

**Growth Regulation of Pancreatic Cancer Cells and Their Normal Cells
of Origin by Nicotinic Acetylcholine Receptors**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
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DEDICATION

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ABSTRACT

Pancreatic cancer is the fourth leading cause of cancer mortality with a five-year survival rate of less than 5 %. It shows no symptoms until it has reached an advanced stage upon which it has metastasized to distant organs limiting therapeutic options. Several studies have identified smoking, alcohol, diabetes and pancreatitis as risk factors for pancreatic cancer. While smoking is a well-documented risk factor for this malignancy, there still remains a controversy on whether alcohol can act as a risk factor itself or cooperatively enhances the effects of other risk factors.

Previous reports provide evidence that nicotinic acetylcholine receptors (nAChRs) and beta-adrenergic receptors cooperatively stimulate the growth and migration of normal and pancreatic ductal adenocarcinoma (PDAC) cells upon binding NNK. Binding of the nicotine-derived nitrosamine ketone (NNK) to nAChRs result in neurotransmitters production regulation. These neurotransmitters in turn act as agonists to β -ARs [beta-adrenergic receptors] thereby activating adenylyl cyclase and the subsequent cAMP dependent signaling pathways. This ultimately results in the stimulation of proliferation, migration and angiogenesis and inhibition of apoptosis.

Using assays for the assessment of adrenaline, noradrenaline, GABA and cAMP production; PKA activity; CREB, Src, Akt and ERK phosphorylation; and cell proliferation and migration, we have identified an autocrine catecholamine loop that is jointly regulated by $\alpha 3$, 5 and 7-nAChRs [alpha 3, 5 and 7-nicotinic acetylcholine receptors] in normal pancreatic ductal epithelial cells (HPDE6-C7) and PDAC cell lines (BxPC-3 and Panc-1) upon stimulation by nicotine or ethanol. This catecholamine loop in

turn acts to stimulate growth of normal and PDAC cell lines via cAMP dependent signaling pathways downstream β -ARs [beta-adrenergic receptors]. This loop however, is interrupted upon γ -amino butyric acid [gamma-amino butyric acid] (GABA) treatment of cells at the level of adenylyl cyclase activation thereby inhibiting nicotine and ethanol-induced stimulatory effects on cell proliferating and migration.

Our study findings therefore suggest smoking and chronic alcohol intake to be risk factors for the development of PDAC. These agents regulate catecholamine and GABA synthesis via modulation of nAChRs thereby stimulating proliferation and migration and inhibiting apoptosis. The observed effects of nicotine and ethanol were all reversed by treatment of cells with GABA, suggesting it as a potential therapeutic agent for the treatment and prevention of this malignancy.

LIST OF ABBREVIATIONS

α 3-nAChR:	Alpha3-Nicotinic Acetylcholine Receptor
α 4-nAChR:	Alpha4-Nicotinic Acetylcholine Receptor
α 5-nAChR:	Alpha5-Nicotinic Acetylcholine Receptor
α 7-nAChR:	Alpha7-Nicotinic Acetylcholine Receptor
α -BTX:	Alpha-Bungarotoxin
β -ARs:	Beta Adrenergic Receptors
AA:	Arachidonic Acid
AC:	Adenylyl Cyclase
AKT (PKB):	Protein Kinase B
AMP:	Adenosine Monophosphate
BPE:	Bovine Pituitary Extract
BxPC-3:	Human Pancreatic Ductal Adenocarcinoma Cell Line
Ca^{2+} :	Calcium ion
cAMP:	Cyclic Adenosine Monophosphate
c-fos:	Nuclear phosphorprotein gene (transcription factor)
c-jun:	Nuclear phosphorprotein gene (transcription factor)

c-myc:	Nuclear phosphorprotein gene (transcription factor)
CREB:	cAMP Response Element Binding Protein
DAG:	Diacylglycerol
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribonucleic Acid
EDTA:	EthyleneDiamineTetraacetic Acid
EGF:	Epidermal Growth Factor
EGFR:	Epidermal Growth Factor Receptor
ERK:	Extracellular-Signal Regulated Kinases
Eth:	Ethanol
EtOH:	Ethyl Alcohol
FBS:	Fetal Bovine Serum
GABA:	γ -amino butyric acid
GPCRs:	G-protein coupled receptors
HBSS:	HEPES Buffered Saline Solution
HCl:	Hydrochloric Acid
hEGF:	Human Epidermal Growth Factor

HPDE6-C7:	Immortalized Human Pancreatic Duct Epithelial Cells
IBMX:	IsoButyl-1-MethylXanthine
JAK:	Janus Kinase
KSFM:	Keratinocyte Serum Free Medium
LSM:	Low Serum Medium
MAPK:	Mitogen-Activated Protein Kinase
MEK:	MAPK/ERK Kinase
nAChRs:	Nicotinic Acetylcholine Receptors
NaCl:	Sodium Chloride
NaOH:	Sodium Hydroxide
Na ₃ VO ₄ :	Sodium Orthovanadate
NCI:	National Cancer Institute
NF-AT:	Nuclear Factor-AT
NF-B:	Nuclear Factor-B
Nic:	Nicotine
NIH:	National Institute of Health
NNK:	4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone

NSB:	Non-Specific Binding
Panc-1:	Human Pancreatic Ductal Adenocarcinoma Cell Line
PBS:	Phosphate Buffered Saline
PDAC:	Pancreatic Ductal Adenocarcinoma
PDEs:	Phosphodiesterase
PI:	Phosphatidylinositol
PIK-3:	Phosphatidylinositol 3-Kinase
PKA:	Protein Kinase-A
PKB (AKT):	Protein Kinase-B
PKC:	Protein Kinase-C
PLA:	Phospholipase-A
PLC:	Phospholipase-C
PLD:	Phospholipase-D
PMSF:	PhenylMethylSulfonyl Floride
RADIL:	Research Animal Diagnostic Laboratory
Raf-1:	Cytoplasmic Serine/Threonine Protein Kinase
Rap1:	Ras-related G-protein

Ras:	Membrane Associated GTP Protein Kinase
RH:	Relative Humidity
Rpm:	Rounds per minute
RTK:	Receptor Tyrosine Kinase
SDS-Page:	Sodium Dodecyl Sulfate-Polyacrylamide Gel
Src:	Non-membrane Associated Tyrosine Kinase
STAT:	Signal Transducers and Activators of Transcription
T3:	Triiodothyronine
TA:	Total Activity
TBS:	Tris-Buffered Saline
TBST:	Tris-Buffered Saline Tween-20
TGF- α :	Transforming Growth Factor-alpha
TGF- β :	Transforming Growth Factor-beta
TNS:	Trypsin Neutralizing Solution
VEGF:	Vascular Endothelial Growth Factor
WHO:	World Health Organization

TABLE OF CONTENTS

CHAPTER I: Introduction	1
Introduction	2
Working hypothesis	5
Specific aims	5
References	6
 CHAPTER II: Literature Review	 9
Abstract	10
Pancreatic cancer overview	11
Nicotinic acetylcholine receptors	15
Beta-adrenergic receptors	17
GABA as a potential therapeutic agent	19
References	23
Appendix	28
 CHAPTER III: Pancreatic Cancer Cells and Normal Pancreatic Duct Epithelial Cells Express an Autocrine Catecholamine Loop That is Activated by Nicotinic Acetylcholine Receptors $\alpha 3$, $\alpha 5$ and $\alpha 7$	 29
Abstract	31
Introduction	32
Materials and Methods	33
Results	40
Discussion	44
References	50
Appendix	54
 CHAPTER IV: Effects of Chronic Nicotine on the Autocrine Regulation of Pancreatic Cancer Cells and Pancreatic Duct Epithelial Cells by Stimulatory and Inhibitory Neurotransmitters	 63
Abstract	65
Introduction	66
Materials and Methods	67
Results	76
Discussion	80
References	86
Appendix	90
 CHAPTER V: Interaction of Chronic Ethanol with Nicotinic Receptor-Mediated Regulation of Pancreatic Cancer Cells and Pancreatic Duct Epithelia	 99
Abstract	101
Introduction	101

Materials and Methods.....	103
Results.....	110
Discussion.....	115
References.....	119
Appendix.....	122
 CHAPTER VI: Conclusion	 130
References.....	135
 VITA	 138

LIST OF FIGURES

CHAPTER II: Literature Review

Figure 1.Schematic diagram showing growth regulation of PDAC cells and normal pancreatic duct epithelia via nAChRs.....	28
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CHAPTER III: Pancreatic Cancer Cells and Normal Pancreatic Duct Epithelial Cells Express an Autocrine Catecholamine Loop That is Activated by Nicotinic Acetylcholine Receptors $\alpha 3$, $\alpha 5$ and $\alpha 7$

Figure 1.Secreted and intracellular stimulatory neurotransmitters levels in cells treated with 1 μ M nicotine from 5 to 30 minutes.....	54
Figure 2.Secreted and intracellular stimulatory neurotransmitters levels in cells treated with nicotine concentrations from 10 pM through 10 μ M for 30 minutes.....	55
Figure 3.ELISA assays assessing stimulatory neurotransmitters levels in response to gene knockdown of nAChRs.	56
Figure 4A-C.Western blots showing effects of gene knockdown on protein expression of nAChR subunits in the presence and absence of nicotine.....	57
Figure 4D.Densitometric analysis of Figures 4A-C.....	58
Figure 5.Western blots assessing phosphorylation of signaling proteins in the presence of acetylcholine, nicotine and NNK.	59
Figure 6.ELISA assays assessing effects of the beta blocker propranolol in the presence and absence of nicotine on signaling proteins phosphorylation	60
Figure 7A.MTT assay assessing the effects of a beta blocker and a nAChR blocker in the presence and absence of nicotine on cell proliferation	61
Figure 7B.Schematic diagram of growth regulation of PDAC and normal pancreatic duct epithelia cells via nAChRs and beta-adrenergic receptors.....	62

CHAPTER IV: Effects of Chronic Nicotine on the Autocrine Regulation of Pancreatic Cancer Cells and Pancreatic Duct Epithelial Cells by Stimulatory and Inhibitory Neurotransmitters

