can exert toxic damage into the pancreas. DBTC dissolved in 100% ethanol and used in 8 mg/kg body weight proportion could attain acute pancreatitis in 3 weeks of interval while periductal fibrosis can be detected within 6 weeks [338-340]. On the other hand, oxidative stress factor ROS known to be proinflammatory observed that it can lead into acute or chronic pancreatitis due to direct damage on cells and activated stellate cells [341]. Repeated injections of caerulein into thirodexin (TRX-1) transgenic mice led attenuated pancreatic fibrosis where thirodexin known as, one of the endogenous antioxidative and anti-inflammatory protein. Likewise, monocyte chemotactic protein 1 (MCP-1), very potent attractant for inflammatory cells, synthesized profibrotic chemokines while exocrine function was preserved. In addition to that, TGFβ also known to be profibrogenic since it can activate stellate cells and eventually promoting fibrosis in different organs. Inhibition of TGFβ signaling ameliorated pancreatic fibrosis while Smad7 negatively regulating that TGFβ signaling resulted as significantly reduced fibrosis in experimental pancreatitis models [342,343]. On the other hand, liver alcohol requires to be metabolized with different mechanism but in pancreas different enzymes required to be involved to metabolize and evade different effects of ethanol [344,345]. Supplementation of alcohol can lead, pancreatic damage through toxic compounds such as ROS which became byproducts of ethanol metabolism and exerts direct effect on pancreatic acinar cells. This process followed with the activation of stellate cells via ROS mediated fibrosis [346]. The duration of alcohol feeding differs among studies between 4 weeks to 16 months but in general, this process leads severe liver damage but it leads changes in pancreatic morphology rather milder functional sufficiency cases in chronic pancreatitis [347]. On the other hand, combinatorial approach of caerulein and alcohol feeding constitutes a synergetic effect and leads upregulated loss of parenchyma, calcification and pancreatic fibrosis [348-350].

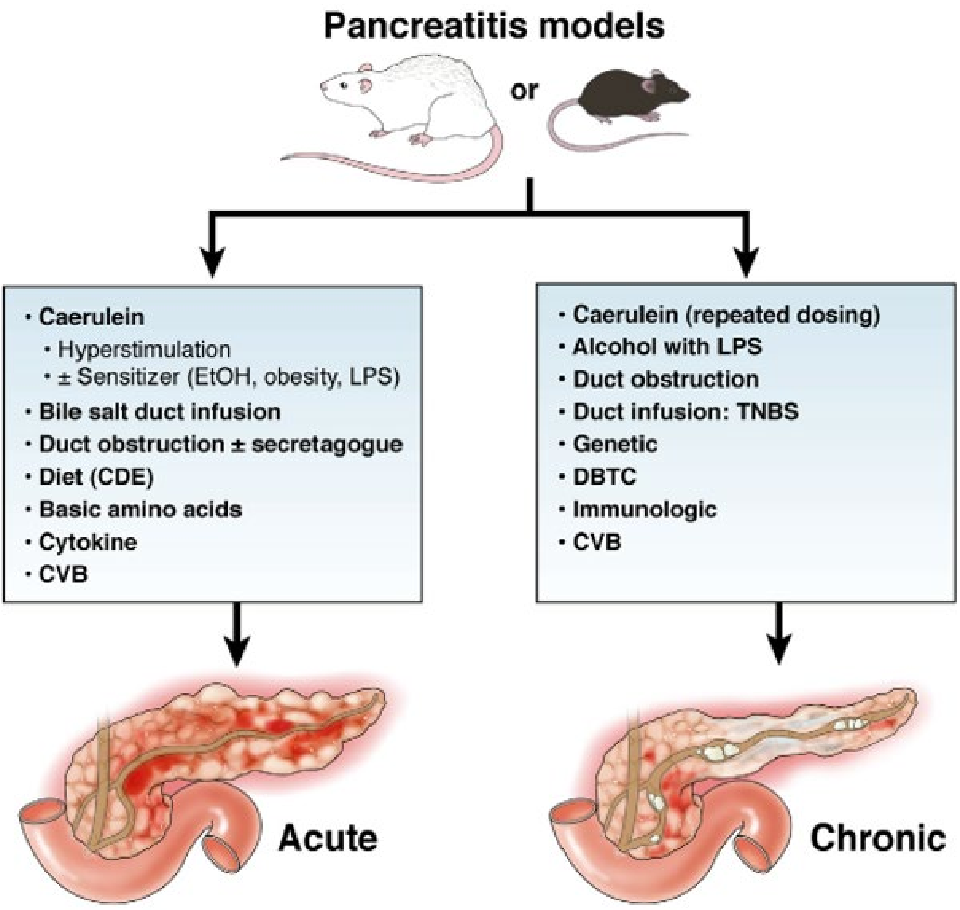



Figure 4 – Pancreatitis Mouse Models

In rats or mice acute pancreatitis (AP) model requires acute inflammation with hemorrhage and edema. AP models mostly constituted with administration of caerulein, bile salt duct infusion and duct obstruction. Chronic pancreatitis (CP) requires chronic inflammation with fibrosis, duct strictures, in some models intraductal stones, irreversible degradation of intraductal acinar cells, loss of endocrine and exocrine functions. Most of the CP models constituted with repeated caerulein dosing, duct infusions, genetically inductions and administration of alcohol and LPS together.

Genetic abnormalities in chronic pancreatitis revealed that activation of trypsinogen is one of the key event during the early phase of genetic manipulations. In this case trypsinogen gene found as a valuable tool to generate genetically engineered animal models while additional focus shifted cystic fibrosis models. WBN/Kob rats are capable of mimicking chronic pancreatitis like lesions with endogenous fashion. In WNB/Kob rats, first changes occurs within the 3 months of age where periductular fibrosis leads into parenchymal loss as an age dependent manner. Over time, endocrine and exocrine functions of the animals also impaired while these rats become diabetic in 60 to 90 weeks of age [351]. It is important to note, chronic pancreatitis only occurs in male rats when androgen synthesis are elevated to a certain level after puberty. On the other hand, use of estradiol leads amelioration in diabetic symptoms and that observation suggested that sex hormones are involved in the development of chronic pancreatitis. Infiltration of inflammatory cells were found highly active in WBN/Kob rats but it was also reversible after glucocorticoid treatment [352,353]. Once trypsinogen is activated within the pancreatic parenchyma, it can also activate other digestive proenzymes in the pancreas. In 1996 Whitcomb et al., reported a rare type of chronic pancreatitis case by administering the arginine-histidine mutation (R122H) along with cationic trypsinogen (PRSS1) which was later correlated with autosomal hereditary pancreatitis phenotype [354]. In a concurrent study missense R122H mutation was also implemented in murine models which displayed acinar cell dedifferentiation according to age and pancreatic fibrosis. In another model, R122H mutation induced with under control of elastase-2 promoter mouse pancreas. Implementation of this model demonstrated elevated levels of lipase without any spontaneous development of chronic pancreatitis. Nevertheless, of that fact, mouse models were given drastically susceptible results to caerulein induced pancreatic development and severe pancreatitis has been observed along with PRSS1 mutation [355,356]. In another model, cystic fibrosis (CF) implemented that leading diminished pancreatic excretory functions up to 90% of the patients. In those cases, a defective chloride channel leads an abnormal chloride passage throughout epithelial cells deficient fluid secretion, thickening within the pancreas [357-359]. Mouse models were also created with the disruption of CFTR gene which displays different features of cystic fibrosis. This model, eventually leads to death after 40 days of intestinal obstruction and ileus [360]. Choosing the right model for the study requires scientific rationale that needs to be carefully consideration. In general, repetitive caerulein model is most widely used while it is an highly reliable, reproducible and easy to perform technique which can be also complemented with different compounds to mediate a further injury, while transgenic or knock-out animals found to be useful to increase the severity of chronic pancreatitis.

# 3 MATERIALS AND METHODS

In this project we have aimed to decipher transcriptional and translational activity of glutamate receptors and especially NMDA receptors took our primary attention since we believed that these receptors might have a role in pain management in CP while they could be a potential therapeutic agent for pancreatic cancer.

## 3.1 In Vitro Experiments

The first part of the experiments were emphasized on in vitro experiments to assess glutamate receptors' transcriptional and translational activity in pancreatic cancer and mouse cell lines. To evaluate activity of those receptors, cells culture were cultured and following a sufficient confluency, RNA and protein isolation was done. After qPCR and WB experiments a promising expression profile observed.

On the second part, siRNA treatments were done on pancreatic cell lines, and after a successful silencing, we have managed to demonstrate it with qPCR application. In further experiments, these manipulated cell lines will be subjected into 3D-migration assay and time-scheduled colony formation assay to evaluate whether they have therapeutic functionalities against cancer.

On the third part, microtome was used to have 2.5 μ slides of both human and mouse pancreas tissue. We have done H&E experiments to demonstrate established successful acute pancreatitis and chronic pancreatitis mouse model morphology. On the other hand, H&E was also used on human samples for correction of the morphology. Following confirmation, IHC staining was done to understand the expression profile of NMDAR1A in human PCa, CP, NP and mouse CP models. CD45 and CD68 markers was also used in human CP tissues.

### 3.1.1 General cell culture protocol

**Materials**

- RPMI 1640 Medium
- DMEM High Glucose Medium
- Penicillin/Streptomycin
- Trypsin
- PBS
- Fetal Bovine Serum
- Non-Essential Amino Acids (NEAA)
- Serological Pipettes
- Pipettor
- T-25 & T-75 & T-150 Cell Culture Flasks
- DMSO
- Trypan Blue
- Thoma Lam
- Light Microscope

**Methods**

According to the cell type, medium was prepared with RPMI 1640 supplemented 10% fetal bovine serum and 1% penicillin/streptomycin or for animal cell line DMEM High Glucose supplemented with 10% fetal bovine serum, 1% penicillin streptomycin and 1% NEAA. Following preparation, frozen cell lines were diffused in water bath, and diluted with prepared complete medium. This procedure followed up with centrifugation at 300 G for 5 minutes. Supernatant was discarded and pellet was resuspended with 1 ml complete medium. Viability and cell number were evaluated with the aid of trypan blue and thoma lam, and according to the number, at least 500.000 cells were seeded onto T-75 flasks and placed into an incubator with 37ºC and 5% $CO_2$ . Cells were investigated under microscope and their proliferation rate was noted. After 2 days of incubation, all the medium contents were discarded, cells were cleaned out with PBS and 10 ml of fresh complete medium was added. Following days, cells investigated until they have reached into confluency. Consequent to confluency, medium was removed and cell were cleaned out with PBS. After removal of PBS, 2.5 ml trypsin was added, and flask placed into incubator with 37ºC and 5% $CO_2$ content for 5 minutes. Cells were investigated under microscope to observe if there any grip. 7.5 ml of complete medium was added onto the flask and all the cell content was placed into 15 ml falcon tube. Centrifugation was done under 300 G for 5 minutes. Supernatant was discarded and pellet was resuspended with 1 ml complete medium. Viability and cell number were evaluated with the aid of trypan blue and thoma lam, and according to the number at least 500.000 cells were seeded onto T-75 flasks and placed into an incubator with 37ºC and 5% $CO_2$. After the passage of cells, cells were investigated under microscope and their proliferation rate was noted. After 2 days, all the medium contents were discarded, cells were cleaned out with PBS and 10 ml of fresh complete medium was added. Cells were observed until they have reached 80% confluency, and from this point, instead of complete medium, their media changed into serum-free medium (SFM). SFM lasted 6 hours for RNA isolation and 24 hours for protein isolation. Upon completion of this procedure, SFM was discarded, and cell were cleaned out with the use of PBS. 2.5 ml trypsin was added, and flask placed into incubator with 37ºC and 5% $CO_2$ content for 5 minutes. Cells were investigated under microscope to observe if there any grip. 7.5 ml of complete medium was added onto the flask and all the cell content was placed into 15 ml falcon tube. Centrifugation was done under 300 G for 5 minutes. Supernatant was removed and cell pellet was kept at -80 ºC for further RNA or protein isolation process.

### 3.1.2 RNA isolation and cDNA library construction

**Materials**

- 2-mercaptoethanol
- RLT Buffer
- RPE Buffer
- %70 and %100 ethanol
- 20-G needle
- gDNA Column
- RNA Easy Column
- Vortex
- Nanodrop
- Centrifuge
- Pipette
- Pipette Tips
- Qiagen RNA Isolation Kit
- Thermo cDNA Conversion Kit
- Thermal Cycler
- PCR Tubes

**Methods**

## RNA isolation protocol

According to the sample number, buffer RLT plus was prepared with the addition of 10 µl of 2-mercaptoethanol to each 1 ml of buffer RLT plus to protect intracellular RNA content against excessive RNase. In this phase RPE buffer was also supplemented with a ratio of 4 ml ethanol (range 96-100%) and 1 ml RPE buffer. Upon collection, cell pellets were additionally processed with 1 ml of PBS wash which follows with centrifugation at 300 G for 5 mins. PBS solution was carefully discarded and 400 µl of RLT Buffer Plus was added onto the cell pellets. After resuspension of pellets with RLT Buffer Plus, 30 seconds of vortex was applied and each cell pellet was passed through 20G needle for 5 times. Centrifuge was applied in maximum speed for 1 minutes. Collected lysate was transferred into gDNA spin columns and a centrifuge was applied for 30 seconds in maximum speed. Following centrifuge, gDNA spin column was discarded and flowthrough elute was supplemented with 400 µl of 70% ethanol. After an adequate pipetting, 700 µl of mixture was transferred into RNAeasy MiniSpin columns and centrifuge was applied in maximum speed for 30 seconds. Flow through was discarded and 700 µl of wash buffer was added onto RNAeasy MiniSpin columns while another centfiuge was applied in maximum speed for 30 seconds. Flow through discarded and 500 µl of RPE buffer was added onto RNAeasy MiniSpin columns while another centrifuge was applied in maximum speed. The RPE addition step was repeated. Following additional centrifuge step with maximum speed, 20 µl of elution buffer was added onto RNAeasy MiniSpin columns and flowthrough RNA was collected. Absorbance value in NanoDrop was evaluated to check purity of isolated RNA. Upon isolation, RNA stored in -80$^{o}$C.

## RT-qPCR and cDNA construction

A master mix was prepared according to the following table 1,

Table 1. Master Mix Preparation for cDNA

| **Component** | **Volume** | |
|---|---|---|
| | **With RNase inhibitor** | **Without Rnase inhibitor** |
| 10x RT Buffer | 2.0 µl | 2.0 µl |
| 25x dNTP Mix (100 mM) | 0.8 µl | 0.8 µl |
| 10x RT random primers | 2.0 µl | 2.0 µl |
| MultiScribe™ Reverse Transcriptase | 1.0 µl | 1.0 µl |
| Rnase Inhibitor | 1.0 µl | - |
| Nuclease-free Water | 3.2 µl | 4.2 µl |
| Total per Reaction | 10.0 µl | 10.0µl |

Upon preparation, master mix was added on 10 µl of RNA samples in a PCR tube. PCR tubes were sealed and spin down was applied to eliminate any air bubbles. The tubes were placed in a thermal cycler with following conditions in the table 2.

Table 2. PCR Conditions for cDNA Construction

| **Settings** | Step 1 | Step 2 | Step 3 | Step 4 |
|---|---|---|---|---|
| Temperature | 25°C | 37°C | 85°C | 4°C |
| Time | 10 mins | 120 mins | 5 mins | ∞ |

In the end of the program, obtained cDNAs were stored in -20ºC.

### 3.1.3 Whole protein extraction protocol & BCA assay

**Materials**

- RIPA lysis buffer
- Phosphotase/Protease Inhibitor
- Ice Bucket
- Vortex
- Centrifuge
- Trypsin/Cell Scraper
- PBS
- 20 G needle
- Sonicator
- BCA Assay Kit (Thermo)
- Pipette tips
- Pipette
- Aluminum
- Incubator
- Spectrophotometer
- Cover plate

**Methods**

## Protein extraction protocol of monolayer and suspension cultured mammalian cells

According to the sample size, RIPA buffer was prepared and supplemented with protease and phosphatase inhibitor tablet. After $5x10^5$ were obtained, 2 ml of PBS was added to get rid of the debris and remnants. Cells were resuspended within the PBS and 300 G centrifuge was applied for 5 minutes. Supernatant was removed and cells were resuspended again with the addition of 1 ml chilled PBS. 20 G was used for 5 times and a centrifugation was done under 600 G and for 5 minutes. Following centrifuge, supernatant was discarded, and 0.5 ml of chilled RIPA lysis buffer was added on each pellet and cells were resuspended. Upon resuspension 30 seconds of vortex was applied, and cells were kept on ice for incubation with RIPA lysis buffer. While incubation was maintained, cells were administrated into sonication process in each 10 minutes cycles; where 5 seconds of sonication was applied with 40% power (4000 watt) and 30 pulse settings. In the end of incubation, samples were centrifuged under 14000 G for 20 minutes. Following centrifuge, supernatants was kept in another collection tube and all samples were kept under -20$^o$C for further analysis.

