ROLE OF GALANIN AND ITS ANTAGONISTS IN EXPERIMENTAL ACUTE PANCREATITIS

A thesis submitted for the degree of Doctor of Philosophy

By

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October 2008

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SUMMARY OF THESIS

The broad aim of the studies described in this thesis was to evaluate the role of neuropeptide galanin in acute pancreatitis (AP). Treatment of AP is mainly symptomatic and supportive and no definitive pharmacological therapy for this disease is currently available.

There are a number of studies in animal models of AP which demonstrate beneficial effect of a pharmacological agent in the management of AP. But most of these studies are limited to single species. The studies presented in the thesis evaluate the role of galanin and several of its antagonists in experimental AP in two different species. The initial part of the experimental work was performed in the possums, using a well established model of AP in the laboratory. Later, the experimental work has been carried out in the mouse.

The overall hypothesis was that galanin plays a major role in the onset and/or progression of AP.

In Chapter 2, the effect galanin or galantide administration, before and after AP induction on severity of AP in the possum model is described. The studies demonstrated that galantide decreased various indices of AP when administered prophylactically and therapeutically.

Chapter 3 outlines studies to determine if administration of galanin or galantide alters pancreatic vascular perfusion (PVP) during AP in the possum model. These studies suggested that in AP there is an initial fall in PVP, which is exacerbated by administration of galanin prior to onset of AP. Conversely, galantide administration prevented this decrease in PVP, and was associated with a rise in PVP through out the duration of the experiment.

Chapter 4 describes preliminary studies on effect of galanin and galantide on pancreatic exocrine secretion. These demonstrated that galantide decreased hyperstimulated pancreatic exocrine secretion, but had no effect on the basal secretion.

The subsequent studies are carried out using the caerulein mouse model of AP. The hypothesis has been tested in three different strains of mice, including a galanin gene knock-out (KO) strain.

Chapter 5 outlines the effect galanin or galantide administration, before and after AP induction on the severity of AP in the caerulein mouse model. These studies revealed that galantide administration both prophylactically and therapeutically decreased the severity of AP in the mouse.

In Chapter 6, the galanin gene KO were used to further test the hypothesis. These studies revealed that AP was less severe in the galanin KO mice, thereby suggesting a role for endogenous galanin in the onset and/or progression of AP.

Chapter 7 describes the effects of various galanin antagonist on the severity of AP in the caerulein mouse model. These studies revealed that galantide and M35 have beneficial effects in AP, i.e. reduced the indices of AP, whereas C7 and M40 had complex effects.

Chapter 8 provides an overview of findings and discussion of their broader ramifications with future recommendations.

Overall, the studies have demonstrated that galanin plays a major role in AP and galanin antagonists may be of potential therapeutic value in the management of AP.

PUBLICATIONS FROM THE STUDIES IN THIS THESIS

Manuscripts

Brooke-Smith ME , Carati CJ, **Bhandari M**, Toouli J, Saccone GTP. Galanin in the regulation of pancreatic vascular perfusion. Accepted for publication in Pancreas, Jan 2008.

Due to intellectual property issues publications were delayed. Presently the following manuscripts are being prepared

Bhandari M, Thomas AC, Carati CJ, Toouli J, Saccone GTP. Galanin antagonism modifies hyperenzymemia and pancreatic vascular perfusion (PVP) changes induced by acute pancreatitis (AP) in a possum model.

Bhandari M, Thomas AC, Carati CJ, Kawamoto M, Toouli J, Saccone GTP. Galanin antagonism ameliorates hyperenzymemia and pancreatic necrosis in caerulein-induced acute pancreatitis in the mouse.

Bhandari M, Kawamoto M, Thomas AC, Carati CJ, Toouli J, Saccone GTP. The galanin knockout mouse is less susceptible to caerulein-induced acute pancreatitis.

Abstracts and Conference Presentations

Abstracts

Saccone GTP, London JA, Woods CM, **Bhandari M**, Carati CJ, Brooke-Smith ME, Toouli J. Acute ethanol modulates pancreatic vascular perfusion in the Australian possum. Proceeding of the Falk Symposium No 143, poster # 42, 2004.

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Bhandari M, Kawamoto M, Thomas AC, Carati CJ, Toouli J, Saccone GTP. The galanin knockout mouse is less susceptible to caerulein-induced acute pancreatitis. J HPB Surgery, in press.

Kawamoto M, **Bhandari M**, Thomas AC, Carati CJ, Toouli J, Saccone GTP. The galanin antagonist M35 but not M40 ameliorates caerulein-induced acute pancreatitis in a mouse model. J HPB Surgery, in press.

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Kawamoto M, **Bhandari M**, Thomas AC, Carati CJ, Toouli J, Saccone GTP. The galanin antagonists galantide and M35 but not M40 ameliorate caerulein-induced acute pancreatitis in mice. Pancreatology, in press.

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Poster - Saccone GTP, London JA, Woods CM, **Bhandari M**, Carati CJ, Brooke-Smith ME, Toouli J. Presenter: GTP Saccone). Acute ethanol modulates pancreatic vascular perfusion in the Australian possum. The Falk Symposium No 143 (Pancreatitis: Advances in pathobiology, diagnosis and treatment), Frieburg, Germany, October, 2004.

Oral - **Bhandari M**, Brooke-Smith ME, Carati CJ, Toouli J, Saccone GTP. (Presenter: M Bhandari). Galanin reduces pancreatic vascular perfusion (PVP) in the Australian possum. AHMR Congress, Sydney, NSW, November 2004.

Poster - London JA, Woods CM, **Bhandari M**, Carati CJ, Brooke-Smith ME, Toouli J, Saccone GTP. Presenter: GTP Saccone). Intragastric but not intravenous ethanol decreases pancreatic vascular perfusion in the Australian possum. APA Chicago, USA, November 2004.

Oral – **Bhandari M**, Thomas AC, Carati CJ, Toouli J, Saccone GTP. (Presenter: M Bhandari). Galanin antagonism ameliorates the severity of caerulein-induced acute pancreatitis in the mouse. Australasian pancreatic Club, Sydney, March, 2006.

Oral - **Bhandari M**, Thomas AC, Carati CJ, Kawamoto M, Brooke-Smith ME, Toouli J, Saccone GTP. (Presenter: M Bhandari). Galanin antagonism reduces hyperenzymemia associated with acute pancreatitis (AP) in a possum model. Digestive Diseases Week, Los Angeles, USA, May 2006.

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Oral - **Bhandari M**, Thomas AC, Carati CJ, Kawamoto M, Brooke-Smith ME, Saccone GTP, Toouli J. (Presenter: J Toouli). Galanin antagonism reduces hyperenzymemia associated with acute pancreatitis (AP) A possum model. International Hepto Pancreato Biliary Association 10th World Congress, Edinburgh, Scotland, September, 2006.

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Poster - Kawamoto M, **Bhandari M**, Thomas AC, Carati CJ, Toouli J, Saccone GTP. The galanin antagonist M35 but not M40 ameliorates caerulein-induced acute pancreatitis in a mouse model. (Presenter: J Toouli). A-PHPB, Fukuoka, Japan, March 2007.

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Poster - **Bhandari M**, Kawamoto M, Thomas AC, Carati CJ, Toouli J, Saccone GTP. Galanin receptor antagonist M35: a potential pharmacological treatment for acute pancreatitis. (Presenter: J Toouli). Digestive Diseases Week, Washington DC, USA, May, 2007.

Poster - Kawamoto M, **Bhandari M**, Thomas AC, Carati CJ, Toouli J, Saccone GTP. The galanin antagonists galantide and M35 but not M40 ameliorate caerulein-induced acute pancreatitis in mice. (Presenter: GTP Saccone). Digestive European Pancreatic Club, Newcastle-Gateshead, UK, July 2007.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Mayank Bhandari, M.B.B.S., M.S
Date:

ACKNOWLEDGEMENTS

I wish to take the opportunity to thank a number of people whose assistance has been instrumental in the studies associated with this thesis.

I am indebted to my supervisors Professor James Toouli, Associate Professor Gino Saccone and Associate Professor Colin Carati for their continuous support, encouragement, constant assistance and critical and constructive feedback during my candidature. I wish to thank them for their useful discussion and comments regarding the preparation of this thesis and associated conference presentations. I am also grateful to Professor A Thomas for his help in histological analysis of slides.

I am also grateful to Ann Schloithe for the technical expertise and advice provided by her on various aspects of surgical skills and interpretation of statistical analyses associated with these studies. The assistance and friendship of staff and students in the Pancreatobiliary Research Laboratory group was especially helpful. In particular I wish to thank Aaron Citti, Masahiko Kawamoto, Charmine Woods and Elizabeth Andrews.

I am also grateful to the staff of Departments of Pathology and Biochemistry for their cooperation in preparation of histological specimen slides and estimation of plasma pancreatic enzymes respectively.

I would especially like to mention the helpful services of the staff in the Animal house and the staff in Medical Illustration and Media, thank you all for your help.

I wish to thank Dr. C.Ormandy from Garvan Institute of Medical Research, NSW for providing us with galanin gene knock- out mice.

I am also thankful to Flinders Technologies, Flinders university of South Australia, Bio Innovation SA and Flinders Medical Centre Research Foundation for the financial support provided for this project.

I wish to thank my wife, family and friends for their patience, encouragement and tireless support during the time taken to complete these studies and thesis.

Finally, I would like to dedicate this work to my beloved mother Mrs. Suman Bhandari.

ABBREVIATIONS

The following abbreviations are used throughout the text, figures and figure legends of this thesis.

ANOVA	Analysis of variance
AP	Acute pancreatitis
BP	Blood pressure
BSA	Bovine serum albumin
Ca++	Calcium
CCK	Cholecystokinin
CCK-8	Cholecystokinin octapeptide
CNS	Central nervous system
CVP	Central venous pressure
GAL-LI	Galanin- like immunoreactivity
GALR1	Galanin receptor 1
GALR2	Galanin receptor 2
GALR3	Galanin receptor 3
GMAP	Galanin message associated peptide
ICAM	Intercellular adhesion molecule
IL	Interleukin
IV	Intravenous
KO	Knock-out
LDF	Laser Doppler fluxmetry
MPO	Myeloperoxidase
NO	Nitric oxide
NOS	Nitric oxide synthase
NFK ß	Nuclear factor kappa Beta
PD	Pancreatic duct
PDP	Pancreatic duct pressure
PVP	Pancreatic vascular perfusion
RNA	Ribonucleic acid

SEC	Secretin
SEM	Standard error of the mean
TNF	Tumour necrosis factor
VIP	Vasoactive intestinal polypeptide
WT	Wild type

STRUCTURE OF THESIS

History of candidature

My candidature for this thesis commenced in April 2004 as a full time student. The literature review was surveyed during 2004. During this year preliminary studies were performed based on the findings of my predecessor, Mark Brook-Smith. Based on these preliminary studies the overall hypothesis and specific hypotheses were defined. The experimental studies were performed initially in the possum during the later half of 2004 to early 2006. Then the studies were undertaken in the mouse from 2006 to mid 2007 to further test the hypothesis. Subsequently during 2007-2008, the thesis was compiled for submission.

Thesis chapters

The structure of this thesis conforms to Flinders University guidelines. This thesis is presented in the following chapters.

Chapter 1 contains an overview of the relevant literature up to the time i completed experimental studies (mid 2007). The literature review has been updated to include key findings that aid in understanding of the pathophysiology of acute pancreatitis. Chapter 1 concludes with the presentation of general hypothesis, followed by the research aims.

Chapters 2-7 describe the experimental studies i.e. aims, methodology, analysis, statistical methods, results and discussion. Each chapter begins with a brief introduction.

Chapter 8 contains general discussion. The purpose of this chapter is to relate the findings to the original hypothesis. This section concludes with suggestions for future research.

Location of figures

To minimise disruption to the text, all figures are located near the end of each chapter.

Chapter 1

Chapter 1: Introduction

1.1 Brief history of the pancreas and acute pancreatitis:

The pancreas was ignored as an organ and as a seat of disease until the early 18th century, perhaps because of its hidden retroperitoneal location and presence of larger intraperitoneal organs such as the liver. It was called 'the hermit organ' by the abdominal surgeons of the early 20th century due to the rarity of operations performed on it.

According to Fitzgerald et al.,[1] Greeks were the first to recognise the pancreas as a distinct organ. Galen gave a vague description of the organ, and termed most inflammatory diseases of the pancreas as 'scirrhous '. The first anatomical description of pancreas was given by Herophilus of Chalcedon (circa 300 B.C.), who was considered the Father of Scientific Anatomy. The name pancreas was coined by Rufus of Ephesus (circa 100 A.D.) from the Greek word pan: all and kreas: flesh or meat. It was so named because of its uniform composition and consistency and lack of bone or cartilage. Vesalius in 1500's gave detailed description of the vascular anatomy of and around the pancreas.

In 1642, Johann George Wirsung described the eponymous main pancreatic duct in the human pancreas, which bears his name. But it was Wirsung's student M. Hoffman, who repeatedly told him of such a duct which he had found in the rooster 1 year earlier[1]. In 1742, Santorini illustrated the accessory duct of the pancreas.

In the 19th century, the physiological functions of the pancreas were defined. The discovery of the function of the pancreas in digestion has been attributed to Claude Bernard. In 1856, he showed that pancreatic juice was equally important for the physiology of the digestive system because before this time gastric digestion was considered as the entirety of digestive physiology. Paul Langherhans (1869), while a medical student, published his inaugural thesis entitled “Contribution to the Microscopic Anatomy of the Pancreas”, in which he first described the structure of islet tissue. These were then named as ‘islets of Langerhans’ by Laguesse in 1893.

Acute pancreatitis (AP) was anecdotally referred to in the 1500’s by Aubert[2] and 1800’s by Friedreich[3]. Reginald Huber Fitz in 1889 first specified signs and symptoms of pancreatitis and defined the disease entity with its gangrenous, haemorrhagic, and suppurative aspects[1]. His description of signs and symptoms of AP created an awareness and interest in the medical field at that time. The presence of a sphincter mechanism at the distal end of the common bile duct was postulated as early as the 1600s by anatomists such as Francisci Glissoni[4] However, it was not until 1887 that Rugero Oddi described the structure that now bears his name, as a smooth muscle sphincter that functions as a regulator of the flow of bile[5]. In the 1930s Boyden, working with fetal material, was able to identify a common bile duct and pancreatic duct sphincter musculature that was distinct from the duodenal musculature[6].

Early 1900's was the era in which interest developed into the understanding of pathogenesis of AP. Interest in AP developed when Opie, who was an instructor in pathology at Johns Hopkins University in 1901, found the cause of death of one of his patients to be due to a stone impacted at the diverticulum of Vater[7]. On finding this, he proposed the "common channel" hypothesis, in which the flow of infected bile into the pancreatic duct occurs from the common bile duct due to a gallstone impacted at the diverticulum of Vater. The first description of gallstone was given by Fitz[8]. Opie's "common channel" hypothesis was then tested by various researchers around the world. Archibald in 1919 demonstrated that by producing spasm of sphincter of Oddi and thus increasing the biliary pressure, AP could be produced. During this period Symmers (1917) studied thirty-one cases with alcoholic indulgence, sudden death, gastric hyperaemia and a necrotic pancreas. He concluded that AP could be due to alcohol intake[9]. At this point alcohol and gallstones were considered as the major causes of AP, but other aetiological factors were postulated and investigated. In 1936, Rich and Duff published experimental studies that suggested pathologic vascular changes caused by escape of pancreatic fragments into the interstitial tissue of the organ, resulted in haemorrhage, digestion and necrosis of the pancreas. In 1976, Kelly suggested that complete bile duct obstruction by gallstone was not necessary to cause AP and partial obstruction could also lead to AP[10]. Extensive study of the pathogenesis of AP indicated that in a small proportion of cases the aetiology is idiopathic.

In 1927, discovery of the serum amylase test by Elman[11] was a great contribution towards differential diagnosis of AP. With the help of this test the milder form of the disease was diagnosed more frequently.

1.2 Anatomy of the human pancreas:

The human pancreas is an elongated structure that lies in the epigastrium and the left upper quadrant of the abdomen. It is situated in the retroperitoneum on the posterior abdominal wall and crosses the transpyloric plane[12]. It weighs approximately 80-100 grams and is soft and lobulated.

The pancreas is divided into the head, neck, body and tail (Fig. 1). The head of pancreas is disc shaped and lies within the concavity of the duodenum (Fig. 2). A part of the head of pancreas extends to the left behind the superior mesenteric vessels and is called the uncinate process. The neck is the constricted portion of the pancreas, which connects the head with the body. It lies in front of the portal vein and the origin of the superior mesenteric artery from the aorta. The body runs upwards and to the left across the midline. The tail passes forwards in the splenorenal ligament and comes in contact with the hilum of the spleen.

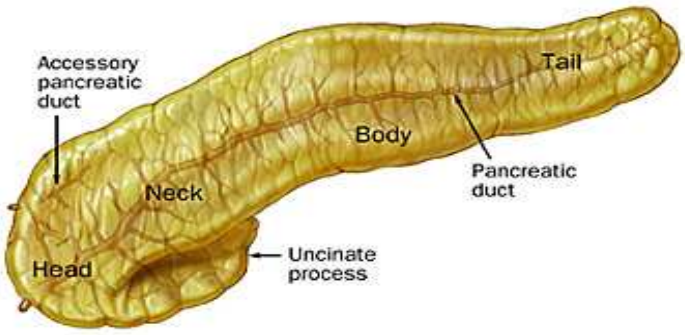


Fig. 1. Structure of the pancreas illustrating its various parts

Referenced from http://hopkins-gi.nts.jhu.edu

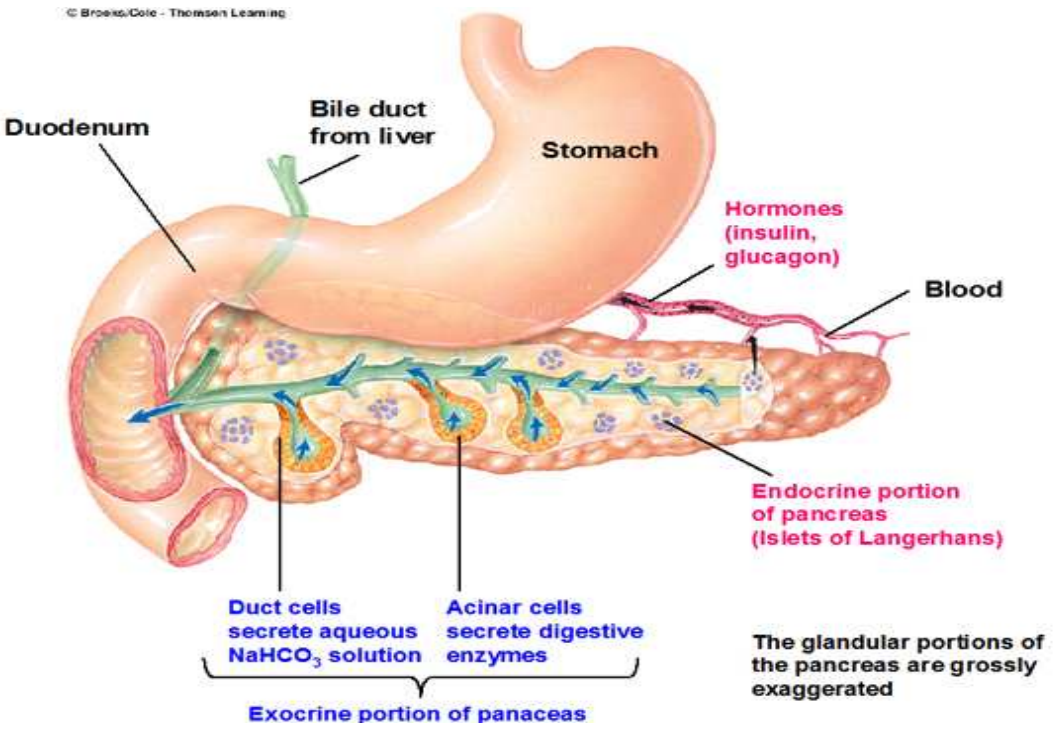


Fig. 2. Diagram of the human pancreas showing the retroperitoneal location and elements of the exocrine and endocrine structures.

Referenced from **www.medicalook.com/.../organs/Pancreas.html**

1.2.1 Anatomical relations of pancreas:

Anteriorly: from right to left: transverse colon and the attachment of transverse mesocolon, the lesser sac and the stomach.

Posteriorly: from right to left: the bile duct, the portal and splenic veins, the inferior vena cava, aorta, the origin of superior mesenteric artery, left psoas muscle, the left suprarenal gland, the left kidney and the hilum of the spleen.

1.2.2 Pancreatic ducts:

The main pancreatic duct begins in the tail and runs along the length of the pancreas (Fig. 1). It receives numerous tributaries along its path. It opens in the second part of the duodenum along with the bile duct at the major duodenal papilla. Sometimes the main pancreatic duct drains separately into the duodenum. The accessory duct of the pancreas when present drains the upper part of the head and then opens into the duodenum above the main pancreatic duct. It opens into the minor duodenal papilla. The accessory duct sometimes communicates with the main pancreatic duct.

1.2.3 Arterial supply of the pancreas:

The pancreas receives arterial supply from the branches of the celiac trunk and the superior mesenteric artery[13].

The head of the pancreas is supplied by several anterior and posterior arcades. The anterior arcade is formed by branches of the anterior superior pancreaticoduodenal artery, which originates from the gastroduodenal artery,

and the anterior inferior pancreaticoduodenal artery, which originates from the superior mesenteric artery or from one of the jejunal arteries. The posterior arcade is formed by the posterior superior pancreaticoduodenal artery , which originates from the gastroduodenal artery, and the posterior inferior pancreaticoduodenal artery, which originates from the superior mesenteric artery or from one of the jejunal arteries.

The neck of the pancreas has a relatively meagre arterial blood supply. Arterial arcades from the gastroduodenal and superior mesenteric arteries, and the vascular supply of the body and tail of pancreas, supply the neck region.

The body and tail of the pancreas is supplied by the dorsal pancreatic and pancreatic arteria magna from the splenic artery. In 25-30% of cases the dorsal pancreatic artery originates from the superior mesenteric or the common hepatic arteries. The inferior pancreatic artery may be a source of major blood supply to the pancreas in 40% of the cases.

1.2.4 Lymphatic drainage of the pancreas:

The lymphatic vessels start within the lobules of the pancreas. These intralobular lymphatic vessels drain into the interlobular lymphatics, which separates the lobules and join with one another to form a larger interlobular lymphatic, also referred to as the collecting vessels. These larger vessels reach the surface of the pancreas and enter a surface network of lymphatic vessels that join upon the surface and converge towards the lymph nodes.

The pancreas has a rich supply of lymph nodes broadly divided into two groups. The lymph nodes lie along the arteries which supply the respective part of the gland. One forms a group around the organ while the second is close to the upper abdominal aorta and its branches.

1.2.5 Nerve supply:

Neural innervation of the pancreas is important for both basal and stimulated pancreatic secretions. Sympathetic and parasympathetic (vagal) nerve fibres supply the pancreas and have profound effects on pancreatic exocrine secretion. Neurotransmitters and neuropeptides within these nerves can exert either stimulatory or inhibitory effects, depending upon the transmitter released. Apart from the gastrointestinal tract, the pancreas is the only peripheral organ that has a significant intrinsic nerve plexus.

1.2.5.1 Extrinsic innervation of the pancreas:

The parasympathetic nerve fibres innervating the pancreas originate mainly from the dorsal vagal nucleus and partly from nucleus ambiguous from the brain[14]. Most of these fibres run in the posterior vagal trunk to the pancreas[15]. The majority of these terminate on the pancreatic ganglia. The pancreas receives its post ganglionic sympathetic innervation from neurons in the celiac and superior mesenteric ganglia.

1.2.5.2 Intrinsic innervation of the pancreas:

The pancreatic ganglia are nervous integration centres of the pancreatic exocrine and endocrine secretion. They receive input from vagal preganglionic, sympathetic post ganglionic, sensory and enteric fibres. Post ganglionic nerve fibres surround almost every acinus, forming a periacinar plexus containing cholinergic, noradrenergic, peptidergic, and nitrergic fibres which predominantly terminate at the acinar cells[16]. Only a subset of fibres project into islets[17].

1.2.5.3 Cholinergic control of pancreatic secretion:

It has been hypothesized that intrapancreatic post ganglionic neurons regulate both enzyme and bicarbonate secretion. These neurons are activated by central input on pancreatic ganglia during the cephalic phase of pancreatic secretion and by vasovagal reflexes initiated by the gastric and intestinal phase stimulation of pancreatic secretion. Acetylcholine released by these neurons acts directly on the mucarinic receptors on acinar cells and/or duct cells to elicit pancreatic secretion[18].

1.2.5.4 Localization of neurotransmitters within the pancreas:

Immunohistochemical techniques have been used to enhance the understanding of pancreatic neural transmission and neurotransmitter localization. There are a number of neurotransmitters which have been identified in the pancreas. Galanin is one of the neurotransmitters identified within the pancreas. I shall later describe galanin in detail as this neurotransmitter will be the focus of my experimental study in AP.

1.2.6 Embryology of the pancreas:

In humans, on about 24th day of gestation the hepatic diverticulum begins to bud from the ventral surface of that part of the primitive digestive tube that is destined to form the duodenum. This hepatic anlage invades the embryonic ventral mesentery and later develops into the liver, gallbladder and the bile ducts.

Some two days later (26th) a similar diverticulum emanates from the dorsal surface of the digestive tube. This forms the dorsal anlage of the pancreas, grows within the dorsal mesentery. On the 32nd day a small ventral pancreatic anlage buds from the hepatic diverticulum. Due to series of rapid developments and rotation of the bile duct, the dorsal and ventral portions of pancreas come in close contact and fuse by the 37th day of gestation. The primitive pancreas contains axial ducts; the dorsal duct arising from the duodenal wall and the ventral duct arises from the common bile duct. The ventral duct (of Wirsung) becomes continuous with the dorsal duct (of Santorini) to form the main pancreatic duct[19].

1.3 Morphology of the pancreas:

The pancreas has both exocrine and endocrine components (Fig. 2). The exocrine portion constitutes most of the pancreas. The endocrine part is composed primarily of islets of Langerhans.

1.3.1 Exocrine pancreas:

The pancreas has a thin, ill-defined fibrocollagenous capsule from which narrow irregular septae penetrate it, dividing it into lobules. Each lobule is composed of

roughly spherical clusters (acini) of secretory exocrine cells. Each acinus has an individual duct which drains into progressively larger ducts.

The acini are composed of protein secreting cells which have a broad base and a narrow apical surface covered by microvilli. The cells are rich in rough endoplasmic recticulum, which is seen in the basal half of the cell. In the apical half of the cell, closer to the lumen, are eosinophilic enzymatic granules, which contain pre-enzymes synthesised by the cell. The pre-enzymes are synthesised by the rough endoplasmic recticulum and packaged in the Golgi apparatus into granules.

The ductal system begins in the acinus. The intercalated ducts from individual acini fuse to form larger interlobular ducts which are lined by columnar epithelium. Interlobular ducts join to form the main pancreatic duct which is lined by tall columnar epithelium containing a number of mucin-secreting goblet cells[20, 21, 22].

1.3.2 Endocrine pancreas:

The neuroendocrine component of human pancreas has three parts:

- Islets of Langerhans.
- Nests or clumps of neuroendocrine cells distinct from islets.
- Single cells scattered within the exocrine and ductular components demonstrable by immunohistochemistry.

Islets of Langerhans:

These are discrete, rounded clusters of cells scattered throughout the pancreas. Islets are most numerous in the tail of the pancreas. They vary in size and number of cells they contain. Individual cells in islets are smaller and paler than the exocrine cells of the acini. They are spherical or polygonal in shape. Each islet has a capillary network which is in contact with each cell. Nests or clumps of neuroendocrine cells are arranged as islets.

Each of these different cell types is associated with a different hormone. The different cell types are as follows[23], [24]:

- B or Beta cells: are approximately 70%. They secrete insulin.
- A or Alpha cells: are approximately 20%. They secrete glucagon.
- D, Delta or Type III cells: are approximately 10%. They secrete somatostatin.
- PP or F cells: are approximately 1-2%. They secrete pancreatic polypeptide.
- Other minor cell types are D-1 or type IV cells which secrete vasoactive-intestinal polypeptide and the EC cells or the enterochromaffin cells which secrete peptides like serotonin, motilin and substance P.

Minor cell types are scattered in exocrine and ductular components. The stellate cells seen in the pancreas have a role in repair of the organ after injury. They contribute to fibrogenesis and regeneration[25, 26]. The islets contain complex network of fenestrated capillaries which merge with capillaries supplying the exocrine component of pancreas.

1.3.3 Insulo–acinar interaction:

The exocrine and endocrine innervations are intimately related anatomically because the nerve fibres that innervate the acinar cells also innervate the islets[27]. Interactions between acinar and islet tissues have been well established through numerous studies that reveal the existence of anatomical and functional relationships between these compartments of the gland. Murakami et al,. reported that a insulo-acinar portal system exists in all mammals. With the help of electron microscopy of vascular corrosion casts, these investigators demonstrated that islets in mouse, rat and guinea pigs were frequently interlobular and the insulo-acinar portal vessels joined directly to lobular capillaries of the acinar tissue. But in humans, monkeys, dogs and cats islets were intralobular[28]. Intravital microscopy has demonstrated that blood flows from the islet through the insulo-acinar portal vessels to the exocrine pancreas and the secretory ducts receive blood from capillary bed of islet and exocrine acini[29,30]. It is well known that pancreatic exocrine secretions are partly under the control of the islets of Langerhans[31]. Pancreata from diabetic patients frequently displays signs of exocrine insufficiency, with reduction of bicarbonate and amylase output in response to secretin/CCK[32, 33, 34]. On the other hand, pancreatic exocrine deficiency caused by chronic pancreatitis is frequently associated with impaired glucose tolerance[35].

Islets are also known to influence ductal secretion. Bicarbonate secretion is related mainly to centroacinar and terminal duct cells. These areas are in contact with neighbouring endocrine tissue[36]. In rat and human pancreas, buds and

single insulin-producing cells are frequently observed associated with small ducts and it is thought that they discharge insulin into the same capillary network that supplies the duct cells[37, 38].

1.4 Physiology of the pancreas:

As outlined earlier, the two major types of tissues in the pancreas are, (1) the acini, which secretes digestive juices into the duodenum; and (2) the islets of Langerhans, which secrete mainly insulin and glucagon directly directly into the blood.

1.4.1 The Exocrine pancreas:

The pancreas secretes about 1 litre of fluid each day[39]. At rest the secretions are plasma like. When flow rates are high an alkaline fluid rich in bicarbonate is secreted. These secretions consist of digestive enzymes secreted by the pancreatic acini and sodium bicarbonate secreted by the small ductules and the large ductules leading from the acini. The combination of both the enzymes and the bicarbonate solution (pancreatic juice) flows through the pancreatic duct into the duodenum via the sphincter of Oddi. The secretion of pancreatic juice is maximum in response to the presence of chyme in the duodenum and upper small intestine and is regulated by neural and hormonal mechanisms.

1.4.1.1 Components of Pancreatic Juice:

Pancreatic juice contains enzymes for digestion of proteins, carbohydrates and fats. It also has bicarbonate ions for neutralising the acidic chyme as well as sodium and chloride ions[40]. The various enzymes required for digestion of proteins are trypsin, chymotrypsin, carboxypeptidases and elastases[41]. A number of these enzymes are synthesized as inactive precursors. Trypsin is normally synthesized in the acinar cell from its inactive form trypsinogen. Trypsinogen is activated by an enzyme, enterokinase. This enzyme is secreted by the intestinal mucosa when chyme comes in contact with the mucosa. Trypsinogen is also autocatalytically activated by trypsin, once it is formed. Chymotrypsinogen and procarboxypeptidases are converted to their active form by trypsin. Carboxypeptidases convert polypeptides into amino acids. The acinar cells also secrete trypsin inhibitor, which prevents activation of enzymes in the resting state and hence pancreatic autodigestion. The enzyme involved in the digestion of carbohydrates is pancreatic amylase. Amylase hydrolyses starch, glycogen and other carbohydrates (except cellulose) to disaccharides and trisaccharides. The enzyme involved in the digestion of fats is lipase. Lipase hydrolyses neutral fat into fatty acids and monoglycerides.

1.4.1.2 Secretion of bicarbonate ions:

The bicarbonate ions are secreted from the duct cells. This secretion is mainly stimulated by the hormone secretin via a cyclic-AMP mediated mechanism. In a

stimulated pancreas the bicarbonate ion secretion can rise to up to 140mEq/liter[42].

1.4.2 Regulation of pancreatic secretion:

The pancreatic secretion is regulated by various hormones. Gastrin, CCK and secretin stimulate pancreatic secretion. Gastrin is secreted during gastric phase of stomach secretion. CCK is secreted by the duodenal and upper jejunal mucosa when acid chyme enters in the intestine. Secretin is secreted by the duodenal and jejunal mucosa when acidic chyme enters the upper small intestine. Acetylcholine is released from parasympathetic (vagus) nerves and from other cholinergic nerves in the enteric nervous system during digestion. Acetylecholine and CCK primarily stimulate the acinar cells leading to more production of digestive enzymes. Secretin on the other hand primarily stimulates ductal cells to secrete bicarbonate and water[43].

1.4.2.1 The phases of pancreatic secretion:

There are three different phases of pancreatic secretion[44]:

1. Cephalic phase: During this phase the neural signals which cause secretion in the stomach also cause acetylcholine release from vagal nerve endings in the pancreas. This leads to moderate amounts of pancreatic enzyme secretion.
2. Gastric phase: In conjunction with neural stimulation of pancreatic secretion, a large amount of gastrin is secreted from the stomach, which stimulates more pancreatic enzyme production.

3. Intestinal phase: As chyme enters the small intestine, the pancreatic secretion increases as a result of increased CCK and secretin release.

1.4.3 Stimulation of bicarbonate secretion by secretin:

Secretin is secreted by S – cells in the mucosa of upper small intestine. Secretin begins to be released as the pH of duodenal contents falls below 4.5. When acid chyme enters the duodenum, it converts prosecretin to active secretin. Secretin binds to secretin receptors resulting in the release of large quantities of bicarbonate ions[45].

1.4.4 Endocrine Pancreas:

The human pancreas has about 1 million islets of Langerhans[46] organised around small capillaries[47]. The islets contain four types of cells namely, alpha, beta, delta and F cells as described previously.

1.4.4.1 Insulin secretion:

Banting and Best first isolated insulin from pancreas in 1922[48]. Most of the insulin formed is released in the portal circulation and hence most is removed in the first pass through the liver. Insulin is synthesised as a larger single polypeptide, preproinsulin. This is cleaved soon after synthesis to proinsulin, which is packaged into the vesicles. The proinsulin is converted to insulin by cleavage of a connecting peptide to form two peptide chains. Insulin is released into the bloodstream by exocytosis in response to increase in blood sugar levels. The half life of insulin is only 6 minutes as it is rapidly metabolised in the liver and kidneys[49].

1.4.4.2 Actions of insulin:

Insulin lowers the blood glucose levels by increasing the uptake of glucose into the muscle and adipose tissue[50]. It increases the synthesis of glucogen, fat and proteins. It also increases the K^+ ion uptake into cells, thereby decreases the serum K^+ ion concentration.

Control of insulin secretion:

An increase in the plasma glucose level is the major stimulus for insulin secretion. Arginine is also a potent stimulator of insulin release whereas adrenaline and somatostatin inhibit insulin release. Glucagon, growth hormone and sulphonylureas (e.g. tolbutamide) increase insulin release. Parasympathetic nerve activity enhances insulin release while sympathetic activity inhibits its release.

1.4.4.3 Glucagon:

Glucagon is a 31 amino acid peptide with a molecular weight of about 3500. As with insulin it is also secreted as a preprohormone which is then converted to prohormone and finally to glucagon. It is a polypeptide secreted in response to low blood glucose levels. Ingestion of protein is the major stimulus to its secretion. The principal target tissue of its action is the liver. It increases blood glucose levels and causes glycogenolysis and gluconeogenesis via a cyclic AMP mediated pathway. It has a lipolytic effect on the adipose tissue and a positive ionotrophic effect on the heart[51].