Figure 1A-C.ELISA assays assessing the effects of nicotine time exposure and $\alpha 4$ -nAChR gene knockdown on GABA levels.....	90
Figure 1D.Western blots showing the effects of gene knockdown on protein expression of the $\alpha 4\beta 2$ -nAChR in the presence and absence of nicotine.....	91
Figure 2.ELISA assays showing the effects of chronic and serial dilutions of nicotine treatments on total GABA and adrenaline levels.....	92
Figure 3.ELISA assays showing the effects of chronic and serial dilutions of nicotine treatments on noradrenaline and dopamine-beta hydroxylase levels.....	93
Figure 4.Western blots assessing protein expression of nAChR subunits in the presence and absence of GABA and nicotine.....	94
Figure 5A.Western blots assessing phosphorylation of AKT, Src, GAD65 and GAD67 in the presence and absence of GABA and nicotine.....	95
Figure 5B.Densitometric analysis of AKT and Src bands presented in figure 5a.....	95
Figure 5C.Densitometric analysis of GAD65 and 67 bands presented in figure 5a.....	96
Figure 6A.ELISA assays assessing phosphorylation of ERK and CREB in the presence and absence of GABA and nicotine.....	97
Figure 6B-C.ELISA assays assessing cell proliferation and migration in the presence and absence of GABA and nicotine.....	98

CHAPTER V: Interaction of Chronic Ethanol with Nicotinic Receptor-Mediated Regulation of Pancreatic Cancer Cells and Pancreatic Duct Epithelia

Figure 1.ELISA assays assessing cell proliferation and migration in the presence and absence of GABA and ethanol.....	122
Figure 2.Western blots assessing protein expression of nAChR subunits in the presence and absence of GABA and ethanol.....	123
Figure 3.Western blots assessing phosphorylation of GAD65 and 67 in the presence and absence of GABA and ethanol	124

Figure 4.ELISA assays showing the effects of chronic and serial dilutions of ethanol treatments on total GABA, adrenaline and noradrenaline levels.....	125
Figure 5.ELISA assays showing cAMP accumulation and PKA activity in the presence and absence of GABA and ethanol.....	126
Figure 6.ELISA assays assessing signaling proteins phosphorylation in the presence and absence of GABA and ethanol.....	127
Figure 7.Western blots assessing protein expression of nAChR subunits in the presence and absence of nicotine and ethanol.....	128
Figure 8.ELISA assays assessing intracellular GABA, adrenaline and noradrenaline levels in the presence and absence of nicotine and ethanol.....	129

CHAPTER I:

Introduction

Introduction

The pancreas is a fish shaped organ behind the stomach that is composed of two different glands [1]. The exocrine gland makes up the larger portion of the pancreas and is responsible for the production of the pancreatic juice which contains enzymes necessary for the digestion of fats and proteins found in food. On the other hand, a small portion of the pancreas composed of islet cells makeup the endocrine gland which is responsible for the secretion of hormones mainly involved in blood glucose regulation [1-3].

Tumors of the pancreas can rise in both the exocrine and endocrine glands. However, most tumors formed in the pancreas are of exocrine origin and are mostly referred to as adenocarcinomas [1,4]. Pancreatic cancer is one of the most aggressive diseases, ranking fourth in cancer mortality in western countries [5,6]. Pancreatic ductal adenocarcinoma (PDAC) make up the majority of pancreatic cancer compromising more than 90 % of all cases [6-8]. Studies have shown that PDAC has a poor five-year survival rate of less than 5 % and morality rate near 100 % within two years of diagnosis [4,6,9,10]. Studies on pancreatic cancer show 265, 000 deaths out of 280, 000 cases in 2008; while estimates by the American Cancer Society predict more than 43,920 new cases and 37,390 deaths from pancreatic cancer in 2012 [11,12].

Several risk factors have been linked to pancreatic cancer. Smoking is a well-documented risk factor for this malignancy with Smokers having a twofold increase in the development of PDAC over non-smokers [13]. Other risk factors associated with this malignancy include chronic alcohol consumption, pancreatitis, diabetes, obesity and

family history [14-18]. The major obstacle to effective clinical outcomes for pancreatic cancer is its delayed diagnosis and resistance to current therapeutics. It shows no symptoms until it has reached an advanced stage upon which it has metastasized to distant organs further complicating its treatment [19,20]. Therefore, further research on pancreatic cancer prevention and therapy are urgently needed.

While older research has focused on gene mutations, recent studies on pancreatic cancer focus on upstream pathways such as nicotinic acetylcholine receptors (nAChRs) and their associated neurotransmitters [21]. These receptors are heterogeneous ionotropic receptors that were initially thought to be restricted to the central nervous system; however, recent studies found them to be expressed in non-neuronal cells such as in the pancreas and the lung [21,22]. Activation of these receptors results in several cellular changes including regulation of the production and secretion of the stimulatory neurotransmitters adrenaline and noradrenaline and the inhibitory neurotransmitter GABA [21,23-26].

The catecholamines produced upon activation of these nAChRs result in the activation of β -adrenergic receptors which are G-protein coupled receptors that activate adenylyl cyclase via the stimulatory G-protein subunit [21,27,28]. Activation of the adenylyl cyclase results in an increase in cAMP levels thereby activating several cAMP dependent pathways which ultimately results in the activation of proliferation and migration of PDAC cells and their normal cells of origin [21,29-31].

While several studies have been consistent on the effects of adrenaline and noradrenaline on PDAC cells and their normal cells of origin, research on GABA is still

controversial. Some studies have found that binding of GABA to its receptor, GABA-B results in the inhibition of this catecholamine-induced pathway via the inhibition of the adenylyl cyclase by the inhibitor G-protein subunit of GABA-B receptor [30]. However, other studies have found that some PDAC cell lines over-expressed the ρ -subunit of GABA-A receptor. Over-expression of this subunit reverses GABA-A's role from being a hyperpolarizing ionotropic receptor to a depolarizing one. Therefore, binding of GABA to GABA-A receptor results in the depolarization of these cell lines thereby increasing intracellular calcium levels which ultimately stimulates cell growth in calmodulin-Calcium dependent mechanism [32].

Using assays for the assessment of cAMP, GABA, adrenaline and noradrenaline production; PKA and MAPK activation; Akt, CREB, Src and ERK phosphorylation; and cell proliferation and migration, this project identified an autocrine catecholamine loop that is co-regulated by α 3, 5 and 7-nAChRs in PDAC cells and their normal cells of origin. This loop activates multiple cellular pathways downstream β -ARs thereby stimulating cell proliferation and migration. Moreover, our data shows that PDAC cells and their normal cells of origin produce GABA via regulation of α 4-nAChR and that treatment of these cells with GABA reverses catecholamine-induced stimulation of growth and migration.

Working hypothesis

The central hypothesis of this project is that ethanol and nicotine alone and in combination promote growth and migration of the immortalized human pancreatic duct epithelial cells (HPDE6-C7) and PDAC cell lines (BxPC-3 and Panc-1) via regulation of nAChRs sensitivity and that these effects were reversed via GABA treatments. Data generated by this project supported this hypothesis and showed that both agents stimulated growth and migration of these cell lines via activation of cAMP dependent pathways downstream the nAChRs.

Specific aims

1. To test the hypothesis that nicotine stimulates the growth and migration of PDAC cells and their normal cells of origin, and to identify its mechanism of action.
2. To test the hypothesis that ethanol stimulates the growth and migration of PDAC cells and their normal cells of origin, and to identify its mechanism of action.
3. To test the hypothesis that GABA reverses nicotine- and ethanol-induced growth and migration of PDAC cells and their normal cells of origin, and to identify its mechanism of action.

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CHAPTER II:
Literature Review

Abstract

Pancreatic cancer is an aggressive disease with poor diagnosis and prognosis. The absence of effective screening tests to detect this malignancy at an early stage is one of the factors responsible for its metastasis. Patients suffering from pancreatic cancer do not show any symptoms until it has reached an advanced stage upon which it has spread to distant organs thereby eliminating surgery as a possible option for therapy. Up to date, there are no effective treatments for pancreatic cancer which makes pancreatic cancer a deadly disease with a 5 year survival rate of less than 5 %. With these horrifying statistics, research on pancreatic cancer took a different approach in studying the mechanisms of this malignancy. While research on the past focused on gene mutations and their downstream signaling pathways, recently, research started to look at up-stream receptors such as nicotinic acetylcholine receptors and how they may be involved in regulating growth of this malignancy.

Nicotinic acetylcholine receptors (nAChRs) are a family of plasma membrane, ionotropic receptors consisting of five subunits. While these receptors were previously thought to be restricted to the nervous system, recent studies have found them to be expressed in most mammalian cells including those of the pancreas. Binding of these receptors to their agonists result in the activation of their associated ion channels which results in several cellular changes including neurotransmitters production. Among the neurotransmitters whose production increase upon activation of the nAChRs are the catecholamine, adrenaline and noradrenaline.

These catecholamines act as agonists to beta adrenergic receptors. These receptors are a family of G-protein coupled receptors which upon activation by adrenaline or noradrenaline, activate adenylyl cyclase and thereby activating cAMP-dependent signaling pathways responsible for growth regulation of pancreatic cancer cells.

While research has failed in finding an effective treatment for pancreatic cancer, recent studies have found the γ -amino butyric acid (GABA) to be an effective anti-cancer agent in vitro and in vivo. This non-essential amino acid is a nutritional supplement found in many plants and fruits including tomatoes, berries and rice. Studies have shown GABA to inhibit pancreatic cancer stimulatory pathways via inhibiting adenylyl cyclase activation through the inhibitory G protein subunit of GABA-B receptor. This agent, therefore, may serve as a potential therapeutic option for the prevention and treatment of pancreatic cancer.

Pancreatic cancer overview

As part of normal growth, cell growth, division and death are governed at several stages of life to ensure production of healthy and normal individual. Once cells start to grow out of control, they initiate cancer [1]. Cancer cell growth differs from normal cell growth in that they continue to grow and divide without any restrictions or limitations due to the absence of effective control centers [1,2]. Moreover, cancer cells in most cases form a mass of cells referred to as tumors. However, not all tumors are considered cancerous. Benign tumors are localized tumors incapable of invading other tissues and most of the time they can be treated via surgical removal of the tumor. On the other hand,

some tumors have the ability to spread and invade other tissues, a process referred to as metastasis. These tumors are referred to as cancers and are considered life threatening due to the complexity of their treatment [1-4].