## BCA protein quantification assay with microplate usage

Upon start, a standard for BCA assay should be prepared as the Table 3 below;

Table 3. BCA standard preparation table

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure (Working Range = 20-2,000µg/mL)

| Vial | Volume of Diluent (µL) | Volume and Source of BSA (µL) | Final BSA Concentration (µg/mL) |
|---|---|---|---|
| A | 0 | 300 of Stock | 2000 |
| B | 125 | 375 of Stock | 1500 |
| C | 325 | 325 of Stock | 1000 |
| D | 175 | 175 of vial B dilution | 750 |
| E | 325 | 325 of vial C dilution | 500 |
| F | 325 | 325 of vial E dilution | 250 |
| G | 325 | 325 of vial F dilution | 125 |
| H | 400 | 100 of vial G dilution | 25 |
| I | 400 | 0 | 0 = Blank |

Calculations of Working Reagent (WR) should be done as follows (25 µl sample should be mixed with 200 µl of WR (1:8 ratio));

(# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required)

After determination of working reagent volume, 50 parts of reagent A and 1 parts of reagent B should be mixed and kept within a aluminum foiled falcon tube and mix should be centrifuged until it gives a clear green color. 25 µl of each standard and unknown substances or replicates should be pipetted into microplate wells and 200 µl of working reagent should be added on top of the samples. Following addition of working reagent 30 seconds plate shaker was used to mix the working reagents and samples. Following this process, plate was covered with aluminum folio and placed into the incubator which works at 37ºC for 30 mins. After the incubation, plate was left to cool down in room temperature for 10 minutes. Measurements were taken with the aid of plate reader at 562 nm.

### 3.1.4 qPCR protocol

**Materials**

- Pipette & Pipette Tips
- dd$H_2O$
- SYBR-Green No-ROX Kit (Bioline)
- Ice Box
- Plate & Plate Film

**Methods**

Obtained cDNAs were measured with spectrophotometer and each cDNA was diluted into 100 ng concentration. According to the Table 4 below, each reagent were added onto the mix within the ice bucket (4 µl for template and 4.4 µl d$H_2O$). In general procedure, conditions in Table 5 was followed in Thermo CFX96 Real-Time qPCR. Upon completion of the reaction, raw data was normalized according to the house-keeping gene of each individual sample.

Table 4. qPCR SYBR No-Rox Reaction

| Reagent | Volume | Final concentration |
|---|---|---|
| 2x SensiFAST SYBR® No-ROX Mix | 10 µL | 1x |
| 10 µM forward primer | 0.8 µL | 400 nM |
| 10 µM reverse primer | 0.8 µL | 400 nM |
| Template | up to 8.4 µL | |
| $H_2O$ | As required | |
| | **20 µL Final volume** | |

Table 5. General qPCR Reaction Cycle

| Cycles | Temp. | Time | Notes |
|---|---|---|---|
| 1 | *95 °C | *2 min | Polymerase activation |
| 40 | 95 °C<br>60-65 °C<br>72 °C | 5 s<br>10 s<br>**5-20 s | Denaturation<br>Annealing<br>Extension (acquire at end of step) |

### 3.1.5 Western blot protocol

**Materials**

- $ddH_2O$
- 30% Acrylamide
- 1.5 M Tris-HCl
- 10%SDS
- 10% APS
- TEMED
- Pipette
- Comb
- Glass Plates
- Holder
- Power Supply
- Tris
- Glycine
- PVDF/Nitrocellulose Membrane
- Semi/Dry Transfer
- Wet Transfer
- TBS
- TBST
- Ponceau S Solution
- Milk Powder
- Coomasie Blue
- Primary & Secondary Antibody
- ECL

**Gel preparation & Gel electrophoresis for WB**

Prior to Western Blot (WB) experiment, gel was prepared according to the desired protein size to be captured. Gels were prepared in different concentration according to the Table 6 below;

Table 6. Western Blot concentration related gel reagents

| Gel % | Water (mL) | 30% acrylamide (mL) | 1.5 M Tris-HCl, pH 8.8 (mL) | 10% SDS (µL) | 10% APS (µL) | TEMED* (µL) |
|---|---|---|---|---|---|---|
| 8% | 4.6 | 2.6 | 2.6 | 100 | 100 | 10 |
| 10% | 3.8 | 3.4 | 2.6 | 100 | 100 | 10 |
| 12% | 3.2 | 4.0 | 2.6 | 100 | 100 | 10 |
| 15% | 2.2 | 5.0 | 2.6 | 100 | 100 | 10 |

*TEMED must be the last ingredient added

Upon solidification of gel, comb was removed from the gel and gel was placed into the transfer buffer to equilibrate buffer pH and electricity with the gel. Each protein sample was prepared with the aid of laemli, mercaptoethanod and $dH_2O$ as in necessary proportions. Samples were vortexed for 30 seconds and a simple benchtop spin down centrifuge was used for 30 seconds. Samples placed onto 95$^oC$ heater block for 5 minutes and placed into benchtop spin down centrifuge for 30 seconds. 20 µl of sample was loaded into each well of the gel, along with the 10 µl of WB ladder. Alignment process was done with 50 V for 40 minutes while separation process was done 100 V for roughly 2 hours of time schedule. Chilled Towbin transfer buffed (25mM Tris; 192 mM Glycine and up to 20% methanol) was used for the transfer process. PVDF transfer membrane was administered into 100% methanol for 30 seconds for the activation process, and all the components of the transfer process was applied as a sandwich method. To accomplish semi-dry transfer, 20 V for 60 minutes was used to accomplish a well-sustained transfer, otherwise for wet-transfer 15 V of voltage and overnight 10-12 hours of transfer was used to have optimal outcome. After transfer, membrane was washed in a shaker with TBS buffer for 3 times and 5 minutes of intervals. Following washing step, Ponceu S solution was used for 5 minutes in a shaker to evaluate success of the transfer. After a successful transfer, blocking was done in a shaker with the use of 5% concentration of skim milk for 1 hour in a room temperature. Following blocking, to get rid of the remnants, membrane administered to washing step in a shaker with the use of TBST for 3 times and 5 minutes of intervals. Primary antibodies with a determined concentration were used on a membrane in a cold room shaker (+4ºC) overnight (10-12 hours). Membrane administered to washing step in a shaker with the use of TBST for 3 times and 5 minutes of intervals. Secondary antibodies were prepared with 10% skim milk blocking supplementation and 1:5000 concentration. Following washing with TBST, secondary antibody was added onto the membrane in a shaker with 1 hour of incubation time. Membrane administered to washing step in a shaker with the use of TBST for 5 times and 7 minutes of intervals. Images were taken in Chemi-Doc instrument and sufficient amount of ECL reagent was used.

### 3.1.6 siRNA protocol

**Materials**

- Opti-MEM
- RPMI SFM
- Lipofectamine (Thermo)
- siRNAs (Thermo)
- 6-well plate
- Serological pipette
- Pipette & Pipette tips
- Centrifuge Tubes
- Vortex
- Microscope
- Incubator
- Live-cell imaging
- Pancreatic cell lines

## Methods

To establish successful siRNA process, provider Thermo's protocol was followed as shown in Table 7 below,

Table 7. Thermo siRNA protocol

| Timeline | Step | Steps | Component | 96-well | 24-well | 6-well |
|---|---|---|---|---|---|---|
| Day 0 | 1 | Seed cells to be 60-80% confluent at transfection | Adherent cells | 1–4 × $10^4$ | 0.5–2 × $10^5$ | 0.25–1 × $10^6$ |
| Day 1 | 2 | Dilute Lipofectamine® RNAiMAX Reagent in Opti-MEM® Medium | Opti-MEM® Medium | 25 µL | 50 µL | 150 µL |
| | | | Lipofectamine® RNAiMAX Reagent | 1.5 µL | 3 µL | 9 µL |
| | 3 | Dilute siRNA in Opti-MEM® Medium | Opti-MEM® Medium | 25 µL | 50 µL | 150 µL |
| | | | siRNA (10 µM) | 0.5 µL (5 pmol) | 1 µL (10 pmol) | 3 µL (30 pmol) |
| | 4 | Add diluted siRNA to diluted Lipofectamine® RNAiMAX Reagent (1:1 ratio) | Diluted siRNA | 25 µL | 50 µL | 150 µL |
| | | | Diluted Lipofectamine® RNAiMAX Reagent | 25 µL | 50 µL | 150 µL |
| | 5 | Incubate | Incubate for 5 minutes at room temperature. | | | |
| | | | Component | 96-well | 24-well | 6-well |
| | 6 | Add siRNA-lipid complex to cells | siRNA-lipid complex per well | 10 µL | 50 µL | 250 µL |
| | | | Final siRNA used per well | 1 pmol | 5 pmol | 25 pmol |
| | | | Final Lipofectamine® RNAiMAX used per well | 0.3 µL | 1.5 µL | 7.5 µL |
| Day 2–4 | 7 | Visualize/analyze transfected cells | Incubate cells for 1–3 days at 37°C. Then, analyze transfected cells. | | | |

### 3.1.7 H&E protocol

**Materials**

- Roticlear
- Ethanol
- $ddH_2O$
- Hematoxylin
- Eosin
- Bluing Reagent

**Methods**

H&E protocol was followed according to the Table 8 in below;

Table 8. H&E protocol

| **Deparafinisation** | **3 x** | **10min** | **RT** | **Roticlear** |
|---|---|---|---|---|
| | | | | |
| **Rehydration** | 3 x | 3min | RT | EtOH 100% |
| | | 3min | RT | EtOH 95% |
| | | 3min | RT | EtOH 70% |
| | | 3min | RT | EtOH 50% |
| | | | | |
| **Wash** | 2 x | 5min | RT | Aqua Dest. |
| | | | | |
| **Staining** | | 3min | RT | Hematoxylin |
| | | | | |
| **Wash** | 2 x | - | RT | Rinse 2 times with Distilled Water |
| | | | | |
| **Staining** | | 15 sec | RT | Bluing Agent |
| | | | | |
| **Wash** | 2 x | - | RT | Rinse 2 times with Distilled Water |
| | | | | |
| **Wash** | 1 x | Dip | RT | 100% ethanol to blot excess-off |
| | | | | |
| **Staining** | | 3min | RT | Eosin |
| | | | | |
| **Wash** | | 3min | RT | 100% ethanol |
| | | | | |
| **Dehydration** | 3 x | 5min | RT | Roticlear |
| | | | | (Last Roticlear overnight or at least 2 hours) |
| | | | | |
| | | | | **Total Duration:** ~90mins |

### 3.1.7 IHC protocol

**Materials**

- Roticlear
- Ethanol
- dd$H_2O$
- Hematoxylin
- Bluing Reagent
- Citrate Buffer
- Microwave
- TBST
- Wet-box
- Dako-Pen
- 3% Hydrogen Peroxide
- 10% Goat Serum
- Primary & Secondary Antibody
- Dab Substrate
- Mounting Medium
- Lam

**Methods**

IHC protocol was followed according to the table 9, throughout this protocol was done with a deparafinisation step which clears the remnants of paraffin via use of roticlear. Than with the rehydration step, tissue was opened with serial alcohol dilutions, and antigen retrieval opens up the epitopes of the antigens for a specific antibody interaction. DAKO pen was used to create a hydrophobic chamber along the tissue to stabilize the liquids on top of the specified area. Blocking step was followed to sustain specific staining conditions, and after the following washing step, primary antibodies with a specified dilution was added and tissue was kept at +4°C in overnight conditions. Following day, after the washing, secondary antibodies were added and incubated for 1 hour at room temperature. After the addition of substrate, tissues were investigated under the microscope for a specified reaction time and counter staining was done with hematoxylin. At last, dehydration step was followed to close up the tissue and mounting was done.

Table 9. IHC protocol

| | | | |
|---|---|---|---|
| Deparafinisation | 3x | 10min | RT |
| Rehydration | 3x | 3min<br>3min<br>3min<br>3min | RT<br>RT<br>RT<br>RT |
| Wash / Shake | 2x | 5min | RT |
| **Antigen retrieval** | | Until boiling | |
| Cooking | | 10min | |
| Cooling | | 20min | RT |
| Wash / Shake | | 5min | RT |

Table 9. IHC protocol (continued)

| | | | |
|---|---|---|---|
| **DAKO Pen + Permeabilization** | | | |
| | | 5min | RT |
| Wash / Shake | | 5min | RT |
| **Hydrogen Peroxidase Blocking** | | 5min | RT |
| Wash / Shake | | 5min | RT |
| **Protein Block** | | 60min | RT |
| **Primary Antibody** | | Overnight | 4°C |
| Wash / Shake | 3x | 10min | RT |
| **Secondary Antibody** | | 60min | RT |
| Wash / Shake | 3x | 10min | RT |
| **Substrate reaction** | | 1.5min | RT |
| **Counter staining** | 2x | dip | RT |
| Wash | | 15min | RT |
| Wash | | 5min | RT |
| Wash | | 15sec | |
| Dehydration | | 3min | |
| | | 3min | |
| | | 3min | |
| | 3x | 3min | |
| | 3x | 5min | |
| Mounting | | | |

### 3.1.8 Cytotoxicity test protocol with CCK8

**Materials**

- SU8686 Cell Line
- T3M4 Cell Line
- Panc1 Cell Line
- 6 well plate
- PBS
- Micropipettes
- RPMI 1640 Medium
- Fetal Bovine Serum
- Penicillin/Streptomycin
- Serological Pipettes
- Pipette and Pipette Tips
- CCK8 Solution
- TCN-201
- Spectrophotometer

**Methods**

In this experiment, cytotoxicity of TCN-201 was evaluated on SU8686, T3M4 and Panc1 cell lines. These cell lines were seeded onto 6 well-plates by 100.000 cells per well. After 2 days of incubation, medium change was done. Following that process, 4 different groups were created with at least 4 different technical replicates with respective order of; no treatment, 3 uM, 15 uM and 30 uM of TCN-201 was added into the wells with the serum free medium. Following TCN-201 treatment, 10 µl of CCK8 solution was added within the medium. With the aid of spectrophotometer, 6-12-18 and 24 hours of absorbance values were recorded.

## 3.2 In Vivo Experiments

On the first phase of in vivo experiments, acute pancreatitis and chronic pancreatitis animal models constituted via use of 50 mg/kg caerulein. After termination of the experiments, organs harvested and these models confirmed morphologically with the aid of H&E staining, blood serum and dry/wet ratio of pancreas.

After the model confirmation, second phase of in vivo experiments initiated via use of TCN-201. In the beginning of the process, we had to understand effective dosage of TCN-201 without any adverse/toxic effects on mice since there was no data on the literature. Following dosage finding study, experiments were done with bigger groups, abdominal pain of the animals were evaluated with Von-Frey filaments and open-field experiments aided us to understand there were no mechanical problems in the motor movement functionality of animals.

### 3.2.1 Constitution of acute and chronic pancreatitis animal models & TCN-201 dosage and preliminary experimental trials

**Materials**

- C57BL/6 Mice
- Insulin Injector
- Isofluorane
- Caerulein
- TCN-201

**Methods**

Acute (Table 10) and Chronic Pancreatitis (Table 11) mouse models were established via use of 50 mg/kg Caerulein intraperitoneal injections. For Acute Pancreatitis these injections were done within a day in hourly fashion and 10 times in a day. On the other hand, Chronic Pancreatitis mouse model, intraperitoneal injections were applied for 8 weeks, 3 days in a week, and 6 times in day with an hour separation between each injection. Von-Frey Experiments were done and reaction to each filament was recorded to assess a pain score. To elaborate, no reaction has given 0 score, while mild reaction took 1 score and severe reaction has given with 2 score while each filament was touched into abdominal area for 10 times. On the other hand, open field experiments were recorded within a square box for 10 minutes time schedule and each specimens relocation and speed was captured.