1.4.4.4 Somatostatin:

Somatostatin exists as 14- and 28- amino acid polypeptides. In the pancreas the 28- amino acid form predominates. It is also seen in various sites of the central nervous system (CNS) where it acts as a neurotransmitter. Somatostatin inhibits the secretion of several hormones, like growth hormone, insulin, glucagon, gastrin, VIP, and thyroid stimulating hormone[52].

1.5 Galanin:

Galanin is the focus of this study on AP, and will be discussed in more detail in the following section.

1.5.1 Discovery of galanin:

Galanin, a 29-amino acid (30-amino acid in humans) polypeptide, was discovered and isolated from porcine upper small intestine by Tatemoto et al., using a procedure that chemically detects peptides with COOH- terminal amides[53]. This peptide was called galanin because of its N-terminal glycine and C-terminal alanine amide residues.

1.5.2 Structure:

The structure of galanin is heterogeneous between species. Two molecular forms of galanin consisting of non-amidated sequences with 19- and 30- amino acid residues have been isolated from the human colon. The N-terminal 15 amino acid part of the galanin molecule is identical in all species that have been investigated so far. The C-terminal amino acid sequences are more species specific. This suggests that the biologically active portion of galanin is the N-terminus. Experimental evidence confirms that many actions of galanin depend

heavily on the N-terminal portion of the molecule. The first two amino acid residues are essential for retention of high biological potencies[54].

1.5.3 Synthesis of endogenous galanin:

Galanin is processed from preprogalanin. In the pig, preprogalanin consists of a 123 amino acid peptide sequence, whereas in the rat, preprogalanin is a 124 amino acid peptide. The processing of preprogalanin gives rise to a signal peptide, a pregalanin message peptide, galanin, and galanin message associated peptide. Galanin message associated peptide is a C-terminally amidated 41 amino acid peptide, which may have pre- and post- synaptic functions[55].

1.5.4 Distribution:

Galanin is widely distributed in the nervous system, both central and peripheral. In the CNS galanin is widely distributed throughout the spinal cord and the brain. In the spinal cord it is present at all levels and has been detected in large amounts in dorsal horn cells, nerve cell bodies intrinsic to the spinal cord, including neurons of lamina II, intermediate dorsal laminae, neurons around lamina X, and in the subpopulations of the lower motor neuron. Galanin containing fibres are most prominent in the lumbosacral regions of the spinal cord[56]. In the brain galanin is widely distributed in the medulla, locus caeruleus, and midbrain. In the forebrain galanin is present in the thalamus, hypothalamus

and magnocellular neurosecretory neurons of supraoptic and paraventricular nuclei.

In the peripheral nervous system galanin is present in the terminal innervation of the gastrointestinal, respiratory, and urogenital tracts, pancreas and the adrenal medulla.

There is abundance of galanin in the gut. Galanin- like immunoreactivity (GAL-LI) is observed throughout the gastrointestinal tracts of humans, pigs, and rats; it is found exclusively in the nerves i.e. myenteric, submucous and the mucous plexus of the gut. No galanin was found in the epithelium of the gut. This suggested that galanin was of nonepithelial origin and may therefore be a gastrointestinal neurotransmitter or neuromodulator. Galanin may coexist with VIP in ganglion cells of the submucous plexus of different species[57].

1.5.5 Pancreatic localization of galanin:

Galanin has been found to be abundant in the innervation of pancreatic blood vessels, islets and acini in a range of species[58,59,60]. In the canine pancreas, GAL-LI has been immunohistochemically localized in nerve fibers predominantly in the islets of Langerhans. In the human pancreas, galanin immunoreactivity has been localized in numerous nerve fibers around glandular acini, ductules and blood vessels and few in the nerve fibers within the islets[61]. In humans GAL-LI has also been demonstrated in the nerve cell bodies in the intrapancreatic ganglia[62]. This suggests that in humans some galanin nerves are intrinsic.

Gel chromatography of pancreatic tissue from dogs, rats, mice, and humans has demonstrated that most GAL-LI correlates with synthetic porcine galanin, implying that in all these species pancreatic galanin is equivalent in size to galanin extracted from the porcine intestine. Studies in our laboratory have demonstrated porcine galanin positive immunoreactive fibres around the blood vessels particularly arterioles of the pancreas[63].

1.5.6 Actions:

Galanin acts by both increasing and decreasing the release of neurohumoral substances and gastrointestinal motility. The actions of galanin in the gut are caused by the activation of the receptors on the smooth muscle and presynaptic neurons. It may also participate in the regulation of absorption, secretion, and blood flow. Bauer et al., [64], described two major actions of galanin, firstly inhibition of postprandial release of neurohumoral substances, namely glucose, insulin, tyrosine, neurotensin, somatostatin and pancreatic polypeptide. Secondly, galanin caused a significant slowing of gastric emptying and caecum-to-colon transit times. In the pancreas, galanin appears to be a primary candidate for the nonadrenergic sympathetic neural effects on pancreatic hormone secretion and is involved in the local regulation of pancreatic endocrine function[65]. In addition to the inhibitory actions of galanin, it also exerts a direct excitatory actions in the gut (table 1). It has been found in anesthetized rats that galanin inhibits pancreatic protein and amylase secretion in both basal and bombesin-, secretin- and CCK- stimulated conditions[66].

Table 1. Actions of galanin in the gut.

Organ	Species	Action	Reference
Motility			
Oesophagus	Opossum	- peristalsis	67
LES	Opossum	+ resting tension	68
Pylorus	Dog	- contraction	69
Small intestine	Rat	+ contraction	70
Small intestine	Human	+ contraction	71
Colon	Guinea pig	- contraction	70
Secretion			
Stomach	Rat, Dog	- acid secretion	72
Pancreas			
Exocrine	Dog	- volume, bicarbonate, protein	73, 74
Endocrine	Dog, mouse, rat	- somatostatin, insulin. + glucagons	75,76,77,78

- Inhibits, + contracts, stimulates or facilitates, LES lower oesophageal sphincter.

1.5.6.1 *Effect of galanin on cardiovascular system:*

Galanin is known to have major cardiovascular effects, and increases blood pressure via a direct effect on the heart and by reduced perfusion in some vascular beds, including splanchnic organs[79,80]. It has a modulating action on

the cardiovascular systems which are species specific. Galanin and the N-terminal fragment galanin(1-15) are involved in central cardiovascular regulation[81]. In cat and possum it is proposed that galanin released from sympathetic nerves inhibits subsequent cardiac vagal actions[82]. In humans, intravenous injection of human galanin decreased sinus arrhythmia[83]. The doses used in this study did not affect blood pressure. In cats galanin significantly decreased blood pressure. In mice galanin contributes to prolonged attenuation of parasympathetic slowing of heart following activation of cardiac sympathetic nerves[84]. The information concerning the effect of galanin on vascular perfusion in specific organs is limited, and its effect on pancreatic vascular perfusion is largely unknown.

1.5.7 Galanin receptors and ligands:

Galanin acts via three known receptors, galanin receptor 1 (GALR1), galanin receptor 2 (GALR2), and galanin receptor 3 (GALR3)[85]. These galanin receptor subtypes have been cloned from both humans and rats. These receptor subtypes belong to the G protein-coupled receptor family. They are widely distributed in central and peripheral nervous systems.

1.5.7.1 Galanin receptor agonists:

a. Peptide type agonists:

The most widely used agonist to study galanin receptors is the endogenous peptide from porcine, rat/mouse and humans. Endogenous galanin has high

affinity for all three receptors[86]. The N-terminal 16 amino acids of galanin are critical for receptor binding since deletion of first 16 amino acids of galanin causes complete loss of its affinity for galanin receptors[87].

b. Galanin fragments and analogs:

Galanin (1-16) and galanin (1-15) have been shown to be highly efficacious galanin receptor agonist *in vitro* and *in vivo*[88,89]. Galanin (2-11) (AR-M1896) has higher affinity for GALR2 and GALR3 as compared to GALR1[90,91].

Endogenously occurring galanin-like peptide carry the N-terminal 1-13 sequence of galanin and exhibit several endocrinological effects similar to galanin. Galanin-like peptide is a high affinity agonist for both GALR1 and GALR2 receptors[92,93].

c. Non-peptide galanin receptor agonists:

Two small galanin receptor agonists, galnon and galmic, have been synthesized[94, 95, 96]. Both compounds are systemically active and substantially more resistant to degradation than endogenous galanin. The drawback of galnon and galmic use is that they have low affinity for galanin receptors and are not receptor specific.

1.5.7.2 Galanin receptor antagonist:

a. Peptide antagonists:

An intact galanin N-terminus is required for the recognition of galanin receptors. A number of chimeric, bi-receptor-recognizing peptides have been synthesized using the N-terminal fragment galanin1-13 and C-terminal portions of some other bioactive peptides (e.g. substance P) in which the receptor recognition site resides in the C-terminal portion. It is important that the C-terminal peptide fragment possess some α-helical structure, which may act to stabilize the conformation of the N-terminal galanin fragment. The various peptide antagonists are Galanin1-12-Pro-substance P_{5-11} amide (Galantide), Galanin1-12-Pro-bradykinin2-9 amide (M35), Galanin1-12-$Pro_{3-}(Ala\text{-}Leu)_2$-Ala amide (M40) and Galanin1-12-Pro-spantide (C7)[97]. By definition these peptides antagonize the actions of galanin. Galanin-mediated inhibition of insulin release from pancreatic islets is fully reversed by galantide. Galantide blocks the inhibitory actions of galanin with high potency. Many antagonist actions of galantide occur in a dose dependent manner. These ligands have partial agonistic properties. It has been shown that galantide, M35 and M40 act as antagonist or as partial agonists at concentrations above 10nM. The various chimeric peptides with antagonistic activity to galanin in different organ systems are summarized in table 2[98].

Table 2. Summary of galanin antagonists actions in different systems.

Antagonist	Pancreas[a]	Spinal cord[b]	Hippocampus[c]	Hypothalamus[d]
M15	+	+	+[*], +[**], +[***]	Not tested
M35	+	+	+[****]	Not tested
M40	Acting as agonist	Not tested	+[*]	+
C7	Acting as agonist	+	Not tested	+
M32	Acting as agonist	+	Not tested	Not tested

[a] antagonism of galaninergic inhibition of glucose induced insulin release.

[b] antagonism of galaninergic effect on flexor response.

[c] [*]antagonism of galaninergic inhibition of evoked release of acetylcholine, [**]antagonism of galaninergic inhibition of protein phosphorylation by PKC, [***]antagonism of galaninergic inhibition of anoxic glutamate release, [****] facilitation of acquisition in the Morris swim maze, probably by revealing galaninergic inhibition.

[d] antagonism of galaninergic effect on feeding behavior.

b. Non-peptide galanin receptor antagonists:

To date little has been published on the development and use of non-peptide galanin receptor antagonists. Spirocoumaranon[99] (Sch 202596) and 2,3-dihydro-2-(4-methyl-phenyl)-1,4-dithiepin-1,1,4,4-tetroxide[100] have been described as non-peptide antagonists. The limitation of these compounds is that they are low affinity compounds. Recently, a non-peptide antagonist to GAL3R has been synthesized, but it also has been a low affinity compound. There is a need for development of non-peptide antagonist for advancement of the field, particularly for future clinical studies.

1.6 Acute Pancreatitis:

AP is defined as an acute inflammatory process of the pancreas usually associated with severe pain in the upper abdomen; in most instances blood levels of pancreatic enzymes, including amylase and lipase, are increased to at least three times the upper limit of normal. The inflammatory reaction in AP results in oedema of the pancreas and extensive local and systemic effects. AP may occur as a new event or as a recurrent condition. In about 85% of cases, the disease takes a mild course with complete disappearance of clinical symptoms in a few days. In a small group of about 15-20% of patients, the disease takes a severe course, necrotising pancreatitis. This may culminate in multi-organ failure and death in about 15% - 40% of these patients[101].

1.6.1 Epidemiology:

The incidence of AP varies around the world. In the USA and UK the incidence ranges from 11 to 23 patients/100,000 population[102, 103, 104]. In view of variable presentation, it is difficult to determine the true incidence as many patients are misdiagnosed and the mild forms may be treated by the family physician as an outpatient for non-specific abdominal pain. There has been an increase in the incidence of AP in the past decade. The main reasons for the increase are increased alcohol consumption, aging population, an increase in gallstone disease and may also be partly due to better diagnostic facilities. The variation in incidence in different parts of the world is probably due to social factors and dietary habits. In the USA, approximately 80% of all causes of AP can be attributed to either gallstones or alcohol[105]. Less common causes which lead to pancreatitis are drugs, infections, trauma, pancreatic ischemia and genetic factors. The first episode of alcohol related AP affects primarily males and the peak incidence is between the age 18 and 30 years, while AP due to gall stones is predominantly seen in females of age 50-60 years[106, 107, 108].

1.6.2 Classification of AP:

A symposium held in Marseilles formed the basis of Atlanta 1992 classification of AP[109]. The following definitions of AP and its complications were agreed.

1. AP: This is an acute inflammatory process of the pancreas, with variable involvement of other regional tissues or remote organ systems. The condition

usually presents with upper abdominal pain accompanied by fever, vomiting, and tachycardia. It is usually associated with raised levels of pancreatic enzymes.

2. Severe AP**:** This is associated with organ failure and/or local complications such as necrosis, abscess, or pseudocyst. These patients have 3 or more Ranson's signs[110] or 8 or more APACHE II (Acute Physiological and Chronic Health Evaluation) points[111].

3. Mild AP: This is associated with mild organ dysfunction and an uneventful recovery.

4. Acute fluid collections: These occur early in the course of pancreatitis and are seen near the pancreas. These lack walls of granulation tissue or fibrous tissue and are seen in 30-50% of patients with the severe form of the disease.

5. Pancreatic necrosis: These are diffuse or focal areas on non-viable pancreatic parenchyma. These are also associated with peripancreatic fat necrosis.

6. Acute pseudocysts: These are collections of pancreatic juice enclosed by a wall of fibrinous or granulation tissue. Formation of pseudocyst requires 4 weeks from the onset of pancreatitis.

7. Pancreatic abscesses: These are circumscribed intra-abdominal collections of pus as a consequence of pancreatitis. These abscesses occur later in the course of pancreatitis, often 4 weeks or more after the onset.

1.6.3 Severity of AP:

The severity of AP has been defined in several ways:

- Predicted severity: Ransons and Glasgow criteria[112] are examples of this. These scoring systems predict prognosis and identify patients who are likely to have worse prognosis and would benefit by intensive care management.
- Actual severity: Atlanta and APACHE systems are examples of this. These systems score local and systemic complications which are used to define actual severity[113].

1.6.4 Etiology:

There are various factors that can trigger the onset of AP. They can be subdivided into toxic-metabolic, mechanical, vascular, infectious and idiopathic. Various factors are enumerated in the table 3 below.

Table 3. Various factors causing AP.

1.	**Toxic and metabolic factors:**
	Alcohol
	Hypertriglyceridemia
	Uremia, hypercalcemia
	Scorpion venom
	Drugs: Azathioprine, frusemide, sulfonamides, valproic acid, methyldopa etc.
2.	**Vascular factors:**
	Cardiogenic shock
	Polyarteritis nodasa
	Atheroembolism
	Hypothermia
	Malignant hypertension
3.	**Mechanical factors:**
	Gallstones
	Trauma
	ERCP
	Benign and neoplastic growth of periampulary region and the pancreas
	Duodenal diverticula, Worm infestation
4.	**Infections:**
	Viruses (Mumps, Coxsackie, adenovirus, HIV)
	Bacterial (Mycobacterium avium, Mycobacterium tuberculosis, Campylobacter, Mycoplasma)
	Worms (Ascaris)
5.	**Autoimmune** (Systemic Lupus Erythematosus, Sjogren's syndrome, Polyarteritis nodosa)
6.	**Genetic** (Cystic fibrosis, Hereditary pancreatitis)
7.	**Idiopathic pancreatitis**
8.	**Postoperative pancreatitis**
9.	**Primary sclerosing cholangitis**
10.	**Sphincter of Oddi dysfunction**

ERCP – Endoscopic retrograde cholangiopancreatography, HIV – Human immunodeficiency virus.

1.6.5 Pathophysiology of AP:

All causes of AP converge at a common point that initiates the events that result in pancreatitis. Various causes of pancreatitis result in similar pattern of disease, but differ in severity and complications. Despite extensive efforts, the cellular mechanisms of AP have not been fully understood, however it has been established that AP is a cascade of events.

The cascade of pathologic events starts with the exposure to a causal factor. The events in AP can be divided into early and late phases. The early phase primarily involves the acinar cells. In the acinar cells there is activation of digestive enzymes. The retention of these digestive enzymes within the acinar cells is the key factor in development of AP. These activated enzymes escape from the zymogen granules and damage the acinar cells. Other early events are generation and release of inflammatory mediators by the acinar cells. The acinar cell also releases certain agents e.g. that cause an increase in vascular permeability which leads to development of oedema within the pancreas. These factors lead to ischaemia of the pancreas, thereby further aggravating pancreatitis.

The early events begin within minutes of exposure to the causal factor and the late events occur several hours later. During this period there is further inflammation of the pancreas and the surrounding tissues. Systemic effects appear such as capillary leak syndrome, fever, and hypotension then begin to appear. The combination of all these may lead to clinical necrosis of the

pancreas via stimulated acinar cell apoptosis and/or necrosis. There is no clear demarcation between the early and late events.

Various mechanisms have been proposed for activation of zymogens. The mechanisms that have received most attention are autoactivation of trypsinogen to trypsin and cleavage of trypsinogen to trypsin by the lysosomal hydrolase cathepsin B[114,115]. Amongst other proposed mechanisms are diminished activities of intracellular pancreatic trypsin inhibitor, proteolytic activation leading to leakage of zymogens and lysosomal enzymes into the cytoplasm, shunting of zymogens into membrane bound compartments that contain active proteases, uptake and processing of secreted zymogens by endocytic pathways, and enhanced susceptibility of zymogens to proteolysis due to oxidation or decondensation[116]. The intrapancreatic activation of trypsinogen to trypsin is the crucial triggering mechanism for AP. Further in the course of pancreatitis are the release of local mediators like cytokines, vasoactive substances, free oxygen radicals etc. which leads to development of microcirculatory disturbances, activation of leucocytes and their infiltration into tissues.

1.6.5.1 Trypsinogen Autoactivation:

Trypsinogen is capable of autoactivation, and the resultant trypsin then further activates trypsinogen to produce more of trypsin[117]. It has been suggested that trypsinogen autoactivation occurs intracellularly along the normal secretory pathway within the cytoplasmic vacuoles that contain lysosomal markers but are

not lysosomes[118]. Recently it has been shown that the conversion of trypsinogen to trypsin in response to supramaximal caerulein remains completely unaffected by presence of specific trypsin inhibitor and thus by the presence or absence of free trypsin activity within the acinar cells. Therefore autoactivation of trypsinogen by cytosolic trypsin may not be an initiating factor for the intrapancreatic proteolytic cascade[119].

1.6.5.2 Role of Cathepsin B in premature digestive protease activation:

Studies have indicated a possible role of lysosomal cysteine protease cathepsin B in premature and intrapancreatic activation of digestive enzymes[120]. The following observations support the role of cathepsin B in AP:

- *In vitro*, cathepsin B can activate trypsinogen[121].
- There is redistribution of cathepsin B into a zymogen granule-containing subcellular compartment during the initial phase of AP[122].
- Lysosomal enzymes colocalize with digestive zymogens in membrane confined organelles during the early course of experimental pancreatitis[123].

1.6.5.3 Diminished activity of intracellular pancreatic trypsin inhibitor:

Under normal conditions, trypsinogen is activated only when it is secreted into the duodenum. Once formed trypsin can also cleave trypsinogen and form more active trypsin. The pancreas has a “safety mechanism” which protects the acinar cell from damage by auto activation of trypsinogen. Pancreatic trypsin inhibitor is present in secretory granules of acinar cells. It binds to the active site of trypsin

in a ratio of 1:1[124]. The molar ratio of pancreatic trypsin inhibitor to trypsin is estimated to be 1:10. When more than 10% of trypsinogen is activated, this inhibitory mechanism is no longer effective. Any disorder in this protective mechanism leads to onset of AP[124].

1.6.5.4 Role of Ca^{++}:

Under physiological conditions, the Ca^{++} ion is an essential second messenger in stimulus-secretion coupling in pancreatic acinar cells[125]. In response to various hormonal signals, the cytosolic free Ca^{++} concentration rises and regulates the exocytosis of digestive enzymes from the apical pole of the acinar cell. The prerequisite for this is that acinar cell maintains a Ca^{++} ion gradient across the cell plasma membrane from low intracellular to high extracellular Ca^{++} concentrations. Pancreatitis induced by caerulein hyperstimulation and duct ligation has been shown to increase intracellular Ca^{++} and disrupt acinar cell Ca^{++} signalling. This is associated with acinar cell vacoulization and the intracellular trypsinogen activation[126,127,128]. There is also evidence that high concentration of intracellular Ca^{++} leads to an acidic pH which is essential for trypsinogen autoactivation. In addition the affinity of pancreatic trypsin inhibitor is decreased at acidic pH[129].

1.6.5.5 Pancreatic acinar cell apoptosis:

Apoptosis has been defined as a physiological or programmed form of cell death that affects scattered cells in a tissue, and has a characteristic and stereotypical morphology, including cell shrinkage, retention of organelles and nuclear chromatin condensation which occur in response to variety of stress related stimuli[130]. Acinar cell death is the hallmark of AP[130]. Acinar cell death occurs by both apoptosis and necrosis. Recent studies have shown that induction of apoptosis reduces the severity of experimental pancreatitis[131,132], while inhibition of apoptosis may worsen it[133]. Several protease families are implicated in apoptosis, most important being the caspases. Caspases are molecular executioners of apoptosis because they bring about most of the morphological and biochemical characteristics of apoptotic cell death[130,134]. Therefore caspase activation may be one of the mechanisms in AP. It has also been shown that caspases negatively regulate necrosis and intra-acinar cell activation of trypsin[135].

There have been reports that there is depletion in neutroplils at the site of inflammation as a result of significant increase in the number of acinar cells undergoing apoptosis[136].

1.6.6 Inflammatory mediators in AP:

Inflammatory mediators play an important role in pathogenesis of pancreatitis and subsequent inflammatory response. Proinflammatory mediators which are involved include TNF-α, interleukin (IL)-1β, IL-6, platelet activating factor (PAF),

ICAM-1, IL-8, monocyte chemoattractant protein-1 and substance P. The anti-inflammatory mediators that play an important role in AP include IL-10, complement component C5a, soluble TNF receptors, IL-1 receptor antagonist, and neutral endopeptidase (NEP). The expression of several of these factors is regulated by transcription factors such as nuclear factor kappa B (NF-κB)[137,138]. NF-κB is one of the factors accelerating local inflammatory response and triggering systemic inflammation. Activation of NF-κB could be related to early events such as trypsin activation. TNF-α and IL-1β levels are elevated during the onset and progress of AP[126,139,140]. Inhibition of these has been shown to attenuate severity of AP in experimental models. IL-6 is produced by different cells in response to stimulation by an endotoxin. It is also produced by periacinar myofibroblasts in response to TNF-α and IL-1β[141]. Plasma levels of IL-6 correlate with haemodynamic abnormalities seen in AP in rabbit[142]. IL-10 is an anti-inflammatory cytokine[139,140]. IL-10 has been found in experimental studies to reduce the extent of inflammation as well as the mortality associated with AP[143]. Two clinical trials involving IL-10 in patients with post endoscopic retrograde cholangiopancreatography induced pancreatitis have shown different effects. One of the trials showed beneficial effect[144] and the other showed no benefit[145]. PAF is proinflammatory mediator; its levels are known to rise during an attack of pancreatitis[146]. Experimental studies have shown that intraperitoneal or intravascular injection of PAF can lead to or increase the severity of AP[147]. It has been reported that trypsin has the ability to up-regulate intercellular adhesion molecule (ICAM)-1, a key vascular endothelial adhesion molecule necessary for transport of leukocytes from the intravascular space into

inflamed tissues[148]. This occurs both in pancreas and lung, but is more pronounced in the lung. Up-regulation of ICAM-1 leads to decreased perfusion of pancreatic microvasculature, presumably because of leukocyte mediated blockage of blood flow**.**

1.6.7 Pancreatic vascular changes in AP:

The influence of ischemia on the pathogenesis of initiation and progression of AP is well recognized[149, 150]. Several experimental studies have analyzed the pancreatic microcirculation in early AP. They have demonstrated that impaired pancreatic microcirculatory perfusion characterizes the development of AP[151, 152]. By contrast, other studies have concluded that pancreatic blood flow remains unchanged during AP[153, 154]. It is argued that measurement of total pancreatic blood flow during AP is not an ideal parameter as it does not take into account the disturbances in blood flow distribution within the gland during the onset and progression of AP. It also does not account for arteriovenous shunting which might result in blood bypassing the capillary bed. Microcirculatory derangements flowing the induction of AP; namely nutritive perfusion failure and microvascular leukocyte adherence, are similar in nature to the microcirculatory events observed subsequent to ischemia and reperfusion seen in other organs such as liver, intestine, striated muscles and skin[149]. A number of experimental studies have analyzed pancreatic microcirculation in AP by intra-arterial injection of methylene blue[155] or india ink[156,157]. They have demonstrated that in severely injured tissue there is vasoconstriction of intralobular arterioles and in less

affected areas there was vessel dilation. This indicates the spatial heterogeneity of the microcirculatory pathology of AP.

Today there is consensus that AP could be the result of ischemia to the pancreatic tissue. Regulation of ischemia and the mechanisms underlying these vascular changes are not completely understood. It has been noted that vascular changes in early stages of AP are likely to lead to severe and necrotizing pancreatitis[158]. Takeda et al., have compared the angiographic abnormalities with perfusion abnormalities by contrast enhanced computerized tomography (CT) in 102 patients with pancreatitis. They found a high incidence of vasospasm in small and medium-sized vessels of the pancreatic bed which led to decreased vascular perfusion. These areas of decreased perfusion developed necrosis as revealed by subsequent CT scans. They also found that mortality was directly related to the degree of vasospasm[158]. It has also been shown that in experimental AP endothelial nitric oxide synthase (e-NOS) is activated, and that elevated e-NOS activity leads to increased blood flow in pancreas. In absence of e-NOS activation, there is no increase in the pancreatic blood flow and hence pancreatitis worsens[159]. There is experimental evidence to support that pancreatic ischemia and hypoperfusion may lead to AP[160]. Andreadis et al.,[161] have shown that in dogs, vasopressin increases pancreatic blood flow and thereby ameliorates the severity in AP. The importance of vascular factors in pathogenesis of acute haemorrhagic AP has been well established[162, 163, 164]. It has been experimentally demonstrated that post ganglionic sympathetic denervation of the pancreas increases the pancreatic

blood flow and perfusion and thereby has beneficial effect on acute haemorrhagic pancreatitis in the dogs[165].

Thus it seems that vascular regulation is important in onset and progression of AP. This will be one aspect of my studies regarding mechanisms underlying AP.

1.6.8 Neural mechanisms in AP:

Both central and peripheral neural pathways are likely to be involved in AP. Experimental studies in rats have shown that central administration of thyrotropin-releasing hormone (TRH) resulted in increased pancreatic blood flow via nitric oxide (NO) dependant mechanisms[166]. Also central intracisternal administration of TRH ameliorated the severity of caerulein induced pancreatitis in rats. The beneficial effect was abolished by sub-diaphragmatic vagotomy or administration of the NOS inhibitor, N(G)-nitro-arginine-methylester (L-NAME). This beneficial effect was not reproduced by intravenous administration of TRH[167] suggesting that there may be a potential centrally regulated mechanism involving the efferent vagus and pancreatic e-NOS production that can modulate pancreatitis through changes in blood flow[168].

Peripherally, neural mechanisms also play an important role in pathogenesis of oedema and the inflammatory process. Stimulation of sensory neurons lead to release of substance P, which binds to post capillary venular neurokinin-1 receptors in the pancreatic circulation, resulting in extravasation of plasma into the interstitium followed by inflammatory cells[169]. Vasoactive intestinal peptide

(VIP) has been noted to have immunomodulatory role. An agonist specific for VIP receptor has shown to accentuate the severity of pancreatitis[170].
These studies show that in AP both central and peripheral neural pathways are involved, but their relative roles in the course of AP are unclear.

1.6.9 Pancreatic vascular perfusion (PVP) and AP:

The significance of changes in PVP during AP is controversial. There are several experimental studies that have shown that vascular changes occur early in AP[171, 172]. These include increase in vascular permeability, extravasation of fluid into perilobular space and there may be development of microemboli within the vessels[173 , 174]. Total pancreatic blood flow has been found to be decreased in some studies of AP induced by intraductal bile infusions in dogs and measured using electromagnetic flowmeter[175], whereas no change was reported by others using radioisotopes and flow meters[176]. It has been reported that administration of phenylephrine, a vasoactive drug, diminishes perfusion of the pancreas and worsens mild caerulein induced AP[177]. Vascular perfusion changes in AP are more obvious as severity of AP increases[178]. Progression from mild to severe form of AP can occur as a result of sudden decrease in PVP. There is substantial experimental and clinical evidence that alteration in vascular perfusion and thereby microvascular failure occurs in severe AP.

One possible source of confusion in this field is that the terms blood flow and perfusion are not interchangeable. Blood flow generally refers to a global

measure of the amount of blood reaching an organ with units of ml/100g/min. On the other hand perfusion relates to the local blood flow in the capillaries of tissue, and the time available for exchange of nutrients from blood stream, and is not necessarily well reflected by measurements in ml/100g/min. There are many techniques to measure pancreatic blood flow. Broadly, there may be measurement of macrovascular flow into the organ or microvascular flow into the tissues. Each technique has its advantage and disadvantage. The macrovascular flow into the organ is assessed by targeting small, medium or large vessels. On the other hand the microvascular flow into the tissue is assessed by targeting the microvasculature of the tissue within the organ. PVP is the assessment of microvacular flow or microcirculation within the pancreas. Thus, I have used Laser Doppler fluxmetry (LDF) to assess pancreatic vascular perfusion in AP.

1.6.9.1 LDF to study PVP:

LDF is based on the Doppler principle, which states that when a waveform is reflected from a moving object it undergoes a shift in frequency; in the case of light it changes its wavelength[179]. LDF is a non-invasive method of measuring tissue perfusion and has been used to measure perfusion in the pancreas[180], [181], [182]. A low intensity visible light from a monochromatic laser is directed at the tissue via a flexible optical fibre which lies in close proximity to another fibre which receive the reflected light. Any red blood cells within the field of illumination will absorb some of this light and reflect the rest. This reflected light, of different frequency is received by the second optical fibre. The difference in

frequency is called the Doppler shift. The extent of the frequency shift and the amount of light returning enables the calculation of red cell flux. This is measured in arbitrary perfusion units in the illuminated volume of tissue per unit time. Various factors can influence measurement of perfusion by LDF such as probe placement, tissue thickness, tissue temperature etc[183]. Apart from the use in the pancreas, LDF has also been used to study the vascular perfusion in the stomach[184], small intestine[185], liver[186], kidney[187], brain[188] etc.

1.6.10 Markers of pancreatic injury:

Over the last decade a spectrum of pancreas-specific markers have been evaluated as predictors of severity of AP. This has been based on the concept that during inflammation of the pancreas, some pancreatic content may spill into systemic circulation. I have used laboratory methods to estimate plasma levels of amylase and lipase, and myeloperoxidase levels in pancreatic tissue as a marker of inflammation and neutrophil infiltration, respectively.

1.6.10.1 Laboratory methods:

Amylase and lipase activity estimation:

It has been well established that markedly elevated plasma levels of pancreatic enzymes are indicators of AP[189]. Amylase and lipase activity are most frequently used clinical parameters used for diagnosing AP. Several studies have concluded that the level of increase in serum amylase and lipase does not correlate with the severity of the disease[190], although these findings are not

universal[191]. Consequently, hyperenzymemia alone is not used to predict severity of AP.

1.6.10.2 *Markers of inflammation:*

Myeloperoxidase levels:

Myeloperoxidase (MPO) is a peroxidase enzyme most abundantly present in neutrophil granulocytes. MPO levels in the tissue reflect the degree of neutrophil inflammation and infiltration[192] and is an accepted marker of AP associated neutrophil infiltration.

1.6.10.3 *Histological assessment of pancreatic injury:*

AP can be characterized histologically in experimental settings. The severity of AP can be graded in the histological sections of the pancreas either by allocating scores to different types of morphological alterations or by determining the proportion of abnormal tissue of the total parenchyma. The former method is based on subjective assessment of histological slides and is suitable for evaluation of both edematous and necrotizing pancreatitis. Histiometeric measurement of necrotic parenchyma can be used only in necrotizing forms of AP. There are various scoring systems used in experimental AP. The scoring systems differ and depend on the model and the severity of AP induced.

In my experimental studies I have used a modified Spormann's scoring system[193] for AP in the possum study and a scoring system modified from Niederau et al.,[194] was used in the mice experiments. Different scoring systems are used because the features of AP are different in these two species and the

two models of AP. The scoring parameters and system will be described in the relevant chapters.

1.7 Experimental models of AP:

There are various models of experimental AP. An ideal model should be easily reproducible, have a similar natural history as that of human disease and a similar response to treatment[195]. It should also be relatively cheap and simple. Current models have contributed greatly to our current understanding of the pathophysiology and cell biology of the disease. They have also become a standard tool for testing new and promising treatments for AP. A brief review of established models of AP follows.

1.7.1 Historical review of experimental models of AP:

Experimental models of pancreatitis have used to study the disease process for more than 150 years. The first experimental model of AP was produced by Bernard in 1856 by injection of bile and olive oil into the canine pancreatic duct[196]. The early models relied mainly on forceful injection at high pressure of various substances such as bile, duodenal contents or activated pancreatic enzymes into the pancreatic duct. In 1862 Pannum[197] injected wax droplets into the pancreatic arteries and hence producing focal ischaemia which lead to haemorrhagic pancreatitis. In 1895 Mouret[198] reported that excessive neural stimulation to the pancreas led to increased vacuole production.

Following this early work there has been variety of models that have been developed considering different etiological factors. There are different ways in which models can be categorised. They can be invasive or non-invasive, depending on whether or not surgical manipulation is required to induce AP. Animal models can also be divided into *in vivo* models and *ex vivo* models.

1.7.2 In vivo techniques to induce experimental AP:

The various *in vivo* techniques used to induce AP can be allocated into six categories: 1. immunological; 2. secretagogue induced; 3. diet induced; 4. duct injection induced; 5. duct ligation induced; 6. microvascular induced.

1.7.2.1 Immunological pancreatitis:

Some immunologic models are invasive and some are non-invasive. The first immunologically induced pancreatitis model was reported by Thal[199]. Thal caused a local Shwartzman – reaction by first infusing the bacterial toxin of Escherichia coli and Meningococci into the pancreatic ducts of rabbits and goats. Twenty four hours later he applied the same toxin intravenously, and a haemorrhagic and necrotizing pancreatitis resulted.

The main drawback of immunologic models is the non-specific nature of the generalised immunological response in other organ systems and high early mortality. The early mortality makes the investigation of pathogenesis and treatment difficult. Because of non-specific nature and difficulty in producing a graded response, immunological models are not commonly used.

1.7.2.2 Secretagogue-induced pancreatitis:

AP may be induced by agents introduced either subcutaneously, intravenously or intraperitonealy in rats and mice. This results in increased proteolytic enzyme secretion to levels that cause pancreatic acinar autolysis[200]. Caerulein (a CCK analogue) may be given intravenously[201] at doses of 5-10µg/kg/h in saline for a period of 4 - 24 h or subcutaneously or intraperitonealy[202] for doses up to 100 µg/kg/h. Progressive interstitial oedema is observed. Caerulein induced pancreatitis in rats produces an adult respiratory distress syndrome-like lung injury. The model allows easily controlled grades of the condition and is self-limiting[203], [204]. It is applicable in rat, mouse and dog. There are structural similarities to surgical AP. The model is useful in studying early acute oedematous pancreatitis and provides information on mechanisms where there may be gut endocrine perturbations in the genesis of pancreatitis. The major drawback of this model in the mice and rats is that frequent blood samples for amylase and lipase estimation are difficult to obtain.