There are many different types of cancers all of which start with out of control growth due to mutations that result in the activation of oncogenes or inactivation of tumor suppressor genes and DNA repair genes. While the initial stage of cancer is the same, different kinds of cancers can behave differently in terms of growth and division rate as well as treatment response [1-4]. Moreover, each type of cancer may have several subtypes depending on the location and type of cells involved. These subtypes present further complication to understanding and treating cancer [1]. For example, lung cancer is divided into four main histological types of cancers: small cell carcinoma, squamous cell carcinoma, adenocarcinoma and large cell carcinoma. Each of these subtypes show different mechanism of development that make it distinguished from other subtypes and is therefore unique in its growth and responsiveness to therapy [5,6].

The pancreas is an interesting organ due to its ability to exert both endocrine and exocrine functions in the body. The endocrine portion of the pancreas produces and releases hormones that act as main regulators of blood glucose levels such as insulin, glucagon and somatostatin. On the other hand, the exocrine gland of the pancreas produces several enzymes cumulatively referred to as the pancreatic juice that play vital role in the digestion of ingested food [1]. Tumors in the pancreas can rise from both the endocrine and exocrine glands. While the tumors rising from islet cells in the endocrine portion of the pancreas are rare, they tend to be very serious and life threatening. These

tumors, referred to as neuroendocrine tumors, tend to cause severe changes in hormonal production and secretion which cause significant changes in blood glucose levels [1]. On the other hand, tumors rising from the exocrine glands make the majority of pancreatic cancer cases [1,2,4]. The majority of exocrine tumors are adenocarcinomas that rise from glandular cells. Over 75 % of pancreatic cancer cases are adenocarcinomas derived from ductal and ductal epithelia and are referred to as pancreatic duct adenocarcinoma (PDAC) [7]. Another type of exocrine tumor is the ampullary cancer which rises in the space where the bile and pancreatic ducts empty into the small intestines. Treatment of all of these exocrine tumors is usually based on the stage of cancer not its exact type. The earlier the stage, the better the chance of tumor removal via surgical interference; however, later stage tend to be more responsive to chemo and/or radiotherapy [1-3].

Epidemiological studies rank pancreatic cancer as the fourth leading cause of cancer death in the world with a morality rate near 100 % within two years of diagnosis [8-10]. It is responsible for the death of 265,000 patients worldwide in 2008 [3,11,12]. Within the past 25 years, pancreatic cancer has a median survival of six months and a five year survival rate of less than 5 % [8,10]. Surprisingly, these studies also show higher rates of pancreatic cancer incidence and mortality in high income areas compared to low income regions [3]. Pancreatic cancer morality in high income countries such as Europe and the U.S has been increasing between 1950s and 80s but has leveled off thereafter in men. Since the 1980s, incidence of pancreatic cancer in men in Europe leveled off at 7.5/100,000 cases while incidence in women rose to 5/100,000 by the early 2000s [3,13,14]. Similar trends were seen in the U.S with morality rates stabilizing in men since

the 1970s and rising in women as time progresses [3,15]. Recent estimates for pancreatic cancer in the United States show 43,920 new cases and 37,390 deaths from pancreatic cancer in 2012 [1].

The normal lifetime risk of developing pancreatic cancer for both men and women in the United States is about 1 in 71 [1]. However, this risk changes upon exposure to risk factors associated with the development of this malignancy. Several risk factors associated with pancreatic cancer include smoking, chronic and hereditary pancreatitis, alcoholism, diabetes and familial cancer syndromes [16-19]. Moreover, while there appears to be no major gender differences with respect to pancreatic cancer incidence, reports show African Americans and older people (over the age of 55) to have higher rates of pancreatic cancer than Caucasians and younger individuals [1].

The aggressive nature of pancreatic cancer lays in its ability to rapidly disseminate to the lymphatic system and distant organs [20,21]. It does not show any symptoms until it has reached an advanced stage upon which it has already metastasized to distant organs [22-24]. The presence of metastasis at the time of diagnosis along with the lack of effective screening methods and effective chemotherapy contribute to the high mortality rate in pancreatic cancer patients [20,21]. Pancreatic cancer is one of the most unresponsive cancers to current therapeutics causing major failure in its treatment [20,21]. Several standard chemotherapeutics have been clinically established such as gemcitabine and FOLFIRINOX for the treatment of pancreatic cancer; however, they only present very minimal increase in survival rate and are not much effective [20,21,25]. Thus, there is no guaranteed way of treating or preventing pancreatic cancer. However,

one can only minimize their risk of developing pancreatic cancer by avoiding exposure to risk factors associated with this disease such as smoking and drinking.

Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) are a family of ion channels located in the plasma membrane [26]. These pentameric receptors can be either homomeric consisting of the same five subunits as in the case of $\alpha 3$, $\alpha 5$, $\alpha 7$, $\alpha 8$ or $\alpha 9$ -nAChRs or heteromeric consisting of combination of subunits from α , β , γ and ϵ as in the case of $\alpha 4\beta 2$ -nAChR [26-30]. Binding of these nAChRs to their agonists causes conformational changes in their subunits opening the receptor ion channels. Opening of the ion channels leads to the inward flow of several cations such as potassium, sodium and calcium; but, the major ion channel associated with nAChRs is the calcium ion channel. Flow of calcium and other cations into the cell non-selectively through the open nAChRs causes the electrical charge inside the plasma membrane to become positive, a process known as depolarization. In turn, this depolarization of these cells stimulates voltage-gated calcium channels leading to additional influx of calcium which leads to several cellular changes including neurotransmitter synthesis and release [31,32].

For decades, the nAChRs were generally thought to be restricted to the nervous system and at neuro-muscular junctions. However, recent studies have found these receptors and their physiological agonist acetylcholine to be expressed in mammalian cells including cancer cells of the pancreas, lung, colon and breast [23,26,33]. These receptors act as regulators of growth of normal and cancer cells by modulating a complex

network involving both stimulatory and inhibitory neurotransmitters [23,26]. Binding of nAChRs to their agonists results in several cellular changes induced by depolarization of the cells which results in regulation of stimulatory and inhibitory neurotransmitters synthesis and release which ultimately act to activate several pathways including cAMP-signaling pathways which ultimately results in stimulation of proliferation, migration and angiogenesis and inhibition of apoptosis (Fig. 1) [23,26]. Several studies on different types of nAChRs have found $\alpha 3$, 5 and 7-nAChRs to be stimulatory nAChRs that stimulate the synthesis of the stimulatory neurotransmitters adrenaline and noradrenaline which ultimately stimulate cellular growth and division. However, studies on $\alpha 4\beta 2$ -nAChR have found it to be an inhibitory receptor involved in the synthesis and release of the inhibitory neurotransmitter GABA thereby inhibiting cancer progression [23,26].

For years it was generally believed that activation of nAChRs requires the release of acetylcholine from the nervous system. However, recent studies have shown that acetylcholine and its synthesizing enzyme, choline acetyltransferase are also expressed in mammalian cells including cancer cells such as those of the pancreas, lung, colon and breast [26,33]. Moreover, recent research on nAChRs have found other agonists to activate $\alpha 3$, 4, 5 and 7-nAChRs including ethanol, nicotine, NNK and NNN [23,26,33,34].

Interestingly, while chronic exposure of most types of cellular receptors to their agonists results in their down-regulation, chronic and acute exposure of nAChRs to their agonists results in their up-regulation [23,26,32,35]. Moreover, exposure of these receptors to nicotine which has a binding affinity to nAChRs that is ten times higher than

acetylcholine results in the up-regulation of these receptors as well [23,26,36]. Interestingly however, research has found nicotine, NNK and acetylcholine to bind with higher affinity to the heteromeric nAChRs, such as the $\alpha 4\beta 2$ -nAChR, than to the homomeric nAChRs such as $\alpha 3$, 5 and 7-nAChRs [26,37,38]. This higher affinity of these agents to the heteromeric nAChRs, particularly $\alpha 4\beta 2$ -nAChR, results in its long-term inactivation or desensitization [26,35]. However, the sensitivity of the homomeric receptors $\alpha 3$, 5 and 7-nAChRs remains unaffected and chronic exposure of these receptors to their agonists results in their hyper-activation [23,26,35]. Therefore, these findings suggest that exposure of nAChRs to nicotine, NNK or even acetylcholine provides a suitable environment for cancer cell growth due to up-regulation of the stimulatory $\alpha 3$, 5 and 7-nAChRs and desensitization of the inhibitory $\alpha 4\beta 2$ -nAChR [26,38-40].

Beta-adrenergic receptors

G protein-coupled receptors (GPCRs) are one of the largest families of cell membrane receptors consisting of an extracellular ligand binding domain, seven transmembrane domains and heteromeric G-protein subunits [41]. The heteromeric subunits are α , β and γ subunits. Binding of a GPCR to its agonist results in the transformation of GDP to GTP, which results in the activation of the receptor and dissociation of the G protein into G_α -GTP and $G_{\beta\gamma}$ subunits. These active subunits in turn can activate ion channels, other receptors and enzymes leading to several cellular reactions induced by the agonist [42-44]. Interestingly, studies have found numerous types of G_α subunits that serve different

functions in the cell. The $G_{\alpha s}$ subunit serves to activate adenylyl cyclase while on the other hand, the $G_{\alpha i}$ inhibits adenylyl cyclase and the $G_{\alpha q}$ activate phospholipase C. Therefore, depending of the type of G_{α} present in the GPCR, ligand binding can induce different cellular reactions within the cell [41,45,46]. Once the ligand leaves the receptor, the GTP is converted to GDP and the subunits of G-protein re-associate with each other to form an inactive G-protein (GDP) bound state [42-44].

Beta-adrenergic receptors (β -ARs) are a family of G protein-coupled membrane receptors expressed in most mammalian cells [47-49]. Studies have found three types of β -ARs expressed in mammalian cells including β_1 , 2 and 3 adrenergic receptors. Beta 1 and 2 are expressed in most mammalian cells with beta 3 being limited to adipose tissues [47-49]. Binding of these receptors to their agonists initiate several signaling cascades via the $G_{\alpha s}$ subunit including the arachidonic acid (AA) pathway, Src/STAT pathway and the adenylyl cyclase / cAMP / PKA / CREB pathway which also transactivates the epidermal growth factor receptor (EGFR) cascade (Fig. 1) [47,50-54].