Table 10. Mouse Acute Pancreatitis Experiment Plan

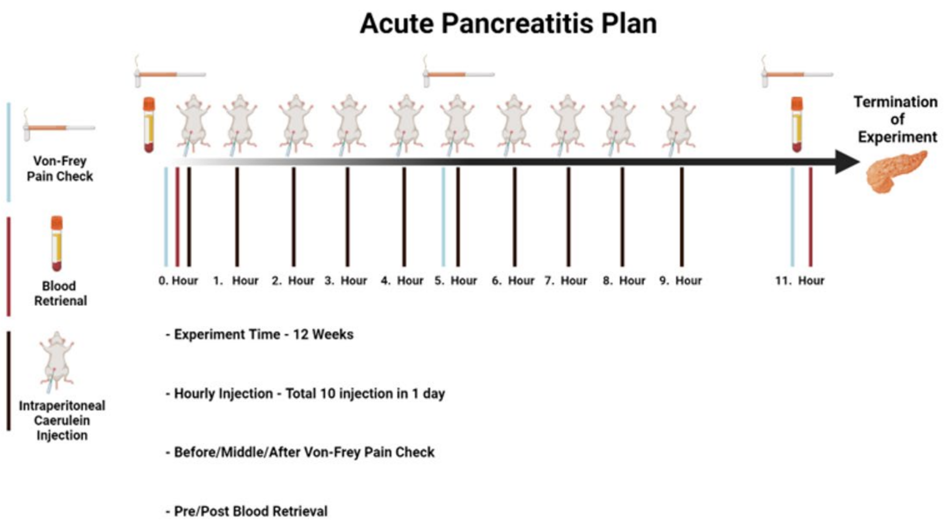
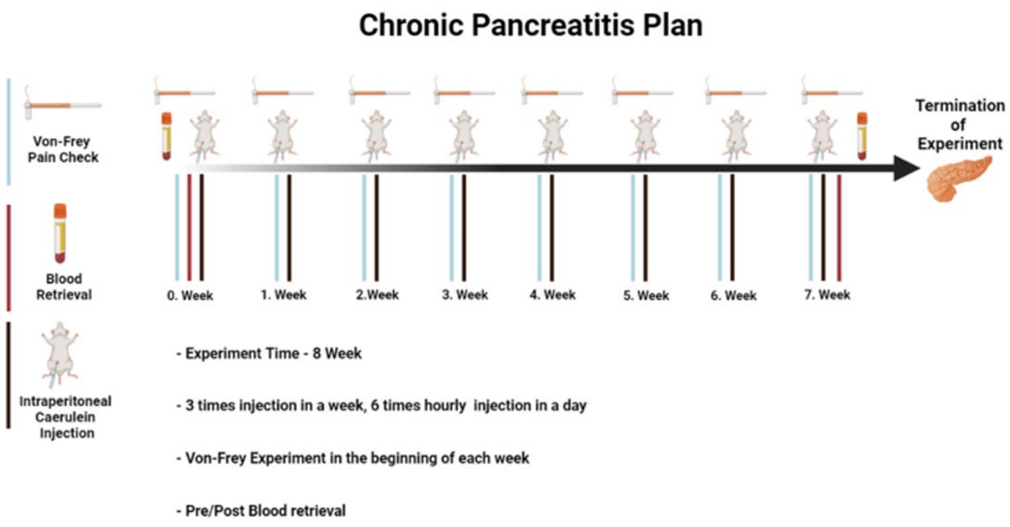



Table 11. Mouse Chronic Pancreatitis Experiment Plan



# 4 RESULTS

## 4.1 qPCR Results of Glutamate Receptors on both human and mouse cell lines and AP & CP mouse tissues

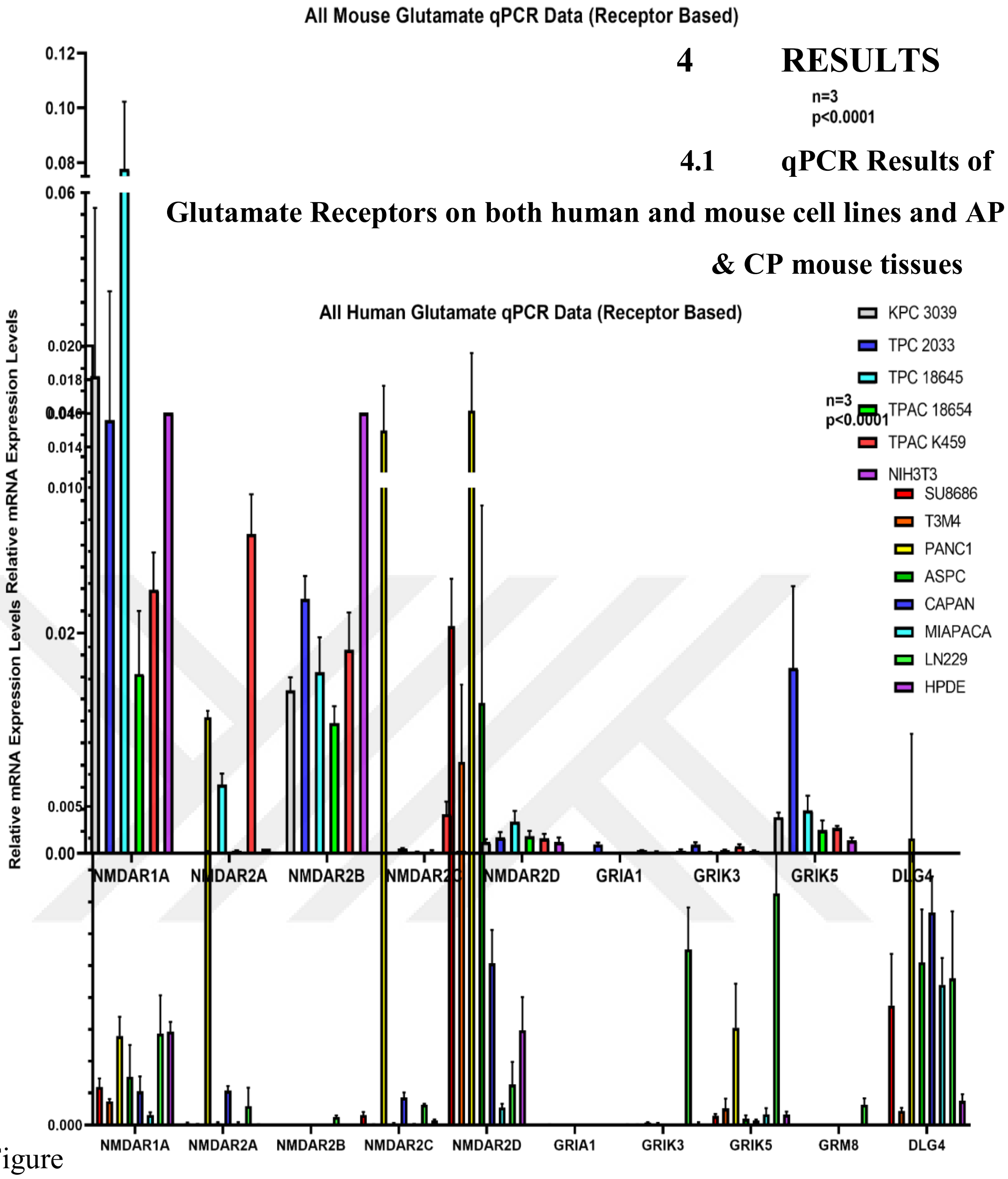



Figure 5 – All Human PCa Cell Lines Glutamate Receptors qPCR Data

Cell culture was done in native conditions of human pancreatic cancer (PCa) cell lines and general glutamate related transcriptional profile was investigated. Among suspected glutamate genes and intermediate gene, especially NMDAR1A, NMDAR2D and DLG4 observed to be highly active in general population of PCa cell lines while other genes also demonstrated specific activity for certain cell lines.

Figure 6 – All Mouse Cell Lines Glutamate Receptors qPCR Data

Cell culture was done in native conditions of mouse pancreatic cancer (PCa) cell lines and general glutamate related transcriptional profile was investigated. Among suspected glutamate genes and intermediate gene, especially NMDAR1A and NMDAR2B observed to be highly activated in general population of PCa cell lines. On the other hand, NMDAR2D and GRIK5 looked less expressive but present in all population of mouse cell lines.

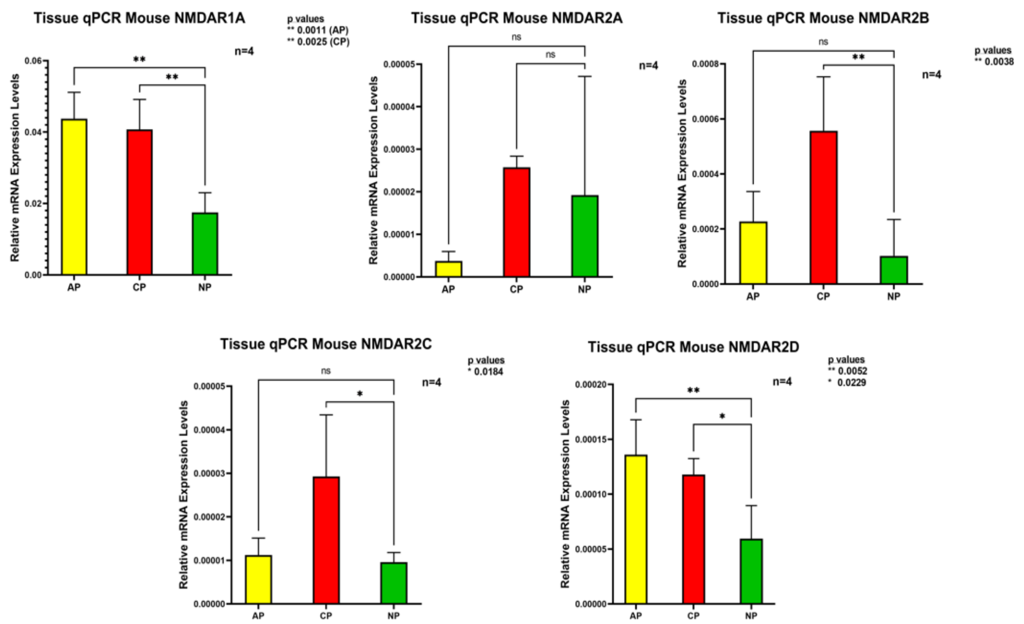



Figure 7 – Established Acute and Chronic Pancreatitis Mouse Models' Pancreas Tissue NMDA Receptor qPCR Results.

Upon termination of experiments pancreas of mice were isolated to obtain general NMDA related transcriptional profile from acute and chronic pancreatitis mouse models. Among suspected NMDARs, results were demonstrated transcriptional similarities with human and mouse PCa cell lines. NMDAR1A, NMDAR2B and NMDAR2D observed to be highly activated when it compared with normal pancreas (NP) tissue. These results indicated that our main target NMDAR1A is upregulated and use of TCN-201 antagonist against NMDAR1A became more important in deciphering neuropathic pain in CP.

### 4.2 Western Blot Results of NMDA Receptors and Underlying Mechanisms in Human Samples, and Human and Mouse PCa Cell Lines

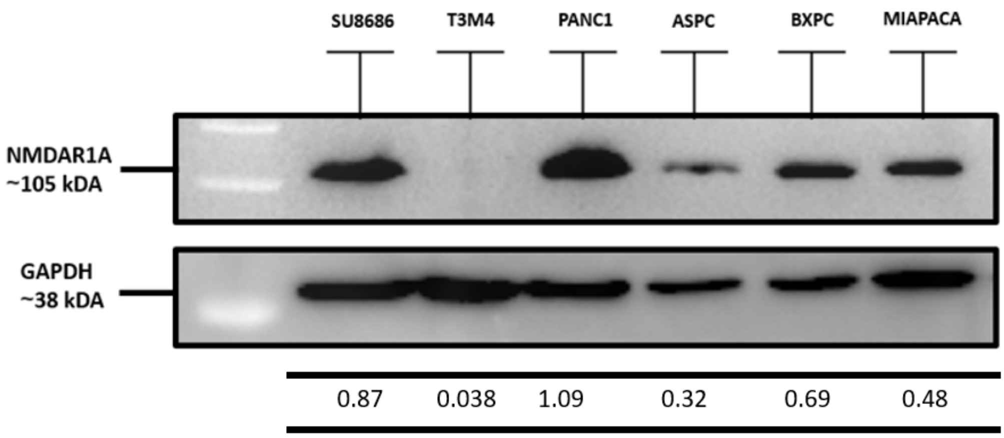



Figure 8 – Preliminary NMDAR1A WB Result in PCa Cancer Cell Lines

Cell culture was done in native conditions of human pancreatic cancer (PCa) cell lines and NMDAR1A translational profile was investigated between pancreatic cancer cell lines. 30 µg of each sample was loaded and GAPDH levels of each sample seem to close to each other which represents a close to equal loading pattern. Each NMDAR1A expression level compared with each samples' individual housekeeping GAPDH to evaluate expression levels of each sample. When each cell line compared, respectively in order, Panc1 demonstrated highest expression level where SU8686 followed in second and in general all PCa cell lines demonstrated expressions in certain levels. When this data compared with the transcriptional data, it demonstrates the same pattern in translational level, whereas T3M4 cell line did not exhibit an expression as seen in transcriptional data.

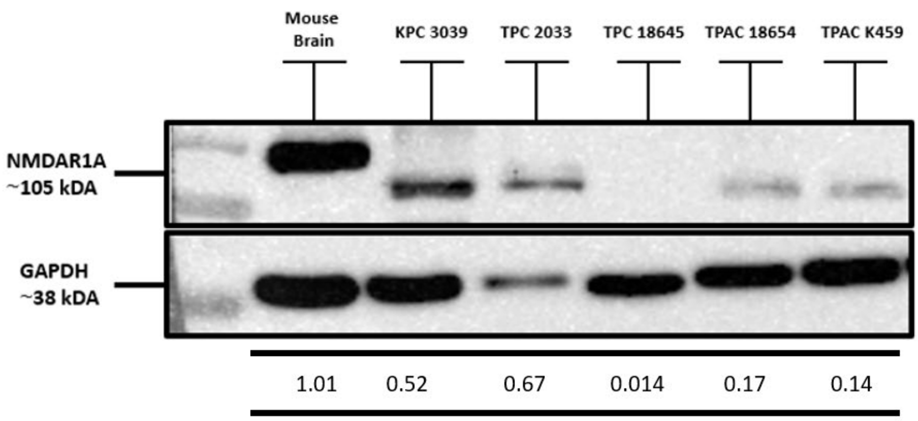



Figure 9 – Preliminary NMDAR1A WB Result in Mouse Cell Lines with brain tissue positive control

Cell culture was done in native conditions of mouse pancreatic cancer (PCa) cell lines and NMDAR1A translational profile was investigated between pancreatic cancer cell lines. 30 µg of each sample was loaded and GAPDH levels of each sample seem to close to each other which represents a close to equal loading pattern, whereas TPC2033 did not exhibited enough concentration in loading. Each NMDAR1A expression level compared with each samples' individual housekeeping GAPDH to evaluate expression levels of each sample. When each cell line compared, respectively in order, as expected mouse brain demonstrated highest level as positive control whereas KPC3039 and TPC2033 shown significant expression levels and TPAC18654 and TPAC K459 followed with respected levels of expression. When this data compared with the transcriptional data, it demonstrates the same pattern in translational level, whereas TPC18645 cell line did not exhibit an expression as seen in transcriptional data.

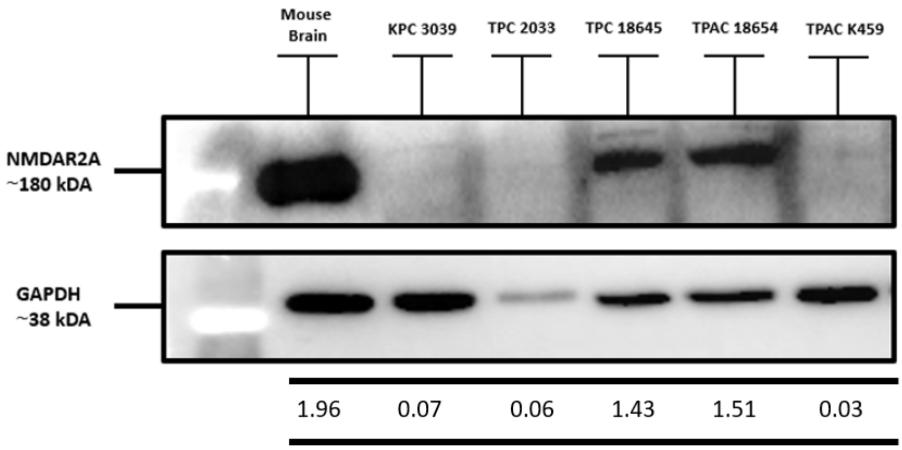
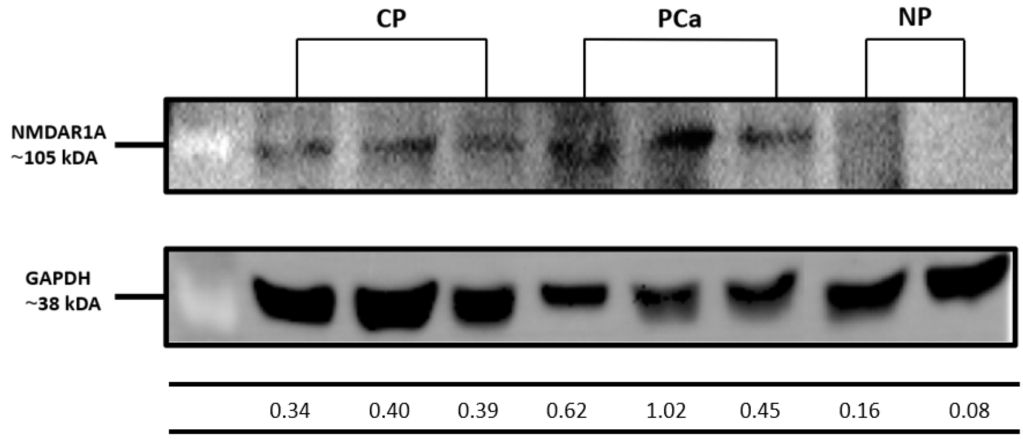



Figure 10 – NMDAR2A WB Result in Mouse Cell Lines with brain tissue positive control

Cell culture was done in native conditions of mouse pancreatic cancer (PCa) cell lines and NMDAR2A translational profile was investigated between pancreatic cancer cell lines. 30 µg of each sample was loaded and GAPDH levels of each sample seem to close to each other which represents a close to equal loading pattern, whereas TPC2033 did not exhibited enough concentration in loading. Each NMDAR1A expression level compared with each samples' individual housekeeping GAPDH to evaluate expression levels of each sample. When each cell line compared, respectively in order, as expected mouse brain demonstrated highest level as positive control whereas TPC18645 and TPC18654 shown significant expression levels while other cell lines did not exhibit any translational expression. When this data compared with the transcriptional data, it demonstrates the same pattern in translational level, whereas TPAC K459 cell line did not exhibit an expression as seen in transcriptional data.