1.7.2.3 Diet-induced pancreatitis:

Acute haemorrhagic pancreatitis with fat necrosis has been induced in female mice using a choline-deficient ethionine supplemented diet[205]. Male mice are insensitive. The sex difference may be due to the deleterious effect of estrogens or a reduced capacity to neutralise active pancreatic enzymes. It is likely that this model is dependent on toxic action of ethionine on the acinar plasmalemma in the absence of the cytoprotective effect of choline. Both gross and histological

appearances of the pancreatic and peripancreatic inflammation, as well as the clinical and biochemical course, resemble the human disease. The mortality rate can be controlled by the amount of feeding[206]. This model has been used in the study of oxygen-derived free radical damage to the pancreas[207]. Its disadvantages are that the amount of injury produced depends critically on the sex, age and weight of mice, and the intake of choline-deficient ethionine supplemented diet.

1.7.2.4 Duct injection-induced pancreatitis:

Reber and co-workers[208] developed a low-pressure ductal perfusion cat model of AP. In this model the pancreatic duct is cannulated in both head and tail of the pancreas; sodium glycodeoxycholate 15mmol/l, a bile salt secretagogue, is introduced at 0.5ml/h for 1 hr to produce acute oedematous pancreatitis. The main pancreatic duct may also be made permeable by intragastric administration of ethanol, the stimulation of pancreatic secretion into an obstructed duct or the creation of acute hypercalcaemia[209]. Perfusion of active pancreatic enzymes into the duct via a catheter results in acute edematous pancreatitis. Retrograde injection of sodium taurocholate into the pancreatic duct has also been used[210], [211]. This model is reliable with low morbidity and mortality rates. Pancreatic juice output and duct pressure can be measured. The main limitations are complexity, expense and low mortality. The low mortality suggests that death cannot be used as an endpoint.

1.7.2.5 Duct ligation-induced pancreatitis:

Pancreatitis can be induced by placing a ligature around the bile duct at its point of entry into the duodenum[212]. Ligation of pancreatic duct alone can also be done[213]. This model shows similarity to various clinical situations like gallstone induced pancreatitis, motility disorders of the sphincter of Oddi, oedema and stricture at the papilla, tumors of papilla and parasites causing obstruction of the pancreatic duct. Duct ligation can also be combined with secretagogue stimulation of the pancreas. Pancreatic ductal pressure can be measured. The model is technically difficult and expensive. A variant of duct ligation model is the closed duodenal loop model of Seidel[214] and Pfeffer et al.,[215]. In this model the first 10cm of duodenum is isolated in an anaesthetized dog by division just distal to pylorus, and between the second and third parts of duodenum. The common bile duct is ligated and a gastroduodenostomy constructed to re-establish gastric outflow. Pancreatic oedema occurs at 4 h and hemorrhagic pancreatitis at 9 – 12 h.

Chen et al., have described pancreatic duct ligation with stimulation of pancreatic exocrine secretion model in the possum[216]. I have used this model in my experimental study in the possum (chapter 2). They have also described a model of severe AP as a result of topical application of carbachol on sphincter of Oddi in the possum.

1.7.2.6 Microvascular-induced pancreatitis:

These models were developed based on the observation that an early event in AP is microvascular changes. Redha et al.,[217] demonstrated that partial occlusion of the arterial supply of the pancreas in the rats could induce acute haemorrhagic pancreatitis. The distal branch of splenic artery is isolated, cannulated and polystyrene microspheres 20µm in diameter are infused. The cannula is then withdrawn and the vessel is ligated. This model may progress to chronic active pancreatitis at the end of 3 weeks. There is minimal endocrine derangement as seen with other models.

1.7.3 Ex vivo techniques:

1.7.3.1 Isolated ex vivo perfused pancreas:

In this canine model, the pancreas with short cuff of duodenum is isolated from the dog and perfusion catheters are placed in the splenic artery, superior mesenteric artery and portal vein[218]. The pancreatic duct is then cannulated. The pancreatic duct is connected to an oxygenated perfused circuit. Gallstone, alcohol and ischaemic pancreatitis may be reproduced respectively by partial duct obstruction with secretin stimulation, free fatty acid infusion and 2h warm ischemia. The disadvantage is that it undergoes functional deterioration within 4h and is cumbersome.

1.8 Project rationale:

As described in **Section 1.5.5**, galanin is an abundant neurotransmitter in the pancreas and is closely associated with the pancreatic blood vessels and has cardiovascular effects (Section 1.5.6), it may play a role in regulation of PVP. Microvascular changes in AP are well documented (Section 1.6.7). Furthermore, recent studies in our laboratory has demonstrated that in the Australian Brush-Tailed possum galanin infusion caused a biphasic response (a decrease followed by an increase) in pancreatic vascular perfusion and a reduction in conductance (the inverse of vascular resistance, i.e. perfusion divided by blood pressure). The effect of the antagonist galantide is also biphasic, but is the reverse to that of galanin i.e. an increase followed by a decrease in pancreatic vascular perfusion[219]. This suggests that the effect of galanin on pancreatic vascular perfusion is via a local effect on vessels within the pancreas. Taken together, these findings raise the possibility that galanin may play a role in AP, and this warrants further investigation.

1.9 Overall hypothesis:

Galanin plays a major role in the onset and/or progression of AP.

1.9.1 Specific hypotheses:

1. Exogenous galanin will increase and exogenous galantide will decrease the severity of AP.

2. Galanin mediates the pancreatic hypoperfusion in AP and this will be reversed by treatment with galantide.

3. Galanin will increase and galantide will decrease the pancreatic exocrine secretion.

4. Galanin knockout (KO) mice will be less susceptible to AP as compared to their wild type (WT) littermates.

5. Galanin antagonists M35, M40 and C7 will decrease the severity of AP in a caerulein mouse model.

1.9.2 Aims:

The aims of the proposed studies were to determine:

1. The effect galanin or galantide administration, before and after AP induction on severity of AP in the possum model (Chapter 2).

2. If administration of galanin or galantide alters PVP during AP in the possum model (Chapter 3).

3. If exogenous galanin or galantide alters the basal and hyperstimulated pancreatic exocrine secretion in the possum (Chapter 4).

4. The effect galanin or galantide administration, before and after AP induction on the severity of AP in the caerulein mouse model of mild AP (Chapter 5).

5. If severity of caerulein-induced AP in galanin knock (KO) mice is different to their wild type (WT) littermates (Chapter 6).

6. If administration of M35, M40 and C7 at AP induction alters the severity of AP in the caerulein mouse model (Chapter 7).

Chapter 2

Chapter 2: Effects of exogenous galanin and galantide administration in a possum model of mild AP

2.1 Introduction:

As described in **Section 1.8**, administration of exogenous galanin infusion produced a biphasic response (a decrease followed by an increase) in PVP and a reduction in conductance. The effect of the antagonist galantide was also biphasic, but the reverse to that produced by galanin[220]. These data are consistent with a direct effect of galanin on PVP via a local effect on vessels within the pancreas. In addition PVP is perturbed during AP, particularly during the early phase[220]. These observations lead to the hypothesis that **galanin plays a key role in the changes in PVP observed in the early stages of AP** and thus may be responsible for onset/progression of AP.

2.1.1 Specific Hypothesis

Exogenous galanin will increase and exogenous galantide will decrease the severity of AP.

2.2 Specific aims:

To determine the effect of galanin or galantide administration, before and after AP induction on severity of AP in the possum model.

2.3 Methodological considerations:

The possum has been identified as a good model to study pancreatic functions as the anatomy and the blood supply of possum pancreas is similar to the human pancreas[221],[222],[223]. My initial experimental study has been in the possum model of mild AP, which involves pancreatic duct ligation and exocrine hyperstimulation with cholecystokinin (CCK) and secretin[224]. This is a well established model of AP in our laboratory. The AP studies are carried out in two parts; 'prophylactic' and 'therapeutic' drug administration groups. In the 'prophylactic' drug administration group, the peptides galanin and galantide are administered prior to the onset of AP. These experiments are designed to assess the role of these peptides in modulation of early events in AP. In the therapeutic drug administration experiments, the peptide galantide is administered 2 and 3 hours post onset AP in separate protocols. The rationale of including the therapeutic protocol was firstly, to assess the role of galantide in AP after the early events have taken place. Secondly, the time course of AP in the animal model is compressed compared to the humans. Hence administration of galantide 2 or 3 hours post onset AP may simulate a clinical situation which would be comparable to the probable time at which a patient suffering from AP would arrive to the emergency department for treatment.

2.4 Methods

2.4.1 Possums and animal ethics approval

This study was approved by the Animal Welfare Committee of the Flinders University (approval numbers 560/03, 603/05). Australian Brush-tailed possums

were used with permission from National Parks and Wildlife Service of South Australia.

Australian Brush-tailed possums (*Trichosurus vulpecula*) of either sex and weighing 1.5 -3 kg were used in these experiments. All procedures related to the possums were carried out according to the principles outlined in the Australian Code of Practice for Animal Care and Use. All animals were fasted overnight but free access to water was allowed.

2.4.2 Pharmaceutical agents

Animals were anaesthetized with intramuscular ketamine (Ketelar; Park Davis, Caringbah, NSW, Australia) and Xylazine (Rompun; Bayer, Botany Bay, NSW, Australia). Anaesthesia was maintained with intravenous infusion of thiopentone (Pentothal; Abbott, Kurnell, NSW, Australia) for the duration of experiment. Sulphated CCK octapeptide (CCK-8) and secretin (porcine, Auspep, Parkville, Victoria, Australia or American Peptide Company, Inc. Sunnyvale, CA, USA) were used to stimulate exocrine secretion of the pancreas. Galanin and galantide (Auspep Pty Ltd, Victoria, Australia or American Peptide Company, Inc. Sunnyvale, CA, USA) was administered to alter AP. All the animals were euthanized with pentobarbitone sodium (Ilium Pentobarb, Troy Laboratories Pty Ltd., Smithfield, NSW, Australia) at the end of experiment.

2.4.3 Experiment set up

After the anaesthesia induced by ketamine (20mg/kg) and xylazine (10mg/kg), the possums were shaved with an electric razor over the neck, abdomen and the inguinal regions. The possums were then placed on a heating blanket to maintain the body temperature at 37^0C (Harvard Apparatus, Holliston, Massachusetts, USA). A tracheostomy was performed and the possum was ventilated with a small animal ventilator (Harvard Apparatus, Holliston, Massachusetts, USA). The left femoral vein was cannulated with two cannulae (1.05mm OD, 0.35mm ID) for delivery of anaesthetic drug (thiopentone at an initial dose of 2ml/kg/h and then titrated to maintain anaesthesia for the duration of experiment) and normal saline. Normal saline was administered at 4 ml/h. Left femoral artery was cannulated with a cannula (1.5mm OD, 0.9mm ID) and it was connected to a pressure transducer (Transpac IV, Abbot Ireland, Sligo, Republic of Ireland) to continuously measure blood pressure. Two cannulae (1.5mm OD, 0.9mm ID) were placed into the right femoral vein; one was advanced into the inferior vena cava in the thorax and connected to a pressure transducer to monitor central venous pressure (CVP), as an indicator of fluid overload and the other cannula was used for infusion of drugs. The right femoral artery was also cannulated (1.5mm OD, 0.9mm ID) to collect blood samples for enzymatic analysis. A urinary catheter (infant feeding tube size 12Fr) was inserted into the bladder to measure urine output. The abdomen was opened by a midline incision. With minimal handling of tissues, the duodenum with the sphincter of Oddi is placed on a platform and stabilised with the help of ophthalmic pins. The pancreatic duct component of the sphincter of Oddi was identified with the aid of

a dissecting microscope and opened with micro-scissors. It was then canulated (1.5mm OD, 0.9mm ID) for 4-5mm and ligated with 5-0 Prolene. The cannula was then connected to a pressure transducer to record the PDP and provide a "functional ligation". The abdomen was then closed with interrupted sutures.

2.4.4 Protocol for inducing AP and collection of data:

Following the completion of the experimental setup, the preparations were allowed to stabilise for 15 minutes. Then synthetic CCK-8 and secretin were dissolved in saline containing 0.01 % bovine serum albumin (BSA) (Sigma®, St. Louis, MO, USA.) was administered as bolus injection (1ml) over 1min via the femoral vein, at 30 minute intervals over the first 5h. The initial dose of CCK-8/secretin was 0.5µgm/kg. This was followed by increasing doses from 1 to 5µgm/kg **(Figure 2.1)**. The final dose was given 5h after pancreatic duct ligation, and the experiment was terminated 3h later.

At hourly intervals a blood sample (700µl) was collected from the right femoral artery for analysis of plasma amylase and lipase activities. The first sample of blood (baseline) was collected at the time of cannulation of the pancreatic duct. At the end of the experimental protocol the possum was euthanized by injecting 1ml of 500mg pentobarbitone sodium. The pancreas was then harvested for histological assessment of tissue damage as follows. The entire pancreas was fixed overnight in 10% buffered formalin. It was then divided into head, neck, body and tail regions and separately embedded in paraffin blocks. Four micron

sections were cut and stained with Hematoxylin and Eosin. These were then histologically scored by an independent, experienced pathologist who was blinded to the experimental protocol using the modified Spormann's scoring system **(Section 2.4.9)**.

2.4.5 Protocol for prophylactic administration of galanin or galantide:

The experiment setup and induction of AP was as described above **(Section 2.4.4)**. Galanin or galantide were administered intravenously 15 minutes prior to pancreatic duct ligation as a bolus injection (0.5nmol/kg and 1.5nmol/kg respectively) in BSA saline, and were continued as an infusion (2nmol/kg/h) for the duration of experiment **(Figure 2.2)**. The above doses of galanin and galantide were selected as they demonstrated PVP changes in normal animals.

2.4.6 Protocol for therapeutic administration of galantide:

The experiment setup and induction of AP was as described above **(Section 2.4.4)**. This group included administration of galantide post onset AP**.** Galantide was administered intravenously 2 h post onset AP as a bolus, and was then continued as an infusion for the duration of experiment, using the doses described above **(Figure 2.3)**. In separate group of animals, galantide was administered intravenously 3 h post onset AP as described above **(Figure 2.4)**.

2.4.7 Study groups

The study comprised of the following groups of animals (**Table 2.1**):

Table 2.1. Various groups in the study.

Study group	Number of animals, n
AP alone	10
AP + prophylactic administration of galanin	5
AP + prophylactic administration of galantide	6
AP + therapeutic (2hr post onset) administration of galantide	5
AP + therapeutic (3hr post onset) administration of galantide	5

2.4.8 Parameters measured

The various parameters measured were BP, PDP, CVP, urine output, plasma amylase and lipase activities and histological assessment of pancreatic damage.

2.4.8.1 BP, PDP and CVP measurements:

The BP, PDP and CVP were continuously recorded on a Maclab recording system (ADInstruments Pty Ltd., Castle Hill, NSW, Australia) using Chart software version 4.2.2. The analysis was done off-line.

2.4.8.2 Urine output:

The total urine output for the duration of experiment was measured in ml and was expressed as the volume per kg body weight of the animal.

2.4.8.3 Plasma amylase and lipase activities:

Plasma amylase and lipase activities were assessed by collecting blood sample in ethylenediaminetetraacetic acid tubes. It was then centrifuged at 2000 rpm for 5 minutes at room temperature. The plasma was then collected and stored at -20°C. For estimation of plasma amylase, AMYL α – Amylase liquid according to IFCC reagents (Roche/Hitachi, Germany) were used for enzymatic colorimetric assay. Lipase-PS^{TM} reagents (Trinity, Biotech, St. Louis, USA) were used for quantitative, kinetic determination of pancreatic lipase activity. Both assays were performed on a centrifugal analyser (Cobas Bios, F Hoffmann-La Roche, Basle, Switzerland). Both amylase and lipase activities were expressed as U/L.

2.4.9 Histological examination:

Pancreatic damage was histologically assessed by using a modified Spormann scoring system[225]. This scoring system assesses the degree of pancreatic damage by allocating a score for oedema, parenchymal inflammation, fat inflammation, peripheral necrosis, fat necrosis and parenchymal necrosis as shown in **Table 2.2**. The histological scoring was done by histopathologist who was blinded to various treatment groups.

Table 2.2: Histological scoring for early AP - modified Spormann's scoring criteria.

Histological feature	Score and description
Oedema	1 – Focally increased between lobules 2 – Diffusely increased between lobules 3 – Tense acini, widely separated lobules
Fat inflammation	1 – mild 2 – moderate 3 – severe
Parenchymal inflammation	1 – mild 2 – moderate 3 – severe
Peripheral necrosis	1 – mild 2 – moderate
Fat necrosis	3 – <2/section 5 – 3-5/section 7 - >5/section
Parenchymal necrosis	3 – focal <5% 5 – and/or sublobular <20% 7 – and/or lobular >20%

The possum model used in this study produced mild AP and the most prominent histological feature of this model is oedema [226]. Previous work in the laboratory showed an increase in the pancreatic wet weight dry weight ratio in this model, consistent with increased oedema. To more precisely quantify interstitial oedema within the pancreas a point scoring system was used **(Figure 2.5)**. This

is a quantitative morphometry method[227]. From each histology slide 5 randomly microscopic fields (40X magnification) were selected and imaged using a digital camera (DP-70, Olympus Japan). A grid of 25 points was randomly superimposed on each image using Microsoft Flash software. The number of grid points falling over inter-acinar (non-cellular) space was counted. An oedema score was generated as the average percentage of points falling over the inter-acinar space per 25 points of the grid. This was considered as an adequate sample size. Data for all the animals per group was then combined and subjected to statistical analysis.

2.4.10 Analysis:

After stabilization period of 15 min, baseline value of BP and PDP were taken by averaging a 1 min period just prior to ligation of pancreatic duct in the AP group and prior to administration of bolus doses of galanin or galantide in the prophylactic administration groups. This period was stable and representative of at least 10 min of the previous data. Subsequently every 15 minutes, the mean of a one minute interval was analysed for the duration of experiment. Care was taken to ensure that the one minute period was representative, and avoid analysis of data where boluses of drugs or saline were administered and where the readings were momentarily unstable. The BP and PDP are expressed in mmHg. If there was a fall in blood pressure to below 40 mmHg, the experiment was aborted, as at this level vascular collapse had occurred.

The plasma amylase and lipase activities of the AP group and prophylactic galanin and galantide administration groups are expressed as units per litre (U/L). When the AP group was compared to the therapeutic galantide administration groups, these activities were expressed as percentage change from the time when galantide was administered.

2.4.11 Statistical analysis:

Statistical analyses were performed using the raw data. All the data were checked for normality. If the data were normally distributed, then multiple comparisons were made using analysis of variance (ANOVA). When data was not normally distributed, a non parametric Mann Whitney U test was used to determine significant differences. A p value of 0.05 or less was considered significant. All the data is expressed as mean ± SEM unless otherwise stated.

2.5 Results

2.5.1 BP:

The BP was stable prior to the onset of AP and then fell during the 1st h in all the groups ($p<0.05$) and then stabilised (**Figure 2.6 top**). The BP in galanin or galantide administration groups was not significantly different from the AP group.

2.5.2 PDP:

PDP gradually increased to 15-20 mmHg after ligation of the PD at the end of the 1st h. This was statistically significant ($p<0.05$) (**Figure 2.6 bottom**). PDP was then steady throughout out the course of the experiment. There were no

significant differences in the PDP displayed by any of the group during the course of the experiment.

2.5.3 Plasma enzyme activities:

2.5.3.1 AP group:

In the AP group the plasma amylase activity gradually increased as AP was induced (**Figure 2.7 top**). In the AP group the mean plasma amylase activity prior to AP induction was 398±46 U/L. At 2-3h post AP induction the plasma amylase activity had increased by 190%, then plateaued and at 7h there was a trend toward a decrease.

The mean plasma lipase activity prior to AP induction was 35±11 U/L. This gradually increased as AP was induced (**Figure 2.7 bottom**), reaching a peak (35 fold) at 2 h post AP and then plateaued.

2.5.3.2 AP plus prophylactic galanin group:

In the prophylactic galanin administration group, the changes in the plasma amylase activity were similar to that seen in the AP group (i.e. 175% rise by 2h post AP induction; **Figure 2.7 top**). The changes in the plasma lipase levels were more dramatic i.e. by 2h post AP induction there was about 220 fold rise (**Figure 2.7 bottom**).

2.5.3.3 AP plus prophylactic galantide group:

There was no rise in plasma amylase activity in the prophylactic galantide administration group (**Figure 2.7 top**). There were significant differences in plasma amylase activity in the prophylactic galantide administration group

compared to the AP group ($p<0.05$). In contrast, in the prophylactic galantide administration group, the rise in plasma lipase was only 4 fold (**Figure 2.7 bottom**). There was significant difference in plasma lipase activity when prophylactic galantide administration group was compared to the AP group ($p<0.05$).

2.5.3.4 AP plus 2 and 3h post onset AP galantide administration groups:

When galantide was administered at 2 hours post onset AP, the subsequent rise in plasma amylase activity was suppressed compared to the AP alone group ($p<0.05$; **Figure 2.8 top**). When galantide was administered 3h post onset AP there was no significant fall in the plasma amylase activity compared to the AP group (**Figure 2.9 top**).

Galantide administered at 2 and 3h post AP induction in separate groups, resulted in significantly lower plasma lipase activity ($p<0.05$, **Figure 2.8 bottom and 2.9 bottom**).

2.5.4 Histology:

2.5.4.1 Modified Spormann's scoring:

There were no significant differences in the histological scores of any group with modified Spormann's scoring system (**Table 2.3**).

Table 2.3: Summary of mean histological score in different experimental groups.

Study group	Number of animals, n	Score (Mean ± SEM)
AP	10	4.9±0.8
AP + prophylactic administration of galanin	5	6.8±0.7
AP + prophylactic administration of galantide	6	5±0.5
AP + therapeutic (2h post AP onset) administration of galantide	5	8.2±0.8
AP + therapeutic (3h post AP onset) administration of galantide	5	4.4±1.7

2.5.4.2 Point scoring for oedema:

Point scoring analysis for oedema showed that the groups with prophylactic and therapeutic administration of galantide had significantly less oedema than the AP group (**Table 2.4**). There was no significant difference in the oedema between the groups which received galantide. On the other hand, prophylactic galanin administration did not result in significant difference in oedema when compared to the AP alone group.

Table 2.4: Summary of point scoring analysis for edema in possum pancreas in different experimental groups.

Study group	Number of animals, n	Score (Mean ± SEM)	P value (Vs. AP)
AP	10	5.05±0.57	
AP + prophylactic administration of galanin	5	3.5±0.15	0.59
AP + prophylactic administration of galantide	6	2.1±0.47	0.006
AP + therapeutic (2h post AP onset) administration of galantide	5	2.5±0.67	0.019
AP + therapeutic (3h post AP onset) administration of galantide	5	1.3±0.49	0.000

2.5.5 Urine output

The urine output was not significantly different in either of the groups for the duration of the experiment (**Table 2.5**).

Table 2.5: Summary of mean urine output in different experimental groups.

Study group	Number of animals, n	Mean urine output (ml/kg)
AP alone	10	22.1±1.8
AP + prophylactic administration of galanin	5	25.5±4.4
AP + prophylactic administration of galantide	6	26.4±4.8
AP + therapeutic administration of galantide (2hours post AP)	5	17.9±2.09
AP + therapeutic administration of galantide (2hours post AP)	5	27.6±1.68

2.6 Discussion:

These data showed that prophylactic treatment of AP with galantide significantly ameliorated plasma hyperenzymemia. Furthermore, therapeutic treatment of AP with galantide showed the similar trend in plasma enzyme activities. This was significant when galantide was administered at 2h post onset AP. On the other hand prophylactic treatment of AP with galanin produced a significant increase in the AP-induced plasma lipase activity but had no effect on the AP-induced plasma amylase activity. The histological damage to the pancreas induced by AP, as assessed by the modified Spormann's score, was not altered by galantide or galanin administration. The point scoring analysis of pancreatic oedema showed that both prophylactic and therapeutic treatment of AP with

galantide significantly decreased interstitial oedema in the pancreas, whereas galanin had no effect. Thus these data are consistent with a role of galanin antagonist in AP and demonstrate that galantide treatment ameliorated the severity of AP in this model.

AP is a complex multi-step process. AP induced hyperenzymemia is due to either increased secretion of pancreatic enzymes from the acinar cells and/or as a result of increased release from damaged acinar cells. It is unclear how galanin might modulate one or more of these steps. Galanin could be involved in the induction and/or progression of AP by modifying pancreatic exocrine secretion or via alteration in PVP or a combination of both. Little is known about the effect of galanin on exocrine pancreas. Whether galanin plays a role in the regulation of exocrine pancreatic secretion and whether this effect is mediated via a direct action on acinar cells or indirectly via an influence on insulin secretion is not clear.

The source of galanin which mediates AP is unclear. As indicated in Chapter 1, galanin is present in the nerves and a subpopulation of islets of the pancreas. Galanin release could result in increase in the plasma levels of galanin. The plasma level of galanin has not been measured in the possum plasma. A relevant method for the quantitation of galanin in possum plasma has not been published and to my knowledge does not exist. One potential difficulty is the likely short half-life of galanin in plasma. Due to the time constraints of the

candidature this estimation was not attempted, however it could be undertaken in future studies.

Galantide decreased AP induced hyperenzymemia due to either a decrease in damage to the acinar cells or a decrease in exocrine secretion from the acinar cells. The damage to the acinar cells in AP depends on the type of model used in the study. In the mild model of AP, interstitial oedema is an early indicator of acinar cell damage. If acinar cell damage is due to microcirculatory changes then the AP-induced changes in the microcirculation should be modified by galanin and galantide. Recent studies[228] showed that galanin and galantide perturb PVP in possums. Thus, there may be a link between galanin, vascular perfusion and hypoxia-induced increased enzyme secretion in AP, this is further explored in Chapter 3.

If galantide decreased hyperenzymemia is due to decrease in secretion from the acinar cells then galanin should increase pancreatic exocrine secretions normally. The literature regarding the effect of galanin on exocrine secretion is unclear. Galanin is reported to inhibit pancreatic enzyme secretion in some studies[229, 230]. Where galanin has been reported to modify pancreatic exocrine secretion, the mechanisms involved have not been clearly described. Guerineau et al.,[231] showed that galanin evokes transient rises in intracellular Ca^{++} concentration in GH3/B6 pituitary cells of rats. As Ca^{++} transients are associated with normal secretion, this effect of galanin is consistent with increased secretion. However, Ahren et al., showed that galanin has a dual action on

calcium ion concentration in rat insulin producing RIN m5F cells[232]. Galanin initially increased intracellular Ca^{++} concentration then decreased it. Hence it will be interesting to study the effects of galanin and galantide on pancreatic exocrine secretion in the possum, this is addressed in Chapter 4.

There were significant differences between the plasma amylase and lipase activities in the group which received therapeutic (2h post onset AP) administration of galantide when compared to the AP group. On the other hand, there was no significant difference in plasma amylase levels between therapeutic galantide administrations at 3 hours post onset AP when compared to the AP group, but the plasma lipase activity was significantly lower. This would suggest that if galantide was administered earlier in the course of AP, it may be helpful in preventing the disease process and that there may be some dissociation in the effect of galantide on plasma enzymes when administered late in the course of the disease. Hence it appears that galantide affects the early events in AP. Conversely, galanin appears to modulate early events in AP.

There were no significant differences in the parameters of histological assessment of different groups using the modified Spormann's scoring system. This was not surprising because the mild AP induced in this model is oedematous pancreatitis. In the modified Spormann's scoring system oedema is one of six scoring parameters consequently the score contributed by oedema is relatively low as compared to the fat and parenchymal necrosis. Because of this limitation with the modified Spormann's scoring system, point scoring was used

to quantify pancreatic oedema. With point scoring for pancreatic oedema prophylactic as well as therapeutic administration of galantide in AP resulted in significantly less oedema. This begs the question on the effect of galanin and galantide on a more severe model of AP, this is addressed in Chapter 5.

The initial fall in BP within the 1st h in all the groups was statistically significant when compared the baseline levels. The reason for this fall could be due to the early events that occur in AP or as a result of CCK-SEC administration. It has been reported that CCK administration is associated with fall in the arterial BP[233] which could be due to splanchnic vasodilation or a systematic effect resulting in decreased heart rate. Both secretin[234] and CCK[235] have been shown to increase pancreatic blood flow. The supersaturation of CCK receptors could explain why the fall in BP did not continue during the experiment. In the galanin and galantide administration groups there was no modification in the initial fall in BP, this could also be due to supersaturation of CCK receptors or a combination of it and vasoactive effects of galanin or galantide. This could suggest that at the doses these drugs were used in the study were unable to overcome the AP-induced effects on BP.

The nervous system, via the vagus nerve, controls inflammation by decreasing the release of tumour necrosis factor-α from endotoxin stimulated macrophages. This anti-inflammatory effect is mediated by an interaction of acetylcholine with macrophage cholinergic nicotinic receptors. Hence there may be a role of nicotinic anti-inflammatory pathway in attenuating inflammation and injury during

experimental AP[236]. Galanin often coexists with other neurotransmitters such as acetylcholine, vasoactive intestinal polypeptide and nitric oxide synthase in the nerve endings and may act pre-synaptically to inhibit the release of these neurotransmitters[237, 238, 239,240]. It is possible that galanin acts to inhibit the release of acetylcholine, and attenuates the nicotinic anti-inflammatory pathway in experimental AP. Hence galantide administration may reduce this effect and limit acinar cell damage during experimental AP.

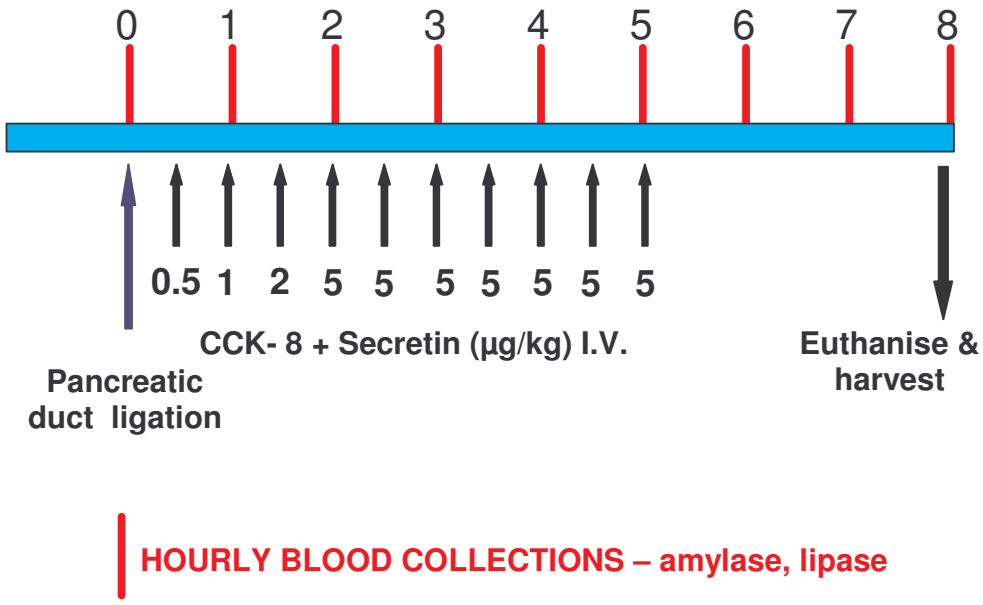
In general, nuclear factor kappa ß (NFK ß) mediated cytokine release occurs as part of the cellular response to injury. One consequence of this may be up-regulation of galanin receptors, which in turn exacerbates abnormal pancreatic secretion by increasing the responsiveness of the acini to galanin. In the dextran sulphate sodium induced model of colitis in mice, in which galanin receptors are up-regulated via a NFK ß mechanism; activation of these contributes up to half the excess colonic chloride and fluid secretion seen in this model[241]. Thus, galanin may have a role in directly increasing pancreatic secretion in AP, perhaps because of AP-induced up-regulation of its receptors.

2.7 Conclusion

The results indicate that galanin may play a role in induction of AP in the possums. The beneficial effect of galantide in AP may be due to either increase in the PVP (**Chapter 3**) or decrease in exocrine secretion (**Chapter 4**) or a combination of these. It will also be interesting to know if galanin and galantide modulate AP in a more severe model (**Chapter 5**). If so, depleting endogenous

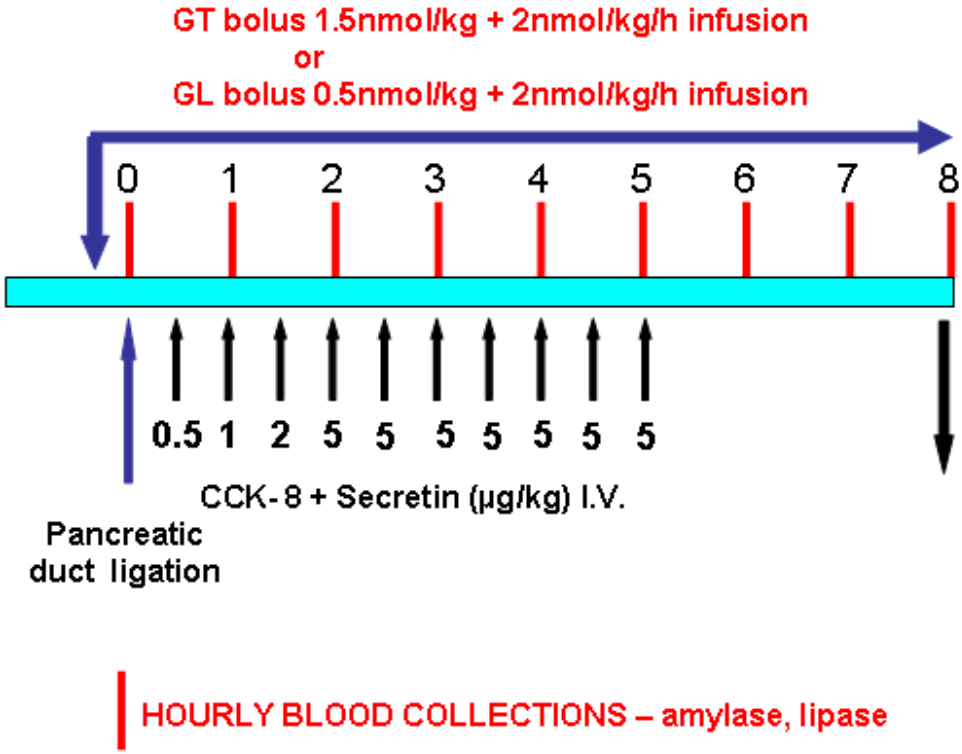
galanin should result in less severe AP (**Chapter 6**). There are various antagonists of galanin, and it would also be interesting to define their role in AP (**Chapter 7**).

Figure 2.1. Schematic representation of protocol to induce AP in the possum.



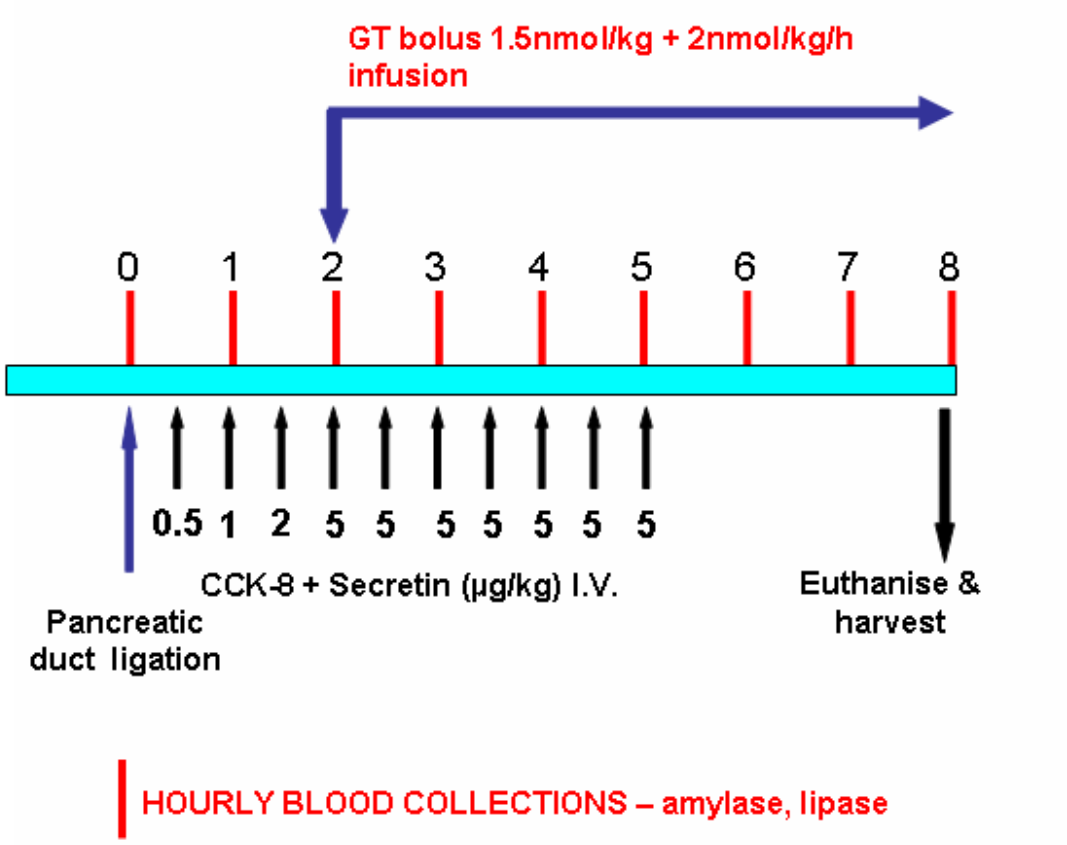
AP was induced by serial injections (30min intervals upto 5h) of cholecystokinin octapeptide (CCK-8) and secretin after pancreatic duct ligation. Blood samples were withdrawn at hourly intervals for estimation of plasma amylase and lipase activity. At the end of 8h the animal was euthanized and pancreas was harvested for histological assessment of severity of AP .