Intensive studies on β -ARs mechanism of action have found the stimulatory catecholamine stress neurotransmitters, adrenaline and noradrenaline, to be the physiological agonists of these receptors with adrenaline preferentially binding to beta 2 and noradrenaline to beta 1 [23,47,53,54]. These neurotransmitters are produced and secreted from the adrenal medulla. Moreover, recent research has found these neurotransmitters to be also regulated by nAChRs [26,53]. Thus, exposure of cells to nicotine, acetylcholine or NNK leads to up-regulation of α_3 , 5 and 7-nAChRs which in turn stimulates catecholamine synthesis. These catecholamines in turn activate the β -ARs

initiating the pathways listed above [26,53,55]. While nicotine and acetylcholine can indirectly activate the β -ARs via up-regulating of nAChRs, studies have found that NNK can directly activate β -ARs via acting as an agonist to these receptors thus providing an additional up-regulation of the signaling cascade [49,53].

With the significant role β -ARs serve in regulation of cancer and normal cell growth, research have focused on blocking these receptors as a potential therapeutic option against cancer growth. Studies have shown that growth of both human pancreatic duct adenocarcinoma cells and pancreatic duct epithelial cells to be stimulated by β -ARs agonists which ultimately result in the stimulation of proliferation, migration and angiogenesis and inhibition of apoptosis in cAMP dependent manner. Interestingly, studies by our laboratory have shown that the use of beta-blockers which have been safely used as cardio-vascular therapeutics such as propranolol can inhibit these growth stimulating pathways by blocking the activity of β -ARs thereby inhibiting adenylyl cyclase activation and the subsequent cAMP synthesis [23,53,56,57]. These results therefore suggest beta blockers as potential therapeutic options for the treatment and prevention of cancer.

GABA as a potential therapeutic agent

Gamma-amino butyric acid, also known as GABA, is a non-essential amino acid that helps maintain a proper balance between body and mind. It is the major inhibitory neurotransmitter in the central nervous system that helps losing excess body fats and has anti-aging properties [40,49,58,59]. Moreover, GABA is an FDA approved supplement

found in many fruits and vegetables including almonds, nuts, oats, brown rice, berries, tomatoes and grapes that is widely used to ease anxiety and promote sleep. In addition, several studies have found GABA to exert anti-tumor activity preventing proliferation and migration of cancer cells in the pancreas and lungs [49].

For decades, it was widely believed that GABA production was restricted to the brain, recent studies have found this neurotransmitter and its receptors to be expressed in several peripheral tissues and organs such as the pancreas and lungs [58]. In the pancreas, GABA is synthesized in β -cells from glutamate in a reaction catalyzed by both isoforms of glutamic acid decarboxylase (GAD), GAD65 and GAD67 in the presence of pyridoxal as a cofactor [40,49].

In the pancreas and lungs, studies by our laboratory have found GABA production to be regulated by $\alpha 4\beta 2$ -nAChR. Activation of this receptor results in an increase GABA synthesis and secretion by both PDAC and pancreatic duct epithelial cells [40,49]. However, interestingly, exposure of this receptor to nicotine, NNK or ethanol induces desensitization of the receptor until removal of the agent. This desensitization in turn results in a reduction of GABA levels which provides an environment suitable for cancer growth [23,40,49].

Studies have found GABA to be associated with 3 different types of receptors: the ionotropic receptors GABA_A and GABA_C, and the G protein-coupled receptor GABA_B which exists as two isoforms termed GABA_BR1 and GABA_BR2 [49,60]. Both of the GABA_B receptors are G protein-coupled receptors coupled to the adenylyl cyclase inhibiting G-protein subunit, G_{oi}. Studies on these receptors have found the GABA_A and

GABA_C ionotropic receptors to mediate excitatory functions upon binding to GABA while the GABA_B receptors mediate GABA's inhibitory functions via the G_{oi} subunit. The G_{oi} subunit of the GABA_B receptors act as powerful control for the cAMP-dependent signaling pathways that drive proliferation, migration and angiogenesis of PDAC and lung adenocarcinomas [49]. These GABA_B receptors act as a physiological brake to counteract the entire cAMP dependent stimulatory signaling activated by β -ARs (Fig. 1). Binding of GABA or baclophen to the GABA_B receptor, resulted in the inhibition of adenylyl cyclase via its G_{oi} subunit. This in turn reduced intracellular cAMP levels and phosphorylation of important signaling proteins downstream the β -ARs that are involved in cell proliferation, migration and angiogenesis such as ERK, Src, CREB and Akt [40,49,61].

While several studies have shown GABA to be a promising agent for the prevention and therapy of PDAC via GABA_B signaling, other studies have found GABA to have stimulatory effects on these PDAC cell lines. These studies have shown that several PDAC cell lines overexpress the π subunit of GABA_A receptor, and only in these cell lines that overexpress this subunit that GABA stimulate PDAC [62]. Binding of GABA to the GABA_A receptor in cells that overexpress the π subunit results in the activation of calcium ion channel which depolarizes the cells. This depolarization in turn results in the activation of voltage gated calcium channels further increasing calcium levels. Calcium in turn binds to calmodulin which in turn activate several stimulatory pathways including the mitogen activated protein kinase (MAPK) pathway [62].

Despite these discrepancies in the effects of GABA in cancer growth, studies in support of GABA as a therapeutic agent are far more frequent than those indicating otherwise. In addition, studies by proton magnetic resonance of the brain have also shown that smoking significantly reduced GABA levels which is in accord with our findings in PDAC cells [62,63]. Although it has shown significant anti-tumor activity in vitro and in vivo in PDAC and lung cancer, GABA anti-tumor effects have not been tested clinically yet [40,49]. Thus, more research is needed to study the potential therapeutic ability of this agent to help in the treatment and prevention of the fourth leading cause of cancer deaths, pancreatic cancer [8,10].

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Appendix

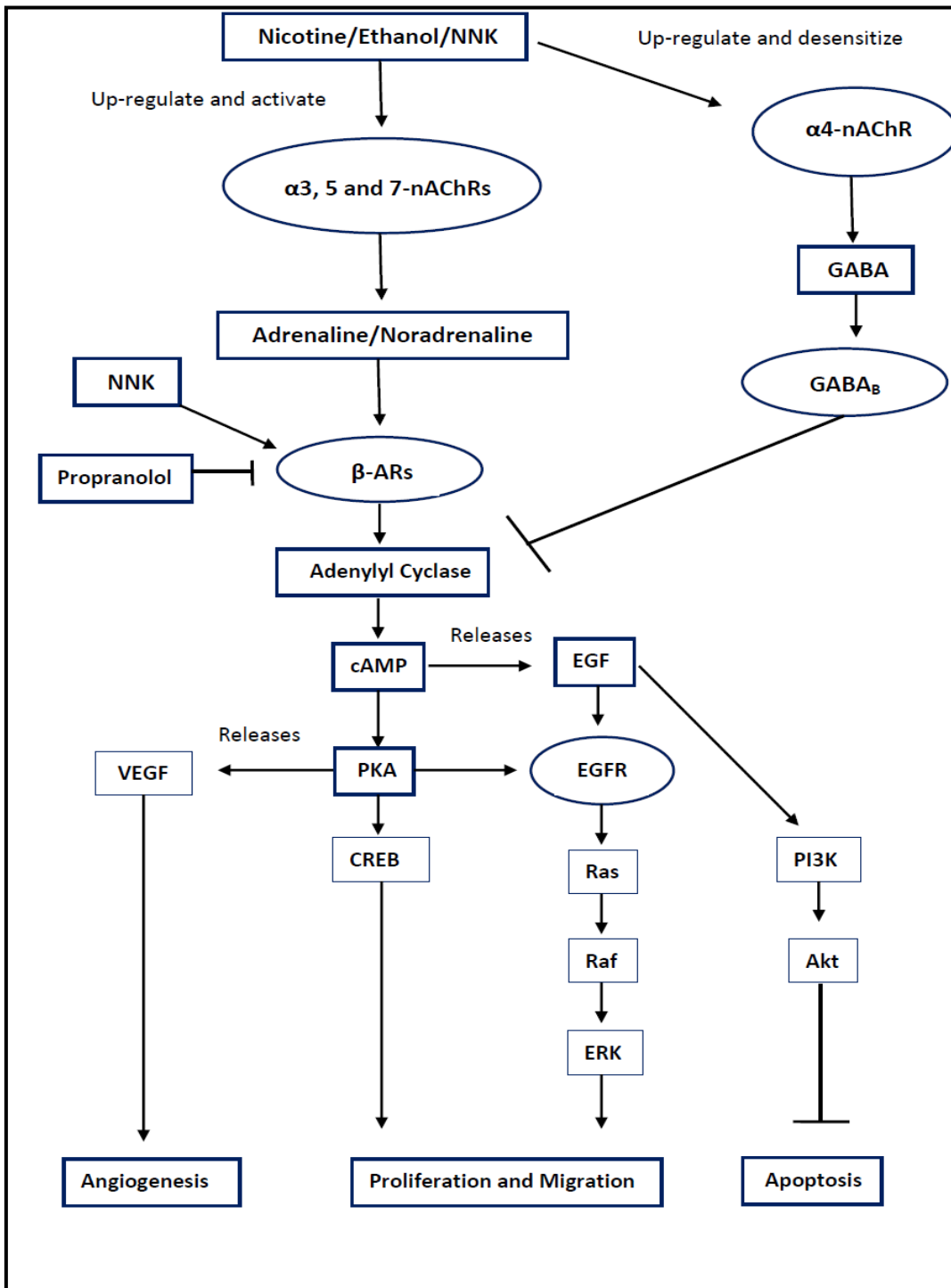


Figure 1. Schematic diagram showing growth regulation of PDAC cells and normal pancreatic duct epithelial cells via nicotinic acetylcholine receptors (nAChRs).

CHAPTER III:

Pancreatic Cancer Cells and Normal Pancreatic Duct Epithelial Cells Express an Autocrine Catecholamine Loop That is Activated by Nicotinic Acetylcholine Receptors $\alpha 3$, $\alpha 5$ and $\alpha 7$

CHAPTER III

Brief explanatory statement

This chapter is a slightly revised version of a manuscript that has been published in “Molecular Cancer Research” on February 2012.

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The use of “we” in this chapter refers to the contributions of the co-authors and I in the project. My contributions in this paper include (1) running all experiments relevant to the paper, (2) analysis and interpretation of the data, (3) statistical analysis of all data, (4) photographic and graphic documentation of results, (5) manuscript writing. Co-authors contributions to this paper include experimental design and writing editorial assistance by Dr. Schuller and technical assistance by Dr. Hussein Al-Wadei.