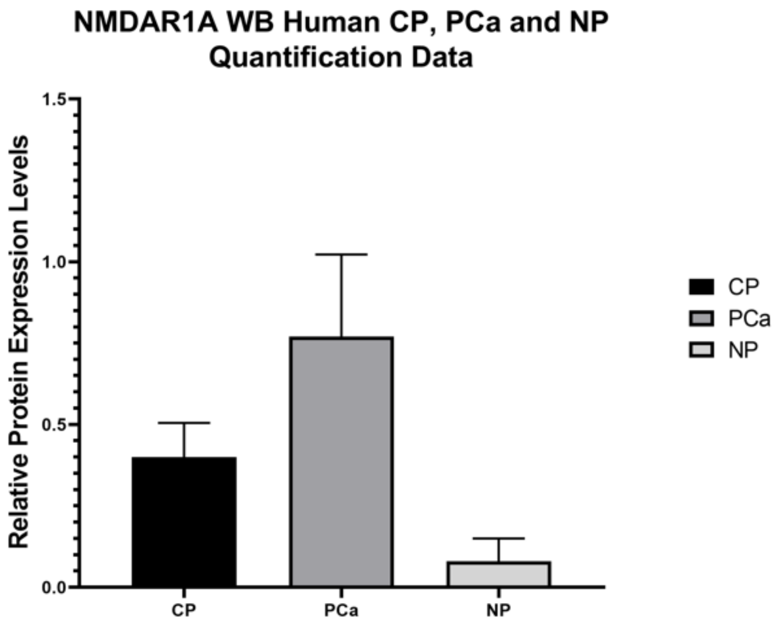




Figure 11 – Preliminary NMDAR1A WB Result in CP, PCa and NP Tissues of Patients

In this experiment, whole protein isolation was done from chronic pancreatitis (CP), pancreatic cancer (PCa) and normal pancreatic (NP) tissues. (On top) 50 µg of each sample was loaded and GAPDH levels of each sample seem to close to each other which represents a close to equal loading pattern. Each NMDAR1A expression level compared with each samples' individual housekeeping GAPDH to evaluate expression levels of each sample. When each tissue compared, despite individual differences of each patient, expression level of each group demonstrated a close similarity. When groups compared in quantification data (on the bottom) PCa and CP demonstrated significantly high expression in NMDAR1A levels. This data also correlates with the human IHC results and looks similar with the transcriptional qPCR mouse data.

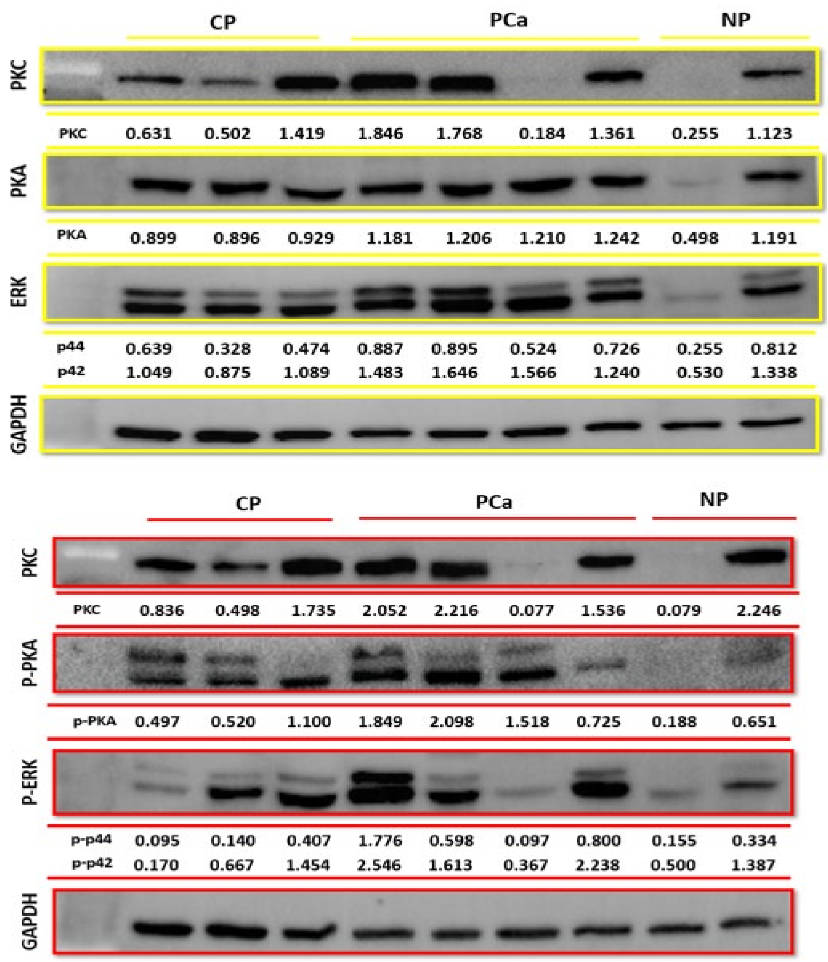



Figure 12 – Preliminary WB Result of PKC, PKA, ERK, p-PKC, p-PKA and p-ERK in CP, PCa and NP Tissues of Patients

In this experiment, aim was to investigate underlying mechanisms of NMDA receptors. To accomplish that goal, whole protein isolation was done from chronic pancreatitis (CP), pancreatic cancer (PCa) and normal pancreatic (NP) tissues. 50 µg of each sample was loaded and GAPDH levels of each sample seem to close to each other which represents a close to equal loading pattern. Each protein of interests' expression level compared with each samples' individual housekeeping GAPDH to evaluate expression levels of each sample. When each tissue compared, in some cases individual differences of each patient stepped up, but expression level of each group has given a preliminary idea about the underlying mechanism. When phosphorylation levels of PCa and CP compared with the NP tissues, PCa and CP groups demonstrated significantly expression in p-PKC, p-PKA and p-ERK levels. This data was expected to be in this manner, since overexpression of NMDA receptors leads into the activation and phosphorylation of underlying mechanisms and starts a feed-forward loop.

### 4.3 Preliminary siRNA Results

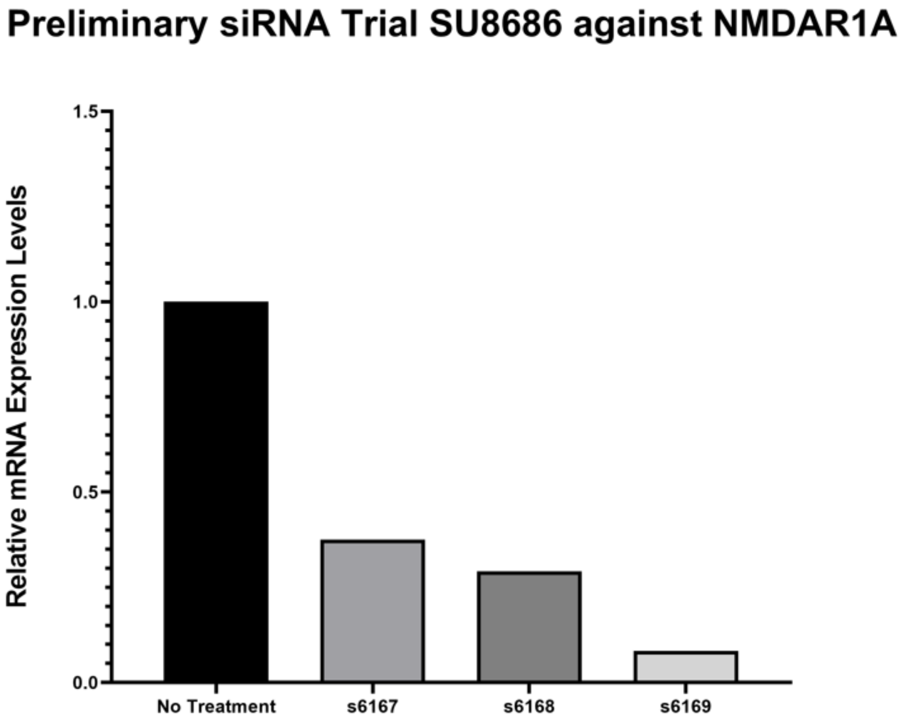



Figure 13 – Prelimanary siRNA Results on SU8686 Cell Line

In this experiment, different siRNAs were used to evaluate their efficiency within the SU8686 cell line. Cells were seeded onto 6 well plates, after 1 day of incubation with complete medium, cells have reached at least 70% of confluency. After this point, three replication was done for each group of without siRNA (negative control), s6167, s6168 and s6169 siRNA administrated groups. After 2 days of incubation with siRNAs, cells were acquired and upon RNA isolation and cDNA conversion, a qPCR assay was done. When it was compared with control group, siRNA administration demonstrated a successful intervention in the expression of NMDAR1A levels and especially s6169 had superior effects when it is compared with the other siRNA administrated groups.

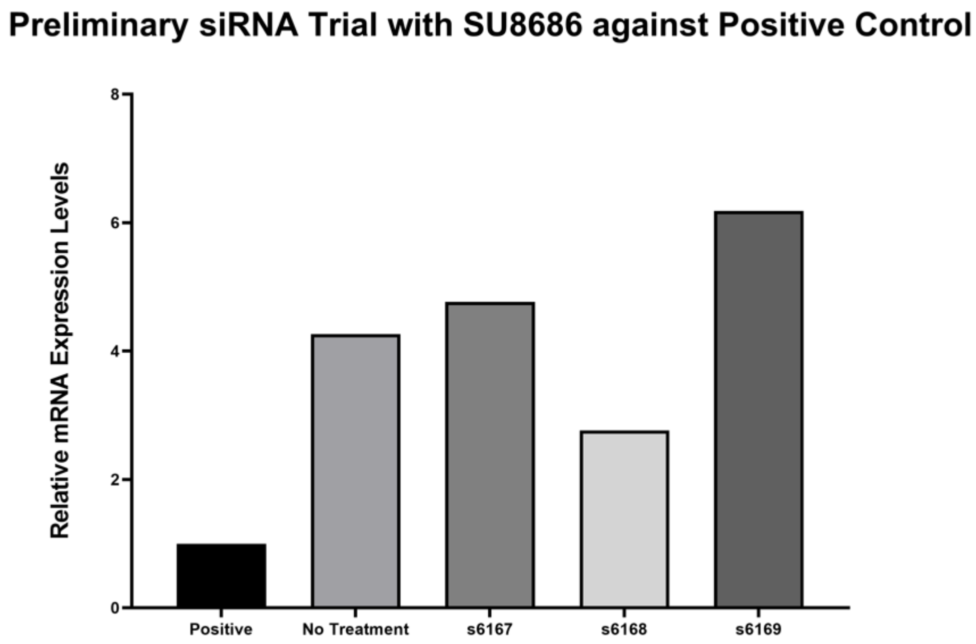



Figure 14 – Preliminary siRNA Result with Positive Control

In this experiment, different siRNAs were used to evaluate their efficiency within the SU8686 cell line. Cells were seeded onto 6 well plates, after 1 day of incubation with complete medium, cells have reached at least 70% of confluency. After this point, three replication was done for each group of siRNA for GAPDH (positive control), without siRNA (negative control), s6167, s6168 and s6169 siRNA administrated groups. After 2 days of incubation with siRNAs, cells were acquired and upon RNA isolation and cDNA conversion, a qPCR assay was done. When it was compared with positive control group, as expected other groups demonstrated a similar expression level as negative control groups. In addition to that, positive control group demonstrated a significant reduction in GAPDH expression which demonstrates that the protocol of siRNA intervention is optimized and works under the selected conditions.

### 4.4 Preliminary In-Vitro TCN-201 Administration Related Cytotoxicity Results

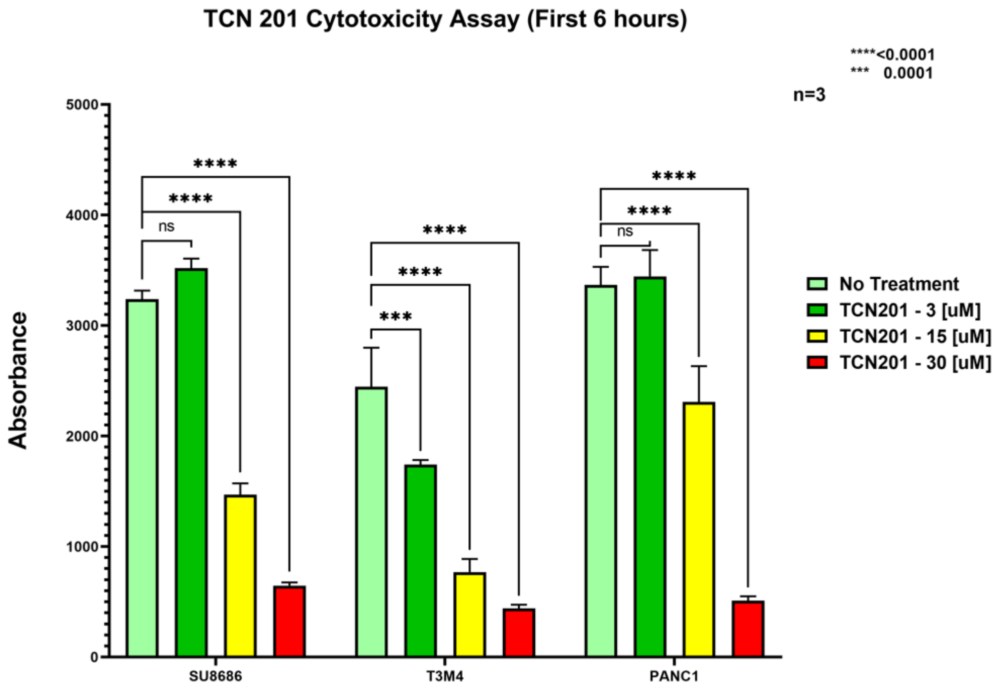



Figure 15 – First 6 hours of cell viability/proliferation rate upon TCN-201 Treatment with 3, 15 and 30 uM concentrations

In this experiment, SU8686, T3M4 and Panc1 cells were seeded and cultivated for 24 hours prior to start this study. 4 different groups were established, 1 negative control group with no treatment, and 3 different groups of TCN201 treatment, respectively 3 uM, 15 uM and 30 uM of concentrations while CCK-8 was added to evaluate the proliferation levels. In the first 6 hours, 15 and 30 uM of TCN-201 treatment seems to be highly effective on retardation in proliferation capacity of PCa cell lines.

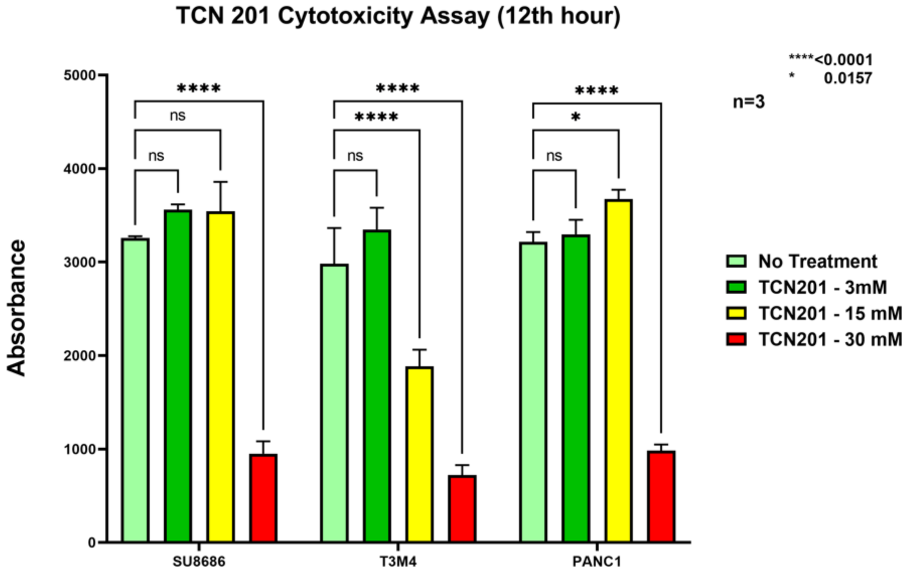



Figure 16 – 16 hours of cell viability/proliferation rate upon TCN-201 Treatment with 3, 15 and 30 uM concentrations

In this experiment, SU8686, T3M4 and Panc1 cells were seeded and cultivated for 24 hours prior to start this study. 4 different groups were established, 1 negative control group with no treatment, and 3 different groups of TCN201 treatment, respectively 3 uM, 15 uM and 30 uM of concentrations while CCK-8 was added to evaluate the proliferation levels. After 16 hours, 30 uM of TCN-201 treatment still looks highly effective on retardation in proliferation capacity of PCa cell lines.