Figure 2.2. Schematic representation of protocol to induce AP with prophylactic galanin or galantide administration.



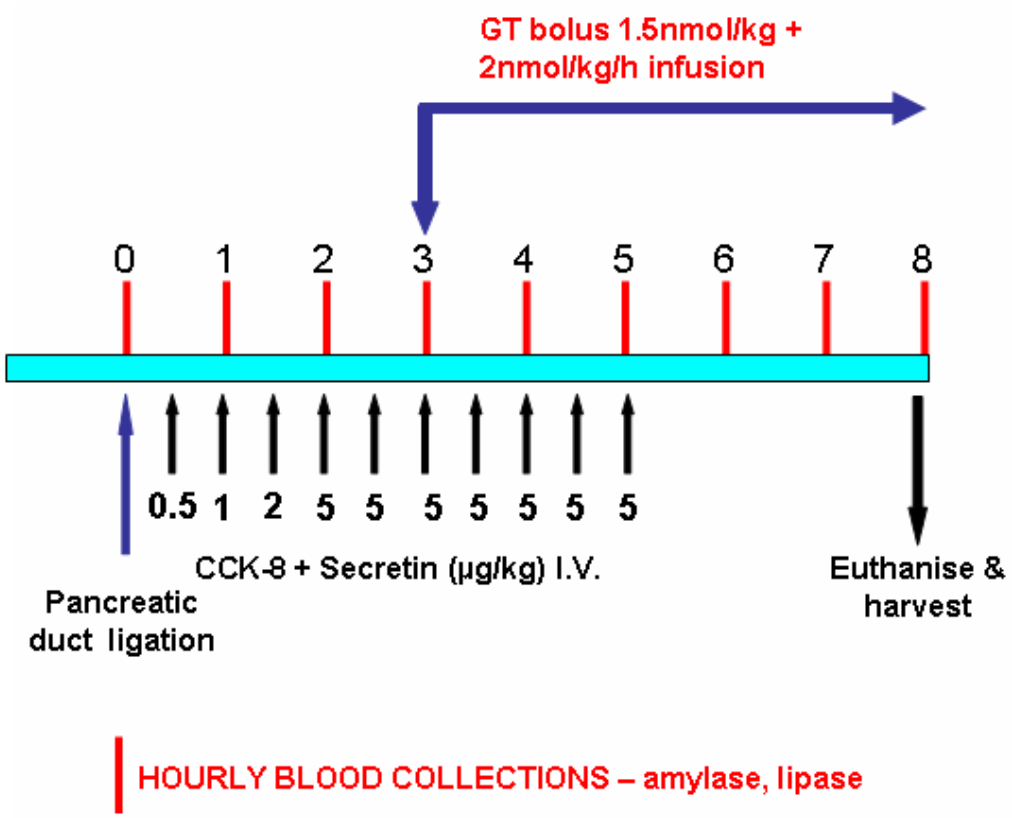
AP was induced as outlined in fig 2.1. Peptides galanin (GL) or galantide (GT) were administered prophylactically 15 min prior to pancreatic duct ligation. Blood samples were withdrawn at hourly intervals. At the end of 8h the animal was euthanized and pancreas was harvested.

Figure 2.3. Schematic representation of protocol to induce AP with therapeutic galantide administration (2 h post onset AP).



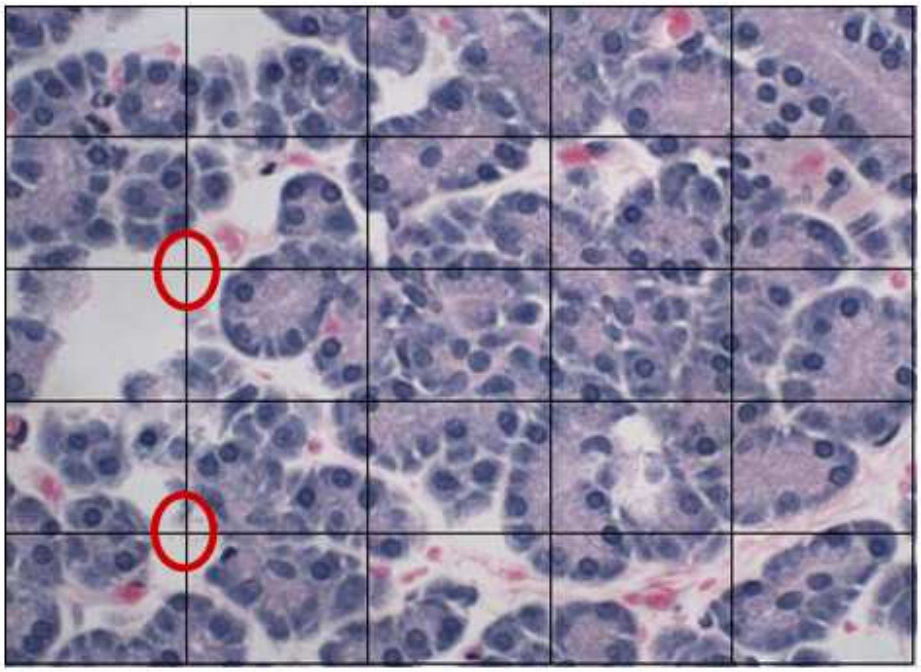
AP was induced as outlined in fig 2.1. Galantide (GT) was administered 2 h post onset AP. Blood samples were withdrawn at hourly intervals. At the end of 8h the animal was euthanized and pancreas was harvested.

Figure 2.4. Schematic representation of protocol to induce AP with therapeutic galantide administration (3 h post onset AP).



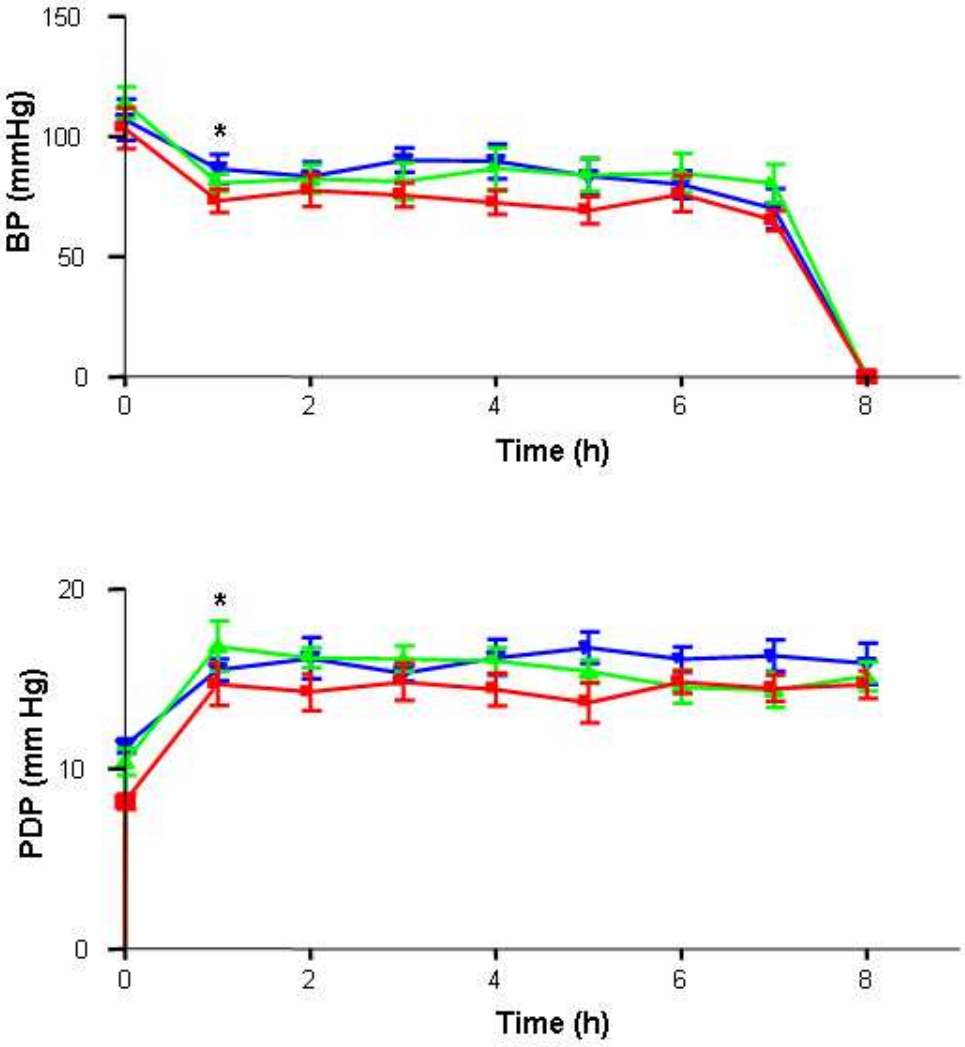
AP was induced as described in fig 2.1. Galantide (GT) was administered 3 h post onset AP. Blood samples were withdrawn at hourly intervals. At the end of 8 h the animal was euthanized and pancreas was harvested.

Figure 2.5. Representative histological image used for point scoring of edema.



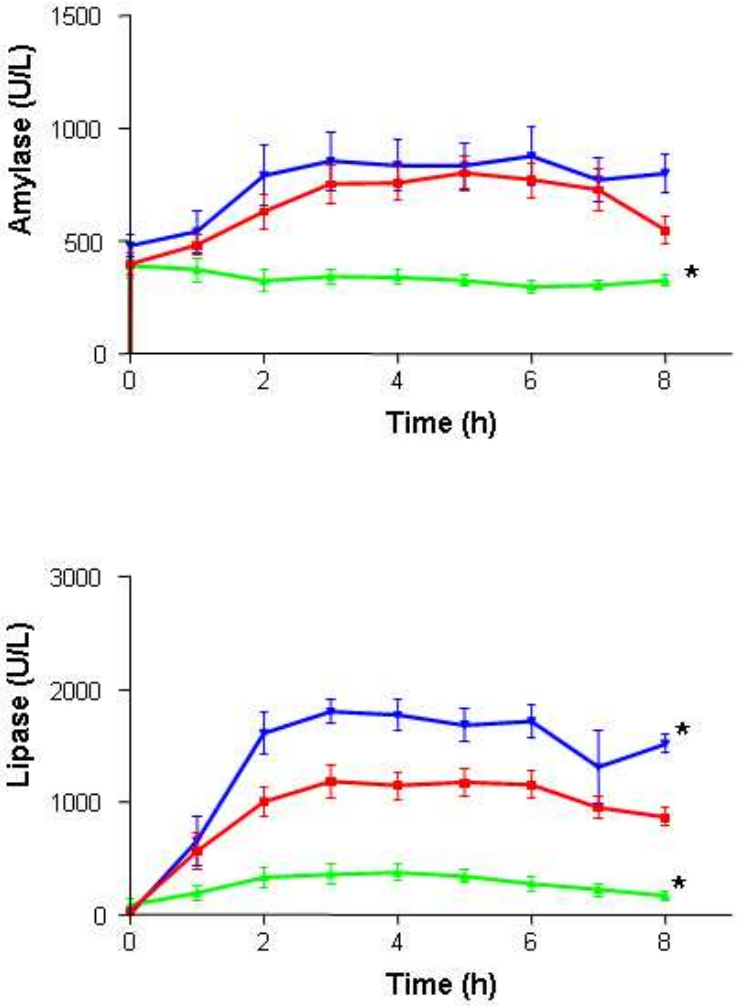
Representation of one digital image from a possum pancreas field taken at a magnification of X40, as described in the text, with a grid of 25 points (5x5), representing 25 μm^2, superimposed over the digital image. The presence under a grid point of interstitial edema (red circle) is highlighted.

Figure 2.6. Change in blood pressure (BP) and pancreatic duct pressure (PDP) during AP with and without prophylactic treatment with galanin or galantide.



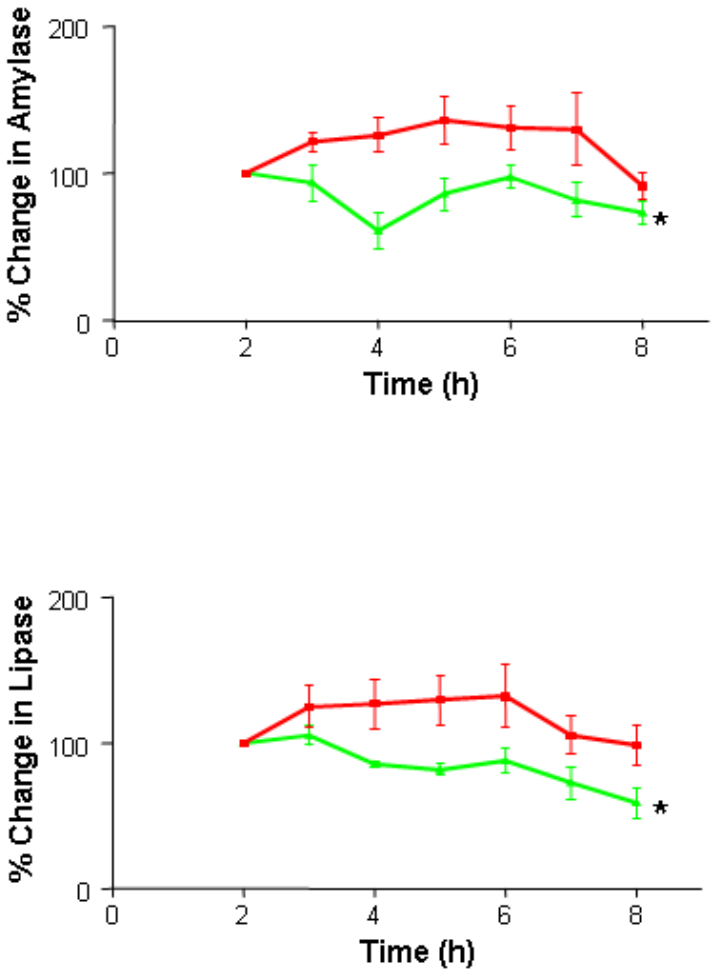
BP (top) and PDP (bottom) in different groups - AP (red), galanin (blue) and galantide (green) administration. There is a significant fall in BP in the first hour in the three groups. This is followed by a stable BP throughout the course of the experiment which is not significantly different between the groups. At 8 h the BP falls in all the groups when the animals were euthanased. The PDP gradually increases during the first hour and then stabilized. There is no significant difference in PDP displayed by all groups. Data is presented as mean ± SEM (n = 5-10) ($p > 0.05$ compared with the AP group, ANOVA). * $p < 0.05$ compared to the value at 0 h.

Figure 2.7. The time course of plasma amylase and lipase activity during AP with and without prophylactic treatment with galanin and galantide.



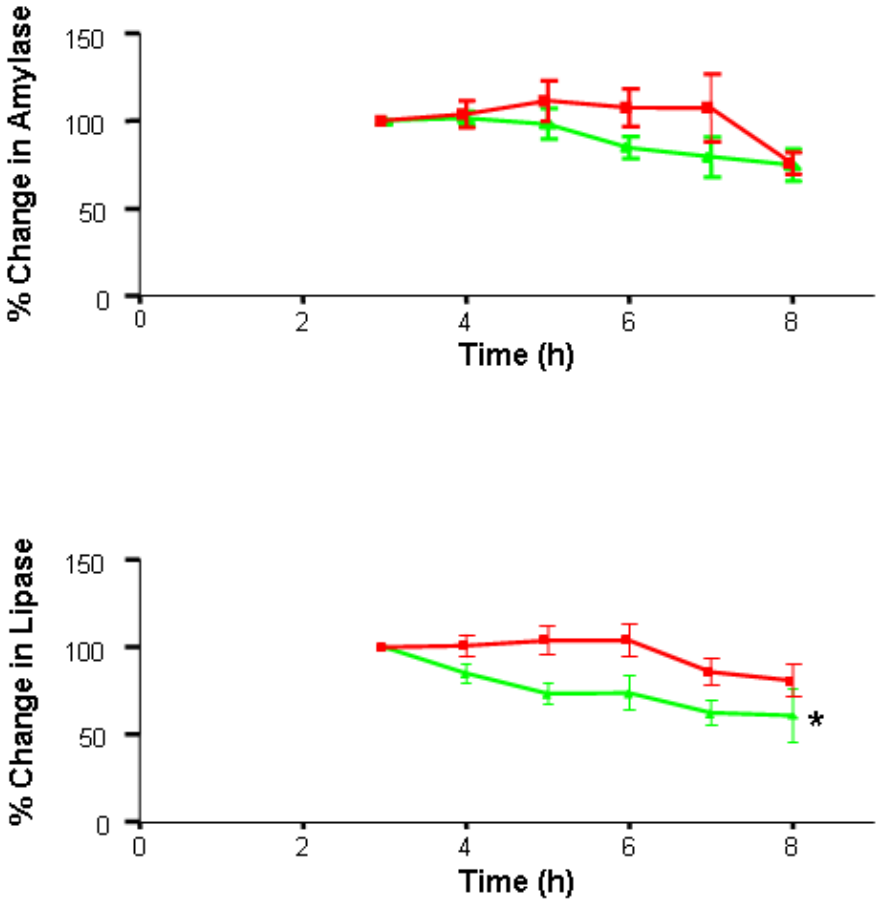
Plasma amylase (top) and plasma lipase (bottom) activities in different groups - AP (red), galanin (blue) and galantide (green) administration are shown. There is a rise in plasma enzyme activities on induction of AP. When GL is administered, the rise in plasma amylase activity is similar to that measured in the AP group but the rise in plasma lipase activity is significantly greater than in the AP group. When GT is administered, the rise in both plasma amylase and lipase activities are significantly decreased. Data is presented as mean ± SEM (n = 5-10) (*$p < 0.05$ compared with the AP group, ANOVA).

Figure 2.8. The time course of plasma amylase and lipase activity during AP with and without therapeutic treatment (2 h post onset AP) with galantide.



The change in amylase (top) and lipase (bottom) activities expressed as percentage of the mean value at 2 h in different groups - AP (red), and galantide (green) administration. In the AP group the plasma enzymes continue to increase and then plateau. When GT is administered at 2 hours post onset AP, there is a significant fall in the plasma enzyme activities as compared to the AP group. Data are presented as mean ± SEM (n = 5-10) (* $p < 0.05$ compared with the AP group, ANOVA).

Figure 2.9. The time course of plasma amylase and lipase activity during AP with and without therapeutic treatment (3 h post onset AP) with galantide.



The change in amylase (top) and lipase (bottom) activities expressed as percentage of the mean value at 3 h in different groups - AP (red) and galantide (green) administration. In the AP group the plasma enzymes activities plateaued during the remainder of the experimental period. When GT is administered at 3 hours post-AP onset, there is no significant fall in the plasma amylase activity as compared to the AP group. In contrast there is a significant decrease in the plasma lipase activity seen with GT administration when compared to the AP group. Data are presented as mean ± SEM (n = 5-10) (*$p < 0.05$ compared with the AP group, ANOVA).

Chapter 3

Chapter 3: Effects of galanin and galantide on Pancreatic Vascular Perfusion (PVP) in the possum model of mild AP.

3.1 Introduction:

As outlined in **Section 1.6.9**, AP is associated with changes in PVP. Several experimental studies have shown that pancreatic microcirculatory changes occur during early AP[242]. These microcirculatory changes include a combination of vasoconstriction, neutrophil accumulation and release of various vasoactive peptides[136]. Progression from mild to severe AP may be due to abrupt changes in PVP. As the severity of AP increases, perfusion changes become more obvious[243].

As discussed in **Chapter 1**, galanin is particularly prominent around blood vessels and especially arterioles in and around the pancreas[244], but its role in PVP during AP is unclear. Galanin is also known to cause pressor effects on blood pressure in the possum[245]. Brooke-Smith et al.,[246] have demonstrated that during AP in the possums, there is an initial fall in PVP, which is followed by an increase in PVP. Given that AP is associated with decreased PVP and development of pancreatic necrosis, galanin may have a potential role in the pathogenesis of AP.

The pancreas has a rich vascular supply to all its regions. It is not known whether different regions of pancreas respond in a uniform way to ischemia

induced by AP. Most experimental studies have measured changes in PVP in AP in one of the region of the pancreas. This approach may have not provided a complete description of perfusion changes in the entire pancreas. Consequently measurement of perfusion changes simultaneously in different regions, namely, head, body and tail of the pancreas will provide more information regarding the changes in AP in the entire pancreas and may reveal if particular regions are more susceptible to AP.

The data described in **Chapter 2**, suggests that galanin may play a role in AP. One possible mechanism is that galanin may mediate microcirculatory changes evident in AP.

3.2 Specific hypothesis:

Galanin mediates the pancreatic hypoperfusion in AP and this will be reversed by treatment with galantide.

3.3 Aim:

To determine, if administration of galanin or galantide alters PVP during AP in the possum model.

3.4 Methods:

Possum's were used in the study as the previous experimental studies were carried out in them. Laser Doppler fluxmetry was used to measure the PVP in

the possums for reasons discussed in **Chapter 1 Section 1.6.9** and also that in the past this technique has been used to study PVP in the possum's in our laboratory.

3.4.1 Pharmaceutical agents:

These are the same as described in **Section 2.4.2.**

3.4.2 Protocol for experimental setup:

The surgical set up was similar to that described in **Section 2.4.3**. The Laser Doppler probes were placed on different regions of the pancreas as described below.

3.4.3 Pancreatic vascular perfusion measurement:

Laser Doppler fluxmetry (**Section 1.6.9.1**) was used to measure the PVP in the head, body and the tail of the pancreas. PVP was continuously measured using a Perimed Periflux 5001 (Perimed, Järfälla, Sweden) and was recorded on a Maclab recording system (ADInstruments, Castle Hill, NSW, Australia). Prior to placement of laser Doppler probes, the pancreas was isolated by retracting the stomach and liver superiorly and colon and small intestine inferiorly. A gauze swab was placed between the pancreas and left kidney by carefully lifting the spleen with the tail of pancreas, to ensure the laser Doppler light did not penetrate to the deeper tissue and measure perfusion of the deeper tissues, i.e. only PVP was measured. Laser Doppler probes (probe 407 with miniholder PH 07, fibre separation 0.25mm, wavelength 780 nm) were then placed on head, body and tail of the pancreas (one probe per site). Care was taken to place the probe on the head of the pancreas away from the site of PD ligation. All the

probes were placed in such a position so as to minimize the recording of artifacts such as those due to respiratory movement or excessively high perfusion resulting from placement of probes directly over major vessels. Moist gauze pieces were then placed on the exposed pancreas to prevent it from drying. An equilibration period of 15 minutes was allowed before taking baseline readings.

3.4.4 Induction of acute pancreatitis:

The protocol to induce pancreatitis was similar to that described in the previous **Section 2.4.4**. Galanin or galantide were given as a prophylactic administration in the doses as described in **Section 2.4.5**.

3.4.5 Parameters measured:

The following parameters were measured,

1. PVP in the head, body and tail of the pancreas
2. BP
3. PDP
4. Plasma levels of amylase and lipase activities

3.4.6 Analysis:

PVP, BP and PDP were continuously recorded. These physiological parameters were allowed to stabilise for 15 minutes. Baseline value of PVP, BP and PDP were taken by averaging a 1 minute period prior to administration of first dose of

CCK-8 and secretin in the AP group. In the galanin or galantide administration groups the baseline value of PVP, BP and PDP were taken by averaging a 1 minute period prior to administration of the respective bolus doses. Subsequently every 15 minutes, the mean of a 1 minute interval was analysed for the duration of experiment. The mean PVP data was also calculated by averaging the perfusion values of head, body and tail of the pancreas. PVP was expressed as percentage change from the baseline value. Conductance was analysed as the mean PVP divided by BP for each corresponding interval. The conductance was then expressed as the percentage change from the baseline. The BP and PDP values were expressed as percentage change from the baseline value. Care was taken to avoid analysis of the recordings where boluses of drugs or saline were administered and where the readings were unstable. If there was a fall in blood pressure to below 40 mm Hg, the experiment was aborted, as at this level vascular collapse would occur. The animals were then euthanized as described in **Section 2.4.4**. Samples of pancreas were not collected for histological analysis because the surface of pancreas was crusted as a result of Laser Doppler probes.

Plasma amylase and lipase activities were analysed and expressed as described in **Chapter 2 Section 2.4.8.3**.

3.4.7 Statistical analysis:

Statistical analysis was done using the raw data as described in **Section 2.4.11**.

3.5 Results:

3.5.1 BP:

There were no significant differences in the BP in the three groups for the duration of the experiment. The BP was similar to that described in **Chapter 2** There was about 20%-30% fall in the BP in the first hour in all the groups and after that it remained steady during the course of the experiment. The initial fall in BP was statistically significant when compared to the baseline levels in different groups ($p<0.05$).

3.5.2 PDP:

On cannulation of the pancreatic duct the PDP was found to be initially 5-8mmHg and gradually increased to 15 to 20 mmHg during the course of AP and then remained steady. There was no significant difference in PDP of either group. The PDP changes were similar to that described in Chapter 2.

3.5.3 PVP:

In the AP group there was an initial fall in PVP in the first hour by about 15% below the baseline values (**Figure 3.1 top**). PVP then increased gradually to reach the baseline levels by 1 h and continued to rise. At 4 h PVP was approximately 35% above the baseline level and then stayed above the baseline. In the AP plus galanin administration group, there was a fall in the PVP by 30% which did not recover throughout the duration of the experiment. In the AP plus galantide administration group, a sustained rise in PVP was evident.

The PVP in this group increased to approximately 105% above the baseline level. There was significant difference in PVP in galanin and galantide administration groups when compared to the AP group ($p<0.05$). There was no regional difference between the vascular perfusion of the head, body or tail of the pancreas in any group (**Figure 3.2**).

3.5.4 Pancreatic vascular conductance:

In the AP group the pancreatic vascular conductance per unit time showed a trend to increase from the baseline level but this was not significantly different (**Figure 3.1 bottom**). In the AP plus galanin administration group the pancreatic vascular conductance was similar to the baseline value and was not significantly different from the AP group. On the other hand with AP plus galantide administration group the conductance increased to about 220% above the baseline levels, peaked by 3 h and was then steady for the duration of the experiment ($p<0.05$ compared with the AP group).

3.5.5 Plasma enzymes:

The plasma enzyme levels were similar to that seen in the earlier experiments as described in **Section 2.5.3**.

3.6 Discussion:

These data suggest that in possum, AP is associated with rise in plasma amylase and lipase activities and a fall in PVP earlier in the course of AP which

is followed by a rise to above the baseline level. When galanin is administered in AP, the plasma amylase and lipase activities are significantly higher than those in the AP group and there was a prolonged and sustained fall in PVP. In contrast with galantide administration prior to induction of AP, amylase and lipase activities were significantly lower than the activities in the AP alone group and was accompanied by a prolonged and a sustained increase in PVP which was significantly greater than that observed in the AP alone group.

Microcirculatory disturbances can lead to AP. Severe AP is associated with both intrapancreatic and extrapancreatic vasospasm and decreased perfusion leading to ischemic damage and necrosis of the pancreas[247].

The initial fall in BP within the first hour in all the groups was statistically significant when compared the respective baseline levels. This fall was also noted and discussed in **Chapter 2**, **Section 2.6**.

In the AP group there was an initial fall in PVP, followed by a rise above baseline. This initial fall could have been as a result of decreased BP observed in the 1st h followed by a vascular rebound which increased the blood flow and hence perfusion of the pancreas. Alternatively, the initial decrease in PVP may be due to local vasoconstrictive effects, followed by vasodilation due to reactive hyperaemia, or by subsequent action of vasodilatory agents such as NO. It has been shown that NO is a potent pancreatic vasodialator[248] and has a protective effect[249],[250] in several animal studies of AP, possibly by increasing pancreatic

microvascular blood flow. It may also have deleterious effect[251] possibly due to oxidative stress or vasodilatation and organ hypoperfusion.

The results also suggest that overall there are no regional differences in PVP in various groups. In the tail of the pancreas of AP and galantide administration groups, PVP overlaps at 3 hours the underlying basis of this is unclear. This lack of regional differences in PVP would suggest that in spite of rich vascular supply to the pancreas; all regions are equally susceptible to injury during AP.

Galanin is a known vasoconstrictor and decreases PVP in possums at the dose used (**Section 1.8**). The galanin administration group demonstrated a fall in PVP which failed to recover. When galantide was administered with AP there was an increase in PVP to above baseline. This effect was immediate and sustained. This effect is consistent with galantide behaving as an antagonist to galanin receptors and suggests a role for endogenous galanin in regulating PVP.

Vascular conductance was calculated to assess the effects of galanin and galantide on pancreatic vasculature allowing for changes in BP during the course of the experimental period. In the AP group the vascular conductance showed a gradual increase over time consistent with either generalised developing vasodilation occurring over the duration of experiment or it may be due of CCK-induced splanchnic vasodilation. The conductance in the galanin treated group was not statistically different from the AP group. This suggests that at this dose, galanin has no effect on the PVP or that the relevant galanin

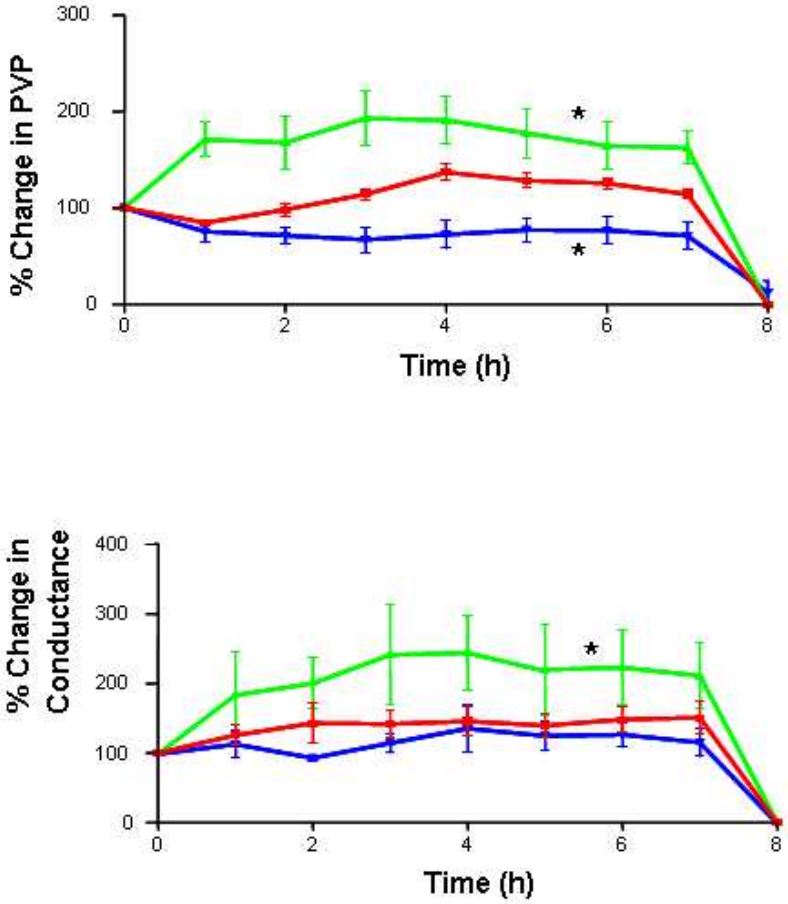
receptors in the pancreas are saturated and the endogenous galanin effect is maximal. The conductance in the galantide treated group was significantly greater than that in the AP group. This would suggest that galantide was causing vasodilation in the pancreatic vasculature. This vasodilatory effect of galantide could be due to either direct galanin receptor antagonism at the level of the blood vessels (endothelium and/or smooth muscle) or via secondary mechanisms e.g. less vasoconstriction resulting from reduced tissue damage.

The effects of galanin and galantide treatment on the plasma amylase and lipase activities is consistent with these agents influencing pancreatic exocrine secretion. It is not known whether galanin or galantide have any effect in pancreatic secretion in the setting of AP. As outlined in Chapter 1 Section 1.5.6, the reported effects of galanin on pancreatic exocrine secretion are not consistent. It is important to know whether galantide-induced, the fall in the plasma enzymes is the result of improved PVP and hence an improvement in pancreatitis or is the result of its direct action on the pancreatic secretion or a combination of both. To further answer this question experimental studies are required to define the role of galanin and galantide on basal and stimulated pancreatic exocrine secretion. Preliminary studies addressing this question are described in **Chapter 4**.

3.7 Conclusion

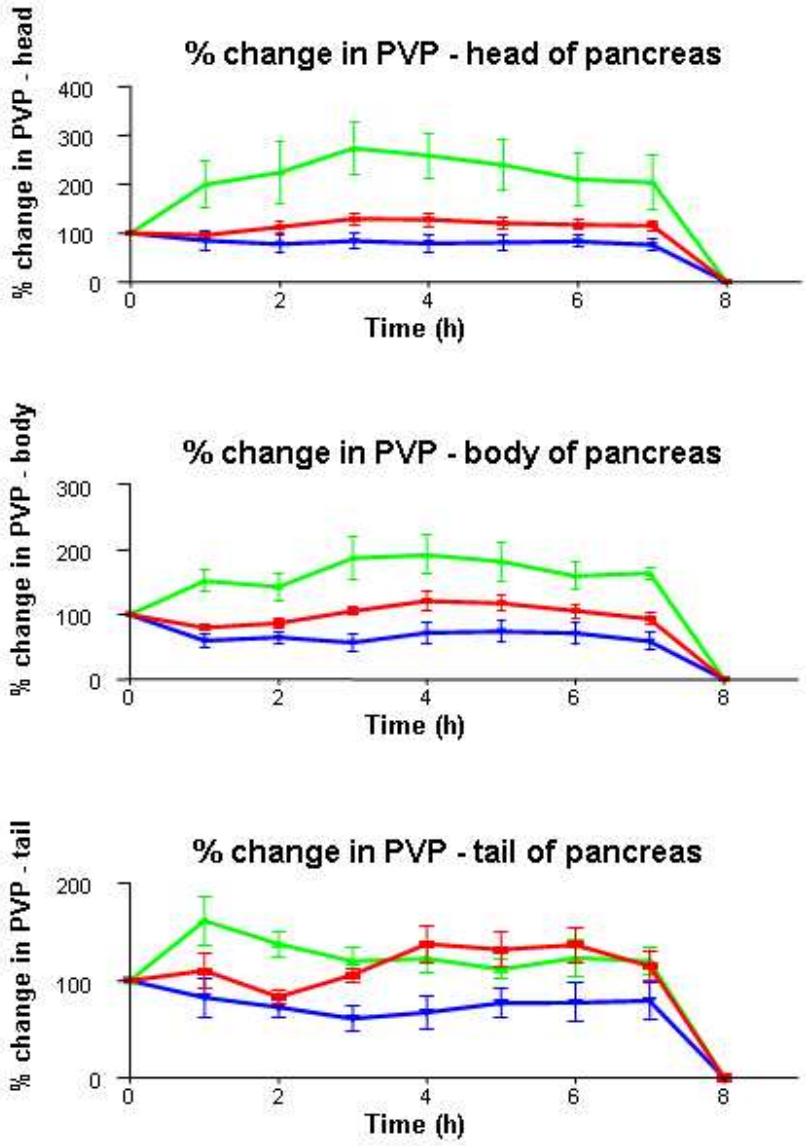
AP is associated with an initial fall in PVP, which is exacerbated by administration of galanin prior to onset of AP. Conversely, galantide administration prevents this decrease in PVP, and is associated with a rise in PVP through out the duration of the experiment. The changes in PVP are similar in different regions of the pancreas. Hence these findings suggest that galantide is reversing the microcirculatory disturbances seen in the early AP at least in part via its effect on PVP.