Abstract

Pancreatic cancer is the fourth leading cause of cancer deaths in developed countries. Smoking is an established risk factor for this malignancy but the underlying mechanisms are poorly understood. Previous reports have provided evidence that nicotinic acetylcholine receptors (nAChRs) and beta-adrenergic receptors (β -ARs) stimulate the growth and migration of pancreatic cancer cells. But a potential cooperation of these two receptor families in the regulation of pancreatic cancer has not been studied to date. Using two pancreatic cancer cell lines and immortalized pancreatic duct epithelia in vitro, our current data show, that all three cell lines synthesized and released the catecholamine neurotransmitters noradrenaline and adrenaline upon exposure to nicotine and that this activity was regulated by $\alpha 3$, $\alpha 5$, and $\alpha 7$ -nAChRs. In accord with the established function of these catecholamines as β -AR agonists, nicotine-induced cell proliferation was blocked by the β -AR antagonist propranolol. Nicotine-induced proliferation was also abolished by the $\alpha 7$ -nAChR antagonist α -bungarotoxin while catecholamine production in response to nicotine was blocked by gene knockdown of the $\alpha 3$, $\alpha 5$, and $\alpha 7$ -nAChRs. The nicotinic agonists acetylcholine, nicotine, and its nitrosated carcinogenic derivative NNK induced the phosphorylation of CREB, ERK, Src and AKT and these responses were inhibited by propranolol. Our findings identify this hitherto unknown autocrine catecholamine loop as an important regulatory cascade in pancreatic cancer that may prove a promising new target for cancer intervention.

Introduction

Pancreatic cancer is the fourth leading cause of cancer deaths with a mortality rate near 100 % within two years of diagnosis [1]. The major impediment to effective clinical outcomes for this malignancy is its delayed diagnosis and resistance to existing cancer therapeutics [2,3]. Novel strategies for more successful prevention and therapy of pancreatic cancer are therefore urgently needed.

Pancreatic ductal adenocarcinoma (PDAC) accounts for the majority of pancreatic cancer cases and is thought to arise from pancreatic duct epithelia [3]. Smoking is a documented risk factor for pancreatic cancer [4], with smokers demonstrating a twofold increase in the risk of developing PDAC [5]. Tobacco smoke is composed of over 4000 chemicals, including the nicotine-derived carcinogenic nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [6]. It has been shown that NNK causes PDAC in rats [7] and hamsters [8]. While nicotine acts as the primary psychoactive component responsible for smoke addiction, it is thought to be non-carcinogenic. However, several laboratories have reported that nicotine activates numerous cellular signaling pathways downstream of nicotinic acetylcholine receptors (nAChRs), resulting in the stimulation of cell proliferation, angiogenesis, and metastasis of several cancers, including PDAC [9-12].

Nicotinic acetylcholine receptors constitute a heterogeneous family of ion channels that were initially thought to be restricted to the central and peripheral nervous system. However, recent studies have identified the expression of this receptor family in numerous non-neuronal cells, including the pancreas [13]. In the nervous system and

adrenal glands, nAChRs regulate the synthesis of the catecholamine neurotransmitters noradrenaline and adrenaline and their release into the extracellular environment and systemic circulation [14,15]. Both catecholamines are agonists for β -adrenergic receptors (β -ARs). Binding of adrenaline or noradrenaline to these receptors activates the stimulatory G protein ($G\alpha_s$) which in turn activates adenylyl cyclase, the single rate limiting step for the formation of intracellular cAMP [16,17]. Moreover, studies by our laboratory have shown that PDAC cells express β -ARs with β_2 -ARs predominating, and that cAMP signaling downstream of these receptors stimulates the proliferation and migration of these cells [18-20].

In the current experiments, we have tested the hypothesis that analogous to their function in the CNS, nAChRs regulate the synthesis and release of noradrenaline and adrenaline in PDAC cells and pancreatic duct epithelial cells and that this autocrine catecholamine loop activates multiple cellular pathways that are overexpressed in pancreatic cancer.

Materials and Methods

Chemicals, primers and antibodies

Lipofectamine 2000 Reagent, stealth-183 for the CHRNA7 gene, stealth-1079 for the CHRNA3 gene, stealth-873 for the CHRNA5 gene, stealth-1973 for the CHRNA4 gene, stealth RNAi Negative Control Low GC Duplex, and Opti-MEM I reduced serum medium 1X were all purchased from Invitrogen Corporation (Carlsbad, CA, USA). The primer used to interfere with the α_7 subunit mRNA was sense, GGA AGC UUU ACA

AGG AGC UGG UCA A and antisense, UUG ACC AGC UCC UUG UAA AGC UUC C. The primer used to interfere with the $\alpha 3$ subunit mRNA was sense, GCU CUU CCA UGA ACC UCA AGG ACU A and antisense, UAG UCC UUG AGG UUC AUG GAA GAG C. The primer used to interfere with the $\alpha 5$ subunit mRNA was sense, GGG AGC AAA GGA AAC AGA ACC GAC A and antisense, UGU CGG UUC UGU UUC CUU UGC UCC C. The primer used to interfere with the $\alpha 4$ subunit mRNA was sense, GAC CGC AUC UUC CUC UGG AUG UUC A and antisense, UGA ACA UCC AGA GGA AGA UGC GGU C.

The TE Buffer 1X was purchased from Promega Corporation (Madison, WI, USA). The 2-Cat Elisa Kits were purchased from Rocky Mountain Diagnostics Incorporation (Colorado Springs, CO, USA). ELISA kits for Akt [pS473], ERK1/2 [pTpY185/187], and CREB [pS133] were purchased from Invitrogen Corporation (Carlsbad, CA, USA). The c-Src Kinase assay was purchased from MBL International (Woburn, MA, USA).

The antibodies Akt (60 kDa), p-Akt (60 kDa), Src (60 kDa), p-Src (60 kDa), ERK $\frac{1}{2}$ (44/42 kDa), p-ERK $\frac{1}{2}$ (44/42 kDa), p-CREB (43 kDa), anti-rabbit and anti-mouse were all purchased from Cell Signaling (Danvers, MA, USA). The primary antibodies anti-CREB (43 kDa) and anti-nicotinic acetylcholine receptor alpha4 (55 kDa) were purchased from Millipore (Billerica, MA, USA). The nicotinic acetylcholine receptor subunits $\alpha 7$ (56 kDa), $\alpha 3$ (57 kDa), $\alpha 5$ (53 kDa), and β -actin (42 kDa) antibodies were purchased from Abcam (Cambridge, MA, USA). Nicotine and propranolol were both purchased from Sigma-Aldrich (St. Louis, MO, USA). The site-selective $\alpha 7$ -nAChR

antagonist, α -bungarotoxin, was purchased from Calbiochem (Gibbstown, NJ, USA). The lysis buffer used to extract proteins along with Pierce ECL western blotting substrate were purchased from Thermo Scientific (Rockford, IL, USA).

Cell culture

The human pancreatic ductal adenocarcinoma cell lines Panc-1 and BxPC-3 were purchased from the American Type Culture Collection (Manassas, VA, USA). The immortalized human pancreatic duct epithelial cell line, HPDE6-C7, was clonally established after transduction of the HPV16-E6E7 genes into primary cultures of pancreatic duct epithelial cells and was a kind gift from Dr. Tsao (Division of Cellular and Molecular Biology, Department of Pathology, Ontario Cancer Institute/Princess Margaret Hospital, University of Toronto, Toronto, ON, Canada). All cell lines have been authenticated at the beginning of the current study by RADIL (Research Animal Diagnostic Laboratory, Columbia, MO, USA) by species-specific PCR evaluation.

The Panc-1 cell line was maintained in DMEM medium supplemented with 10 % Fetal Bovine Serum (FBS). BxPC-3 cells were maintained in RPMI 1640 medium supplemented with 10 % FBS. HPDE6-C7 cells were maintained in Keratinocyte Serum Free Medium (KSFM) supplemented with 25 mg / 500 ml Bovine Pituitary Extract (BPE) and 2.5 μ g / 500 ml Epidermal Growth Factor (EGF) (GIBCO Invitrogen Corporation, Grand Island, NY, USA). All cell lines were grown without antibiotics in an atmosphere of 5 % CO₂, 99 % relative humidity, and 37 °C.

Analysis of intracellular and secreted adrenaline and noradrenaline

All three cell lines were maintained in their respective complete medium until reaching 65 % confluence, at which time they were switched to basal medium for 24 hours starvation. Cells were then switched into fresh basal media and were divided into two groups. The first groups of cells were either untreated or treated with 1 μ M nicotine for 1, 5, 15, or 30 minutes. The second groups of cells were either untreated or treated with 10 pM, 500 pM, 1 nM, 500 nM, 1 μ M, or 10 μ M nicotine for 30 minutes. The culture media, containing secreted catecholamines were then collected in 15 ml test tubes. The cells which contained synthesized intracellular catecholamines were lysed and harvested into 1.5 ml eppendorf tubes after a one time wash with warm 1X PBS. Quantitative analyses of intracellular and secreted adrenaline and noradrenaline of five samples per treatment group were conducted using 2-Cat ELISA kits following the vendor's recommendations. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Gene knockdown of the $\alpha 3$, 4, 5, and 7-nAChRs

Cells from all three cell lines were grown for 24 hours in their respective complete media. At that time, cells were switched to Opti-MEM I media and were divided into several groups. Groups 1 and 2 from each cell line were left untreated in Opti-MEM I media for 24 hours. Group 3 was transfected for 24 hours with stealth RNAi Negative Control Low GC Duplex. Groups 4 and 5 were transfected with either stealth-

183, 1079, 873, or 1973 for the CHRNA7, 3, 5, and 4 genes respectively for 24 hours in Opti-MEM I media. Once the 24 hours transfection was complete, all cells were switched into their respective basal media. Groups 1, 3, and 4 were left untreated for 30 minutes in basal media whereas groups 2 and 5 were treated with 1 μ M nicotine for 30 minutes in basal media. All transfections were done using Lipofectamine 2000 reagent following the instructions of the manufacturer. Cell lysates were then harvested and collected in 1.5 ml eppendorf tubes after one time wash with warm 1X PBS for adrenaline and noradrenaline analyses by immunoassays as described above. The transfection efficiency was monitored by western blots using α 3, 4, 5 and 7-nAChRs as primary antibodies and actin as a loading control following the procedure outlined below. Following background subtraction, mean densities of 2 rectangular areas of standard size per band from three independent westerns were determined and mean values and standard deviations (n = 6) of protein expression were calculated.