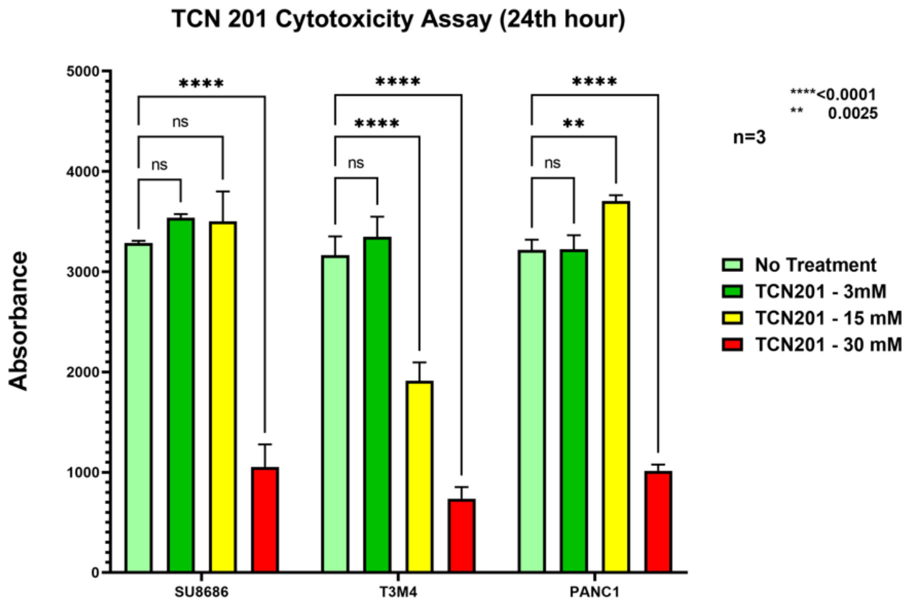



Figure 17 – 24 hours of cell viability/proliferation rate upon TCN-201 Treatment with 3, 15 and 30 uM concentrations

In this experiment, SU8686, T3M4 and Panc1 cells were seeded and cultivated for 24 hours prior to start this study. 4 different groups were established, 1 negative control group with no treatment, and 3 different groups of TCN201 treatment, respectively 3 uM, 15 uM and 30 uM of concentrations while CCK-8 was added to evaluate the proliferation levels. After 24 hours, 30 uM of TCN-201 treatment still looks highly effective on retardation in proliferation capacity of PCa cell lines.

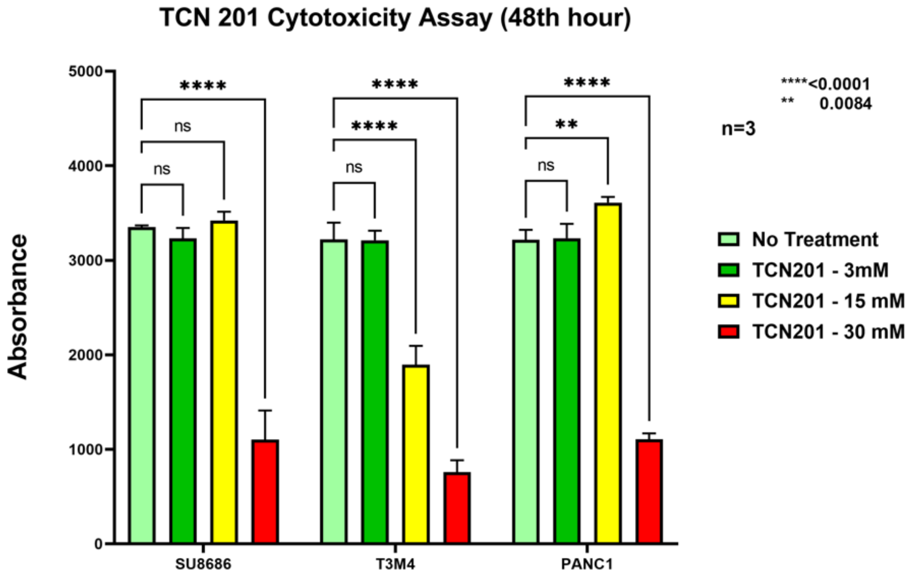



Figure 18 –48 hours of cell viability/proliferation rate upon TCN-201 Treatment with 3, 15 and 30 uM concentrations

In this experiment, SU8686, T3M4 and Panc1 cells were seeded and cultivated for 24 hours prior to start this study. 4 different groups were established, 1 negative control group with no treatment, and 3 different groups of TCN201 treatment, respectively 3 uM, 15 uM and 30 uM of concentrations while CCK-8 was added to evaluate the proliferation levels. After 48 hours, 30 uM of TCN-201 treatment still looks highly effective on retardation in proliferation capacity of PCa cell lines.

## 4.5 H&E Application and Confirmation of AP and CP Mouse Models

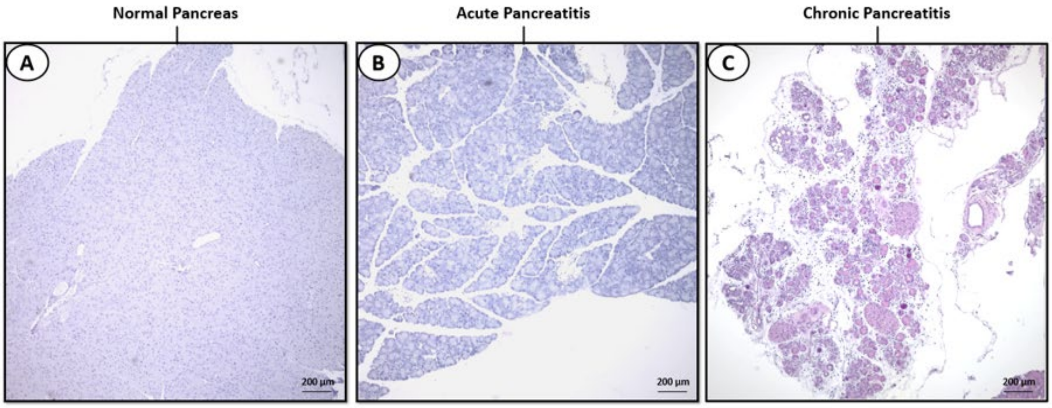



Figure 19 – Mouse Tissue H&E Staining of Normal Pancreas Comparison with Established Acute and Chronic Pancreatitis Models

In this experiment, normal pancreas was isolated by scarification of 16 weeks old mouse, while other tissues were obtained after the acute pancreatitis (Table 11) and chronic pancreatitis (Table 12) experiments that previously described in the methods section. After the isolation of pancreas, tissue process has been done to paraffinize the tissue and 2.5 micron of slides were taken onto positive charged lams. H&E staining procedure was followed carefully, and images taken under a light microscope. When results compared with (A) normal pancreas tissue (negative control); (B) while acute pancreatitis has demonstrated edema and immune cell infiltration, (C) chronic pancreatitis demonstrated severe edema, disruption of acinar cells, immune cell infiltration.

## 4.6 IHC Staining Results of NMDAR1A, CD45 and CD68

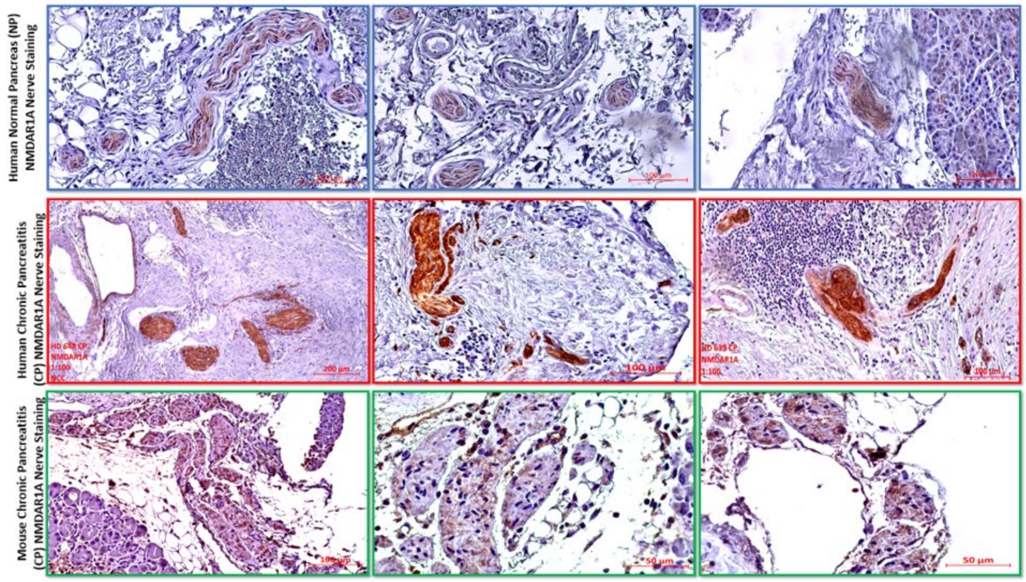



Figure 20 – IHC NMDAR1A Nerve Staining Result Comparison

All the tissues were obtained and followed a tissue processing for preservation and paraffinization. Each tissue was cut in the thickness of 2.5 micron of slides were taken onto positive charged lams. IHC protocol was followed with the use of NMDAR1A antibody and hematoxylin for counter staining. In all human and mouse pancreas NMDAR1A staining especially observed in the nerves. When it is compared with normal pancreas (NP) human tissues (top section) and human CP tissues (middle section), observed nerve staining was clearly evident in CP tissues. This result demonstrated that NMDAR1A is activated in CP patients and it is also present in CP mouse tissues while NP tissues of mice did not exhibit any NMDAR1A staining.

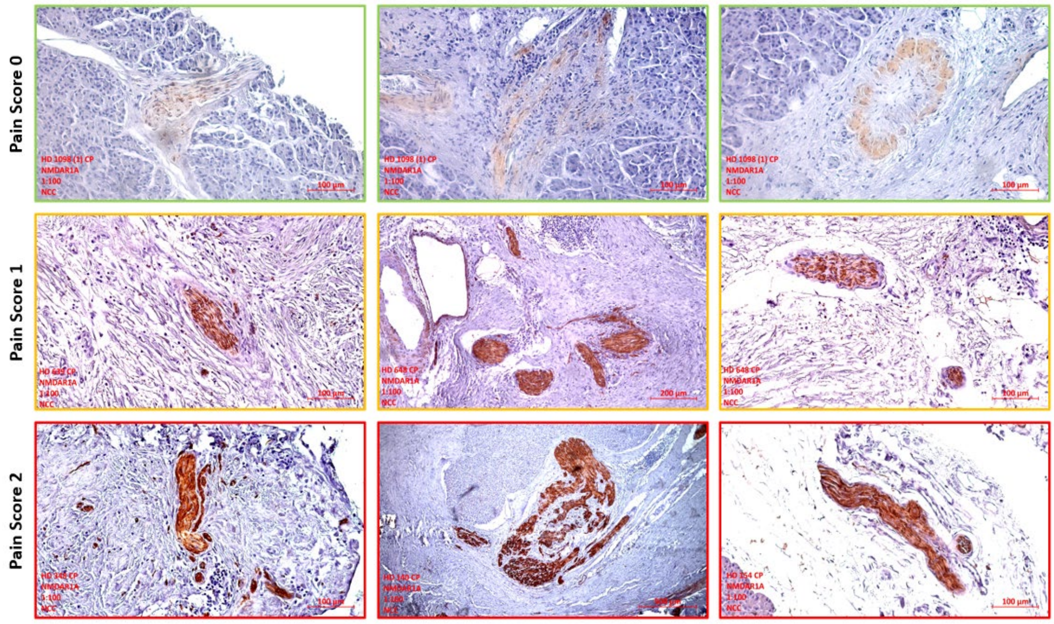



Figure 21 – IHC NMDAR1A Staining for Human PC Tissues

All the tissues were obtained and followed a tissue processing for preservation and paraffinization. Each tissue was cut in the thickness of 2.5 micron of slides were taken onto positive charged lams. IHC protocol was followed with the use of NMDAR1A antibody and hematoxylin for counter staining. According to the pain scores of patients, NMDAR1A staining within the nerves has significantly increased in the intensity and it has shown correlation with the increase in pain score. These results demonstrated that NMDAR1A might have a significant role in neuro-pancreatic pain in CP patients.

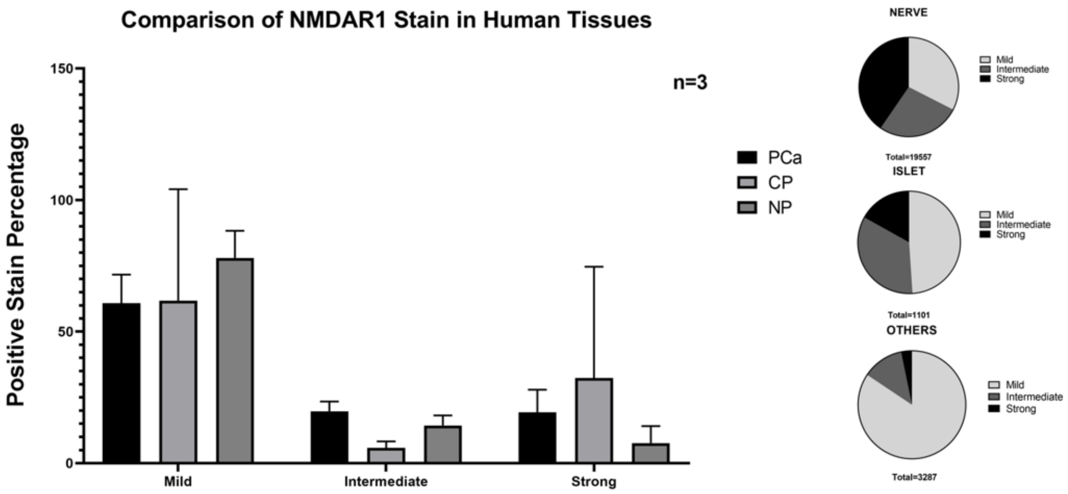



Figure 22 – IHC NMDAR1A Preliminary Staining Analysis

After each IHC staining, stained tissues were scanned and general staining profile of PCa, CP and NP was investigated with the help of QuPath program. On the left; PCa, CP and NP Tissues Compared with Stain Intensity. When it compared with each other, strong staining against NMDAR1 antibody especially observed in PCa and CP tissues when it was compared with NP tissues which mostly had mild staining profile. On the other hand, pie chart demonstrated that most of the staining was exhibited within the nerves with a total count number 19557, while islets had 1101 and other unrelated portions had 3287 total count numbers. This data has shown good statistical significance since most of the staining of NMDAR1A recognized within the nerves of the tissues, and expression of the NMDAR1A increased in PCa and CP conditions.

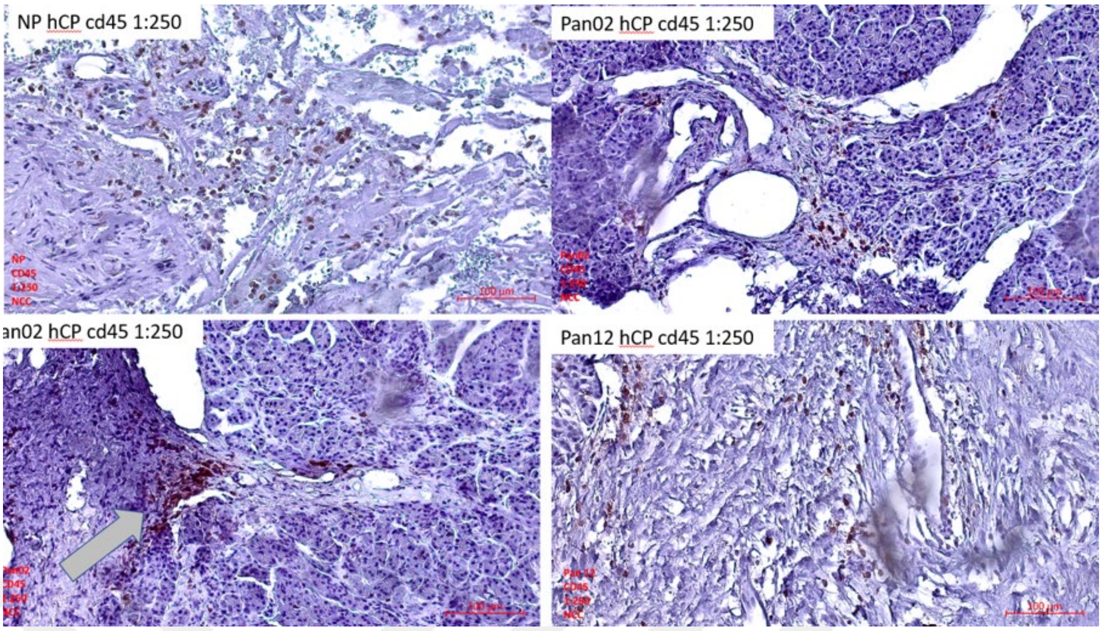



Figure 23- CD45 IHC Staining on Human CP Tissues

All the tissues were obtained and followed a tissue processing for preservation and paraffinization. Each tissue was cut in the thickness of 2.5 micron of slides were taken onto positive charged lams. IHC protocol was followed with the use of CD45 antibody and hematoxylin for counter staining. As demonstrated in the human CP tissues; immune cell infiltration observed within the tissues