Figure 3.1. The change in PVP and conductance during AP with and without prophylactic treatment with galanin (GL) and galantide (GT).



The change in PVP (top) and conductance (bottom) are expressed as percentage of the mean value of the PVP from the head body and tail of the pancreas in the respective group - AP (red), galanin (blue) and galantide (green) administration. AP is associated with an initial fall in PVP which then recovers to baseline by 2hours and then rises above the baseline. Galanin administration is associated with a sustained fall in PVP which is significantly less than that observed in the AP group. In contrast, galantide administration is associated with increase in PVP which is significantly more than that in the AP group ($p<0.05$). AP is associated with an increase in pancreatic vascular conductance. Galanin administration is associated with no change in vascular conductance; galantide administration is associated with a significant increase in pancreatic vascular conductance ($p<0.05$). This suggested that galantide has a local vasodialatory action on the pancreatic vasculature. Data is presented as mean ± SEM (n=4-6). * $p < 0.05$, ANOVA.

Figure 3.2. The change in PVP in the head, body and the tail of the pancreas during AP and with or without prophylactic treatment with galanin (GL) and galantide (GT).



The change in PVP is expressed as percentage of the baseline value of the head body and tail of the pancreas in that group - AP (red), galanin (blue) and galantide (green) administration. The PVP changes in the head, body and tail of the pancreas were not significantly different in the respective groups. The fall in PVP to zero in each group at 8 h is due to euthanasia. Data is presented as mean ± SEM (n=4-6).

Chapter 4

Chapter 4: Effects of galanin and galantide administration on basal and hyperstimulated pancreatic exocrine secretion.

4.1 Introduction:

The data presented in the preceding chapters suggest that galanin and galantide treatment can modulate the AP-induced changes in hyperenzymemia, PVP and interstitial oedema within the pancreas. It is not known however, if some of these effects are due to altered pancreatic secretion. In this chapter preliminary studies were performed to determine the effects of exogenous galanin and galantide on basal and hyperstimulated pancreatic exocrine secretion *in vivo*.

4.2 Hypothesis:

Galanin will increase and galantide will decrease the pancreatic exocrine secretion.

4.3 Aims:

The aim of this study was to determine, if exogenous galanin or galantide alters the basal and hyperstimulated pancreatic exocrine secretion in the possum.

4.4 Methods:

The initial experiments were designed to measure the basal secretion rate with and without the administration of exogenous galanin or galantide. Preliminary

experiments were then designed to a dose of CCK-SEC in the hypersecretion range, 0.5 - 5µg/kg/h, which produced a stable and elevated exocrine secretion rate. This dose range of was selected as it was comparable to what was used to induce AP as described in the previous **Section 2.4.4**. Subsequent experiments were designed to ascertain if hyperstimulated pancreatic exocrine secretion could be influenced by administration of exogenous galanin or galantide at doses known to influence PVP and AP in the possums.

4.4.1 Pharmaceutical agents:

The pharmacological agents used in this part of the study are described in **Section 2.4.2**.

4.4.2 Basal pancreatic exocrine secretion study:

The possums were anaesthetized as described in the **Chapter 2 Section 2.4.2**. The experimental set up was the same as described in **Section 2.4.3**. After the cannulation of PD, the cannula was left open to collect pancreatic juice. After an initial stabilization period of 15 minutes, the pancreatic juice was collected every 30 minutes for the next 3.5 hours (**Figure 4.1**).

In separate groups of animals after the initial stabilization period galanin or galantide were administered as detailed in **Figure 4.1**.

4.4.3 Hyperstimulated pancreatic exocrine secretion study:

The experimental setup was similar to as described above. In the control group after an initial stabilization period of 15 minutes, the pancreatic juice was collected at half hourly intervals for the remainder of the experimental period. The basal pancreatic exocrine secretion was collected for 60 min. Then CCK-SEC intravenous infusion at 5µg/kg/h was commenced and continued for the remainder of the experiment (**Figure 4.2**). The study groups received either galanin or galantide 1.5 h after the onset of the CCK-SEC infusion. The galanin or galantide infusion was continued for a further 1.5 h and then terminated. Pancreatic juice was collected at 0.5 h intervals for a further 1 h.

The quantity of the juice collected per 0.5 h was measured gravimetrically and then stored at -20℃ for subsequent assessments of amylase and lipase activities and total protein.

4.4.4 Study groups:

The study groups in the basal pancreatic exocrine secretion study includes the control group in which basal secretion was measured (n=5), the exogenous galanin administration group (n=4) and the exogenous galantide administration group (n=4).

The various study groups in the hyperstimulated pancreatic exocrine secretion study to examine the quantity, enzyme and protein content of pancreatic exocrine secretion include the control group which received only CCK-SEC

(n=4) and the exogenous galanin and galantide administration groups (n=4 each) in which CCK-SEC was also administered.

4.4.5 Parameters analyzed:

1. The pancreatic exocrine secretion rate expressed as ml/30 min.
2. Pancreatic amylase and lipase enzyme activities in the pancreatic juice.

The plasma amylase assay kit (Roche/Hitachi Mannheim, Germany) and lipase assay kit (Sigma, St. Louis, USA) were used in conjunction with Hitachi Autoanalyzer (Roche/Hitachi Mannheim) to estimate pancreatic juice amylase and lipase activities.

3. Estimation of total protein in pancreatic juice:

BSA (Sigma) dissolved in saline (1mg/ml) was used to generate a standard curve. The BSA solution was aliquoted in 2,4,6,8 and 10µl in the wells of a micro-titre plate. The total reaction volume was 200µl consisting of 40µl of Bio-Rad reagent, various volumes of either BSA or diluted pancreatic juice and saline. The light absorbance was detected at 595nm using a micro plate reader Bio-Rad model 680 (Bio-Rad Lab, NSW, Pty, Ltd.). As pancreatic juice was rich in protein, all the samples were diluted 1:300 in normal saline prior to assay and compared to the standard curve. All the samples were assayed in duplicate and the values averaged. The protein content of pancreatic juice was expressed as mg/ml juice.

4.4.6 Analysis:

The baseline values for amylase and lipase activities and total protein secreted were obtained by averaging the basal values (usually 2) and the data is expressed as percentage change from the baseline value.

4.4.7 Statistical analysis:

The statistical analyses were performed as described in the **Section 2.4.11**.

4.5 Results:

4.5.1 Basal exocrine secretion:

The average basal exocrine secretion rate was approximately 0.197 ± 0.04 ml/30 min after stabilization. There was however no significant difference in the rates of secretion with and without galanin or galantide administration (**Figure 4.3**).

4.5.2 Hyperstimulated exocrine secretion:

Pancreatic secretion increased by 360% of the baseline rate following the onset of CCK-SEC stimulation in the possum model ($p<0.05$) (**Figure 4.4**). The stimulated secretion rate peaked at 30 minutes post CCK-SEC infusion onset and remained stable for the remainder of the experimental period. There was no significant difference in the stimulated secretion rate when galanin was administered. In contrast, galantide administration at 2.5 h resulted in a significant fall (by 28%) in the rate of secretion at 3.5 h ($p<0.05$) and when

galantide infusion was terminated there was a gradual recovery of rate of secretion to the level equivalent to that in the control group at 5 h.

4.5.3 Pancreatic amylase and lipase activities:

In the 3 groups where secretion was hyperstimulated, the amylase activity in the pancreatic juice increased from the baseline levels by 4-10 fold (**Figure 4.5**). Secretion peaked at 1 hour post CCK-SEC infusion onset and then declined to near baseline levels by 3 hours despite the continuous CCK-SEC infusion. In the galanin or galantide infusion groups there was a trend for the amylase activity to fall, but this was not significantly different from the activity in the control group. The lipase activity showed a similar trend to that of amylase activity with no significant difference in the enzyme activity in either the galanin or galantide treated groups compared to the control group (**Figure 4.5**).

4.5.4 Pancreatic total protein secretion:

The total protein concentration of the pancreatic juice showed a similar profile to that of the pancreatic enzymes with no significant difference in the concentration with the galanin or galantide administration compared to the CCK-SEC control group (**Figure 4.5**).

4.6 Discussion:

These data suggest that the basal rate of pancreatic exocrine secretion is not altered by galanin or galantide administration. However, galantide caused a

significant and reversible decrease in the hyperstimulated pancreatic exocrine secretion rate. There were no differences in the amylase or lipase activities and the total protein secretion between either group when compared with CCK-SEC hyperstimulation only group. This suggests that galantide given during CCK-SEC hyperstimulation resulted in lower volume of secretion but similar amounts of enzymes and proteins as compared to other groups.

In the hyperstimulated exocrine study, the control group (CCK-SEC) was associated with an increase in amylase and lipase activities, which peaks at 1 hour after commencement of infusion and then declined. This decline, while CCK-SEC is still being infused, could be due to either less enzymes secreted as a result of secretory block or secondary to zymogen exhaustion. The decline could also be due to reduced enzyme activity resulting from enzyme inactivation e.g. by trypsin. However, trypsin activity was not measured in the pancreatic juice.

CCK-SEC also increased the protein output initially, which then declined, perhaps for similar reasons to those mentioned above. With administration of galanin or galantide there was no difference in the protein output, but galantide administration resulted in decreased secretion rate of the pancreatic juice. Therefore, with galantide administration one would expect an increase in protein concentration. But, there was no difference in the total protein output measured. Hence, it may be possible that galantide is acting on the ductal cells to decrease their secretion of pancreatic fluid, without affecting enzyme or protein production by the acinar cells.

Acinar cell secretion can be induced by ingestion of food and or administration of secretagogues, which initiates multiple endocrine, neurocrine and paracrine mechanisms that regulate release of appropriate amounts of digestive enzymes. Stimulation with secretagogues such as CCK and secretin activate signal transduction pathways. CCK stimulates acinar cell secretion by activating inositol triphosphate (IP_3) / diacyl glycerol signalling pathways, thus leading to increased cytosolic Ca^{++} and protein kinase C activity. Secretin on the other hand stimulates secretion by elevating intracellular cyclic adenosine mono phosphate (cAMP) and thereby activating protein kinase A[252]. Secretin administration is associated with high volume, bicarbonate rich pancreatic juice production while CCK administration is associated with low volume, enzyme rich pancreatic juice production.

It would seem that galantide may act on secretin signal transduction pathway i.e. inhibits elevation of cyclic adenosine mono phosphate and thereby protein kinase A activity in the acinar cell leading to decreased quantity of pancreatic juice produced. It may also be possible that galantide is acting as an antagonist on galanin receptors on the ductal epithelium, but there is no evidence in the literature about the presence of galanin receptors on the duct epithelium. However, there is evidence regarding the presence of galanin positive nerve endings in the exocrine pancreatic panrenchyma[253], although the localization of these relative to acinar cells, islets, ducts or blood vessels is uncertain.

The literature concerning the effects of galanin on carbachol- and CCK-stimulated pancreatic amylase secretion from isolated pancreatic acini are contradictory[254, 255, 256]. Kashimura et al.,[256] have reported a direct and selective inhibitory action of galanin on carbachol-stimulated amylase release and the disappearance of this inhibition following pre-treatment with pertussis toxin. Their results suggest the presence of galanin receptors on rat acinar membranes. On the other hand, Herzig et al.,[255] demonstrated no direct inhibitory effect of galanin on CCK-8- and carbachol- stimulated amylase secretion on isolated pancreatic acini. Runzi et al.,[257] have shown that the effect of galanin on pancreatic exocrine secretion was dose dependent. They concluded that galanin significantly enhanced the CCK-8-stimulated amylase secretion in low doses (0.001 – 0.1pmol/ml) but high concentration (1-100pmol/ml) did not alter CCK-8-stimulated amylase secretion.

Insulin is known to potentiate pancreatic enzyme secretion via the insuloacinar axis[258, 259, 260]. Galanin is known to affect the endocrine pancreas by inhibiting the release of insulin and somatostatin and augmenting the release of glucagon[261, 262, 263]. Hence the inhibitory effect of galanin on pancreatic exocrine pancreatic secretion could be mediated via an indirect action on β-cells of the endocrine pancreas.

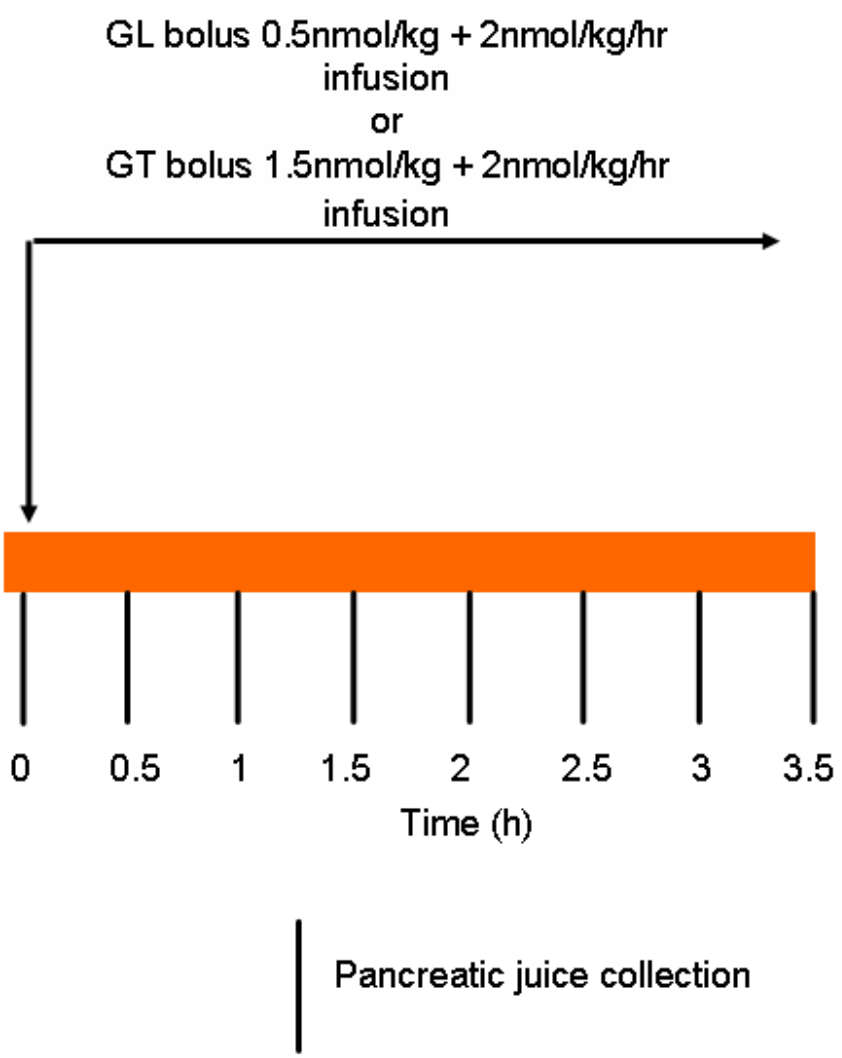
The present study must be considered preliminary only. As evident from the protocol, galanin or galantide were administered after commencing CCK-SEC infusion. The data indicate that even before galanin or galantide were

administered there was a fall in enzyme and protein levels consistent with depletion of exocrine secretory products or more likely secretory block, probably because of supra-physiological dose of CCK-SEC. Thus, one explanation for the observation that galanin and galantide did not affect the enzyme and protein secretion could be that enzyme secretory mechanisms were “exhausted”. It has been demonstrated by Kern et al., that in rats with supramaximal dose of caerulein there was an almost complete reduction of volume and protein output from the cannulated main pancreatic duct[264]. This was attributed to autodigestion of the pancreas. Various studies have used CCK-SEC to stimulate pancreatic exocrine secretion[256, 257, 265] in a manner similar to this study, but the dose has been considerably smaller. Therefore, it may be recommended for future studies to use a range of doses and protocols for administration of CCK-SEC, galanin or its antagonists.

4.7 Conclusion

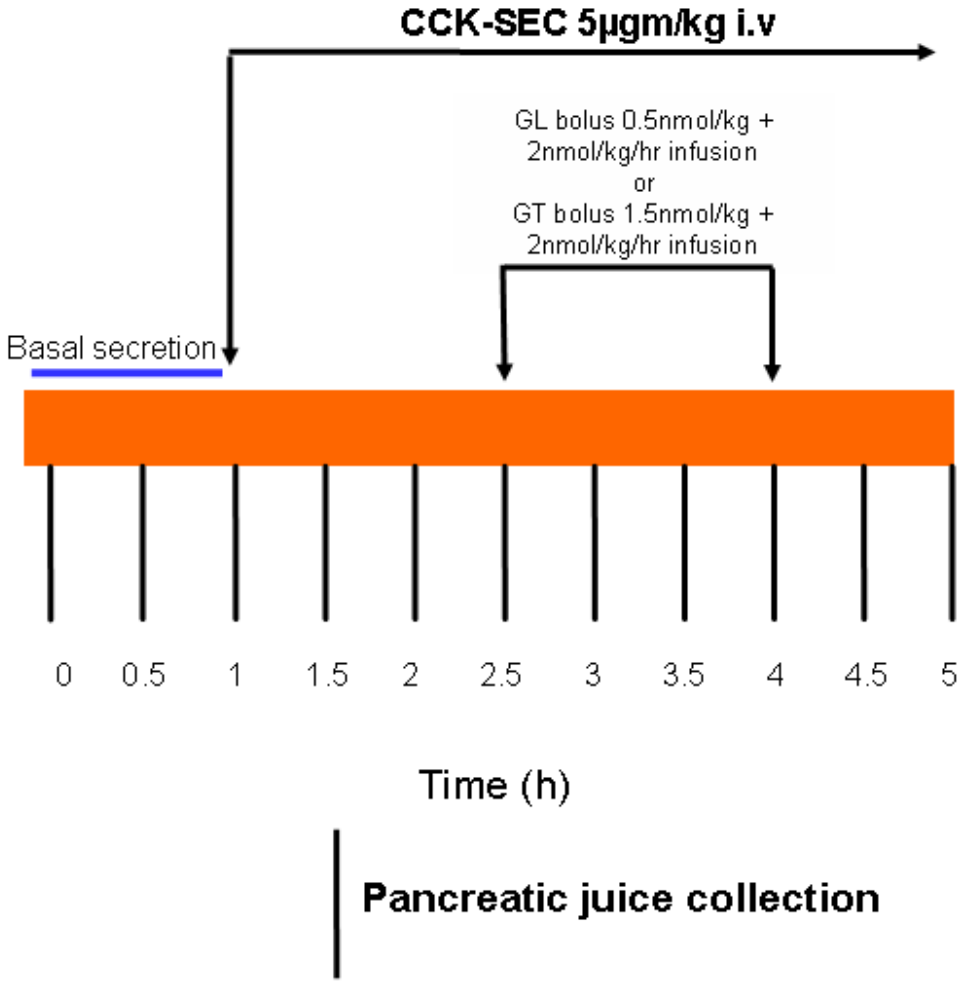
In view of the present findings it is difficult to derive any firm conclusion from this pilot study. It appears that galantide treatment alters ductal cell secretion. Further studies are required to define the role of galanin and galantide on pancreatic exocrine secretion.

Figure 4.1 . Schematic representation of protocol for collection of basal secretion of pancreatic juice in the possum.



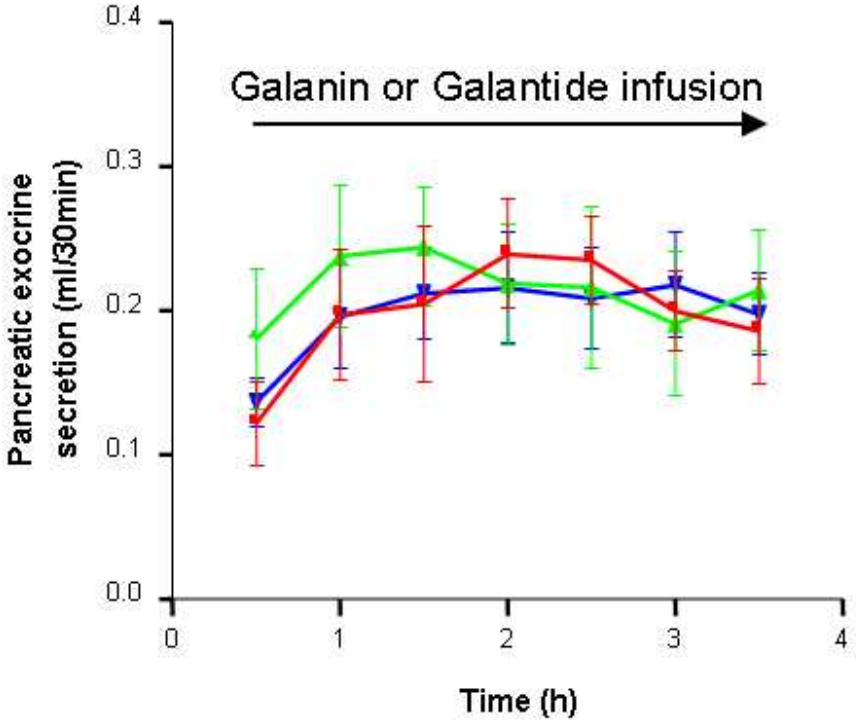
Pancreatic juice was collected at 30min interval. The basal pancreatic secretion was measured before administration of galanin (GL) or galantide (GT) infusion as indicated.

Figure 4.2 . Schematic representation of protocol for collection of hyperstimulated secretion of pancreatic juice in the possum.



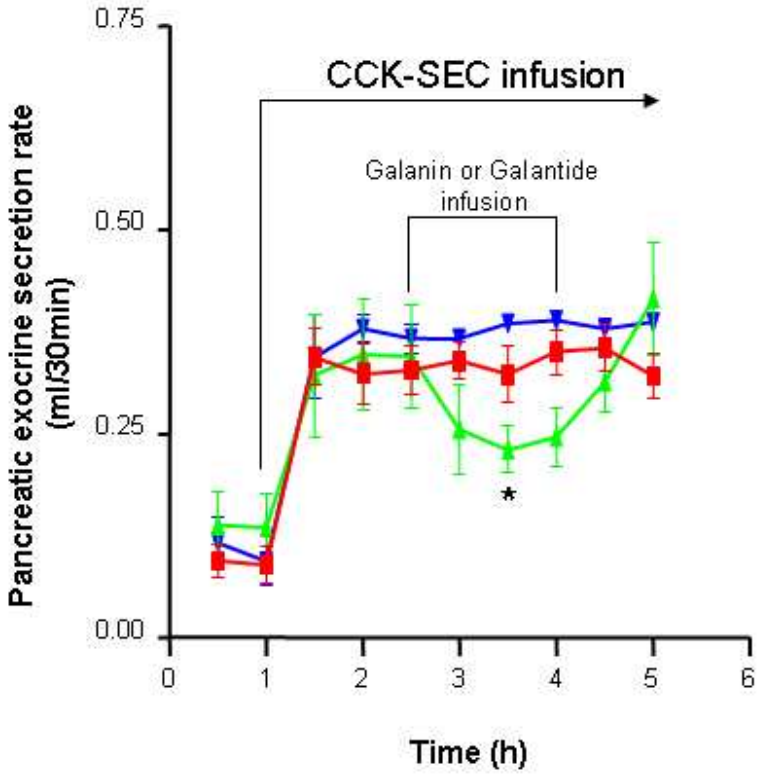
Pancreatic juice was collected at 30min interval. The pancreatic exocrine secretion was hyperstimulated with cholecystokinin (CCK) and secretin (SEC) infusion. Galanin (GL) or galantide (GT) were infused at the dose and rate indicated for a 90 min period commencing at 2.5hr .

Figure 4.3. Effect of galanin and galantide administration on basal pancreatic exocrine secretion rate.



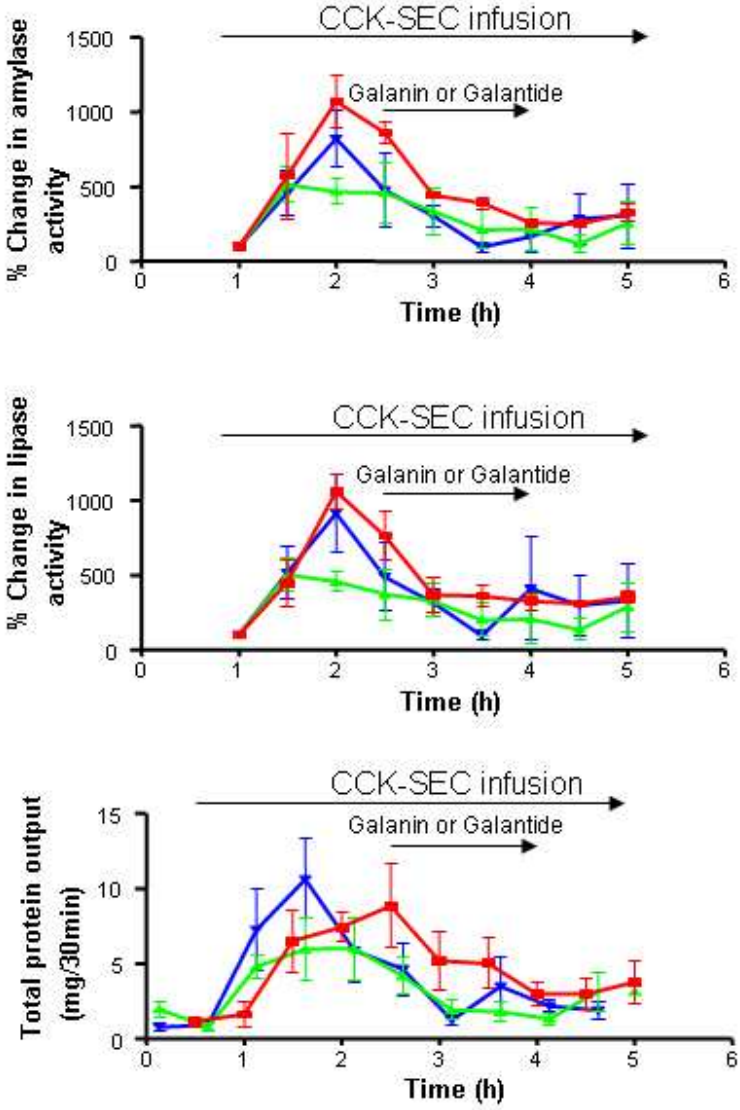
Pancreatic exocrine secretion rate in different groups basal secretion (red), galanin (blue) and galantide (green) is shown. Administration of galanin or galantide did not alter the basal exocrine secretion rate. Data is presented as mean ± SEM (n=4-5) ($p>0.05$ compared with basal secretion group, ANOVA).

Figure 4.4. Effect of galanin and galantide administration on CCK-SEC stimulated exocrine secretion rate.



Effect of exogenous galanin (blue) and galantide (green) on CCK-SEC stimulated exocrine secretion (red) is shown. CCK-SEC administration significantly increased the rate of pancreatic exocrine secretion. Administration of galanin did not alter the rate of hyperstimulated exocrine secretion. Administration of exogenous galantide significantly decreased the rate of exocrine secretion which recovered to the pre-administration rate at 5h. Data is presented as mean ± SEM (n=4) (* $p<0.05$ compared with CCK-SEC group, ANOVA).

Figure 4.5. Percentage change in the amylase and lipase activities and total protein output in the CCK-SEC stimulated pancreatic exocrine secretion with galantide and galanin administration.



Administration of galanin (blue) or galantide (green) did not alter the amylase, lipase activity and total protein output in the CCK-SEC (red) stimulated pancreatic exocrine secretion. Data is presented as mean of percentage change in amylase, lipase activities and total protein ± SEM (n=4) ($p>0.05$ compared with CCK-SEC group, ANOVA).

Chapter 5

Chapter 5: Effects of galanin and galantide administration on AP in the caerulein mouse model.

5.1 Introduction

In **Chapter 3**, the effects of galanin and galantide in the possum model of AP were described. There were significant differences in the pancreatic oedema between the different groups studied, but there was no difference in the degree of necrotic damage in the different groups. This was attributed to the mild AP induced in this particular model. In order to study the effect of galanin and galantide in AP in another model, the caerulein mouse model of AP [266] was selected. This decision was based on the following considerations, firstly the need to determine if galantide's ability to ameliorate AP occurred in a different species and secondly, to confirm the role of endogenous galanin in the induction and/or progression of AP. The mouse model was selected because it is commonly used and produced a more severe form of AP compared to the possum model used in the **Chapter 2**. In addition, galanin and galanin 1 receptor knockout mice are available which could be used in studies to provide further evidence to support (or reject) the overall hypothesis for a role of galanin in development of AP.

Galanin positive immunoreactive nerves have been demonstrated in abundance in the mouse pancreas in association with the blood vessels[267]. This confirms the presence of galanin as a neurotransmitter in the mouse pancreas, but its role in AP has not been studied.

Caerulein is a synthetic decapeptide that was originally isolated from the skin of the Australian amphibian Hyla caerulea. Caerulein shares seven of its eight C-terminal amino acids with the C-terminal octapeptide of cholecystokinin (CCK). Hence it has stimulatory actions on exocrine pancreatic secretions similar to those of CCK. Caerulein induces a mild to severe form of AP proportional to the dose and duration of caerulein application.

Pancreatitis induced by caerulein hyperstimulation has been shown to cause a rise in intracellular calcium and disruption of acinar cell calcium signalling. This is associated with acinar cell vacuolization and intracellular trypsinogen activation seen in early AP[268]. Caerulein administration induces biochemical and morphological changes similar to acute oedematous pancreatitis[269].

The mouse studies described are conducted in three different strains of mice. Firstly, the Balb C strain in which the effects of galanin and galantide in AP were assessed. Then C57BL/6 background mice were used to further define the role of endogenous galanin in AP (**Chapter 6**); finally Swiss strain mice were used to study the role of other galanin antagonists in AP (**Chapter 7**).

5.2 Hypothesis

Exogenous galanin will increase and exogenous galantide will decrease the severity of AP in the caerulein mouse model.

5.3 Aim

To determine, in the Balb C strain of mice, the effect galanin or galantide administration, before and after AP induction on the severity of AP in the caerulein mouse model of AP

5.4 Methods

5.4.1 Animal ethics approval

The mice studies were approved by the Animal Welfare Committee of the Flinders University {project approval number 603/06(b)}. All procedures were carried out with the principles outlined in the Australian Code of Practice for Animal Care and Use.

A day before the experiment the animals were randomly assigned to various experimental groups (**Table 5.1**). A blood sample was collected from each mouse by orbital bleeding under general anaesthesia. This was used to assess the baseline activities of plasma amylase and lipase. All the mice were fasted overnight, but had free access to water.

5.4.2 Animals:

Female Balb C mice 4-6 weeks of age, weighing 15 – 24 g were used in the study.

5.4.3 Pharmaceutical agents:

For orbital bleeding, the mouse were anaesthetized using I.P. injections of ketamine (Parnell lab, Australasia Pty. Ltd, Alexandria, NSW, Australia) and medetomidine (Domitor®, Novartis Animal Health, Auckland, New Zealand). Animals were recovered from anaesthesia by an I.P. injection of atipamazole (Antisedan®, Novartis Animal Health, Auckland, New Zealand). Caerulein (American Peptide Company, Inc. Sunnyvale, CA, USA) was used to induce AP. Galanin and galantide were obtained from the American Peptide Company, Inc. Saline containing BSA (Sigma®, St. Louis, MO, USA) was used as a vehicle to administer peptides. Subcutaneous injection of buprenorphine (Temgesic® Inject, Reckitt Benckiser Healthcare UK Limited, Hull, UK) was used to provide analgesia for the duration of the experiment.

5.4.4 Orbital sinus bleeding:

A day prior to experimentation in mice, blood (100µl) was collected from one orbital sinus using a heparinized capillary tube[270.] Following blood collection the tube was centrifuged at 10000 rpm for 10 min at room temperature. The plasma was collected and stored at -20℃ for subsequent estimation of amylase and lipase activities.

5.4.5 Protocol for induction of AP:

The mice received seven I.P injections of caerulein (50 µg/kg) in 150 µl of 0.9% NaCl at hourly intervals over 6 h. Buprenorphine (0.1mg/kg) was administered subcutaneously at the time of the first caerulein injection. Twelve hours after the

first injection the mice were anaesthetised and post treatment blood sample was collected by orbital sinus bleeding. The mice were then euthanized by exsanguination and the pancreas was harvested for assessment of pancreatic myeloperoxidase (MPO) levels and histological examination to determine the severity of AP. As described in **Section 1.6.10.2**, MPO is commonly used as a marker of neutrophil infiltration associated with AP, hence pancreatic MPO was used to determine degree of inflammation within the pancreas. About 1/4th of the harvested pancreas was stored at -80℃ in the freezer for subsequent MPO activity estimation. The remaining pancreas was fixed overnight in 10% buffered formalin solution (Orion Laboratories Pty ltd, Perth, WA, Australia) for histological examination (**Figure 5.1**).

5.4.6 Prophylactic protocol:

In separate groups of mice together with AP induction, I.P galanin or galantide bolus injections were administered with each caerulein injection. Galanin was administered in 10nmol/kg and galantide in 10, 20 and 40nmol/kg dose in separate groups of mice (**Table 5.1**). The control groups comprised of mice which received saline (150µl), galanin alone 10nmol/kg or galantide alone 10, 20 and 40nmol/kg injections (**Figure 5.1**).

5.4.7 Therapeutic protocol:

In this group, AP was induced as described above but galantide (40nmol/kg) was administered 2 h post onset AP and continued as bolus injections with subsequent caerulein injections.

5.4.8 Study groups:

The various study groups are as described in the **Table 5.1**.

Table 5.1 Study groups.

Study groups	Number of animals, n
AP alone	6
AP + prophylactic galanin 10nmol/kg/h	6
AP + prophylactic galantide 10nmol/kg/h	5
AP + prophylactic galantide 20nmol/kg/h	6
AP + prophylactic galantide 40nmol/kg/h	6
Saline only hourly	7
Galanin 10nmol/kg/h	3
Galantide 20nmol/kg/h	4
Galantide 40nmol/kg/h	7
AP + therapeutic galantide 40nmol/kg/h	5

5.4.9 Pancreas harvest:

The pancreas was harvested for MPO level estimation and histological assessment of severity. A midline laparotomy was performed. The pancreas

was identified and carefully dissected from its attachments to the stomach, duodenum and spleen. Fat and connective tissue were trimmed away. The pancreas was then rinsed with saline and blotted dry on blotting paper. It was then divided and weight of each portion was recorded.

5.4.10 Parameters measured:

5.4.10.1 Plasma amylase and lipase estimation:

Plasma amylase and lipase activities were estimated as described in **Section 2.4.8.3**.

5.4.10.2 Histological examination:

Formalin fixed pancreata were embedded in paraffin blocks. 5µm sections were cut and stained with Haematoxylin and Eosin. The slides were then examined by an independent and experienced pathologist unaware of the experimental details. Fifteen randomly chosen microscopic fields were examined. The histological scoring was performed by assessing the degree of oedema, inflammatory infiltrate, vacuolization and number of necrotic cells as outlined in the **Table 5.2** and was based on the method described by Niederau et al,[267]. Each parameter had a different grade and depending on the grade a score was given. A high score suggested a more severe grade. The modified Spormann's scoring system for AP in the possums was not used in the mice studies because of the difference in the characteristics in AP seen in the mouse i.e. more vacuolization, fat and parenchymal inflammation and necrosis.

Table 5.2. The scoring system used to assess the degree of pancreatic tissue damage.