Assessment of cell proliferation by MTT assay

Cells from the three cell lines were seeded in 6-well plates at a density of 50,000 cells per well in their respective phenol red free complete media. Cells were then left untreated, or treated with 1 μ M nicotine for 72 hours, 200 nM α -bungarotoxin for 72 hours, 1 μ M propranolol for 72 hours, 200 nM α -bungarotoxin for 10 minutes followed by 1 μ M nicotine for 72 hours, or 1 μ M propranolol for 10 minutes followed by 1 μ M nicotine for 72 hours. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) colorimetric assay (Sigma-Aldrich St Louis, MO, USA)

was used to assess cell proliferation following instructions by the vendor. The MTT assay is based on the nicotinamide adenine dinucleotide-dependent enzymatic reduction of the tetrazolium salt MTT to form formazan in metabolically active, viable cells. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 570 nm primary and 650 nm reference wavelengths.

Quantitative assessment of phosphorylation of signaling proteins by ELISA assays

Cells from the three cell lines were left to grow in their respective complete media until reaching 65 % confluence. Cells were then left untreated, or treated with 1 μ M nicotine for 72 hours, 1 μ M propranolol for 72 hours, or 1 μ M propranolol for 10 minutes followed by 1 μ M nicotine for 72 hours in complete media. The cells were then lysed and harvested into 1.5 ml eppendorf tubes after a one time wash with warm 1X PBS. Quantitative analyses of Akt, CREB, Src, and ERK1/2 phosphorylation of five samples per treatment group were conducted using Akt, CREB, c-Src, and ERK1/2 ELISA kits respectively following the vendors' recommendations. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Western blot analysis

Cells from HPDE6-C7, BxPC-3, and Panc-1 were grown in their respective complete medium until reaching 65 % confluence. Cells were then switched to their respective basal media without any supplements or antibiotics for 24 hours starvation.

The cells were then switched to fresh basal media and were divided into three groups. Group 1 was either untreated, or treated with 1 μ M nicotine for 10, 15, 30 or 60 minutes. Group 2 was either untreated, or treated with 1 nM NNK for 10, 15, 30 or 60 minutes. Group 3 was either untreated, or treated with 10 μ M acetylcholine for 10, 15, 30 or 60 minutes. Protein samples were prepared using lysis buffer (50-mmol / L Tris-HCl, 1 % NP-40, 150-mmol / L NaCl, 1-mmol / L phenylmethanesulfonylfluoride, 1-mmol / L Na_3VO_4 , 1-mmol / L NaF, and 1 μ g / mL of aprotinin, leupeptin, and pepstatin). After heat denaturation, protein samples were electrophoresed using 12 % SDS gels (Invitrogen) and blotted onto membranes. The membranes were blocked (5 % nonfat dry milk solution) for one hour at room temperature. Membranes were then incubated overnight at 4 °C with the following primary antibodies: Akt, p-Akt, Src, p-Src, CREB, p-CREB, ERK 1/2, and p-ERK 1/2. The membranes were then washed (0.5 % Tween 20 / TBS) and incubated with their respective fluorescent secondary antibodies for two hours. Protein bands were then visualized with enhanced chemiluminescence reagent (Pierce ECL Western Blotting Detection Substrate).

Statistical analysis of data

GraphPad Instat 3 software (GraphPad Instant biostatistics, San Diego, CA, USA) was used to test significant differences between different treatment groups. Statistical tests used included non-parametric One Way ANOVA and Tukey-Kramer multiple comparison tests. In addition, ImageJ from NIH was used for mean density determination of bands. Data of the immunoassays and MTT assays are expressed as mean and + / -

standard deviation of five samples per treatment group. The EC_{50} values of the concentration-response curves were determined by nonlinear regression analysis. Densitometry data of western blots are expressed as mean values and standard deviations of 2 density determinations per band from three independent westerns per antibody (n = 6).

Results

Effects of nicotine on catecholamine neurotransmitter levels

One important role of the $\alpha 7$ -nAChR in the nervous system is the regulation of the synthesis and release of neurotransmitters, including adrenaline and noradrenaline [14,21]. While our laboratory has previously shown that β -ARs regulate the proliferation and migration of pancreatic cancer cells in vitro [18-20], the function of nAChRs expressed in these cells is poorly understood. We therefore tested the hypothesis that pancreatic ductal adenocarcinoma cells and normal pancreatic duct epithelial cells, synthesize and release their own catecholamine neurotransmitters and that this activity is regulated by nAChRs. In support of this hypothesis, our immunoassays detected noradrenaline and adrenaline in cell lysates as well as culture media of both pancreatic cancer cell lines and the immortalized pancreatic duct epithelial cells. The intracellular as well as secreted levels of noradrenaline and adrenaline increased significantly ($p < 0.0001$) in a time-dependent manner when the cells were exposed to nicotine (1 μ M) for 1 to 30 minutes (Figure 1). The immortalized duct epithelial cell line HPDE6-C7 was less responsive to nicotine than the cancer cells ($p < 0.0001$ at the 30 minute time point) as

indicated by lower levels of intracellular and secreted catecholamines at all-time points investigated (Figure 1). Exposure of the cells for 30 minutes to nicotine at concentrations from 10 pM through 10 μ M additionally revealed concentration-dependent increases in intracellular and secreted catecholamines in all three cell lines (Figure 2). In accord with the differences in responsiveness observed in the time courses (Figure 1), the EC₅₀ values of nicotine for intracellular and secreted noradrenaline and adrenaline were in the nanomolar range for the two cancer cell lines while they were in the 10-100 nanomolar range for HPDE6-C7 cells (Figure 2).

Effects of nAChR knockdown on neurotransmitter levels and nAChR protein

To assess the potential regulatory role of the α 3, α 4, α 5, and α 7-nAChRs for catecholamine synthesis in pancreatic cancer and pancreatic duct epithelial cells, the three cell lines were transfected with stealth α 3, α 4, α 5, or α 7 RNAi constructs. Immunoassays showed that transfections with the α 3, α 5, and α 7 RNAi constructs significantly inhibited ($p < 0.0001$) the stimulatory effect of nicotine on adrenaline and noradrenaline synthesis in each of the three cell lines and reduced catecholamine synthesis below base levels in control cells (Figure 3). These findings confirm regulatory roles of the α 3, α 5, and α 7-nAChRs for catecholamine production by these cells. By contrast, gene knockdown of the α 4-nAChR did not significantly reduce catecholamine synthesis in either cell line (Figure 3). As no reduction in catecholamine synthesis was observed in the cells transfected with the negative control RNAi, these findings indicate

that the cells also produce one or both of the physiological agonists for these nAChRs (acetylcholine, choline) that continuously stimulated base level catecholamine synthesis.

Results of western blots showed significant increases ($p < 0.0001$) in the expression of all investigated nAChR proteins in all three cell lines exposed for 30 minutes to 1 μM nicotine (Figure 4). Furthermore, transfection of these cells with stealth RNAi for each of the investigated nAChR constructs significantly decreased ($p < 0.0001$) their protein expression in cells with and without nicotine exposure (Figure 4). By contrast, transfection of cells with stealth RNAi negative control low GC showed no significant change in the protein expression of this receptor (Figure 4), confirming the specificity of the observed gene knockdowns.

Activation of multiple signaling proteins by nAChR agonists and inhibition by the beta-blocker propranolol

Binding of an agonist to β -ARs activates adenylyl cyclase, leading to the formation of cAMP and phosphorylation of the transcription factor CREB by activated protein kinase A [17]. In addition, activated protein kinase A transactivates the EGFR pathway in pancreatic cancer cells and pancreatic duct epithelia, leading to the phosphorylation of the extracellular signal regulated kinases ERK1/2 [19]. We therefore assessed the phosphorylation of CREB and ERK by western blots in our three cell lines after exposures from 10 to 60 minutes to nicotine (1 μM), NNK (1 nM) or acetylcholine (10 μM). We also monitored the phosphorylation of Src family tyrosine kinases and of the serine / threonine protein kinase B, AKT, because both are frequently overexpressed

in pancreatic cancer [22,23]. As Figure 5 shows, 1 μ M nicotine induced the phosphorylation of all four signaling proteins in both pancreatic cancer cell lines and immortalized pancreatic duct epithelial cells. In accord with findings that the affinity of NNK to the $\alpha 7$ -nAChR is about 1000 times greater than that of nicotine [24], similar responses of the four investigated signaling proteins were observed when the cells were exposed to 1 nM NNK (Figure 5). The physiological nAChR agonist, acetylcholine, binds to these receptors at a significantly lower affinity than nicotine [14]. We therefore used acetylcholine at a 10 μ M concentration when assessing its effects on the phosphorylation status of the signaling proteins under investigation. As shown in Figure 5, at this concentration, acetylcholine had similar inducing effects on all four signaling proteins as nicotine and NNK. The quantitative assessment of nicotine-induced activation of these signaling proteins and the inhibitory effects of propranolol were achieved by immunoassays. As Figure 6 shows, the phosphorylation of ERK, CREB, Src and AKT were each significantly ($p < 0.001$) induced by nicotine and these responses were completely blocked by propranolol ($p < 0.0001$). Propranolol also significantly ($p < 0.01$) reduced base level phosphorylation of these signaling proteins in cells not exposed to nicotine (Figure 6).

Regulation of cell proliferation by neurotransmitter receptors

MTT assays were conducted to evaluate cell proliferation of HPDE6-C7, BxPC-3, and Panc-1 cells induced by nicotine (1 μ M) in the presence and absence of the general β -AR antagonist, propranolol (1 μ M) or the site-selective $\alpha 7$ -nAChR antagonist, α -

bungarotoxin (α -BTX, 200 nM). Cells from each of the three investigated cell lines exposed to nicotine for 72 hours showed significant increases ($p < 0.0001$) in cell proliferation. This response to nicotine was reduced below base levels ($p < 0.0001$) by pre-exposure of cells to either α -BTX or propranolol (Figure 7a), indicating that the $\alpha 7$ -nAChR as well as β -ARs were involved in the observed nicotine-induced cell proliferation. In addition, base level cell proliferation in the absence of nicotine was significantly ($p < 0.0001$) reduced by α -BTX or propranolol (Figure 7a), suggesting regulatory functions of both receptor types in non-exogenously stimulated cells.