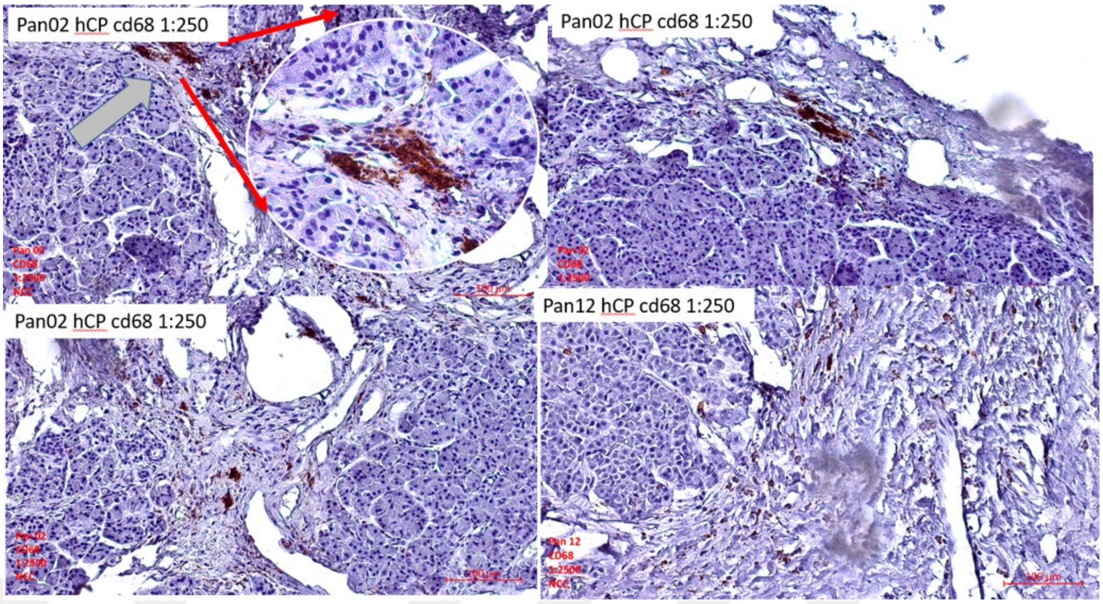



Figure 24- CD68 IHC Staining on Human CP Tissues

All the tissues were obtained and followed a tissue processing for preservation and paraffinization. Each tissue was cut in the thickness of 2.5 micron of slides were taken onto positive charged lams. IHC protocol was followed with the use of CD68 antibody and hematoxylin for counter staining. As demonstrated in the human CP tissues; CD68 is a tumor macrophage marker and within specific areas infiltration of macrophages observed within the tissues

## 4.7 Constitution of AP Mouse Model Results

### Von-Frey Abdominal Pain Scores, Amylase/Lipase Levels, Pancreas Wet/Dry Ratio

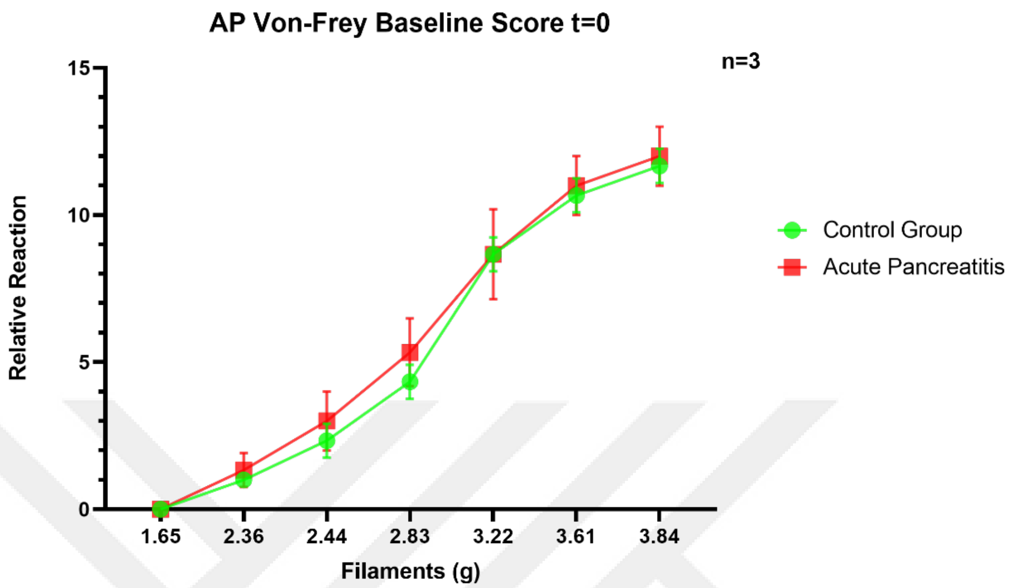



Figure 25 – Baseline Von-Frey Scores of Control and AP Group

In this experiment, 8 weeks old C57BL6 animals were used as control and acute pancreatitis (n=3) groups. Von Frey test was applied on the abdominal area of mice with the aid of Von-Frey filaments that could apply different pressure on a specific area. Each filament was applied for 10 times in abdominal cavity and response scored as follows; no reaction (0), only mild reaction such as awareness of touch (1), high reaction as jumping or retrieving abdomen instantly (2). Before any experimental procedure and caerulein injection, baseline scores were collected for further evaluation of abdominal pain scores. As graphic indicates, in t=0 time (before the experiment), both groups shared a similar reaction to the filaments.

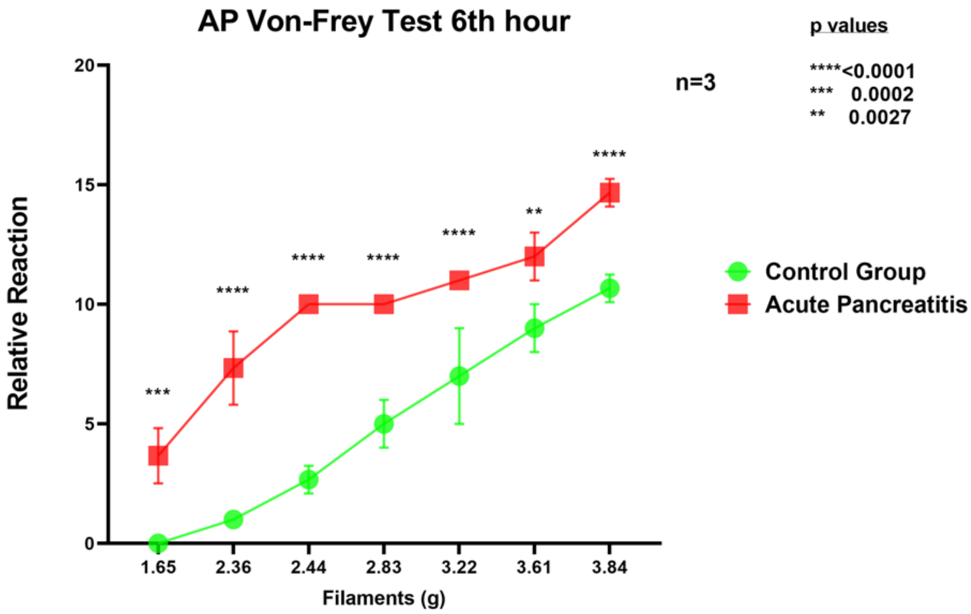



Figure 26 – 6th hour of Von-Frey Scores of Control and AP Group

In this experiment, 8 weeks old C57BL6 animals were used as control and acute pancreatitis (n=3) groups. Von Frey test was applied on the abdominal area of mice with the aid of Von-Frey filaments that could apply different pressure on a specific area. Each filament was applied for 10 times in abdominal cavity and response scored as follows; no reaction (0), only mild reaction such as awareness of touch (1), high reaction as jumping or retrieving abdomen instantly (2). After 6 hours of caerulein injection to the AP group and PBS to the control group, abdominal pain scores were collected. As graphic indicates, in t=6 time (5 injections were done until this point), acute pancreatitis group demonstrated a significant response difference than the control group.

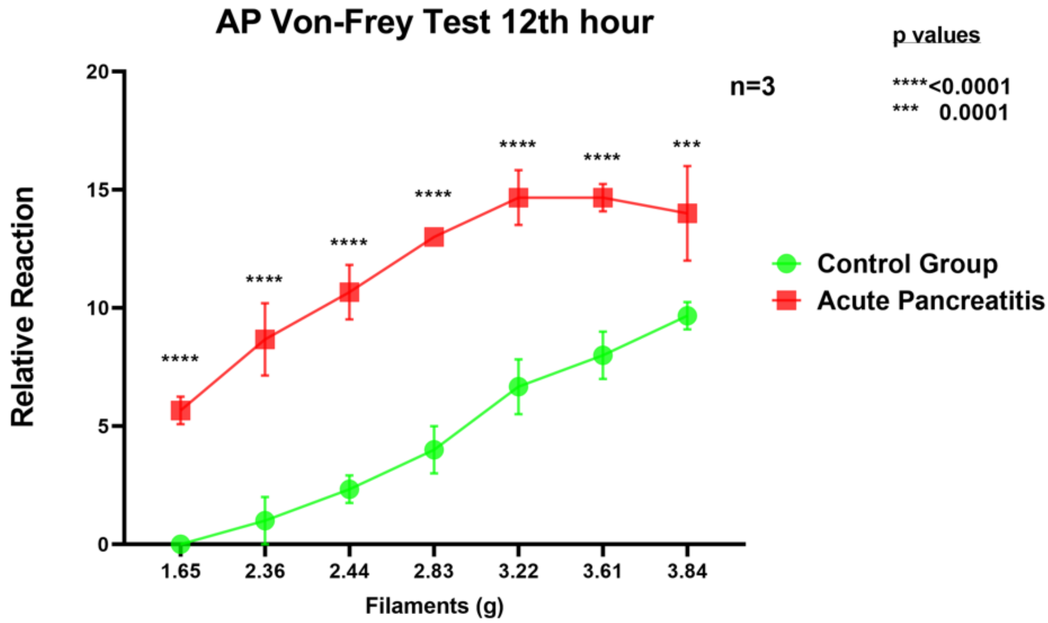



Figure 27 – 12th hour of Von-Frey Scores of Control and AP Group

In this experiment, 8 weeks old C57BL6 animals were used as control and acute pancreatitis (n=3) groups. Von Frey test was applied on the abdominal area of mice with the aid of Von-Frey filaments that could apply different pressure on a specific area. Each filament was applied for 10 times in abdominal cavity and response scored as follows; no reaction (0), only mild reaction such as awareness of touch (1), high reaction as jumping or retrieving abdomen instantly (2). After 6 hours of caerulein injection to the AP group and PBS to the control group, abdominal pain scores were collected. As graphic indicates, in t=12 time (10 injections were done until this point), acute pancreatitis group demonstrated a significant response difference than the control group.

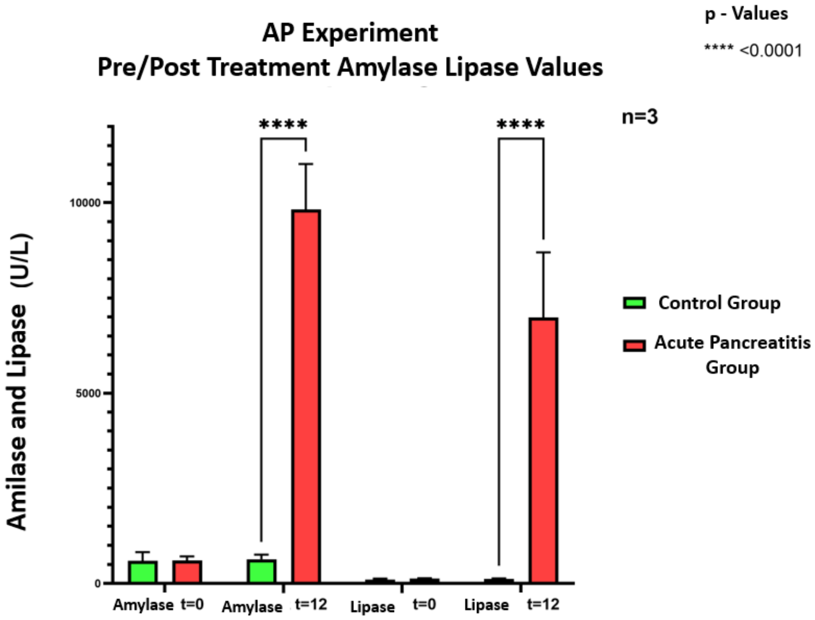



Figure 28 – Pre/Post Treatment Amylase & Lipase Values

In this experiment, 8 weeks old C57BL6 animals were used as control and acute pancreatitis (n=3) groups. Before and after 10 hours of caerulein injection, blood retrieval was done to assess amylase and lipase levels from the serum. As expected, in the AP groups' amylase and lipase levels significantly elevated.

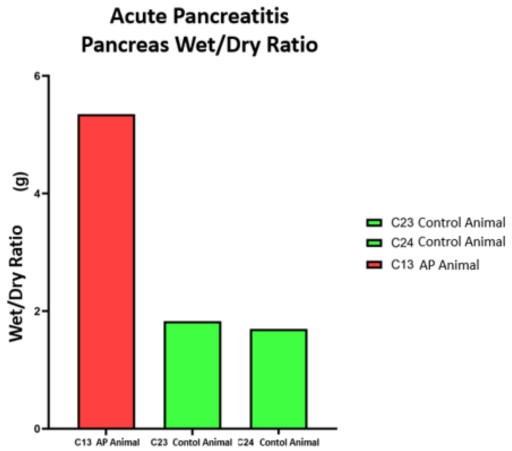



Figure 29 - Pancreas Wet/Dry Ratio

In this experiment, a small amount of pancreas was taken out and it weighted before and after dried out. As expected, with the edema occurrence in AP, this ratio is elevated when it compared with the control group.

### 4.8 Constitution of CP Mouse Model & Treatment with TCN-201

**Open-Field Data, Von-Frey Abdominal Pain Scores & Amylase/Lipase Levels**

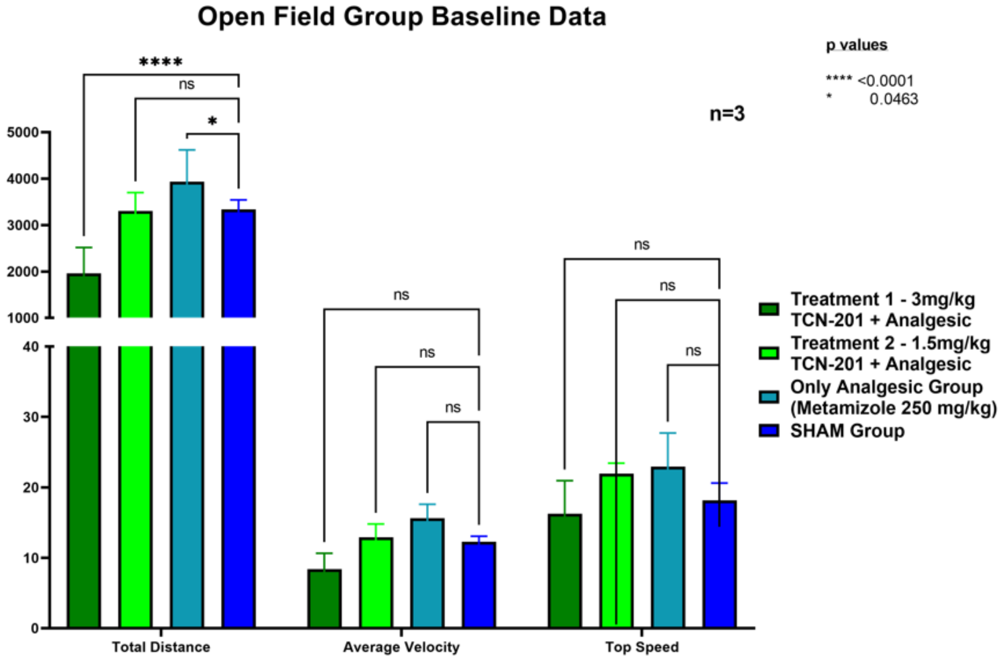



Figure 30 – Baseline Open Field Experiment in Dosage Finding Study

Before experiment, an open-field experiment was administrated among groups to evaluate their locomotive motion. To establish this experiment, each mouse has given been placed 20x20 box, camera assisted tracking program followed up their motion progress for 10 minutes.  In the baseline score (before any experiment) scores of total distance, average velocity and top speed did not demonstrate any significant differences between the groups.

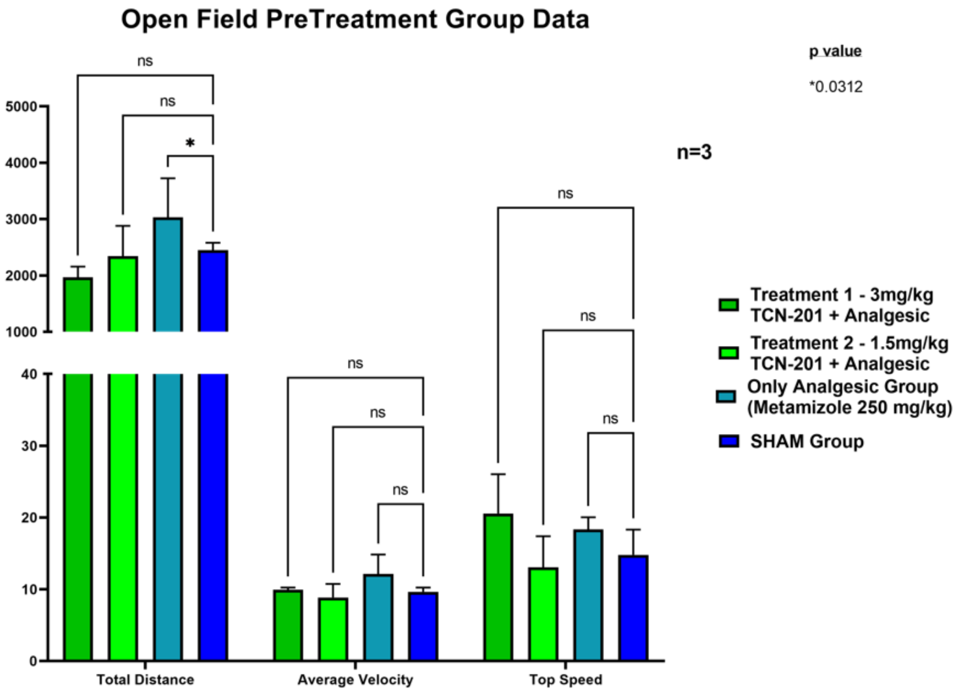



Figure 31 – Pre-Termination Open Field Experiment in Dosage Finding Study

An open-field experiment was administrated among groups to evaluate their locomotive motion. To establish this experiment, each mouse has given been placed 20x20 box, camera assisted tracking program followed up their motion progress for 10 minutes. In the pretreatment timeline (before administration of TCN-201 and metamizole as analgesic), for 2 weeks; each group has been given caerulein injection 3 times in a week and 6 times in a day. Parameters of total distance, average velocity and top speed did not demonstrate any significant differences between the groups.