PARAMETERS	GRADE	SCORE
0edema	Not diffuse – +,++,+++	1
	Diffuse	2
	Severe diffuse	3
Fat inflammation	Mild, moderate, severe	1-3
Parenchymal inflammation	Mild, moderate, severe	1-3
Individual cell necrosis	**Upto10%**	
	< 10cells	1
	10-50cells	2
	50-100cells	3
	100-200cells	4
	200-500cells	5
	500-1000	6
	1000-2000	7
	2000-5000	8
	10-20%	**9**
	20-30%	**10**
	30-40%	**11**
	40-50%	**12**
	>50%	**13**
Vacuolization	**Upto10%**	
	< 10cells	1
	10-50cells	2
	50-100cells	3
	100-200cells	4
	200-500cells	5
	500-1000	6
	1000-2000	7
	2000-5000	8
	10-20%	**9**
	20-30%	**10**
	30-40%	**11**
	>50%	**12**

5.4.10.3 *MPO assay:*

The protocol for MPO extraction was modified from that described by Bhatia et al.,[126]. The frozen samples of pancreata were thawed and homogenised in 1.5ml of ice-cold 50 mM phosphate buffer (pH 6). The homogenate was centrifuged for 10 min in an Eppendorf micro-centrifuge at 14,000 rpm at 4°C. The resulting pellets were resuspended in 1ml ice-cold 50 mM phosphate buffer (pH 6) and re-centrifuged. The pellet was then suspended in ice cold 0.5ml 0.5% hexadecyltrimethylammonium bromide (Sigma®) dissolved in saline. The suspension was subjected to three cycles of freezing/thawing and the homogenate was further disrupted by sonication (Sonifier® B-12, Banson Sonic Power Company, Danbury, Connecticut). Sonication was performed twice for a 5 s period with 10 min between each bout. The solution was then centrifuged for 10 min at 14,000 rpm at 4°C and the supernatant was collected for the MPO assay. All extractions were performed on ice and using ice cold reagents.

The assay was based on the method as described by Bhatia et al.,[271]. The reaction mixture consisted of supernatant (100µl), o-dianisidine dihydrochloride (2mg in 9ml distilled water) and 0.1% hydrogen peroxide (2ml). This mixture was incubated at 25°C and absorbance at 460nm was measured in a Cobas Bio Auto Analyzer. The activity was then corrected for the wet weight of tissue sample used, and the results were expressed as activity (U) per mg of wet weight pancreatic tissue.

5.4.11 Statistical analysis:

The statistical analysis was performed using the statistical package SPSS (version 11.5; SPSS inc., Chicago,III. USA). All data are expressed as mean ± standard error of mean (SEM) of n number of animals. If the data was normally distributed, it was analysed using ANOVA. If ANOVA indicated a significant difference, the data was further analysed using Student's t-test where appropriate. The data that was not normally distributed was evaluated using non-parametric tests; Mann-Whitney rank when data consisted of two groups and Kruskall-Wallis test when 3 or more groups were analysed. Statistical significance was accepted at the $p<0.05$ level. Absence of error bars in any of the figures indicates that the SEM was too small to be illustrated.

5.5 Results

5.5.1 Plasma amylase and lipase activity:

The plasma amylase and lipase activities for various groups are shown in **Figure 5.2**. AP induced a 9 fold increase in plasma amylase activity when compared to its pre treatment level (panel A). Prophylactic administration of galantide (20 and 40nmol/kg/h) inhibited the AP-induced rise in plasma amylase activities by 35 and 38% of the level in the AP alone group (both $p<0.05$). Prophylactic administration of galanin (10nmol/kg/h) and galantide (10nmol/kg/h) were without a significant effect on AP-induced plasma enzyme levels (panel A). All control groups displayed a plasma amylase activity which was significantly

different from that of the AP group, but not different from the pre treatment levels.

Similarly, plasma lipase activity was significantly decreased by treatment with galantide (20 and 40nmol/kg/h) by approximately 40% of that observed in the AP group ($p<0.05$). The plasma lipase activity in AP was unaffected by treatment with galanin and galantide at 10nmol/kg/h (panel C). All control groups displayed plasma lipase activity which was significantly different from that of the AP group (panel D). The plasma lipase activity in the groups receiving galantide (10 and 20nmol/kg/h) were not different from the pre treatment activity, however the activity in the groups receiving 40nmol/kg/h galantide and saline were elevated above the pre treatment groups ($p<0.05$).

When galantide 40nmol/kg/h was administered therapeutically i.e. 2 h post AP onset, there was an apparent fall in the plasma amylase activity but it was not statistically significant. On the other hand, the plasma lipase activity was reduced by 40% of the AP level ($p<0.05$) (**Figure 5.3**).

5.5.2 Pancreatic MPO activity:

Pancreatic MPO activities for various groups are shown in **Figure 5.4**. As expected the MPO activity in the AP group was significantly greater than the control groups. Prophylactic treatment with galantide (10 and 40nmol/kg/h) reduced the MPO activity by approximately 70 and 60% respectively of that in

the AP group ($p<0.05$).The MPO activity of the prophylactic galanin 10nmol/kg/h and galantide 20nmol/kg/h was not significantly different from the AP group.

When galantide 40nmol/kg/h was administered therapeutically there was no significant difference in the MPO activity when compared to the AP group (**Figure 5.5**).

5.5.3 Histological assessment:

The degree of fat and parenchymal inflammation and oedema were similar in all the treatment groups with AP. The mean necrosis score in the AP group was 5.83 (**Figure 5.6**). With prophylactic administration of galantide at 20 or 40nmol/kg/h doses the necrosis score was significantly reduced by 75% and 45% respectively ($p=.0001$ and $p=.031$). In contrast, with prophylactic administration of galanin and galantide 10nmol/kg/h there was no significant difference in the necrosis score compared with that observed in the AP group (**Figure 5.6 and 5.7**).

The vacuolization score was significantly greater for the AP group when compared with that for the control groups but not with the galanin 10nmol group ($p<0.05$). The AP group also had significantly higher vacuolization score than the group which had prophylactic administration of galanin 10nmol/kg/hr ($p<0.05$). With other prophylactic administration groups there was no difference in the degree of vacuolization when compared to the AP group except with

administration of galantide 10nmol/kg/h produced significantly more vacuolization than the AP group ($p<0.05$) (**Figure 5.6**).

The therapeutic administration of galantide reduced the necrosis by approximately 35% of that estimated in the AP group ($p<0.05$) (**Figure 5.8**). On the other hand vacuolization score (**Figure 5.8**) was approximately 50% greater in the therapeutic galantide administration group compared to the AP group ($p<0.05$).

A summary of results of different parameters measured is illustrated in **Table 5.3**.

Table 5.3. Summary of results of various groups in the prophylactic and therapeutic protocol.

Parameter	Prophylactic protocol				Therapeutic protocol
	AP+GL10	AP+GT10	AP+GT20	AP+GT40	AP+GT40
Plasma amylase	↔	↔	↓	↓	↔
Plasma lipase	↔	↔	↓	↓	↓
MPO	↔	↓	↔	↓	↔
Necrosis score	↔	↔	↓	↓	↓
Vacuolization score	↓	↔	↔	↔	↑
Interstitial oedema	↔	↔	↔	↔	↔
Parenchymal inflammation	↔	↔	↔	↔	↔
Fat inflammation	↔	↔	↔	↔	↔

↔ - no change, ↑ - significantly increased, ↓ - significantly decreased. AP+GL10 - galanin 10nmol/kg/h administered in AP, AP+GT10 - galantide 10nmol/kg/h administered in AP, AP+GT20 - galantide 20nmol/kg/h administered in AP, AP+GT40 - galantide 40nmol/kg/h administered in AP. All the results are relative to the AP group.

5.6 Discussion

The data presented in this chapter shows that prophylactic treatment with galantide reduced the indices of AP. On the other hand administration of galanin did not influence the AP induced changes (**Table 5.3**). In addition, the therapeutic administration of galantide also reduced most indices of AP (**Table 5.3**). Hence these data suggest that galantide may be an effective treatment for AP, supporting the findings described in the possum studies.

The mechanism(s) by which galantide has beneficial effects is unclear. As described in **Chapter 3**, galantide does affect PVP and may alter stimulated pancreatic exocrine secretion (**Chapter 4**). Both these actions of galantide could contribute to amelioration of AP. Galantide administration was associated with decrease in AP-induced plasma amylase and lipase activities. This could be due to effects on the secretory mechanism of the acinar cells or as a result of less acinar cell damage. Li et al., have demonstrated that in the mouse pancreas all 3 galanin receptors are expressed, but GalR2 and GalR3 are most abundant[272]. It may be possible that either or both of these receptors mediate secretory processes relevant to AP. On the other hand galantide could ameliorate the PVP changes expected in AP (**Chapter 3**), resulting in less acinar cell damage, and thus less enzyme release into the blood stream.

Galanin acts via its receptor namely Gal1, Gal2 or Gal3. Its effect on pancreas may be as a result of action on one or a combination of its receptors. Galanin receptors belong to G – protein family of receptors. Actions of galanin are

mediated via a high-affinity G_i/G_o protein-coupled receptor involving a number of intracellular mechanisms, namely opening of K^+ATP channels, NO/cGMP-dependent mechanisms, and activation/inhibition of adenylate cyclase[273],[274].

As evident from the results galantide administration in AP was associated with significantly less plasma enzyme levels. The pancreatic enzymes are synthesized, packaged, stored, and released from acinar cells by a process of exocytosis. Most of the packaging of enzymes is done in the Golgi complex. A membrane bound G-protein facilitates the transfer of enzymic proteins from endoplasmic vesicles into the cisterns[275]. Galantide being an antagonist may inhibit the transfer of these enzymic proteins and thereby decreasing the release of pancreatic enzymes and hence attenuating AP.

Steinle et al.,[276] have reported that induction of AP in rats by supramaximal doses of caerulein promotes rapid translocation of NF-κB into the nuclei of acinar cells and this activates transcription of a spectrum of proinflammatory genes and precedes pancreatic injury and inflammation. It has also been reported by Marrero et al.,[277] that Gal 1R is upregulated by NF-κB in the colonic epithelium of mice with inflammatory bowel disease. Hence it may be possible that during caerulein induced pancreatitis there is an up-regulation of galanin receptors in the pancreas. Hence there would be more binding sites available for galanin and this could potentially have adverse effects in AP.

It has been shown that galanin via its action on galanin receptors may lead to reduction in intracellular Ca^{++} concentrations in most cell types and thereby

reduce transmitter release and inhibit other Ca^{++}- dependent processes such as pancreatic secretion[278]. However, the actual mechanism of the effect of galanin on Ca^{++} ion handling in pancreatic acinar cells is currently unknown.

The actions of galanin may also be mediated via cholinergic neural mechanisms leading to inhibition of acetylcholine release[279]. The efferent vagus nerve has been implicated as an important anti inflammatory pathway. Studies have shown that a variety of nicotinic acetylcholine receptors are present on neutrophils and stimulation of these nicotinic receptors inhibit neutrophil migration and hence inflammation[280]. Galanin via its effects on cholinergic suppression may increase neutrophil migration and hence MPO levels and tissue damage as seen in AP.

Prophylactic galantide administration reduced the AP-induced rise in MPO activity. This could be either as a result of less pancreatic damage leading to less neutrophil recruitment or indirectly via its action on ICAM-1. But the evidence regarding this is lacking. Future studies using ICAM-1 knock-out mice and galantide would be of interest.

It has been reported that galanin is able to induce an up-regulation of inflammatory cytokine interleukin-1 α (IL-1α) and IL-8 and increase expression of TNF-α mRNA from keratinocytes[281]. Yasuda et al., have reported an up-regulation in the expression of IL-1α and TNF- α mRNA in AP after pancreatic duct ligation[282]. These mediators are pro-inflammatory and are involved in neutrophil activation and chemotaxis[283]. If galanin up-regulates IL-1α and IL-8 and increases expression of TNF-α mRNA in the pancreas then it could

potentially play a role in the onset or progression of AP by enhancing the migration of neutrophils via IL-1α, IL-8 and/or TNF- α mediated mechanisms.

Jimenez-Andrade et al.,[284] have shown that peripheral galanin increases acute inflammatory pain through activation of protein kinase C intracellular pathways. It is well known that kinases play a crucial role in expression and activation of inflammatory mediators[285]. In AP one of the critical event is disruption of the actin cytoskeleton of the acinar cell[286, 287, 288] . This disruption of the actin cytoskeleton is mediated by protein kinase C dependent pathway[289]. Hence it may be possible that galanin may play a role in AP by leading to pancreatic damage by activation of a protein kinase C dependent pathway.

As discussed in the **Section 3.6**, the beneficial effects of galantide may be mediated in part by release of NO, possibly due to presence of 5- to11-amino acid fragment in galantide that may mimic substance P-like action, causing an endothelium dependent vasodilation. Bivalacqua et al., have shown that penile erection in cats caused by antagonist galantide is mediated by NO/cGMP pathways as this was significantly decreased by NO inhibitor L-NAME. NO produced by endothelial cells is an important regulator of vascular tone i.e. increases capillary blood flow[290,291,292]. It has been shown that the pancreatic blood flow is reduced by 50% during AP in the caerulein model[293]. Hence the presence of endothelial NO may be beneficial. NO also inhibits neutrophil adhesion and accumulation in small vessels[294].

Runzi et al.,[295] have reported that galanin augmented CCK-stimulated amylase release in the isolated perfused rat pancreas. But interestingly Brodish et al.,[296] have reported inhibitory effect of galanin on CCK stimulated dog pancreas. Our findings in the mouse suggest that galanin administration in caerulein induced AP does not augment amylase and lipase release. The possible explanation for these divergent findings is unclear, but may relate to differences in species or model.

Galantide is a chimeric peptide consisting of N-terminal portion of galanin (1-13) and C-terminal portion of substance P. This may result in galantide acting as substance P antagonist. It is known that substance P when released from nerve endings binds to neurokinin-1 receptor on effector cells, increases microvascular permeability, and promotes plasma extravasation from intra vascular to extra vascular space[297]. Pancreatic acinar cells are known to express neurokinin-1 receptor, and substance P has been detected in the pancreas[298, 299]. Studies have also shown that levels of substance P and the expression of neurokinin-1 receptor is increased on pancreatic acinar cells during experimental AP[300]. Hence the beneficial effects of galantide may be as a result of substance P antagonism. Therefore it is important to evaluate other galanin antagonist in AP as they do not have Substance P fragment (**Chapter 7**). This will further establish the role of galanin antagonism in amelioration of AP.

The results of therapeutic administration of galantide in AP were not as dramatic as prophylactic administration. In the therapeutic administration group the MPO

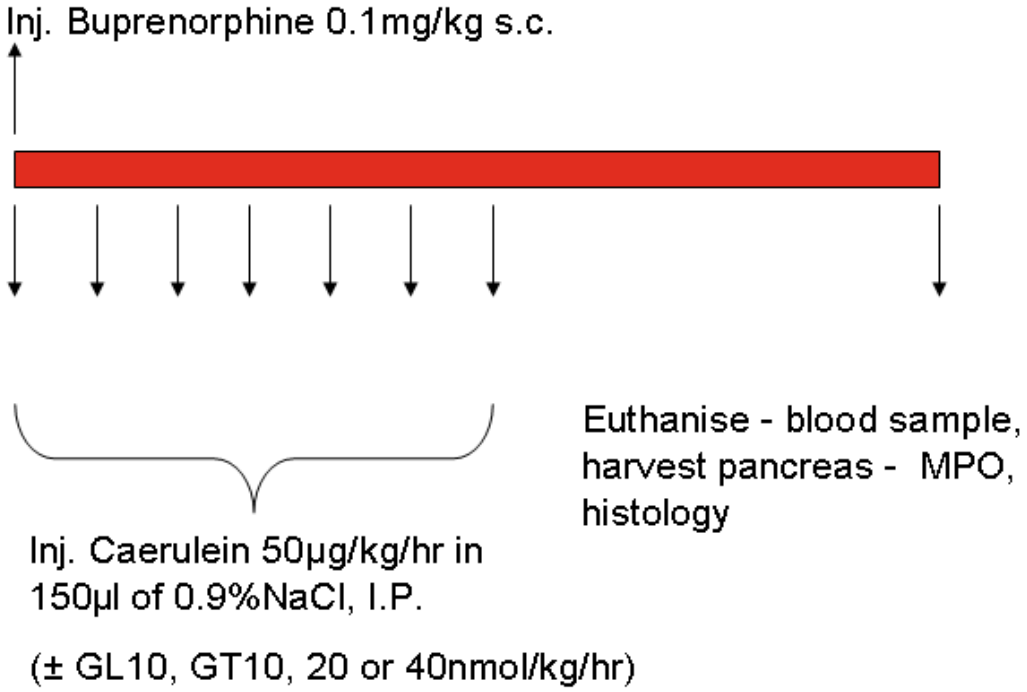
levels were not significantly different form the AP group. This could probably suggest that galantide was acting during the early events in AP.

Interestingly, galantide treatment increased vacuolization in acinar cells, however the significance of this finding is unclear. The true role of vacuolization in the pathogenesis of AP is questionable as this phenomenon, also known as compound exocytosis, is observed in other settings and may reflect physiological event which is likely to increase the release digestive enzymes into the duct[301].

5.7 Conclusion

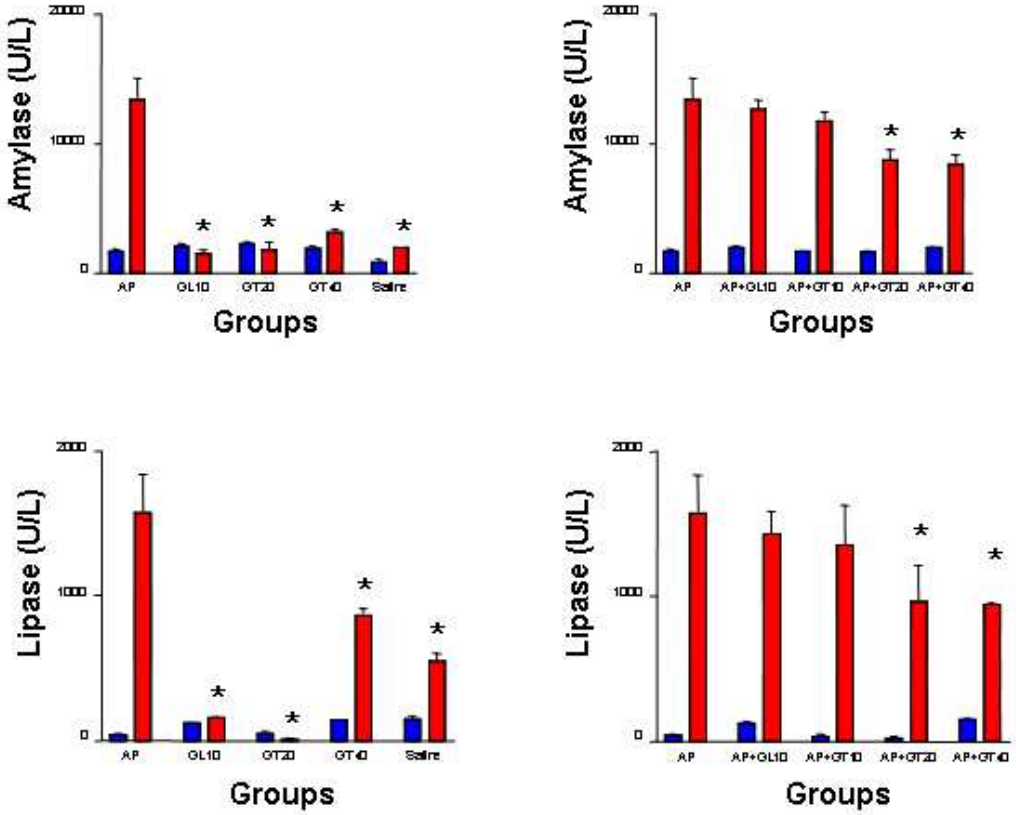
It appears that exogenous administration of galantide has a beneficial effect in AP in the mouse model. This could be due to various mechanisms as described above. Hence galanin was modulating AP in two species, the possum and the mouse. To further strengthen the hypothesis the role of endogenous galanin (**Chapter 6**) and the effects of other galanin antagonists (**Chapter 7**) in AP were studied.

Figure 5.1. Schematic representation of protocol to induce AP in the mouse.



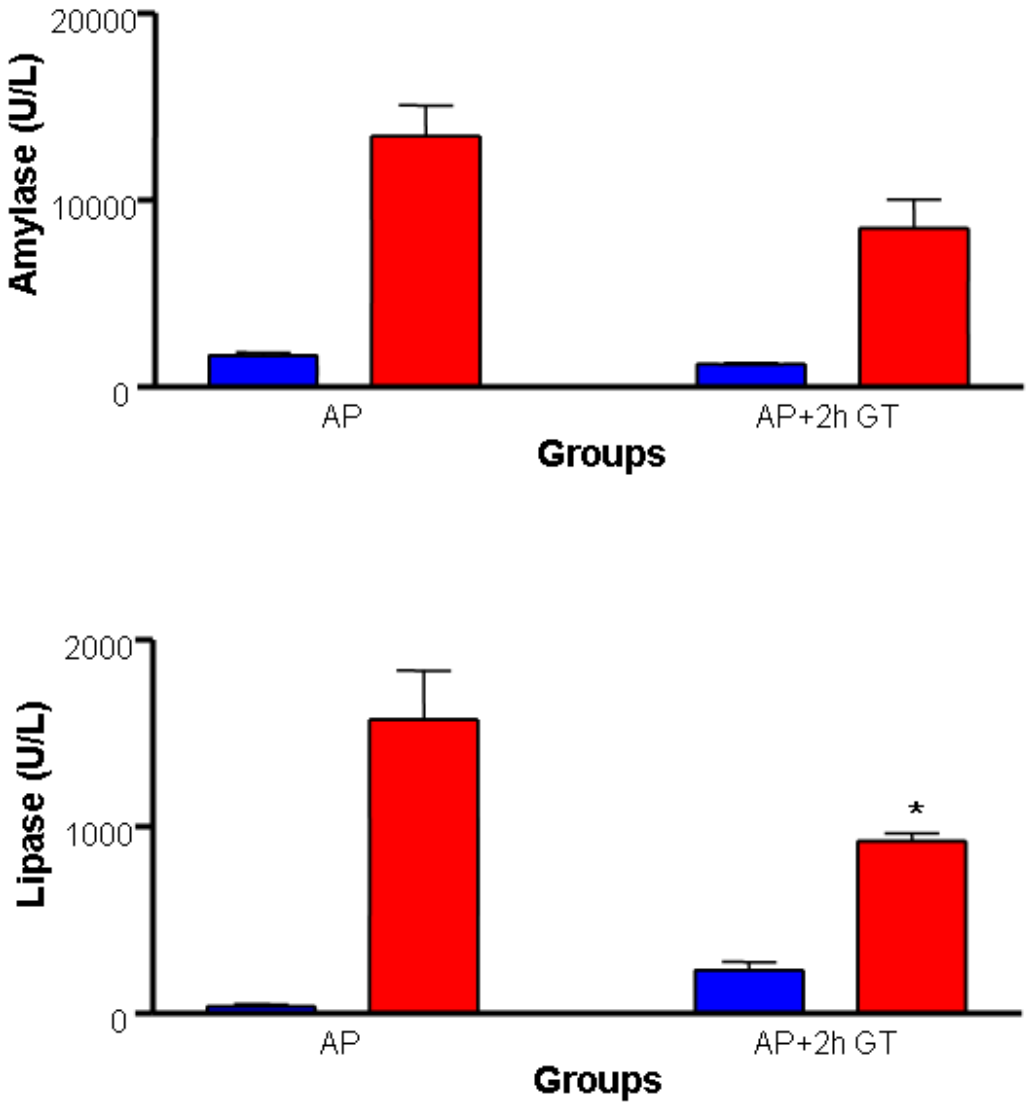
AP is induced by 7 hourly injections of caerulein over six hours. Six hours after the last injection the mouse was euthanized and blood sample collected for plasma enzymes measurement, pancreatic tissue harvested for estimation of myeloperoxidase (MPO) activity and histological assessment of pancreatic damage. Hourly injections of galanin 10nmol/kg/hr (GL10) or galantide 10,20 or 40nmol/kg/hr (GT10, 20 or 40) were administered to mice in appropriate groups. Injection buprenorphine (0.1mg/kg) was administered s.c. with the first dose of caerulein.

Figure 5.2. Effect on AP-induced plasma amylase and lipase activities of exogenous galanin (GL) and galantide (GT) treatment.



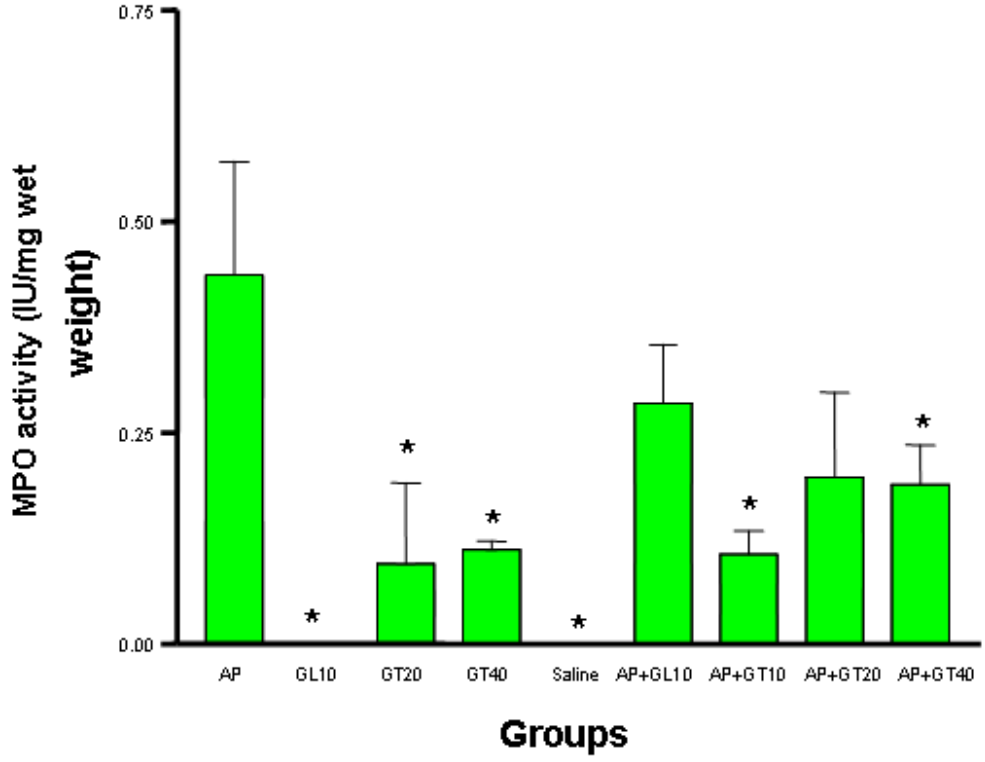
Plasma amylase activity is shown in the top panel, and the plasma lipase activity in the bottom panel. Pre-treatment activity is shown in the blue and the post-treatment activity in red. The AP-induced increase in plasma amylase was significantly decreased by treatment with 20 and 40nmol of galantide (GT20 and GT40 respectively), but not by treatment with 10nmol of GT or galanin (GT10 and GL10, respectively). All control groups displayed a plasma amylase activity which was significantly different from that of the AP group, but not different from the pre-treatment activity (upper panel). Plasma lipase activity was also significantly decreased by treatment with GT20 and GT40 and unaffected by treatment with GT10 or GL10. All control groups displayed a plasma lipase activity which was significantly different from that of the AP group (lower panel). The plasma lipase activity in the groups which received GT10 and GT20 were not different from the pre-treatment activity. However the activity in the GT40 and saline groups were significantly elevated above the pre-treatment levels. Data is presented as mean ± SEM (n=3-7). *$p<0.05$ vs AP is considered as significant.

Figure 5.3. Effect of therapeutic galantide (2 hour post-AP onset) administration on AP-induced plasma amylase and lipase activities.



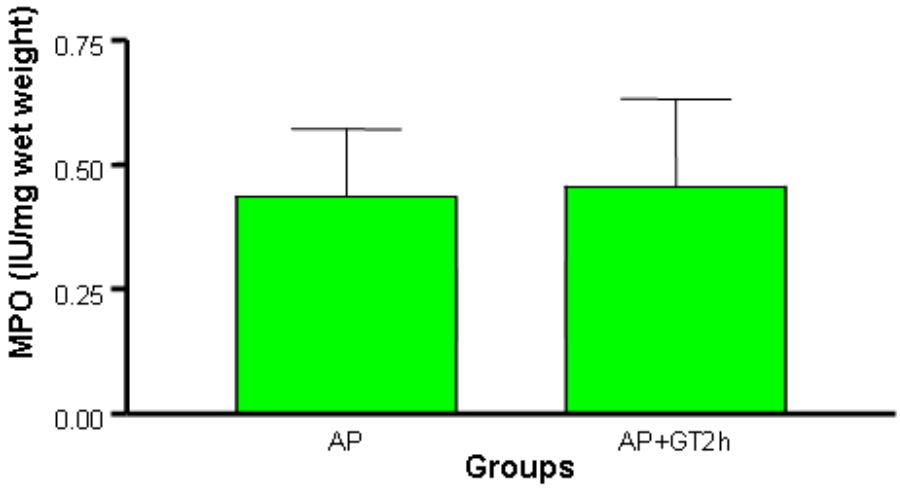
Pre-treatment (blue) of plasma amylase (top) and lipase (bottom) were similar in AP and therapeutic galantide treatment groups. AP increased the amylase and lipase activities. Therapeutic administration of galantide (2h GT) did not alter the AP-induced increase in the amylase activity but significantly decreased the lipase activity. Data is presented as mean ± SEM (n=5-6). *P < 0.05 vs AP is considered as significant, ANOVA.

Figure 5.4. Effect of galanin (GL) and galantide (GT) on AP-induced pancreatic MPO activity.



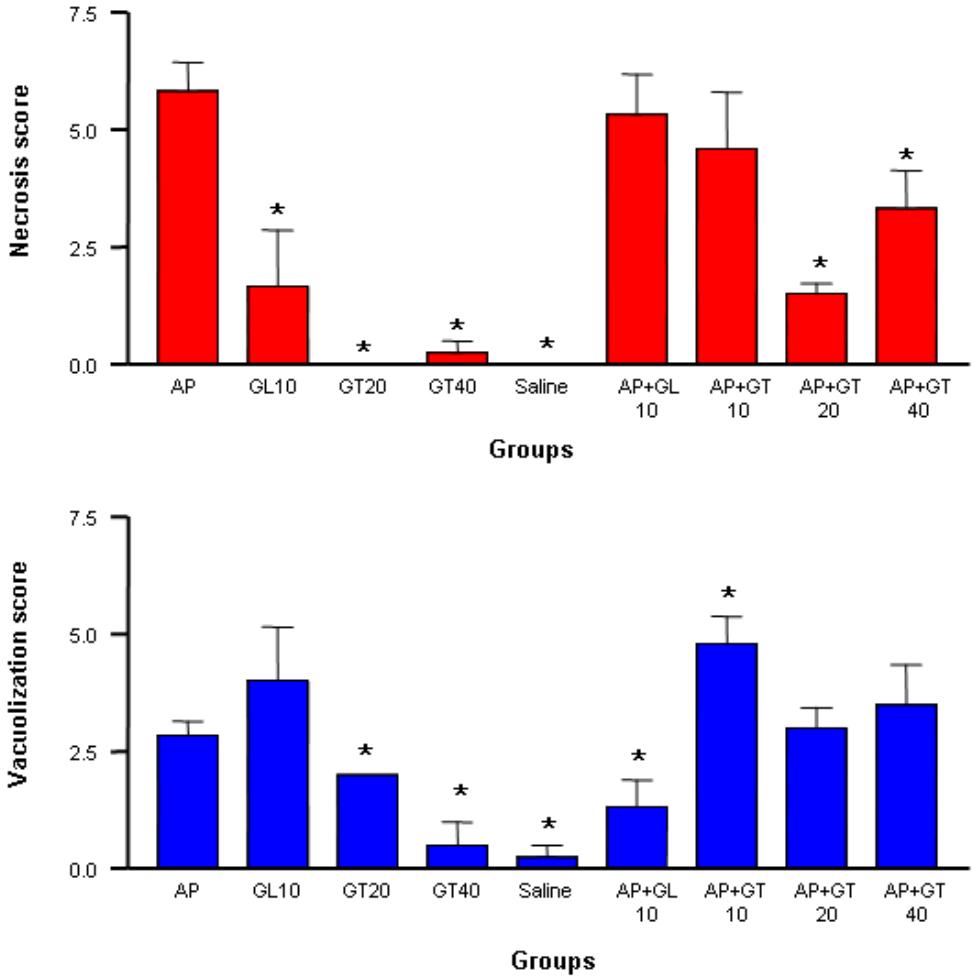
The AP-induced increase in MPO activity was significantly more than the control groups. It was significantly reduced with prophylactic administration of galantide 10 and 40nmol (GT10 and GT40) but was not altered by prophylactic galanin 10nmol (GL10) or galantide 20nmol (GT20). Data is presented as mean ± SEM (n=3-7). *P < 0.05 vs AP is considered as significant, ANOVA.

Figure 5.5. Effect of therapeutic galantide (2 h post-AP onset) administration on AP-induced MPO activity.



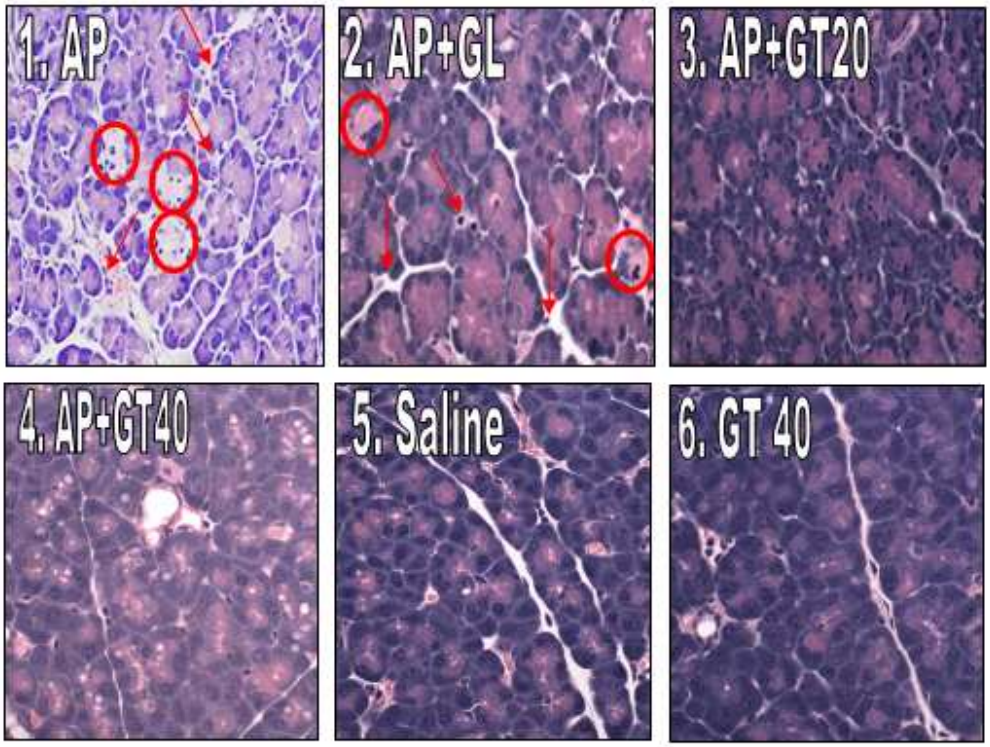
The AP-induced MPO activity was not altered by therapeutic administration of galantide (GT2h) . Data is presented as mean ± SEM (n=5-6). P>0.05, ANOVA.

Figure 5.6. Effect of galanin (GL) and galantide (GT) treatment in AP-induced necrosis and vacuolization in the pancreas.