Discussion

Our data show, for the first time, that pancreatic cancer cells and normal, pancreatic duct epithelia, express an autocrine catecholamine loop that stimulates their proliferation and is jointly regulated by the nAChRs $\alpha 3$, $\alpha 5$, $\alpha 7$ and by β -ARs. The catecholamines noradrenaline and adrenaline, that were synthesized and released by two investigated pancreatic cancer cell lines and a cell line of immortalized pancreatic duct epithelia, are commonly known as stress neurotransmitters because they are synthesized in the adrenal medulla and released into the systemic blood circulation in response to psychological stress [15]. In addition, both of these neurotransmitters are synthesized and released from nerves of the sympathetic [25], thus regulating vital functions in multiple organs. Furthermore, noradrenaline and adrenaline have excitatory and anti-inflammatory functions in the brain where they are synthesized and released by neurons [21]. It is well

documented that nicotine increases catecholamine production at these neuroendocrine sites, thus increasing the systemic levels of noradrenaline and adrenaline [26,27].

Accordingly, a previous study by our laboratory showed a significant nicotine-induced growth promotion of pancreatic cancer xenografts associated with increased levels of noradrenaline, adrenaline and cAMP in blood and xenograft tissues as well as an induction of p-CREB and p-ERK in xenograft tissues. These changes were interpreted as indirect effects of such systemic neuroendocrine responses [28]. Our current in vitro experiments are not influenced by this systemic neuroendocrine effect of nicotine and unequivocally show that two pancreatic cancer cell lines as well as immortalized pancreatic duct epithelia synthesized and released their own noradrenaline and adrenaline in response to nicotine. The important role of the catecholamine neurotransmitters in the observed nicotine-induced stimulation of cell proliferation was confirmed by abolishment of this response by propranolol, an antagonist for β 1- as well as β 2-ARs. As assessed by both, gene knockdown and pharmacological blockage with α -BTX, the α 7-nAChR regulated catecholamine production as well as cell proliferation in response to nicotine. Interestingly, gene knockdown of the α 3 and α 5-nAChRs also significantly reduced catecholamine production in all three cell lines, indicating cooperative function of these two nAChRs with the α 7-nAChR. By contrast, the α 4-nAChR did not appear to participate in the regulation of catecholamine production. These findings are in accord with reports that the α 3, α 5, and α 7-nAChRs cooperate in regulating the proliferation of oral keratinocytes [29] whereas the α 4-nAChR regulates the production of γ -aminobutyric acid (GABA) in human small airway epithelial cells [30]. The α 3, α 5 and

$\alpha 7$ -nAChRs thus function as the upstream regulator of this novel autocrine regulatory loop with β -ARs as the effectors of released noradrenaline and adrenaline (Figure 7b).

Our findings are in accord with recent observations that the $\alpha 7$ -nAChR regulates the synthesis and release of noradrenaline and adrenaline in small airway epithelial cells [30] and colon cancer cells [31] and suggest this autocrine catecholamine loop as a novel target for pancreatic cancer intervention. This interpretation is supported by our findings that multiple phosphorylated signaling proteins that are frequently overexpressed in pancreatic cancer were simultaneously induced in each of the investigated cell lines by exposure to nicotine while the beta-blocker propranolol reversed these responses and additionally reduced the phosphorylation of all investigated signaling protein levels below base levels. These findings indicate that the observed phosphorylation of signaling proteins were events downstream of beta-adrenergic receptors. Inhibitors of ERK, Src, AKT and EGFR tyrosine kinases alone or in combination are currently being explored as 'targeted therapeutics' for pancreatic cancer, an approach that necessitates treatment of the patient with multiple inhibitors [2,3]. Our current data suggest single agent therapy with the beta-blocker propranolol as a novel alternative to this strategy.

While CREB, Src and AKT are traditionally considered downstream effectors of the EGFR pathway in pancreatic cancer [3], in vitro studies with pancreatic cancer cell lines and immortalized pancreatic duct epithelia have shown that ERK is also phosphorylated simultaneously with CREB in response to beta-adrenergic agonists following the PKA-dependent transactivation of the EGFR [19]. In addition, studies in

ovarian cancer cells have identified the phosphorylation of Src in these cells as a cAMP-dependent event in response to stress neurotransmitters [32].

The nitrosated carcinogenic nitrosamine NNK is a nAChR agonist with a thousand-fold higher affinity to the $\alpha 7$ -nAChR than nicotine [24,29]. In the current experiments, exposures of the cells to NNK were therefore conducted at a 1 nM concentration as opposed to 1 μ M nicotine used in the accompanying experiments. In turn, the documented lower affinity of acetylcholine to nAChRs than nicotine [14] was the reason why we exposed our cell lines to 10 μ M acetylcholine. As our western blots show, the induction of the investigated signaling proteins was similar with all three agents at the concentrations used. Exposure of the cells to 1 μ M nicotine for 30 minutes additionally upregulated the protein expression of nAChRs in the investigated three cell lines. This response is in accord with the reported rapid increase of nAChR numbers in response to nicotine or other agonists [33]. Similar effects of nicotine on the $\alpha 7$ -nAChR have been described in the brain and are thought to be caused by post-translational and post-transcriptional mechanisms [34].

Smoking is a documented risk factor for the development of pancreatic cancer [4]. However, this malignancy also develops in a significant number of nonsmokers. While some of these cases are preceded by diabetes or pancreatitis, two additional known risk factors for pancreatic cancer [4,35], our current findings suggest that psychological stress may also contribute to the development and progression of this disease. While in our experiments the production of catecholamines and resulting induction of multiple signaling proteins that regulate cell proliferation, migration and apoptosis was induced by

the exogenous addition of nAChR agonists to the cells, psychological stress triggers a systemic increase in noradrenaline and adrenaline via activation of the pituitary/adrenal system [15]. In analogy to reports that experimentally induced psychological stress promotes the growth and metastasis of ovarian cancer via beta-adrenergic, cAMP-dependent signaling [36], psychological stress may therefore also facilitate the development of pancreatic cancer and promote the progression of this malignancy, thus impairing therapeutic outcomes [37,38]. However, further studies are needed to address this potential aspect of pancreatic cancer regulation.

In summary, our data suggest that the autocrine catecholamine loop expressed in pancreatic cancer cells and in normal pancreatic duct epithelial cells that is jointly governed by the α_3 , α_5 , and α_7 -nAChRs and β -ARs as an important regulatory network that controls multiple signal transduction pathways known to be hyperactive in pancreatic cancer. As shown in Figure 7, this entire cascade could theoretically be inhibited by beta-blockers, or by agents that inhibit the activation of adenylyl cyclase and associated formation of cAMP. While the α_7 -nAChR has been suggested as a drug target for the therapy of non-small cell lung cancer [39], the vital functions of this receptor in the nervous system renders the use of α_7 -nAChR antagonists for cancer therapy problematic. On the other hand, beta-blockers have been safely used for decades as cardiovascular therapeutics and the beta-blocker propranolol prevented the development of NNK-induced pancreatic cancer in hamsters [38]. A recent report has also identified significantly better clinical outcomes in breast cancer patients treated with beta-blockers [40]. These findings are in accord with data that have shown stimulation of breast cancer

cell proliferation by beta-adrenergic agonists in vitro [41,42] . In addition, it has been shown that γ -amino butyric acid (GABA) inhibited the proliferation and migration of pancreatic cancer cells in vitro via GABA-B receptor mediated inhibition of adenylyl cyclase [20]. GABA also reversed the growth promoting effects of nicotine on pancreatic cancer xenografts by reducing tumor cAMP levels [28]. GABA has been safely used as a nutritional supplement for many years and selective GABA-B-receptor agonists are widely used for the pharmacological management of spastic pain after spinal injuries and spinal surgery. Further studies are now warranted to explore the potential usefulness of these agents for the improvement of clinical outcomes in pancreatic cancer therapy.