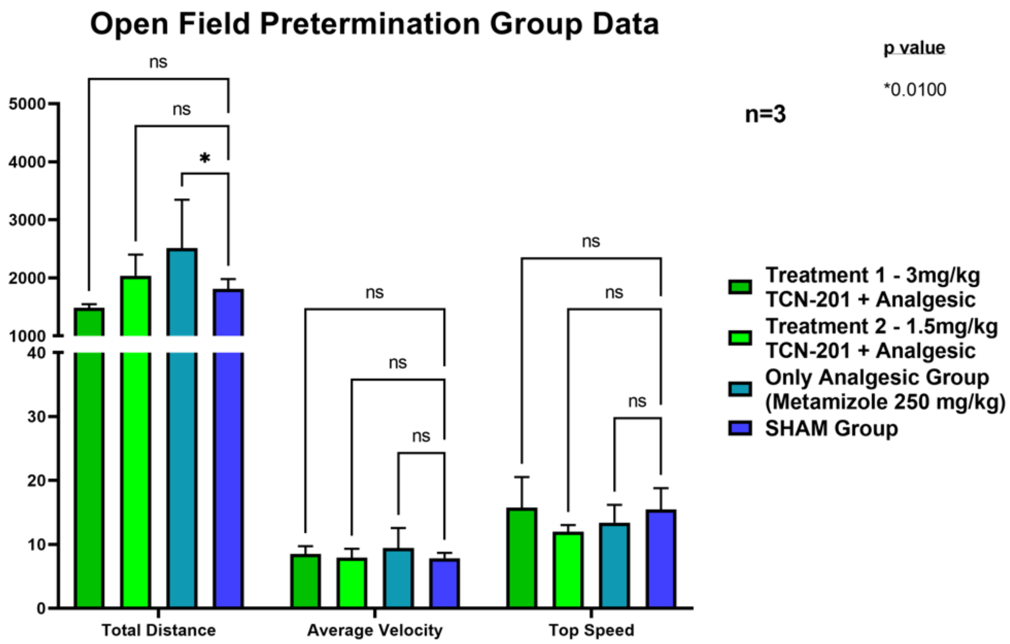



Figure 32 – Pre-Termination Open Field Experiment in Dosage Finding Study

An open-field experiment was administrated among groups to evaluate their locomotive motion. To establish this experiment, each mouse has given been placed 20x20 box, camera assisted tracking program followed up their motion progress for 10 minutes.  In the pretermination timeline (treatment was done and this data was last open field assay), for 6 weeks; each group has been given caerulein injection 3 times in a week and 6 times in a day while TCN-201 treatment was done 3 times in a week and analgesic was given orally within the drinking water. Parameters of total distance, average velocity and top speed did not demonstrate any significant differences between the groups. These results demonstrated, at least for the locomotion and behavior, TCN-201 treatment did not have any toxic effects.

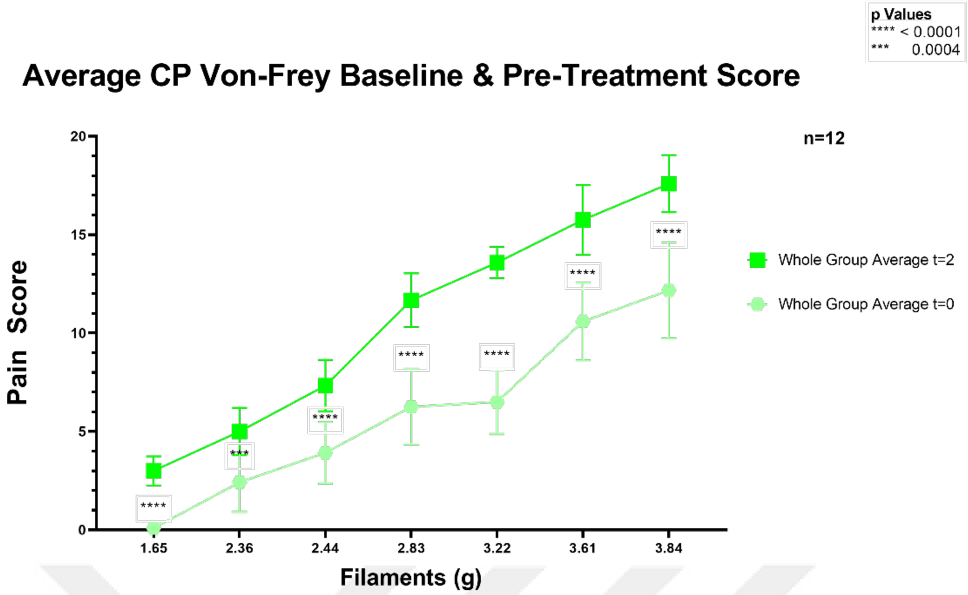



Figure 33 – Baseline and Pre-Treatment Abdominal Pain Scores of CP (Dosage Assay)

In this experiment, 4 different groups were established as sham, analgesic, treatment with TCN-201 (1.5 mg/kg) and analgesic and treatment with TCN-201 (3.0 mg/kg) and analgesic were established with n=3 animals with each group. Von Frey test was applied on the abdominal area of mice with the aid of Von-Frey filaments that could apply different pressure on a specific area. Each filament was applied for 10 times in abdominal cavity and response scored as follows; no reaction (0), only mild reaction such as awareness of touch (1), high reaction as jumping or retrieving abdomen instantly (2). To evaluate difference between baseline (before any experiment t=0) and pretreatment time (before any treatment but 2 weeks of caerulein administration t=2), After 2 weeks of intraperitoneal caerulein injection (t=2 time point), all of the pain scores were observed to be significantly elevated for all groups (n=12) before TCN-201 treatment.

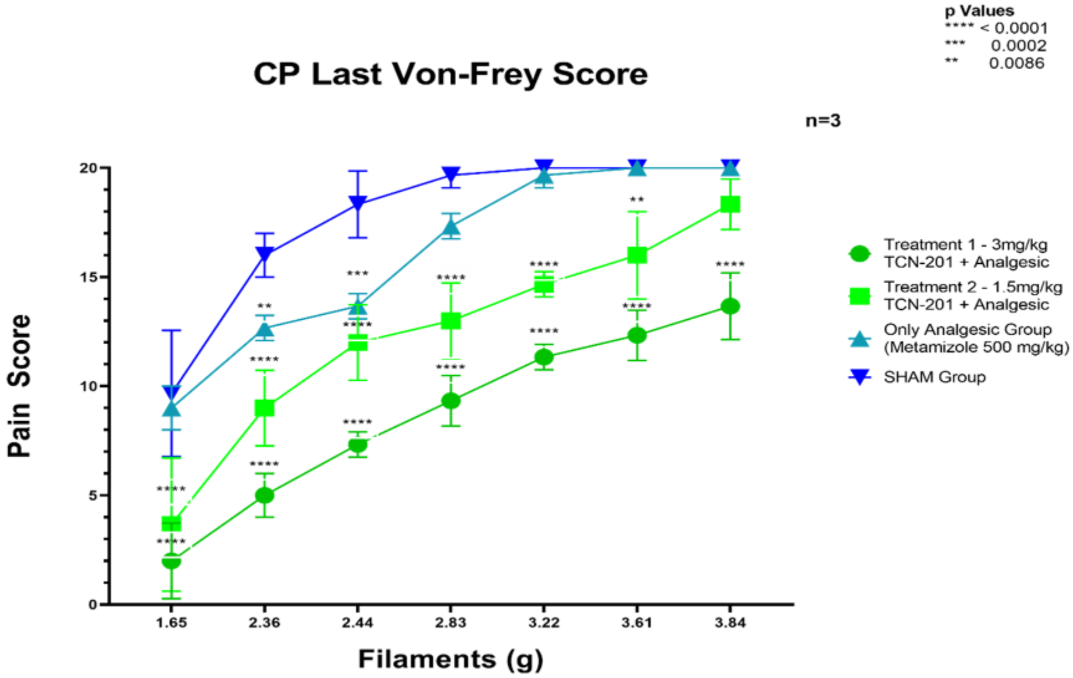
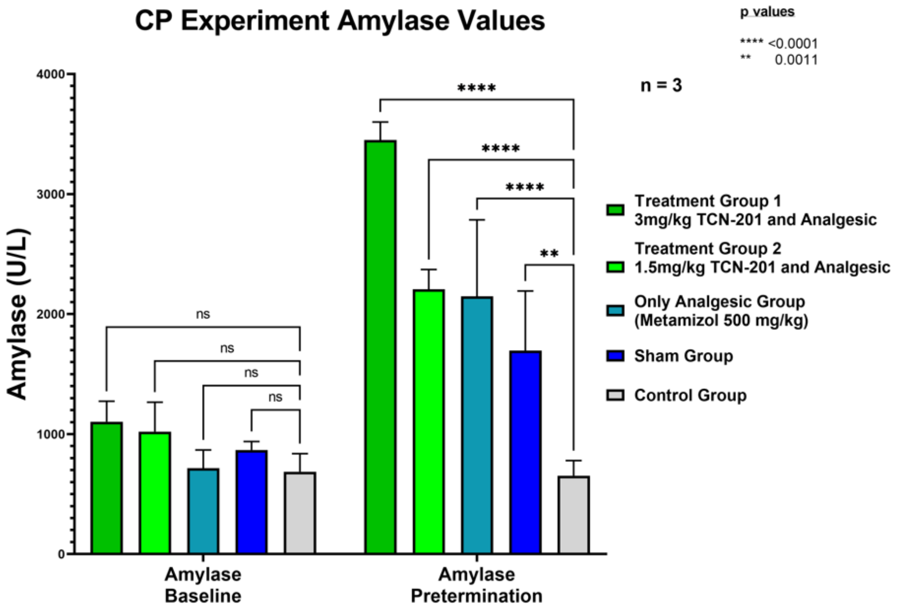



Figure 34 –Abdominal Pain Scores of CP Before Termination of Experiment (Dosage Assay)

In this experiment, 4 different groups were established as sham, analgesic, treatment with TCN-201 (1.5 mg/kg) and analgesic and treatment with TCN-201 (3.0 mg/kg) and analgesic were established with n=3 animals with each group. Von Frey test was applied on the abdominal area of mice with the aid of Von-Frey filaments that could apply different pressure on a specific area. Each filament was applied for 10 times in abdominal cavity and response scored as follows; no reaction (0), only mild reaction such as awareness of touch (1), high reaction as jumping or retrieving abdomen instantly (2). After 8 weeks of CP procedure and 6 weeks of TCN-201 and metamizole treatment period, all 3 treatment groups were compared with Sham group. While analgesic group could not attain a pain relief response, different concentrations of TCN-201 has yielded significant pain relief on caerulein induced CP mouse models.



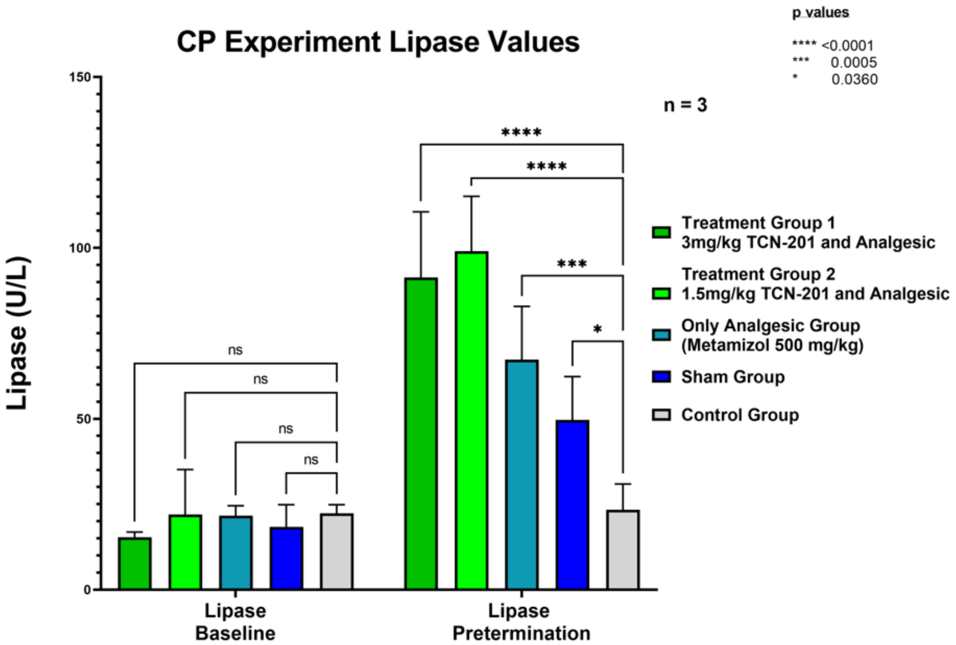




Figure 35 - Amylase/Lipase Levels of CP

Blood was taken from cheek of the animals, in 2 individual time points; before treatment and before termination. When it compared with control group amylase and lipase levels demonstrated a significant increase.

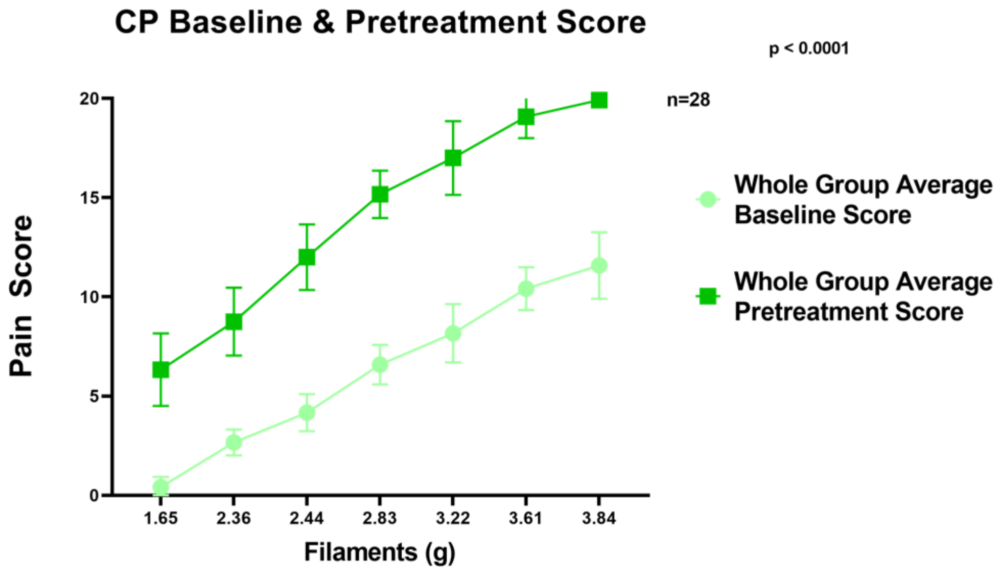



Figure 36 - Baseline and Pre-Treatment Abdominal Pain Scores of CP

In this experiment, 4 different groups were established as sham, analgesic, treatment with only TCN-201 (3 mg/kg) and treatment with TCN-201 (3.0 mg/kg) and analgesic were established with n=7 animals with each group. Von Frey test was applied on the abdominal area of mice with the aid of Von-Frey filaments that could apply different pressure on a specific area. Each filament was applied for 10 times in abdominal cavity and response scored as follows; no reaction (0), only mild reaction such as awareness of touch (1), high reaction as jumping or retrieving abdomen instantly (2). To evaluate difference between baseline (before any experiment) and pretreatment time (before any treatment but 2 weeks of caerulein administration), After 2 weeks of intraperitoneal caerulein injection (pretreatment time point), all of the pain scores were observed to be significantly elevated for all groups (n=28) before TCN-201 treatment.