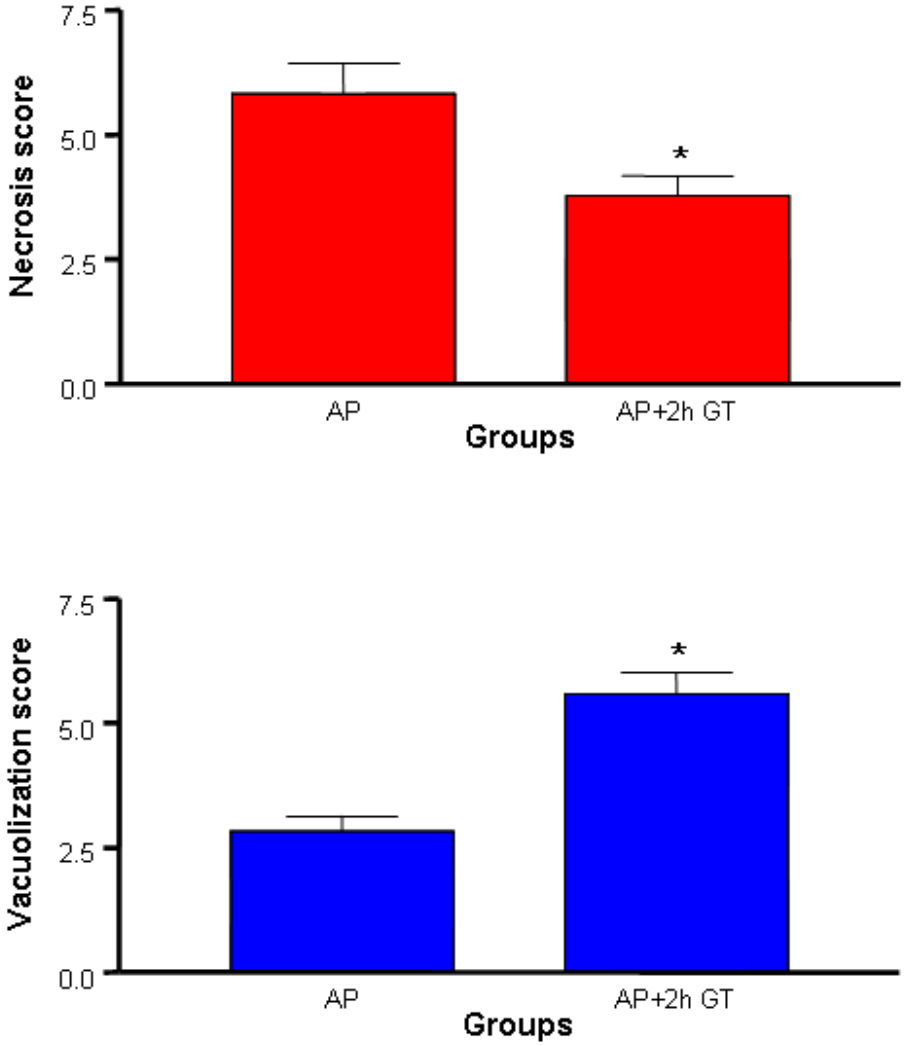
The necrosis score (top) was significantly higher in the AP group as compared to the control groups and groups which received prophylactic administration of galantide 20 and 40nmol (GT20 and GT40). Prophylactic galantide treatment with 20 and 40nmol decreased the AP-induced necrotic damage to the pancreas. Vacuolization score (bottom) of pancreas was significantly more in the AP group as compared to control groups except galanin 10nmol (GL10), which had greater vacuolization as compared to the AP group. The AP-induced vacuolization was significantly reduced by prophylactic administration of galanin 10nmol. In contrast, galantide 10nmol/kg increased vacuolization while galantide (20 and 40nmol/kg) did not alter. Data is presented as mean ± SEM (n=3-7). *P < 0.05 vs AP is considered as significant, ANOVA.

Figure 5.7. Representative Histological Images of mouse pancreas.



The induction of AP (1) resulted in a acinar cell necrosis (red circles) and interstitial edema (red arrows). A similar result is seen when galanin (GL) is administered at the time of AP induction (2). Galantide (GT) 20 and 40nmol/kg administeration at the time of AP induction (3,4) resulted in significantly less necrosis and interstitial edema. Essentially normal acinar cell architecture is seen following saline (5) and GT40 (6) alone administration. Magnification X40.

Figure 5.8. Effect of therapeutic galantide (2h post-AP onset) administration on pancreatic necrosis and vacuolization.



The AP-induced necrosis (upper panel) was significantly reduced by therapeutic administration of galantide (2h GT). In contrast, the AP-induced vacuolization (lower panel) was significantly more in the therapeutic galantide administration group as compared to the AP alone group. Data is presented as mean ± SEM (n=5-6). *P < 0.05 vs AP is considered as significant, ANOVA.

Chapter 6

Chapter 6: Role of endogenous galanin in AP

In **Chapter 5**, the data presented suggested a major role of galanin in AP. In order to test this hypothesis further, an approach which manipulated endogenous galanin was adopted. The galanin knock-out (KO) mice have been described by Wynick et al.,[302]. In this model, a targeting vector was constructed in which a PGK-Neo cassette in reverse orientation was used to replace exons one to five of the galanin gene, removing the signal peptide, the coding region for galanin and most of galanin associated peptide. Thus, these mice are deficient in endogenous galanin. If galanin plays a vital role in AP, then the AP induced in these mice should be less severe than the mice with galanin gene. Li et al., (2006) have demonstrated that in mice there is relatively higher expression of galR3 receptor mRNA as compared to galR2 and galR1 receptor mRNA's. They have also demonstrated that in galanin KO mice the expression of galR1, galR2 and galR3 mRNA was about 40%, 20% and 57% respectively when compared to the corresponding expression in their wild type littermates. Therefore despite the absence of endogenous ligand, expression of the 3 galanin receptor mRNA remains, albeit down-regulated.

6.1 Hypothesis:

Galanin KO mice will be less susceptible to AP as compared to their wild type (WT) littermates.

6.2 Aim

To determine if severity of caerulein-induced AP in galanin KO mice is different to their WT littermates.

6.3 Methods

6.3.1 Animal ethics approval

This study was approved by the Animal Welfare Committee of the Flinders University {project approval number 603/06(b)}. All procedures were carried out with the principles outlined in the Australian Code of Practice for Animal Care and Use.

6.3.2 Animals:

The mice used in this study were on C57BL/6 background as described by Wynick et al.,[303]. These were obtained from Dr. C. Ormandy, Garvan Research Institute (Sydney, NSW, Australia). Wynick et al., constructed a targeting vector in which a PGK-Neo cassette in reverse orientation was used to replace exons one to five of galanin gene. The vector was linearised and electroporated into the E14 embryonic stem-cell line. In total 9 clones were identified in which galanin gene was targeted. These clones were injected into 3.5 day old blastocysts from C57BL/6 mice [302]. These mice were females, 8-14 weeks of age and weighed 15-30 g.

6.3.3 Pharmaceutical agents:

The pharmaceutical agents used in this experimental study were described in **Section 5.4.3**.

6.3.4 Protocol for induction of AP:

AP was induced by 7 injections of caerulein which were administered at hourly intervals as described in **Chapter 5, Section 5.4.5**. The animals were euthanized 6 hours after the last caerulein injection. The control groups comprised of WT and galanin KO mice which received hourly saline (150µl) only injections IP.

6.4 The post-treatment blood sample was collected and pancreas harvested as described in Section 5.4.9.

6.4.1 Study groups:

The various study groups are,

Study groups	Number of animals, n
WT littermates with AP	6
Galanin KO with AP	9
WT littermates saline control	4
Galanin KO saline control	3

6.4.2 Parameters measured:

The following parameters were measured.

6.4.2.1 The plasma amylase and lipase estimation

The plasma enzymes were estimated as described in **Section 2.4.8.3**.

6.4.2.2 Histological examination

The histological examination was performed by the scoring system as described in **Section 5.4.10.2**.

6.4.3 MPO assay

Tissue extraction for pancreatic MPO was similar to as described in **Section 5.4.10.3**.

A different MPO assay to that used in the **Chapter 5** was adopted due to analyser problems. A microplate assay was based on that described by Moore-Olufemi et al.,[304] was developed. The assay used Bio-Rad model 680 microplate reader. This kinetic assay was performed at 25ºC. The MPO activity was expressed as U/mg protein.

6.4.4 Protein assay

In order to estimate the protein concentration in the MPO extracts, the following assay was performed. Extracts were precipitated to eliminate interference due to hexadecyltrimethylammonium bromide[305]. Aliquots (50µl) of MPO extracts and 250µl of ice cold 5% tricholoroacetic acid, were mixed and centrifuged at 10,000 rpm at 4ºC for 2 minutes. The pellet was air dried and then dissolved with 100µl of PBS solution. 5µl of this extract was used in the Bio-Rad protein assay. The standard curve was obtained using BSA as a standard (1mg/ml of PBS) in

aliquots of 2, 4, 6, 8, 10µl with Bio-Rad reagent (40µl) and the total volume adjusted to 160µl with distilled water. Following mixing, the absorbance was detected at 595 nm using BIORAD model-680 microplate reader. All the samples and standards were run in duplicate and the mean values calculated. Using Microsoft Excel the BSA standard curve was obtained, and protein concentration in each TCA extract in mg was calculated for the volume of the extract.

6.4.5 Statistical analysis:

Statistical analysis was performed as described in the **Section 5.4.11**.

6.5 Results

6.5.1 Plasma amylase and lipase activities:

In the WT mice, AP induced a 4.9 fold increase in the plasma amylase activity. On the other hand in the galanin KO mice, AP induced a 3.8 fold increase in the plasma amylase activity when compared to its pre-treatment level (**Figure 6.1**). The post AP amylase activities in the galanin KO were not significantly different from the WT mice ($p = 0.41$). Similarly, in the WT and galanin KO mice the rise in plasma lipase level was about 7.4 and 3.2 fold above the pre treatment levels respectively and these were significantly different ($p = 0.018$).

There were no significant differences in the levels of pre treatment activities of plasma amylase or lipase in different groups (**Figure 6.1**). After induction of AP

in the WT and galanin KO mice there were significant rises in the levels of plasma enzymes when compared to their pre treatment levels ($p<0.05$). Saline treated WT and galanin KO mice did not show any significant differences in the activities of plasma enzymes from their pre-treatment levels.

6.5.2 Histological assessment:

There were significant differences in parenchymal inflammation, necrosis and vacuolization between different groups, but no significant differences in oedema and fat inflammation between different groups.

Oedema score was significantly greater in WT mice with AP was compared to KO saline control group and WT saline control mice group (both $p<0.05$). But the oedema score in WT and KO mice with AP was not significantly different from each other. The oedema score in KO mice with AP was significantly higher than the score in the KO saline control group and WT saline control mice group (both $p<0.05$, **Table 6.2**).

The parenchymal inflammation score in the WT mice with AP was significantly greater than in the KO mice group with AP, the KO saline control group and the WT saline control group ($p<0.05$). There were no significant differences in the parenchymal inflammation score between the KO mice with AP, KO saline control and WT saline control groups (**Table 6.2**).

Table 6.2. Oedema and parenchymal and fat inflammation scores in various groups.

Parameter	WT-AP	KO-AP	WT-saline control	KO-saline control
Oedema score	1 ± 0	1 ± 0	0 ± 0*	0 ± 0*
Parenchymal inflammation score	0.8 ± 0.2	0.22 ± 0.14	0.2 ± 0.2	0.2 ± 0.2
Fat inflammation	1 ± 0	1 ± 0	1 ± 0	1 ± 0

WT-AP Wild type littermates with AP, KO-AP knock-out mice with AP, WT-saline control wild type littermates saline control group, KO-saline knock-out saline control group. Data presented is mean ± SEM. * No oedema. The WT-saline and KO-saline groups had no oedema.

Necrosis and vacuolization scores were 3.8 and 1.3 fold greater in the WT mice with AP as compared to the galanin KO mice with AP. These scores were significantly greater in the WT group in which AP was induced (for both $p<0.05$; **Figure 6.2**). The WT and galanin KO saline control groups had no necrosis and vacuolization. Representative histological images are shown in **Figure 6.3**.

6.5.3 Pancreatic MPO activity:

The WT mice with AP had pancreatic MPO activity 23 times higher than that in the galanin KO mice with AP ($p<0.05$; **Figure 6.4**). The MPO activity was significantly less in both KO and WT saline control groups. The MPO activity in the KO mice with AP was comparable to that in the KO and WT saline control groups.

6.6 Discussion:

The data presented above shows that galanin KO mice were associated with significantly reduced levels of lipase, pancreatic oedema, parenchymal inflammation, necrosis, vacuolization and pancreatic MPO activity compared with WT littermates. This is consistent with less severe AP in the KO mice.

The KO mice with AP appeared to have lower levels of amylase and lipase, but this was not as dramatic as the reduction noted in the previous studies with exogenous galantide administration. This suggests that the release of these enzymes occurs via a mechanism other than a galantide dependent pathway e.g. via the cytokine up-regulation pathway.

The parenchymal inflammation in the KO mice with AP was significantly less than the WT mice with AP, suggesting the role of galanin in inflammatory pathway.

The MPO activity in the KO mice with AP was very low as compared to the WT mice with AP. This suggests a role of galanin in neutrophil chemotaxis and/or activation, either directly, or as a consequence of reduced severity in galanin KO animals.

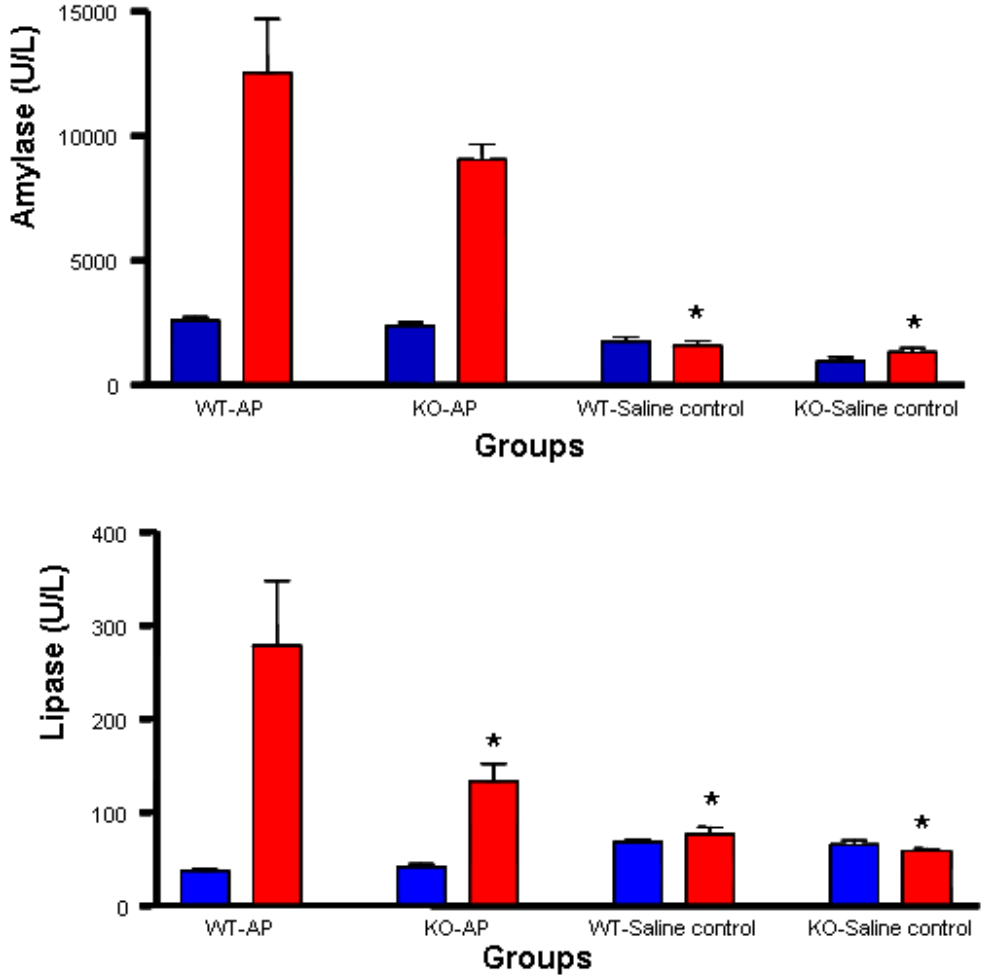
Both the necrosis score and vacuolization scores were significantly lower in the KO mice as compared to the WT mice with AP. The effect on necrosis was more dramatic than on vacuolization. The reason for this is unclear, but is presumably related to lesser severity of AP in mice deficient in galanin.

Li et al.,[306] have studied the galanin receptor expression in the galanin KO mice, and they have found that in galanin KO mice all three galanin receptors are expressed, with GalR3 receptor relatively more expressed than GalR2 and GalR1 receptors. The fact that there is galanin receptor expression in the KO mice could suggest that galanin receptors may play a role in induction of AP.

It will be interesting to know the effect on AP in galanin KO mice on administration of exogenous galanin and galantide. These experimental studies could further strengthen the hypothesis.

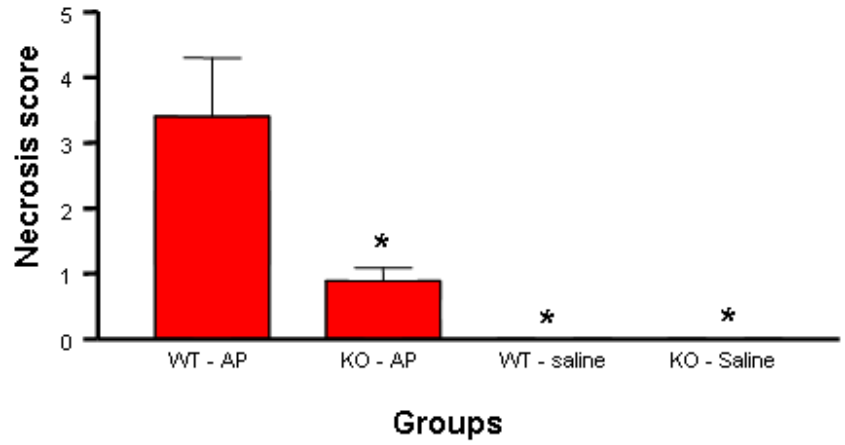
The findings described in this chapter suggest that endogenous galanin plays a role in AP in mice.

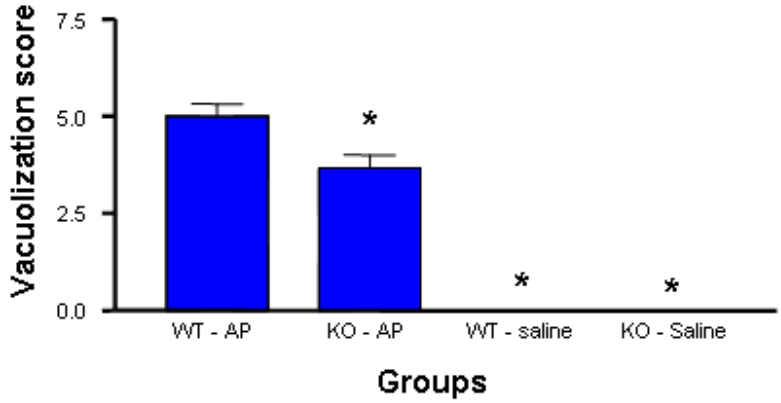
Figure 6.1. Effect on AP-induced plasma amylase and lipase activity in wild type (WT) and knock-out (KO) mice on AP induction.



Plasma amylase activity is shown in the upper panel and lipase activity in the lower panel. Pre-treatment enzyme activity is shown in blue and post treatment enzyme activity is shown in red. Pre-treatment amylase and lipase activities were similar in all the groups. There was a significant rise in the post-treatment plasma amylase and lipase activities in the wild type (WT-AP) and knock-out (KO-AP) groups. The rise in plasma lipase levels in the WT-AP group was significantly more as compared to the KO-AP group. Data is presented as mean ± SEM (n=3-9). *P < 0.05 vs WT-AP, ANOVA.

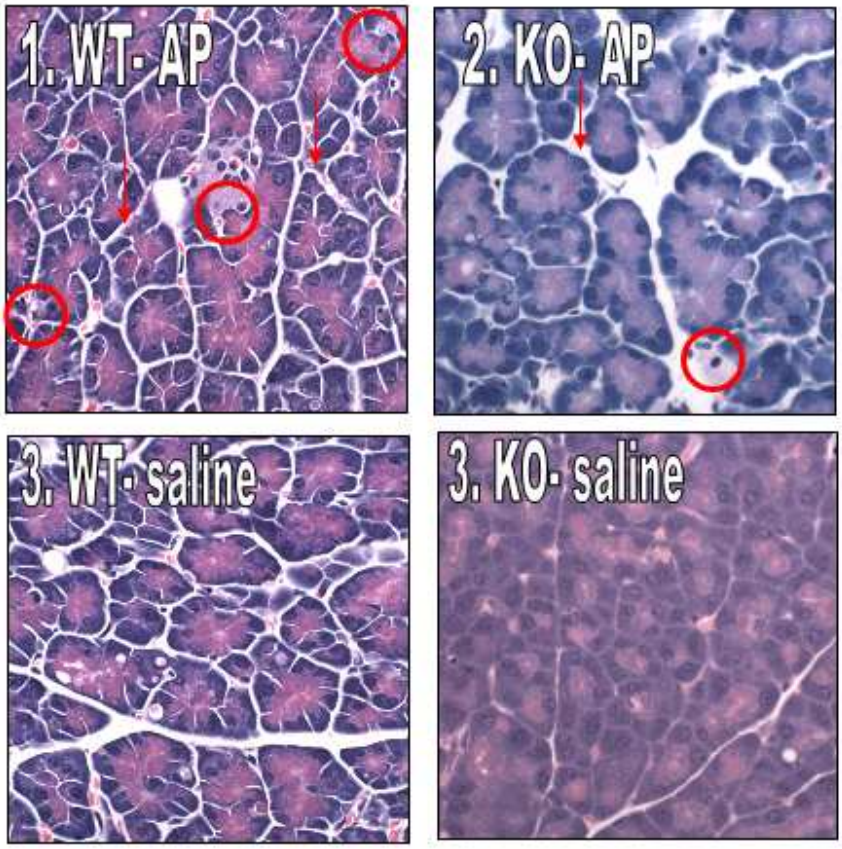
Figure 6.2. Effect on AP-induced necrosis and vacuolization scores in wild type (WT) and knock-out (KO) mice on AP induction.





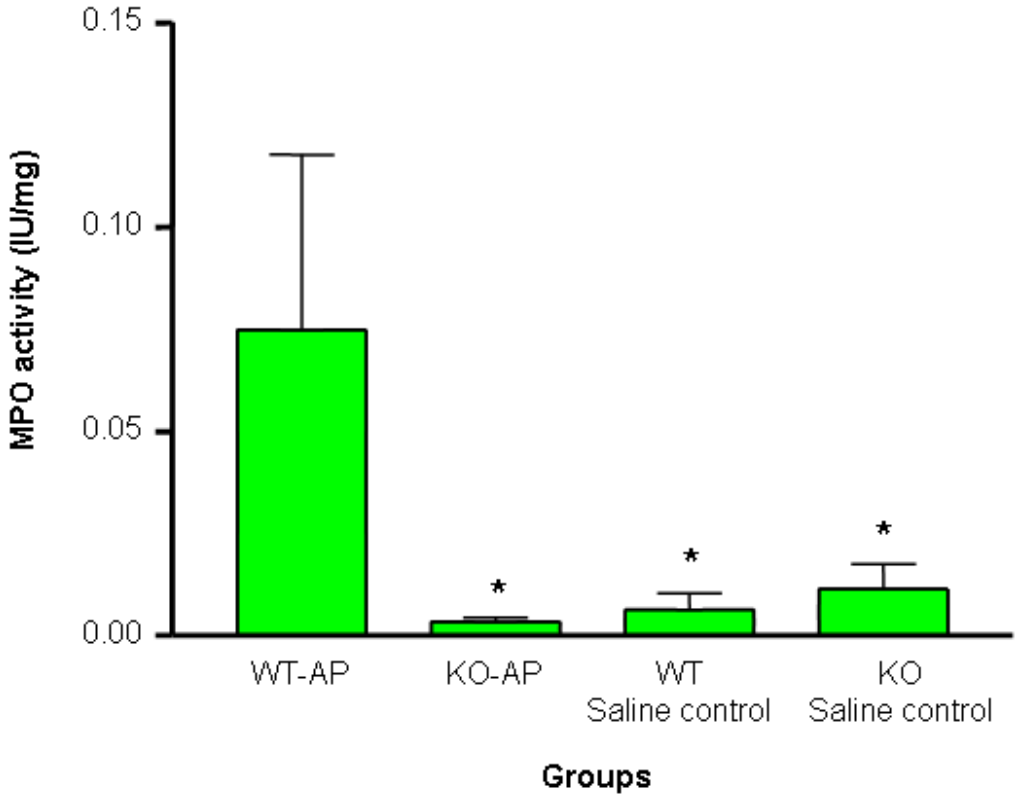
Necrosis score is shown in the upper panel and vacuolization score in the lower panel. There was a rise in the necrosis and vacuolization scores in wild type (WT) and knock-out (KO) mice on AP induction. The degree of necrosis and vacuolization in the WT-AP was significantly more than the KO-AP group and the control groups. The necrosis score suggested more histological damage to the pancreas. Data is presented as mean ± SEM (n=3-9). *P < 0.05 vs WT-AP is considered as significant, ANOVA.

Figure 6.3. Representative Histological Images of mouse pancreas.



Induction of AP (1) in wild type (WT) mice resulted in a acinar cell necrosis (red circle) and interstitial edema (red arrow). When AP was induced in the galanin knock-out (KO) mice there was significantly less necrosis and edema (2). Almost entirely normal acinar cell architecture is seen in WT and KO saline control groups (3,4). Magnification X40.

Figure 6.4. Effect on AP-induced MPO activity in wild type (WT) and knock-out (KO) mice.



The pancreatic MPO activity was significantly high in the wild type (WT-AP) group as compared to the KO group. The MPO activity in the KO-AP was similar to that measured in the control groups. Data is presented as mean ± SEM (n=3-9). *P < 0.05 vs WT-AP, ANOVA.

Chapter 7

Chapter 7: Effects of M35, M40 and C7 in caerulein mouse model of mild AP

7.1 Introduction

In **Chapter 5**, I have shown that administration of galantide had beneficial effects in AP in the Balb C strain of mice and that galanin plays an important role in AP. In this section I evaluate the potential therapeutic value of other antagonists of galanin in the Swiss strain of mice. A different strain of mice was used in this study to further asses if the effects of exogenous galanin and galantide were strain specific or not.

As described in **Chapter 1**, several non-selective antagonists of galanin are commercially available. I have evaluated galantide, M35, M40 and C7 in my experimental study as using these antagonists of different receptor selectivities, I might be able to determine which receptor types are involved in AP and also these antagonists are readily available. In addition, the effect of exogenous galanin on the severity of AP was also evaluated. There is limited information on the effect of these chimeric peptides on pancreatic exocrine secretion, but there is no information on their effect on the severity of AP.

7.2 Hypothesis

Galanin antagonist's galantide, M35, M40 and C7 will decrease the severity of AP in a caerulein mouse model.

7.3 Aim

To determine if administration of galantide, M35, M40 and C7 at AP induction alters the severity of AP in the caerulein mouse model.

7.4 Methods

7.4.1 Animal ethics approval

As outlined in **Section 5.4.1** the study was approved by the Animal Welfare Committee of the Flinders University (project approval number 603/06(b)). All procedures were carried out with the principles outlined in the Australian Code of Practice for Animal Care and Use.

7.4.2 Animals:

Male Swiss strain of mice, 4-6 weeks of age, weighing 15-30 grams were used in the study.

7.4.3 Pharmaceutical agents:

The pharmaceutical agents were similar to those described in **Section 5.4.3**. M35, M40 and C7 were obtained from American Peptide Company, Inc. Sunnyvale, CA, USA.

7.4.4 Protocol for induction of AP:

This was same to as described in **Section 5.4.5**. These antagonists were evaluated using the prophylactic treatment protocol only. The doses of M35, M40 and C7 were based on the doses of galantide used in the Chapter 5.

7.4.5 Study groups:

The various study groups with number of animals in each group are shown in the **Table 7.1.**

Table 7.1. Study groups.

Study groups	Number of animals, n
AP alone	6
AP + prophylactic galanin 10nmol/kg/h	6
AP + prophylactic galantide 40nmol/kg/h	6
AP + prophylactic M35 20nmol/kg/h	6
AP + prophylactic M35 40nmol/kg/h	6
AP + prophylactic M40 20nmol/kg/h	6
AP + prophylactic M40 40nmol/kg/h	5
AP + prophylactic C7 20nmol/kg/h	9
AP + prophylactic C7 40nmol/kg/h	9
Saline only hourly	4
Galanin 10nmol/kg/h	4
Galantide 40nmol/kg/h	4
M35 40nmol/kg/h	4
M40 40nmol/kg/h	4
C7 40nmol/kg/h	6

7.4.6 Parameters measured and statistical analyses:

These were performed as described in **Section 6.4**.

7.5 Results

7.5.1 Plasma enzymes:

The pre treatment activities of plasma amylase and plasma lipase (**Figure 7.1 and 7.2**) were not significantly different from each other in different groups. There was a significant rise in the post treatment activity of plasma amylase and lipase in the groups which had AP induced.

In the AP group, the plasma amylase and plasma lipase levels increased by 13 and 23 fold respectively (**Figure 7.1 and 7.2**).

With administration of galantide (40nmol/kg/h) in acute pancreatitic animals the plasma amylase and lipase activities increased by 4 and 14 fold respectively. These levels were significantly lower than the post treatment plasma enzyme activity seen in the AP alone group ($p=0.01$).

When M35 (20nmol/kg/h) was administered in acute pancreatitic animals the plasma amylase and plasma lipase activities increased by 7 and 18 fold respectively. This rise in the plasma enzymes was not significantly different from the AP group ($p>0.05$). In contrast when M35 (40nmol/kg/h) was administered in AP animals there was a rise in plasma amylase and plasma lipase activity by 3 and 5 fold only. This increase in post treatment levels of plasma amylase ($p=0.017$) and lipase ($p=0.004$) was significantly lower than the AP alone group.

When M40 (20 and 40nmol/kg/h) were administered in acute pancreatitic animal the plasma amylase and plasma lipase activities increased by approximately 8 and 16 fold respectively. This increase in plasma enzyme levels was not significantly different from the AP alone group ($p>0.05$).

When C7 (20nmol/kg/h) was administered in acute pancreatitic animals the plasma amylase and plasma lipase increased by 9 and 23 fold respectively. This rise in the plasma enzymes was not significantly different from the AP group ($p>0.05$). In contrast when C7 (40nmol/kg/h) was administered in AP animals there was a rise in plasma amylase and plasma lipase levels by 6 and 10 fold only. This increase in post treatment levels of plasma amylase ($p=0.013$) and lipase ($p=0.032$) was significantly lower than the AP alone group (**Figure 7.1 and 7.2**).

When galanin (10nmol/kg/h) was administered in acute pancreatitic animals the plasma amylase and lipase activities increased by 11 and 35 folds respectively. This was not significantly different from the post treatment levels in the AP alone group ($p>0.05$ for both amylase and lipase).

The post treatment activities of plasma enzymes in the groups receiving M35 (40nmol/kg/h) and C7 (40nmol/kg/h) in AP animals were not significantly different from each other.

The control groups which received only galanin, galantide, M35, M40 or C7 did not show any significant differences in the post treatment levels of plasma amylase when compared to their pre treatment levels. All the control groups except C7 had post treatment plasma lipase levels not significantly different from the pre treatment levels. The control group which received only C7 had post treatment plasma lipase 3 fold higher than the pre treatment level ($p<0.05$).

7.5.2 MPO activity:

The MPO activity increased (2.5-10 fold) in the AP group (**Figure 7.3**). The AP-induced MPO activity was significantly reduced with the administration of galantide (40nmol/kg/h), M35 (20 and 40nmol/kg/h) and M40 (20 and 40nmol) ($p<0.05$ for all). In contrast, the AP- induced MPO activity was not significantly different when galanin (10nmol/kg/h) and C7 (20nmol/kg/h) were administered ($p>0.05$ for both). On the other hand the AP-induced MPO activity was significantly higher with administration of C7 (40nmol/kg/h) ($p<0.05$).

The control groups which received galanin, galantide, M35, M40 or saline alone had significantly lower MPO activity as compared to the AP alone group ($p<0.05$). But the control group which received only C7 (40nmol/kg/h) had MPO activity which was not different to that seen in the AP alone group ($p>0.05$).

7.5.3 Histological assessment:

7.5.3.1 Oedema:

The AP alone group was associated with histologically evident oedema. The AP-induced oedema was not altered by administration of other peptides ($p>0.05$ for all). The control groups also had similar degree of oedema as the AP alone group except that when M40 (40nmol) was administered, it was associated with significantly less oedema than seen in AP, but more than seen in saline controls ($p<0.05$) (**Table 7.2**).

7.5.3.2 Fat inflammation:

The AP alone group was associated with fat inflammation compared to the saline control group. The AP-induced fat inflammation was not altered by administration of other peptides ($p>0.05$). The fat inflammation in the control groups was not significantly different from the AP alone group ($p>0.05$). The AP-induced fat inflammation in the in the C7 40nmol/kg/h administration group was significantly more than the control groups which received M35 and saline only ($p<0.05$) (**Table 7.2**).

7.5.3.3 Parenchymal inflammation:

The AP alone group was associated with parenchymal inflammation. The AP-induced parenchymal inflammation was not altered by administration of other

peptides (p>0.05). The parenchymal inflammation in the AP alone group was significantly more than the control groups (p<0.05) (**Table 7.2**).

Table 7.2. Oedema and parenchymal and fat inflammation scores in various groups

Groups	Oedema	Fat inflammation	Parenchymal inflammation
AP	1.16±0.16	1±0	1.3±0.2
AP+GL10	1±0	1±0	1±0
AP+GT40	1±0	0.83±0.16	0.66±0.2
AP+M35(20)	1.16±0.16	1.1±0.16	1.1±0.1
AP+M35(40)	1±0	0.66±0.2	0.66±0.2
AP+M40(20)	1.16±0.16	1±0	1±0
AP+M40(40)	1±0	1±0	1.6±0.16
AP+C7(20)	1.11±0.2	0.88±0.11	0.77±0.14
AP+C7(40)	1±0	1±0	1.1±0.1
GL10	0.75±0.25	0.75±0.47	0.25±0.25
GT40	1±0	1±0	0±0*
M35(40)	0.5±0.28	0.5±0.28	0±0*
M40(40)	0.25±0.25	1±0	0±0*
C7(40)	0.5±0.22	0.83±0.3	0.16±0.16
Saline only	0±0*	0.5±0.28	0±0*

AP acute pancreatitis, GL10 galanin 10nmol/kg/h, GT40 galantide 40nmol/kg/h, M35(20) M35 20nmol/kg/h, M35(40) M35 40nmol/kg/h, C7(20) C7 20nmol/kg/h, C7(40) C7 40nmol/kg/h. * no oedema or inflammation. Data is presented as mean ± SEM.

7.5.3.4 Vacuolization:

The AP group was associated with vacuolization within the acinar cells (**Figure 7.4**). The AP-induced vacuolization was significantly reduced with administration of M40 20 and 40nmol/kg/h (p<0.05). In contrast, the AP-induced vacuolization was not altered by administration of other peptides. The vacuolization score in the AP group was significantly greater than the control groups (p<0.05) (**Figure**

7.4). On the other hand, there was not any significant differences in the vacuolization scores amongst the various control groups ($p>0.05$).

7.5.3.5 Necrosis:

The AP group was associated with necrosis of the acinar cells (**Figure 7.4 and 7.5**). The AP-induced necrosis was significantly reduced by administration of galantide 40nmol/kg/h and M35 40nmol/kg/h ($p<0.05$ for both). In contrast the AP-induced necrosis was not affected by administration of other peptides. The AP-induced necrosis was significantly more than the control groups ($p<0.05$). On the other hand, there was not any significant differences in the necrosis scores amongst the various control groups ($p>0.05$).

Table 7.3. Summary of results.

Parameter	AP+GL10	AP+GT40	AP+M35(20)	AP+M35(40)	AP+M40(20)	AP+C7(20)	AP+C7(40)
Amylase activity	↔	↓	↔	↓	↔	↔	↓
Lipase activity	↔	↓	↔	↓	↔	↔	↓
MPO activity	↔	↓	↓	↓	↓	↔	↔
Necrosis	↔	↓	↔	↓	↔	↔	↔
Vacuolization	↔	↔	↔	↔	↓	↔	↔
Oedema	↔	↔	↔	↔	↔	↔	↔
Fat inflammation	↔	↔	↔	↔	↔	↔	↔
Parenchymal Inflammation	↔	↔	↔	↔	↔	↔	↔

AP Acute pancreatitis, GL10 galanin 10nmol/kg/h, GT40 galantide 40nmol/kg/h, M35(20) M35 20nmol/kg/h, M35(40) 40nmol/kg/h, C7(20) C7 20nmol/kg/h, C7(40) C7 40nmol/kg/h, ↓ significantly less than AP, ↔ not significantly different from AP.