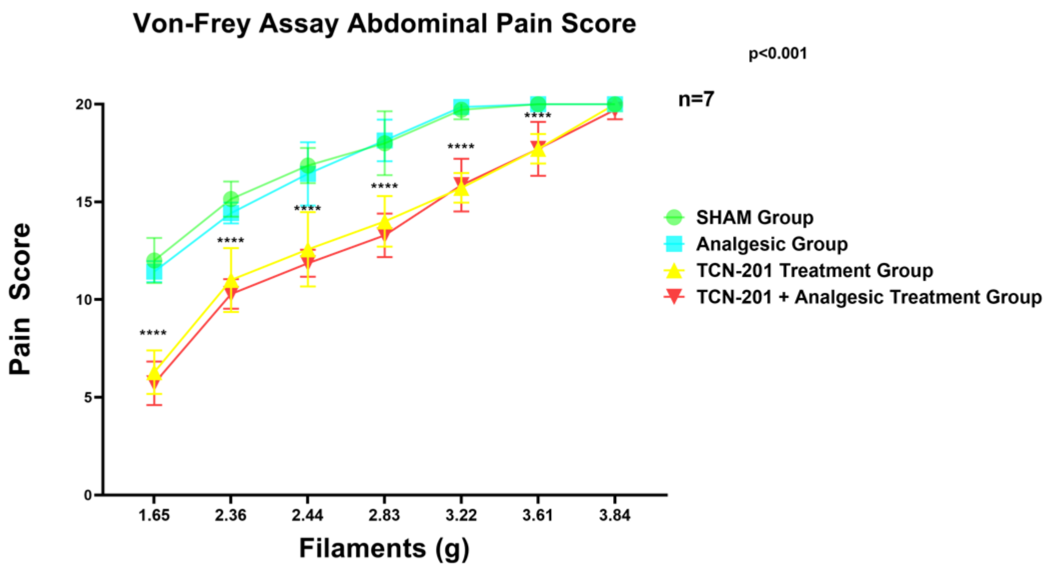



Figure 37 - Abdominal Pain Scores of CP Before Termination of Experiment

In this experiment, 4 different groups were established as sham, analgesic, treatment with only TCN-201 (3 mg/kg) and analgesic and treatment with TCN-201 (3.0 mg/kg) and analgesic were established with n=7 animals with each group. Von Frey test was applied on the abdominal area of mice with the aid of Von-Frey filaments that could apply different pressure on a specific area. Each filament was applied for 10 times in abdominal cavity and response scored as follows; no reaction (0), only mild reaction such as awareness of touch (1), high reaction as jumping or retrieving abdomen instantly (2). After 8 weeks of CP procedure and 6 weeks of sole TCN-201 use and TCN-201 supplemented with analgesic metamizole treatment period, all 3 treatment groups were compared with Sham group. While analgesic group could not attain a pain relief response, use of TCN-201 has yielded significant pain relief on caerulein induced CP mouse models while supplementing TCN-201 with analgesic did not give a synergetic effect.

# 5 DISCUSSION

Treating pain in CP is still one of the biggest challenges of gastroenterology. Unfortunately, most of the CP patients suffers from recurrence of unbearable abdominal pain (back pain in some cases) and opiate treatment dependency despite endoscopic or surgical interventions. While conventional analgesic treatments could not attain an expected treatment, and there is no viable opioid based therapy options for patients which may ameliorate the pain and enhance quality of life of patients. To comprehend these changes in molecular levels, consensus should be shifted into action potential related reprogramming capability of central and peripheral neuronal levels, susceptible nociceptors, and neurotransmitters. At this point, deciphering role of neuropathic pain related changes, molecules and neurotransmitters became significantly important. To have a better understanding of the underlying mechanism neuropathic pain in CP, this thesis focused on glutamate related NMDA receptor. To decipher this correlation certain investigation was done on NMDA receptors and NMDA's downstream effectors. In a previous research done by our group, Ekin et al., his group tried to elaborate neurotransmitter and neuro-enzyme profile of the intrapancreatic nerves of CP and pancreatic cancer (PCa) patients. Their investigation focused on SP, CGRP, VIP and nNOS on human normal, CP and PCa tissues. They have found out nNOS expression was significantly increased in CP tissues, and was also correlated with pain and neuritis score of CP patients. Following these findings, they have used caerulein induced CP mouse model, and nNOS specific inhibitor NPLA to assess the abdominal pain in mouse. When it is compared with no treatment group, results of their experiments were satisfactory in pain management and this pathway looked promising for an applicable therapy option [42]. Mechanistically, with the pain occurrence, release of presynaptic glutamate activates ionotropic and metabotropic receptors. In this case, activated NMDA receptors, allows intracellular $Ca^{2+}$ intake, and this $Ca^{2+}$ accumulation can activate $Ca^{2+}$/Cd pathway, leading activation of neuronal nitric oxide synthetase (nNOS) and leading to the production of protein kinases (PKs) and nitric oxide (NO). Following this process, NO also works as feedback loop, extracellularly activating the presynaptic end and leading release of glutamate. When we look at the just one point of this pathway, inhibition of NMDAR may also inhibit intracellular accumulation of $Ca^{2+}$ with that, which may affect $Ca^{2+}$/Cd pathway activation. Beside of $Ca^{2+}$/Cd pathway, inhibition of NMDA receptors may also inhibit phosphorylation of protein kinase A (PKA) and protein kinase C (PKC). This is an important factor since both phosphorylated PKC and PKA, in latter stages also phosphorylates NMDA receptors which leads more $Ca^{2+}$ intracellular influx. On the other hand, inhibition of NMDA may also lead inhibition significant pathways such as; MEK-ERK-MAPK, CaMK II, PI3K-AKT-GSK3β, NO-HIF1α-VEGF, Ca/NFkB and mTOR. In the previous researches these pathways demonstrated significant pain management and effectiveness  in cancer therapy.

In the preliminary part of this thesis, aim was the evaluation of transcriptional and translational activation of Glutamate receptors, which especially focused on NMDA receptors. Since there was not any commercially available CP cell line, PCa cell lines were used and these cell lines profile also compared with the CP. In human PCa cell lines, among suspected glutamate genes and intermediate gene, especially NMDAR1A, NMDAR2D and DLG4 (scaffolding protein) observed to be highly active in general population of PCa cell lines while other genes also demonstrated specific activity for certain cell lines.  This result has revealed that, transcriptionally NMDA activation has an important role in human PCa which can be a case in neurotrophic pain related diseases. In addition to that, scaffolding protein DLG4 (PSD95) was seen highly active in conveying receptor activation (Figure 5). Following this result, other investigation was done to evaluate whether there was a similarity between human and mouse cell lines. These mouse cell lines were specific to PCa lineages, and these results also yielded similar results with human PCa cell lines. The only difference was they have exerted more NMDAR1A and NMDAR2B activation while NMDAR2D was not evident as human PCa cell lines but it was present in all groups (Figure 6). This result has revealed that, transcriptionally mouse models were also relying on NMDA receptor activation, and it was mostly complementing with the results of human PCa transcriptional expression results. After the establishment of caerulein-induced AP and CP mouse models, prior to termination of experiments pancreas of mice were isolated to obtain general NMDA related transcriptional profile from acute and chronic pancreatitis mouse models. Among suspected NMDARs, results were demonstrated transcriptional similarities in the pattern with previously investigated human and mouse PCa cell lines. NMDAR1A, NMDAR2B and NMDAR2D observed to be highly activated when it compared with normal pancreas (NP) tissue. These results indicated that our main target NMDAR1A excessively upregulated when it was compared with NP tissue and the use of TCN-201 antagonist against NMDAR1A became more important in deciphering neuropathic pain in CP (Figure 7). Existence of NMDA expression levels were expected since glutamate is the one of the most principal mediators of the fast excitatory neurotransmitter in CNS that controls the cognition, memory learning, sensory information, emotions, memory retrieval and motor coordination. In fact, pain mediated excessive release of glutamates role in enhancing the expression of NMDA was an important discovery in these results to understand the role of NMDA neuropathic pain. To assess these finding further, human and mouse PCa cell lines and CP, PCa and NP tissue protein lysates were isolated. Preliminary Western Blot results has shown that for NMDAR1A, when each cell line compared, respectively in order, Panc1 demonstrated highest expression level where SU8686 followed in second and in general all PCa cell lines demonstrated expressions in certain levels. When this data compared with the transcriptional data, it demonstrates the same pattern in translational level, whereas T3M4 cell line did not exhibit an expression as seen in transcriptional data (Figure 8). On the other hand, for the mouse cell lines NMDAR1A seen highest level in mouse brain as positive control whereas KPC3039 and TPC2033 shown significant expression levels and TPAC18654 and TPAC K459 followed with respected levels of expression. When this data compared with the transcriptional data, it demonstrates the same pattern in translational level, whereas TPC18645 cell line did not exhibit an expression as seen in transcriptional data (Figure 9). NMDAR2A for the mouse tissues yielded highest level in the mouse brain as positive control whereas TPC18645 and TPC18654 shown significant expression levels while other cell lines did not exhibit any translational expression. When this data compared with the transcriptional data, it demonstrates the same pattern in translational level, whereas TPAC K459 cell line did not exhibit an expression as seen in transcriptional data (Figure 10). When tissue lysates of CP, PCa and NP tissues were isolated, When each tissue compared, despite individual differences of each patient, expression level of each group demonstrated a close similarity. When groups compared in quantification data (on the bottom) PCa and CP demonstrated significantly high expression in NMDAR1A levels. This data also correlates with the human IHC results and looks similar with the transcriptional qPCR mouse data (Figure 11). Also, this experiment was done with PCa, CP and NP tissues to evaluate underlying mechanisms of NMDA, when each tissue compared, in some cases individual differences of each patient stepped up, but expression level of each group has given a preliminary idea about the underlying mechanism. When phosphorylation levels of PCa and CP compared with the NP tissues, PCa and CP groups significantly demonstrated expression in p-PKC, p-PKA and p-ERK levels. This data was expected to be in this manner since overexpression of NMDA receptors leads into the activation and phosphorylation of underlying mechanisms and starts a feed-forward loop (Figure 12). Following these results, another experiment was designed to investigate to inhibit NMDAR1A overexpression activity via the use of siRNAs. Preliminary siRNA use on SU8686 cells yielded a good silencing result (Figure 13/14). For further investigations, this modified cells are going to be used in our 3D-migration assays to evaluate nerve and inflammatory cell relations. As previously described, NMDAR1A found to be highly active in both cell lines and tissues. Another research also done to decrease overexpression levels of NMDAR1A/2A receptors and TCN-201 was effectively used on the PCa cell lines. As mentioned before, previous studies not only demonstrated that NMDA receptors could play an important role in pain management, but also researchers reported that NMDA receptors may have therapeutic effects on cancer cell lines. To evaluate that, TCN-201 was used on SU8686, T3M4 and Panc1 PCa cell lines. Within 6 hours, 15 uM and 30 uM TCN-201 concentrations were effective in retardation of PCa proliferation capacity (Figure 15). In the following hours 30 uM TCN-201 concentration has seen as reliable in inhibition cell proliferation (Figure - 16/17/18).

In the in-vivo part of the experiments, both caerulein-induced AP and CP mouse models were established in our laboratory. To have a confirmation for the model, the pancreas of the animals were paraffinized and 2.5 micron slides were prepared. To investigate morphology, H&E procedure was followed. AP and CP pancreas mouse tissues were compared with normal mouse pancreas tissue. This comparison demonstrated that acute pancreatitis has demonstrated edema and immune cell infiltration while chronic pancreatitis model had severe edema, disruption of acinar cells and immune cell infiltration (Figure 19). All of the morphological findings were satisfactory, to move on next step in animal studies. On the other hand, IHC studies were conveyed on human NP, CP PCa and animal NP, CP tissues. In all human and mouse pancreas NMDAR1A staining especially observed in the nerves. When it is compared with normal pancreas (NP) human tissues (top section) and human CP tissues (middle section), observed nerve staining was clearly evident in CP tissues. This result demonstrated that NMDAR1A is activated in CP patients and it is also present in CP mouse tissues while NP tissues of mice did not exhibit any NMDAR1A staining (Figure 20). These results were also complementary to our previous qPCR and WB results. In another IHC study, CP patients were selected according to their individual pain scores and NMDAR1A staining was applied to evaluate relation of pain sensation with NMDAR1A expression. According to the pain scores of patients, NMDAR1A staining within the nerves has significantly increased in the intensity and it has shown correlation with the increase in pain score. These results demonstrated that NMDAR1A might have a significant role in neuro-pancreatic pain in CP patients (Figure 21).

On the other hand, staining analysis was done to compare PCa, CP and NP tissues. The strongest activation was found on CP tissues and PCa tissue nerve stainings were also found to be significantly better than NP tissues. Strong staining generally observed within the nerve areas. This data has shown good statistical significance since most of the staining of NMDAR1A recognized within the nerves of the tissues, and expression of the NMDAR1A increased in PCa and CP conditions. (Figure 22). To evaluate immune cell infiltration and macrophages, CD45 and CD68 were use in human CP tissues where significant stainings were for within the infiltrative specific ares of the CP tissues (Figure 23/24). Constitution of AP mouse model was done via intraperitoneal 50 mg/kg caerulein injection, 10 times/hourly in a day. Before the beginning of the experiment a baseline abdominal pain score was taken from the animals with the aid of Von-Frey filaments. This procedure was also followed up in the $6^{th}$ and $12^{th}$ hours of the experiment. As the experiment progresses, a meaningful abdominal pain difference was observed between control and acute pancreatitis group (Figure 25/26/27). In addition to that, blood was taken before beginning and terminating experiment. Plasma samples were investigated against amylase and lipase which were found significantly elevated in AP groups (Figure 28). Also pancreas dry/weight ratio helped us to understand whether edema after AP truly established (Figure 29). In all four aspects; morphology, pain and amylase/lipase levels, wet/dry ratio of the pancreas were successful in demonstrating of valid caerulein-induced AP mouse model. Following success in both AP and CP mouse models, we have shifted our focus on the use of TCN-201 NMDAR1A/2A antagonist to relieve pain in caerulein-induced CP mouse models. While I was administrating this experiment, open field experiments were also done to evaluate whether TCN-201 concentration has any adverse effects on treated mouse groups. Thankfully, treatment groups did not show any less locomotive motor activities than other groups which was very important since NMDA is an effective glutamate receptor, and it also conveys different responsibilities since it has expression levels in all the parts of the brain (Figure 30/31/32). To assess pain sensation in caerulein induced CP mouse models, Von-Frey Filaments were used in each week of the experiments and results were recorded carefully. Before the experiment, the abdominal pains of all groups were investigated to establish a general baseline abdominal score for whole groups. After 2 weeks of Caerulein administration, another Von-Frey experiment was done to evaluate pain changes in whole groups After 2 weeks of intraperitoneal caerulein injection, all of the pain scores were observed to be significantly elevated for all groups (n=12) before TCN-201 treatment (Figure 33). After 8 weeks of CP procedure and 6 weeks of TCN-201 and metamizole treatment period, all 3 treatment groups were compared with Sham group. Following weeks, TCN-201 treatment (3 mg/kg and 1.5 mg/kg) was given with analgesics, only the analgesic group (metamizole 500 mg/kg) and Sham group were established. Treatment with TCN-201 yielded a significant pain relief response in caerulein induced CP mouse groups, while only analgesic group could not attain significant response when it is compared with Sham group (Figure 34). All animals were survived until the end of experiment with no reported adverse effects. 3 mg/kg TCN-201 administration was dramatically more effective in pain relief than 1.5 mg/kg TCN-201 administrated group. As previously discussed analgesic group was not satisfactory in pain relief of CP, whether it is not known if it contributed to TCN-201 effectiveness in this experiment. Also this experiment was repeated with a larger CP animal group. In this experiment, 4 different groups were established as sham, analgesic, treatment with only TCN-201 (3 mg/kg) and treatment with TCN-201 (3.0 mg/kg) and analgesic were established with n=7 animals with each group. Von Frey test was applied on the abdominal area of mice with the aid of Von-Frey filaments that could apply different pressure on a specific area. Each filament was applied for 10 times in abdominal cavity and response scored as follows; no reaction (0), only mild reaction such as awareness of touch (1), high reaction as jumping or retrieving abdomen instantly (2). To evaluate difference between baseline (before any experiment) and pretreatment time (before any treatment but 2 weeks of caerulein administration), After 2 weeks of intraperitoneal caerulein injection (pretreatment time point), all of the pain scores were observed to be significantly elevated for all groups (n=28) before TCN-201 treatment (Figure 36). After 8 weeks of CP procedure and 6 weeks of sole TCN-201 use and TCN-201 supplemented with analgesic metamizole treatment period, all 3 treatment groups were compared with Sham group. While analgesic group could not attain a pain relief response, use of TCN-201 has yielded significant pain relief on caerulein induced CP mouse models while supplementing TCN-201 with analgesic did not give a synergetic effect (Figure 37).

# 6 CONCLUSION

The main emphasis of this thesis was evaluation of NMDA receptors' role in pain relief in CP. To achieve this goal, in vitro experiments were done to understand the transcriptional and translational role of these receptors. qPCR and Western Blot results demonstrated NMDA receptors were highly active in both human and mouse cell lines. In addition to that, RNA was isolated from established caerulein-induced AP and CP mouse pancreas tissues. qPCR results acquired from tissue were also complimentary with our previous mouse cell line data. With these outcomes, NMDA receptors have taken our attention for further investigations. To comprehend the locational expression profile of NMDA receptors' NP, CP, PCa human tissues, and NP, CP mouse tissues were used in IHC staining. After staining with NMDAR1A, it is highly active in CP and PCa when compared with NP. Histological analysis also demonstrated strong NMDAR1A staining was frequently found on the nerves of CP tissues. This result also has given us a hint about correlation between NMDAR1A activation and pain relation. After histological and physiological confirmation of both caerulein-induced AP and CP models, we have shifted our focus onto pain relief the via use of TCN-201 antagonist. With the help of Von-Frey Filaments, abdominal pain scores were recorded between two treatment (3.0 mg/kg and 1.5 mg/kg TCN-201), one analgesic and one sham group. When compared with the sham and analgesic group, the effects of TCN-201 were significant in pain relief. Moreover, any of treatment groups did not demonstrate any adverse effects. With these significant results, our group applied TÜBİTAK 1001 to demonstrate effectiveness of NMDA related pain relief on a larger animal cohort.

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# 8 APPENDIX

## APPENDIX 1

Ethical approval for collection of tissues.

Ethical approval for collection of tissues (continued).

**APPENDIX 2**

Ethical approval for caerulein induced CP constitution and treatment.

Ethical approval for caerulein induced CP constitution and treatment (continue).

# 9 CURRICULUM VITAE