7.6 Discussion

These data suggest that galantide and M35 (both 40nmol/kg/h), when administered at the induction of AP, significantly decreased the indices of AP. On the other hand when galanin, M35 (20nmol/kg), M40 or C7 (20nmol/kg) were administered at AP induction no significant differences in hyperenzymemia and necrosis score were evident. C7 (40nmol/kg/h) when administered at AP-onset only reduced the AP-induced hyperenzymemia. Galantide, M35 and M40 when

administered at AP-induction reduced the AP-induced MPO activity. Whereas vacuolization within the acinar cells was only reduced by M40 treatment. The mixed results suggests the non-specificity of the peptides used.

Kisfalvi I Jr et al.,[307] studied the effects of galanin antagonists M35 and C7 on the rat exocrine pancreas under urethane anaesthesia; they concluded that these chimeric peptides behaved as galanin agonist. However, the effect of these two agents on the severity of AP demonstrated in this study is not consistent with their findings. This could be due to various factors. Firstly, there is species difference in the two studies. Secondly, Kisfalvi and colleagues used different route and doses of these agents.

As described earlier M35 and C7 are chimeric peptides, and hence these peptide antagonists could theoretically interact with receptors apart from galanin receptors and thus complicate the interpretation of the data. But this is not the case with galanin receptor antagonist M40, its C-terminal fragment consists of amino acids which are not homologous to any known peptide therefore there are no known receptors for its C-terminal fragment. Hence M40 would interact with galanin receptor surface or surrounding area of the membrane to increase the affinity of the antagonist and thus to increase the ability to displace galanin[308].

It has been shown in the rat hypothalamus that galantide and M35 have higher affinities to galanin receptors 1, 2 and 3 than galanin itself, whereas C7 has similar affinity and M40 a lower affinity[309].

M35 as described earlier is a chimeric peptide in which C-terminal sequence is homologous to portions of bradykinin. It is known that M35 possesses ≈ 100-fold higher affinity than M40 to act and block the CNS, spinal cord and pancreatic actions of galanin[310]. M35 may also act as a bradykinin receptor antagonist and inhibit actions of bradykinin. Bradykinin is a vasoactive proinflammatory peptide that induces relaxation of vascular smooth muscle in arteries and arterioles and promotes adhesion molecule expression, leukocyte sequestration, and the formation of interendothelial gaps and protein extravasation in postcapillary venules[311]. These actions may aggravate AP. If M35 acts as bradykinin antagonist, it may ameliorate AP by inhibiting bradykinin activity in AP. A future study evaluating the efficacy of therapeutic administration of M35 in AP should be undertaken.

Bartfai et al.,[312] have shown that M40 failed to antagonize galanin mediated inhibition of the glucose induced insulin release in isolated mouse pancreatic islets and acts as a weak agonist at galR2 receptors in the pancreas. It is possible that there is failure of M40 peptide when administered in AP to act as galanin antagonist at pancreatic galanin receptors. This may be due to large excess of spare galanin receptors in the pancreas. If this is true then, M40 peptide with its lower affinity can never occupy all galanin receptors in presence of competing endogenous galanin. M40 may also have galanin agonistic activity at pancreatic galanin receptors and hence does not seem to ameliorate AP. Bartfai et al.,[312] demonstrated that M40 showed no galanin agonist activity in the

CNS, but in this AP study it appears to behave as an agonist. This could suggest that there may be chemical differences between galanin receptors in the CNS and the pancreas. It was interesting to see that M40 when administered in AP significantly decreased the pancreatic MPO activity but increased acinar cell vacuolization when compared to the AP alone group. This has been difficult to explain. Its effect on acinar cell vacuolization could be due to an increase in intracellular calcium ion by some mechanism.

C7 as described earlier has C-terminal sequence homologous to a fragment of the substance P antagonist, spantide. C7 could theoretically produce biological activities as a result of its binding to both galanin and substance P receptors. Spantide is an anti-inflammatory peptide that blocks the inflammatory effects of substance P by competitively binding to neurokinin (NK) receptors[313], thus acts as a tachykinin receptor antagonist. The combination of the N-terminal fragment of galanin and pro-spantide may act as an antagonist of spantide at neurokinin receptor leading to more inflammation. Hence, C7 may act as a chemotactic agent for neutrophils. C7 when administered in AP has produced effects similar to that of substance P i.e. increases pancreatic damage. It has also been shown that C7 has high affinity for substance P receptors in the rat hypothalamus[314]. Hence the effect of C7 on MPO levels and necrotic damage to the pancreatic tissue may be due to substance P like effect.

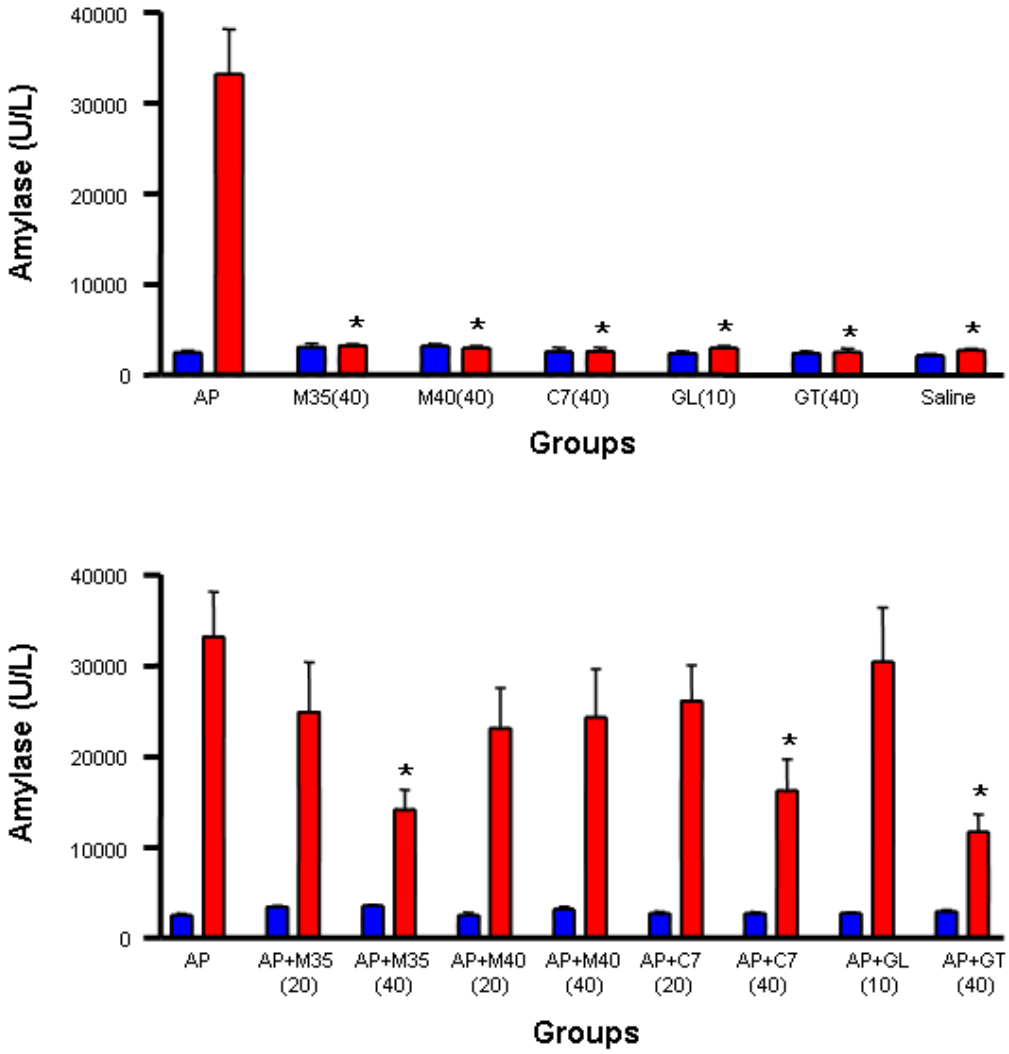
Our data suggest that, it is difficult at this stage to comment on the galanin receptors involved in AP. This would need further studies. The data also provide

little direct evidence of galanin action, rather that galanin is one amongst a number agents involved in the mechanisms active in AP, and these ‘antagonists’ probably interact with several of these mechanisms.

7.7 Conclusion

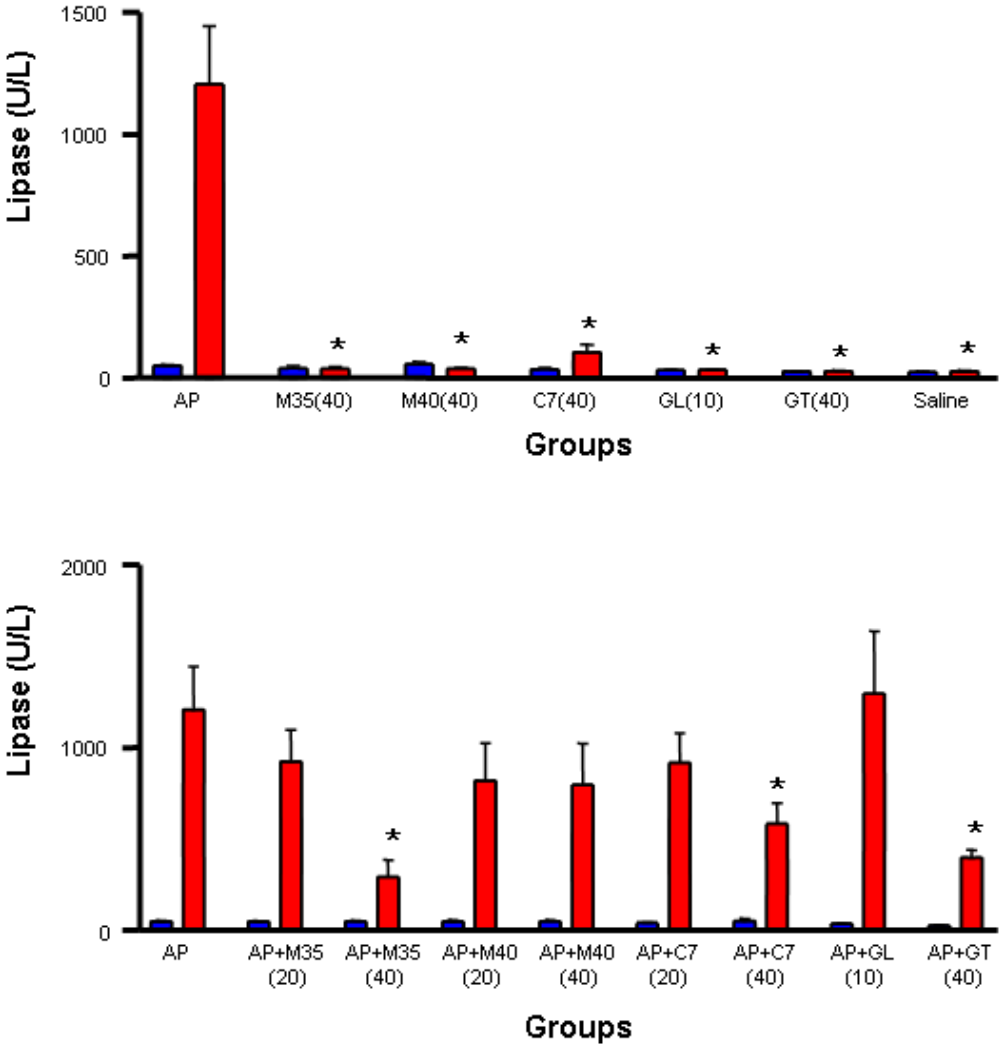
The data indicate that prophylactic administration of galantide and M35 have a beneficial effect in AP induced in the caerulein mouse model.

Figure 7.1. Effect on AP-induced plasma amylase activity on galanin (GL), galantide (GT), M35, M40 and C7 treatment.



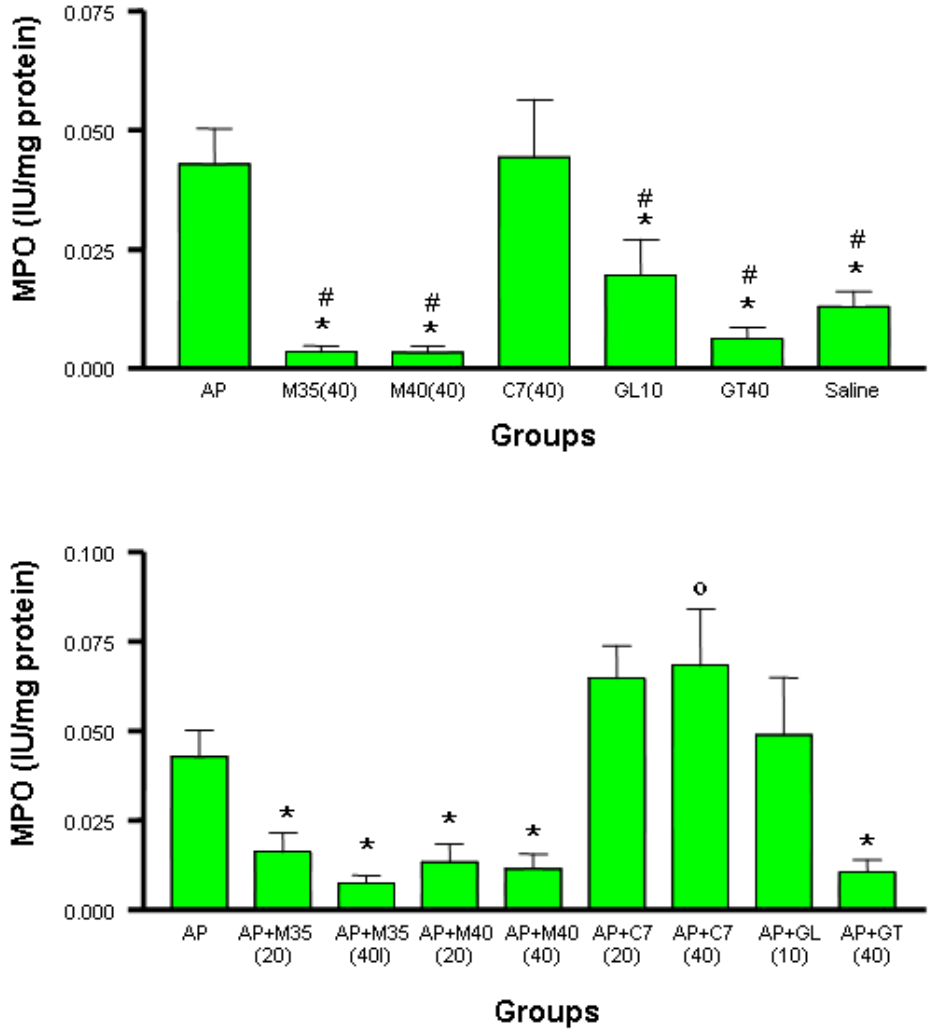
Pre-treatment activity is shown in the blue and the post-treatment activity in red. Pre treatment activity of amylase is similar in all the groups. All control groups displayed a plasma amylase activity which was significantly different from that of the AP group, but not different from the pre-treatment activity (upper panel). The AP-induced increase in plasma amylase was significantly decreased by treatment with 40nmol/kg of M35, galantide 40nmol (GT40) and C7, but not by treatment with 10nmol/kg of galanin (GL10), 20nmol/kg of M35, M40 and C7 (lower panel). Data is presented as mean ± SEM (n=4-9). *P < 0.05 vs AP is considered as significant, ANOVA.

Figure 7.2. Effect on AP-induced plasma lipase activity on galanin (GL), galantide (GT), M35, M40 and C7 treatment.



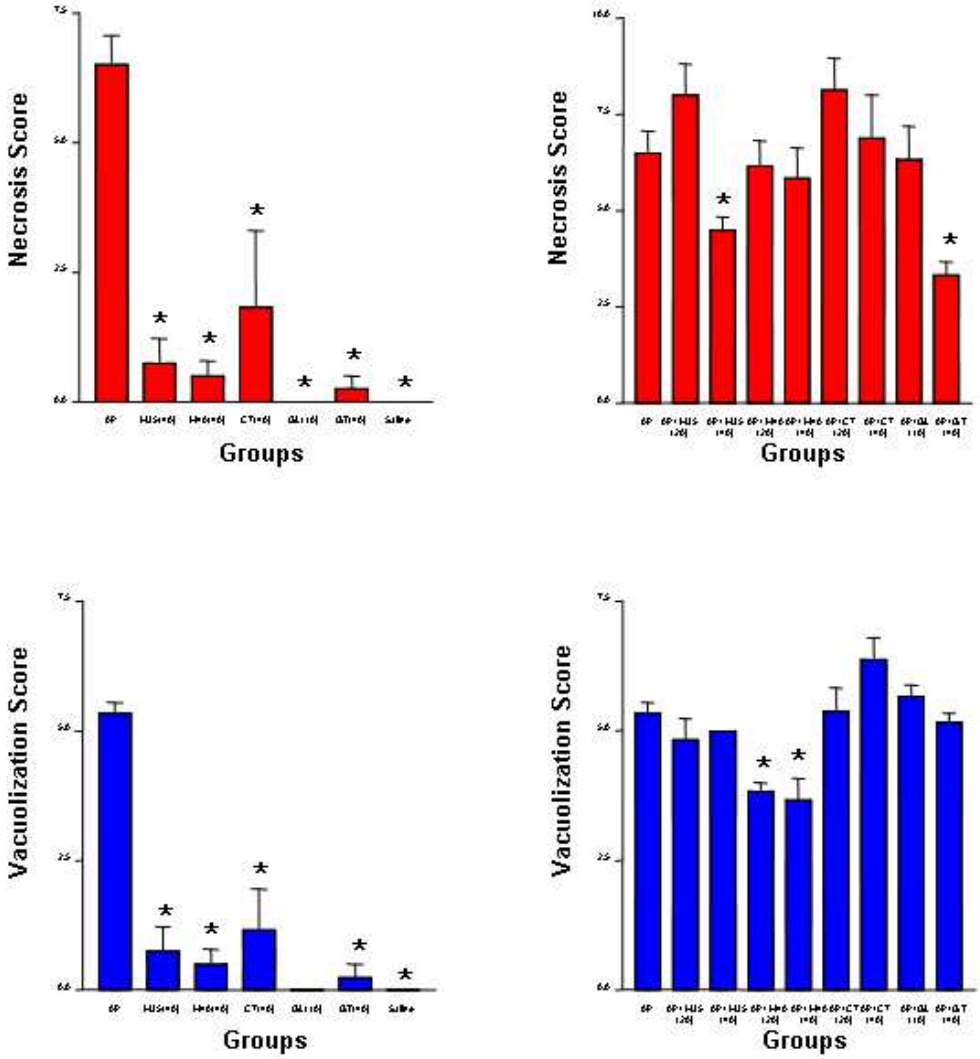
Pre-treatment lipase activity is shown in the blue and the post-treatment activity in red. Pre treatment activity of lipase is similar in all the groups. All control groups displayed a plasma lipase activity which was significantly different from that of the AP group, but not different from the pre-treatment activity (upper panel). The AP-induced increase in plasma lipase was significantly decreased by treatment with 40nmol/kg of M35, galantide (GT40) and C7, but not by treatment with 10nmol/kg of galanin (GL10), 20nmol/kg of M35, M40 and C7 (lower panel). Data is presented as mean ± SEM (n=4-9). *P < 0.05 vs AP is considered as significant, ANOVA.

Figure 7.3. Effect on AP-induced pancreatic Myeloperoxidase (MPO) activity on M35, M40, C7, galanin (GL) and galantide (GT) treatment.



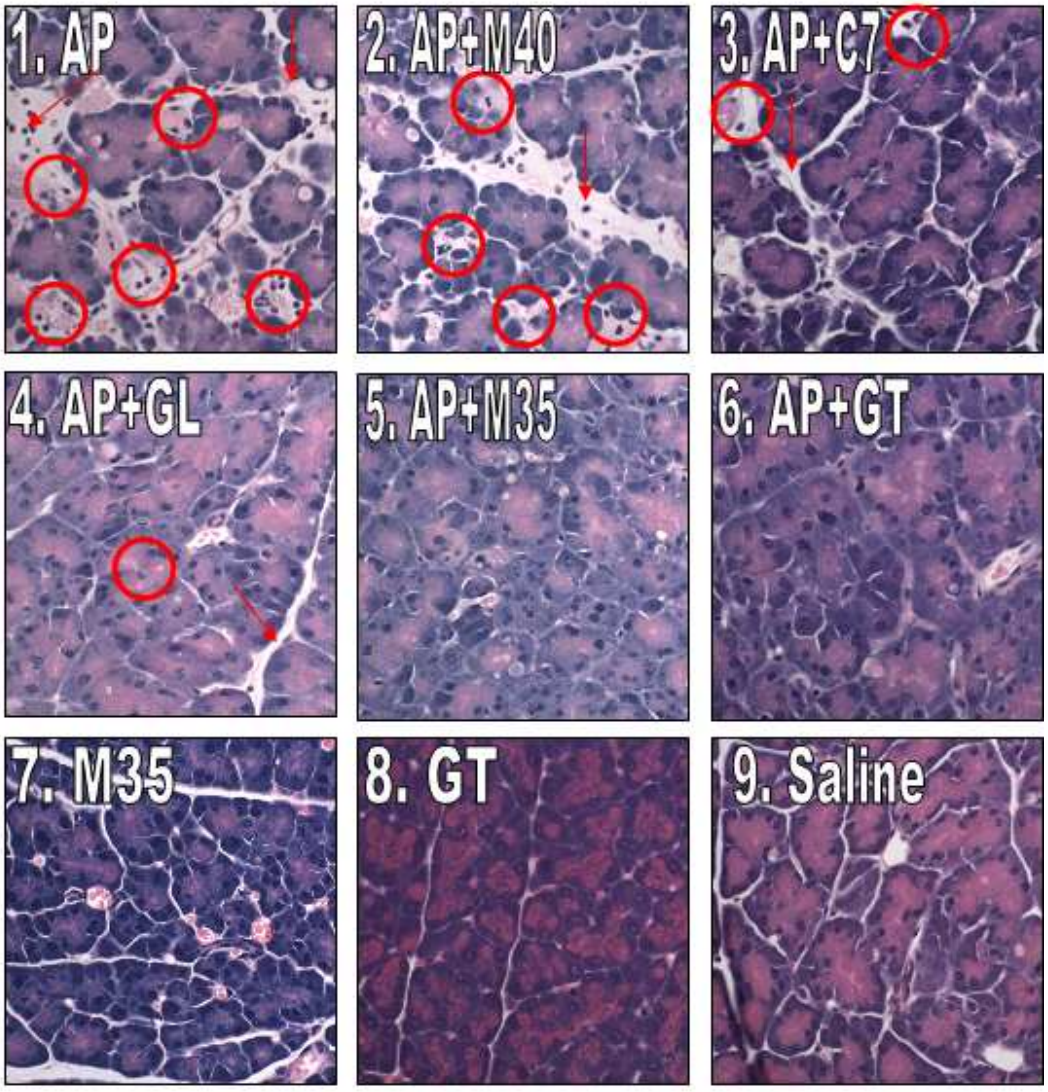
The AP-induced increase in the MPO activity was significantly higher than the control groups which received 10nmol/kg galanin (GL), 40nmol/kg M35, M40, galantide (GT40) and saline but not form C7. In the control groups, C7 treatment was associated with significantly higher MPO activity than other control groups (upper panel). The AP-induced MPO activity was significantly reduced by treatment with 20 and 40nmol/kg of M35, M40 and GT. In contrast the AP-induced MPO activity was significantly increased with treatment of 40nmol/kg C7. While 20nmol/kg of C7 and 10nmol/kg of GL did not alter the AP-induced MPO activity (lower panel). Data is presented as mean ± SEM (n=4-9). *,ºP < 0.05 vs AP, #P < 0.05 vs C7 is considered as significant.

Figure 7.4. Effect on AP-induced pancreatic necrosis and vacuolization scores on M35, M40, C7, galanin (GL) and galantide (GT) treatment.



AP-induced rise in necrosis score was significantly more than the control groups (upper left panel). The AP-induced necrosis score was significantly decreased by treatment with 40nmol/kg M35 and galantide (GT40) while M40, GL and C7 treatment did not affect the AP-induced necrosis (Upper right panel). AP-induced rise in vacuolization score was significantly more than the control groups (lower left panel). The AP-induced vacuolization score was significantly decreased by treatment with 20 and 40nmol/kg M40. On the other hand M35, GL and C7 treatment did not affect the AP-induced vacuolization (lower right panel). Data is presented as mean ± SEM (n=4-9). *P < 0.05 vs AP is considered as significant, ANOVA.

Figure 7.5. Representative Histological Images of mouse pancreas.



The induction of AP (1) resulted in a acinar cell necrosis (red circle) and interstitial edema (red arrow). A similar result was seen when M40 (40nmol), C7 (40nmol) and galanin 10nmol (GL), were administered at the time of AP induction (2,3,4). When M35 (40nmol) and galantide (GT) 40nmol were administered at the time of AP induction (5,6), it resulted in significantly less necrosis. Almost entirely normal acinar cell architecture was seen in M35 (40nmol), GT (40nmol) and saline control groups (7,8,9). Magnification X40.

Chapter 8

Chapter 8: General discussion

As each preceding chapter has included a detailed discussion, this chapter provides an overview of the findings and a discussion of their broader ramifications. Today, the treatment for AP is symptomatic and supportive. There is no pharmaceutical intervention available that will halt or reverse the disease process, so there is a pressing need for its development.

8.1 Overview of findings

The studies reported in this thesis have established that,

1. Galanin exacerbates AP in the possums and galanin KO mice.
2. Galantide ameliorates, some indices of AP in possums and in three strains of mice.
3. Galantide alters the AP-induced changes in microcirculation in the possum.
4. Galanin-KO mice have less severe caerulein-induced AP.

Collectively these data implicate a role for galanin in the pathogenesis of AP. The cascade of events that underlie the pathogenesis of AP is incompletely understood **(Figure 8.1).** The Galanin antagonists, galantide and M35 may act at one or more points in the cascade leading to AP. Their actions could be direct on the pancreas or may influence AP indirectly via other mechanisms.

8.2 General conclusions and future studies

The source of endogenous galanin that participates in AP remains to be determined. As described in **Chapter 1 section 1.5.5**, galanin is abundantly found in the pancreas. It has been localized to the intrapancreatic neurons and a subset of islets. However, in AP, galanin may be produced at an extrapancreatic site and then delivered to the pancreas. There is considerable indirect evidence that the sympathetic innervation of the pancreas is activated during acute stress and influences the endocrine pancreas. It seems likely that the classical sympathetic neurotransmitter, noradrenaline, acts in concert with peptide co-transmitters, such as galanin and neuropeptide Y. These are released during the stimulation of pancreatic sympathetic nerves and are capable of influencing islet function or pancreatic blood flow[315]. Hence endogenous galanin from sympathetic nerves may modulate AP, directly via its action on the pancreatic blood vessels and/or indirectly by influencing the islets and/or acinar cells. One experimental approach which could be used to determine if galanin from sympathetic nerves does play a role in AP could be to use sympathectomized animals. The severity of AP in sympathectomized animals should be less than that observed in normal animals.

The neurotransmitter substance P is present in the sensory nerves of the pancreas. It has been implicated in the pathogenesis of AP[316]. As both substance P and galanin could be released during the onset and or progression of AP, they may interact to modulate the severity of AP. Future experimental

studies evaluating possible interactions between substance P and galanin in AP should be considered.

The evidence regarding the presence, sub-types and location of galanin receptors on various cell types within the pancreas or on blood vessels of the pancreas is lacking. Galanin receptors have been isolated from the beta cells[317]. Future experimental studies using real time RT-PCR on isolated acinar, ductal and endothelial cells to quantify mRNA transcripts could provide some useful information. This would enable the quantification and comparison of the distribution of different galanin receptor mRNAs. In addition, Northern blot analysis and *in situ* hybridization studies could clarify the differential expression patterns of the 3 receptors on various cell types. Other members of our laboratory have carried out immunohistochemical studies using mouse pancreas and commercially available galanin receptor antisera, but the results have not been very promising as currently available antibodies are not specific (i.e. high background labeling).

The pancreatic vascular perfusion studies in the possum suggest the possibility of galanin receptors on perivascular nerves and/or vascular smooth muscle in the pancreas. These receptors could be identified with the help of immunohistochemistry using appropriate antibodies.

It is also not clear which galanin receptor/receptors are involved in AP, as galanin antagonist selectivity for galanin receptors is poorly characterized.

Future experiments with specific galanin receptor-KO mice could address this question. This would then enable receptor specific antagonist to be used to treat AP. In our laboratory preliminary studies with M871 (selective GalR2 antagonist) and SNAP (specific GalR3 antagonist) have been undertaken in experimental AP in mice (G. Saccone, personal communication). The preliminary data suggest that M871 decreased the MPO activity i.e. affects the neutrophil function and/or recruitment, thereby implicating a role for GalR2. Other preliminary data have shown that SNAP administration in AP decreased plasma enzymes and MPO activity, thereby suggesting a role for GalR3 on several cell types.

In addition to acinar cells other cell types involved in AP include neutrophils and other inflammatory cells, endothelial cells, ductal cells etc. The expression of galanin receptors by these cells is not known. These could also be the sites of action of galanin antagonists (**Figure 8.1**). As demonstrated from the mouse studies described in **Chapter 7**, when galantide and M35 were administered prophylactically there was a decrease in the MPO activity. This is consistent with decrease in the expression of chemokines or ICAM-1 on endothelial cells, leading to reduced neutrophil infiltration. The potential role of ICAM-1 could be evaluated by measuring the effect of galanin and its antagonists on the severity of caerulein induced-AP in ICAM-1 knock-out mice. On the other hand when galantide was administered therapeutically in mice, the MPO activity was similar to the AP group. It may be that galantide affects the neutrophil activation state or

acts early in the inflammatory cascade. A future study to define the presence of galanin receptors on the neutrophils may help to resolve this question.

Considering the potential beneficial effects of galantide for the treatment of AP, additional studies are required to investigate optimum dosage, duration, and route of administration. Such studies could employ the same or more severe AP model as described in Chapter 1 section 1.7. Other studies could examine the effect of galantide when administered orally or subcutaneously.

The drawback of the studies with antagonists described in Chapter 5 and 7 are (a) possible peptidase sensitivity, and hence short biological half-life and (b) when applied in high concentration, possible agonist-like effects due to their intact N-terminus which might mask the true effectiveness of antagonists and/or influence the interpretation of the data. Despite these shortcomings one possible clinical advantage is that because of their relatively large size (compared to the non-peptide molecules) they should not cross the blood-brain barrier and hence should be free from CNS side effects. Conversely, probable short half-life and poor selectivity of these peptide antagonists highlights the need for development of more stable analogues and specific non-peptide galanin receptor antagonists (although these could potentially have the disadvantage of crossing the blood-brain barrier).

Due to time constraints the following future studies are recommended:

1. Explore the possible interaction between substance P and galanin in the pathogenesis of AP.

2. *In-vitro* studies examining the effect of exogenous galanin and galantide on exocrine secretion using pancreatic acini.

3. Measurement of pancreatic cytokine levels and transcription factor activation in acinar cells in a more severe model of AP in the mouse to define the intracellular signaling pathways.

4. Experimental studies to determine if the severity of AP in galanin KO mice is influenced by the administering exogenous galanin.

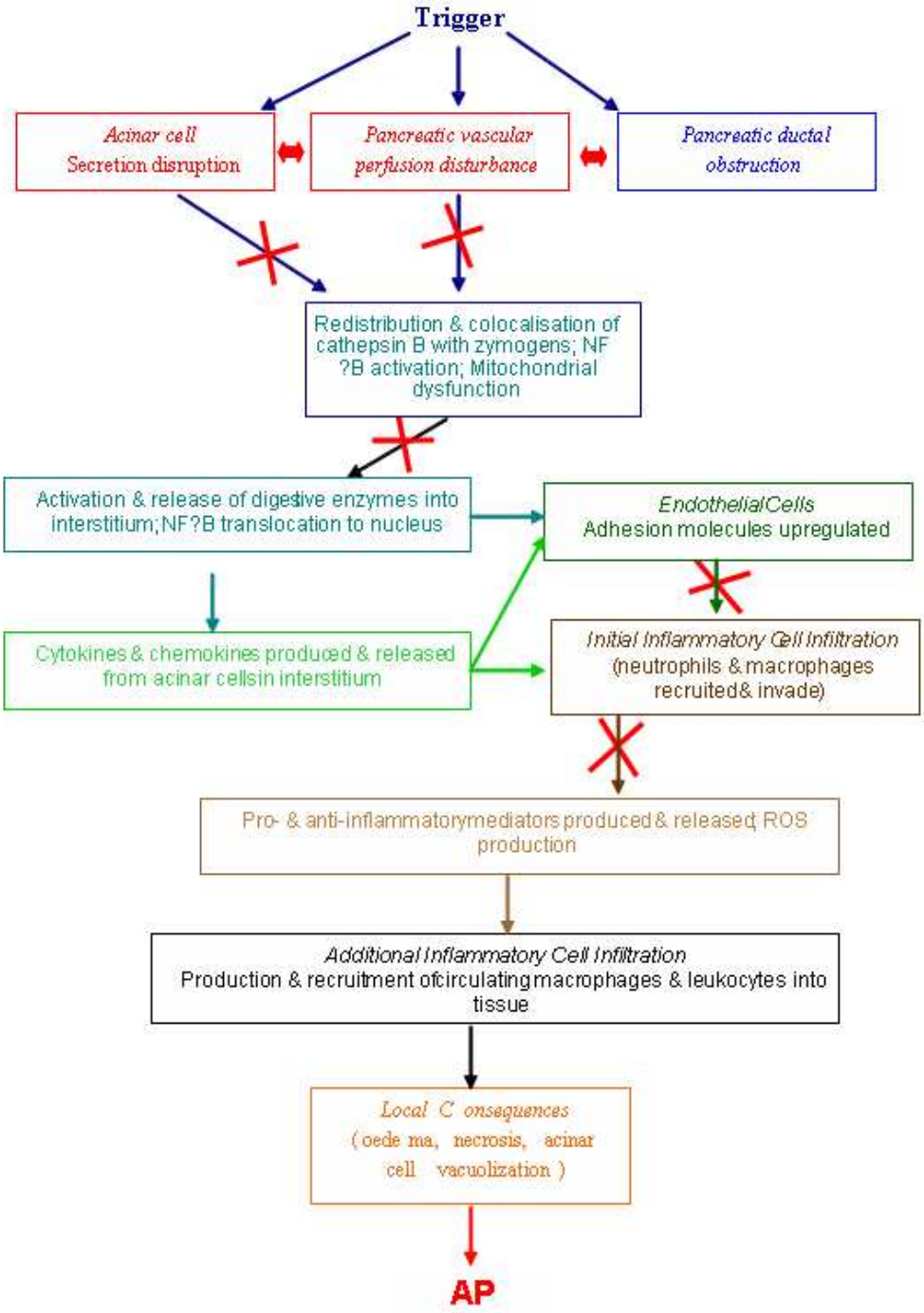
5. Determine the efficacy of therapeutic administration of M35 in the caerulein mouse model of AP.

In conclusion, at present there is no specific pharmacological treatment of AP. Many experimental drugs have ameliorated AP in the animal models but have failed in the humans. This could suggest the different nature of disease in humans. Galanin antagonists may be the exception and could potentially be a drug for treatment of AP. The studies described in this thesis show that at least galantide has several diverse and encouraging effects i.e.

1. Is effective in reducing the severity of AP in two different models of AP that use different species.
2. Modifies AP-induced pancreatic vascular perfusion changes and reduces hyperstimulated pancreatic exocrine secretion.
3. Is effective in reducing the indices of AP when administered both prophylactically and therapeutically.

Prior to considering phase 1 trials, the effects of various galanin antagonists should be evaluated in more severe models of AP. If these are promising, then toxicity studies to show their safety should be conducted in animals.

Figure 8.1. Flow chart illustrating the pathophysiology of AP and potential events where galanin antagonist may be acting.



X possible site of galanin antagonist action. (NF-κB= nuclear factor κB; ROS= reactive oxygen species).

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