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Appendix

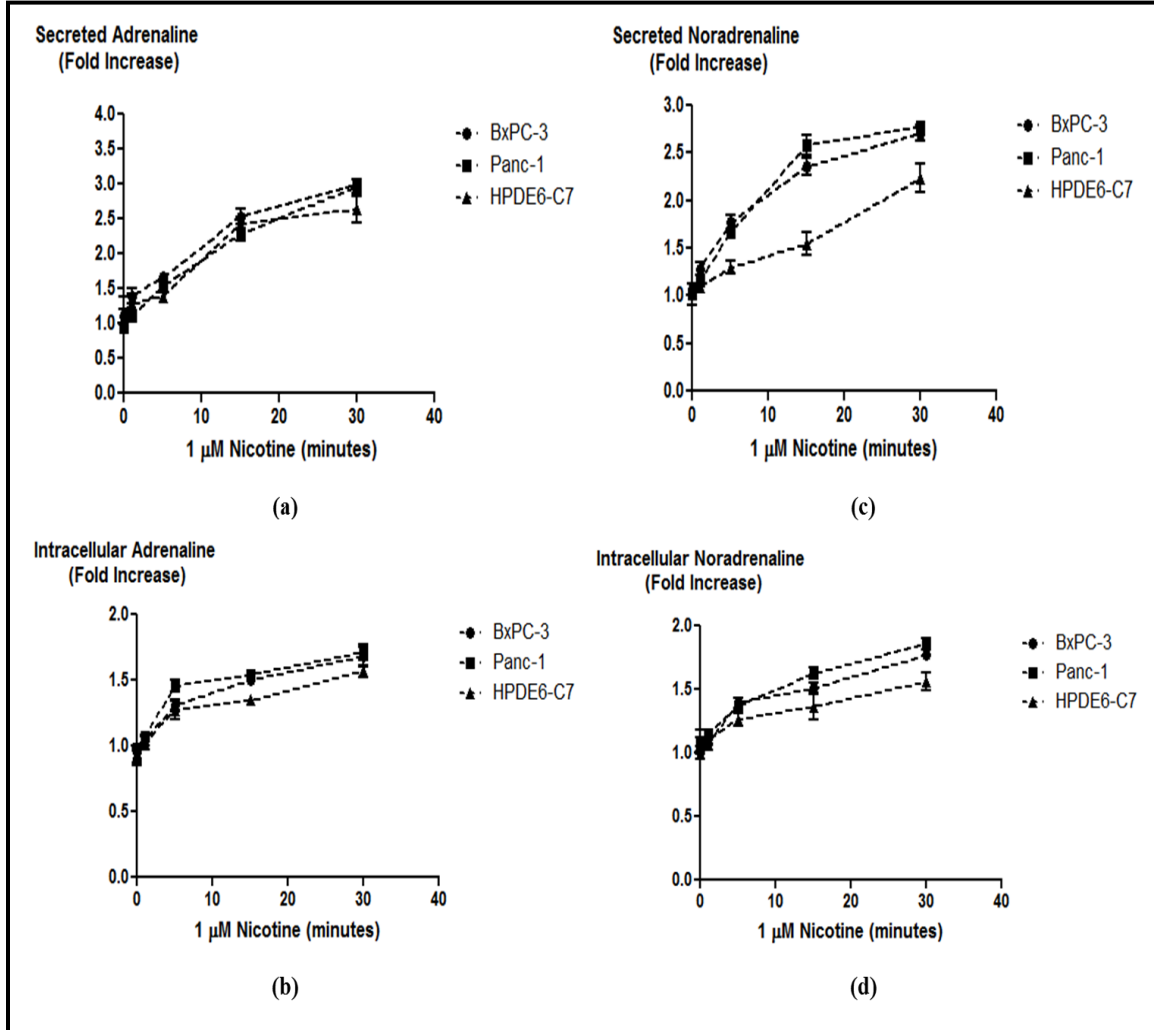


Figure 1. Secreted (a) and intracellular (b) adrenaline levels; and secreted (c) and intracellular (d) noradrenaline levels in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 1 μ M nicotine from 5 to 30 minutes. Representative data points are mean and $\pm$ SD from five samples per treatment group.

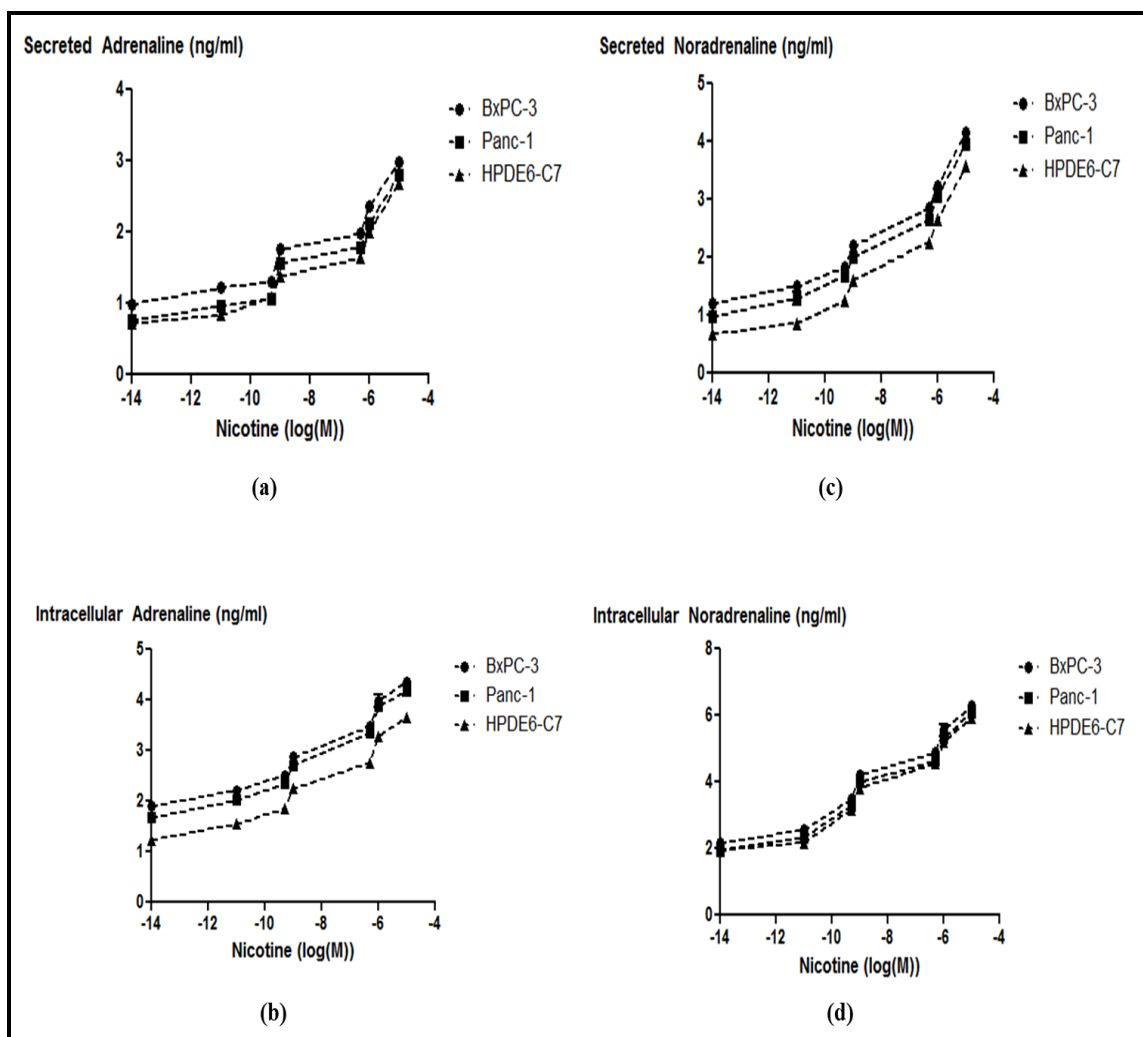


Figure 2. Secreted (a) and intracellular (b) adrenaline levels; and secreted (c) and intracellular (d) noradrenaline levels in HPDE6-C7, BxPC-3 and Panc-1 cells treated with nicotine concentrations from 10 pM through 10 μ M for 30 minutes. Representative data points are mean and $\pm$ SD from five samples per treatment group.

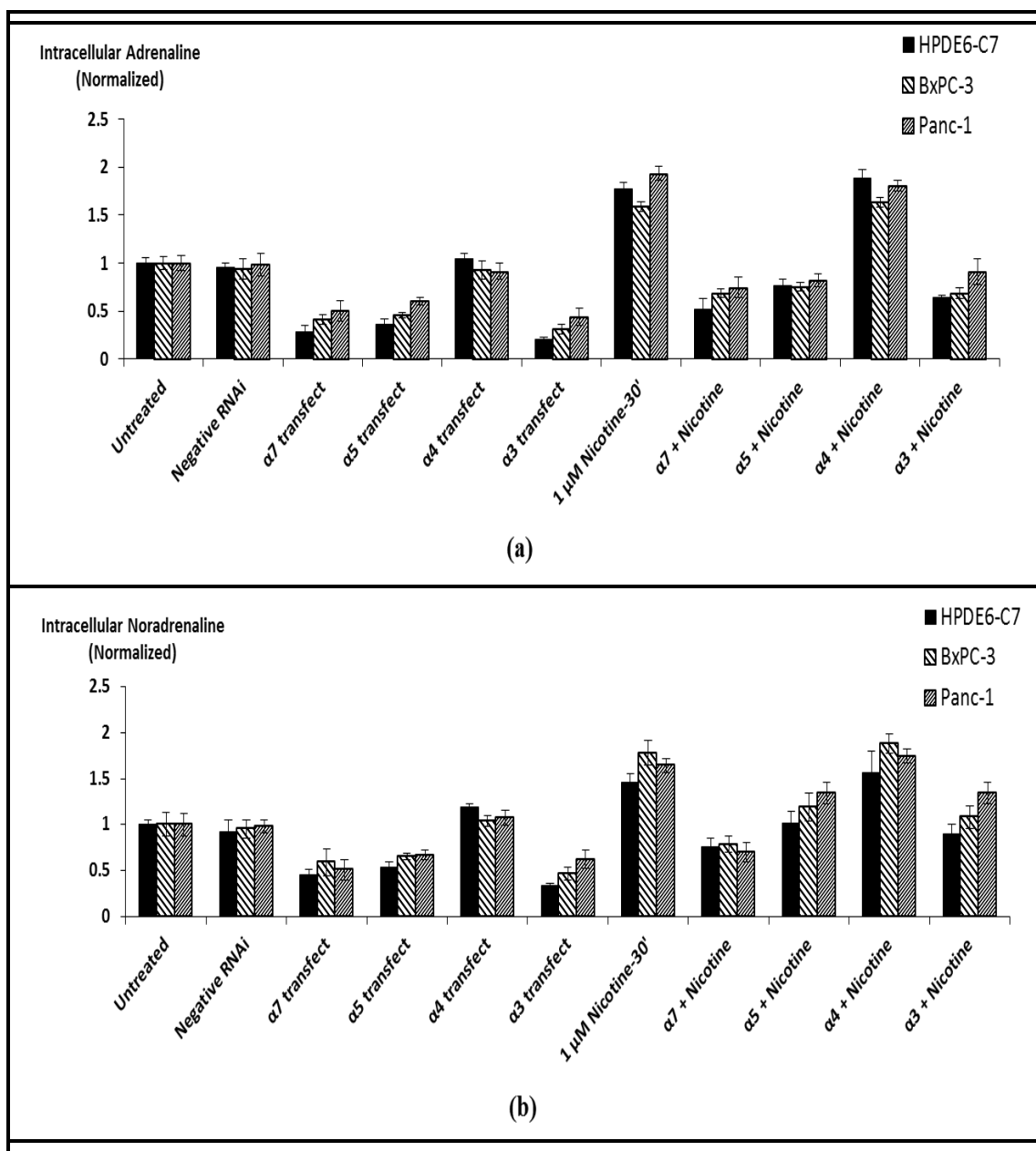


Figure 3. Assessment of the role of nAChR subunits in catecholamine production. Receptor knockdown of $\alpha 3$, 5, and 7 significantly ($p < 0.001$) reduced nicotine induced stimulation of intracellular (a) adrenaline and (b) noradrenaline. By contrast, $\alpha 4$ receptor knockdown showed no significant effect on catecholamine production. The columns in the graphs are mean and $\pm$ SD from five samples per treatment group.

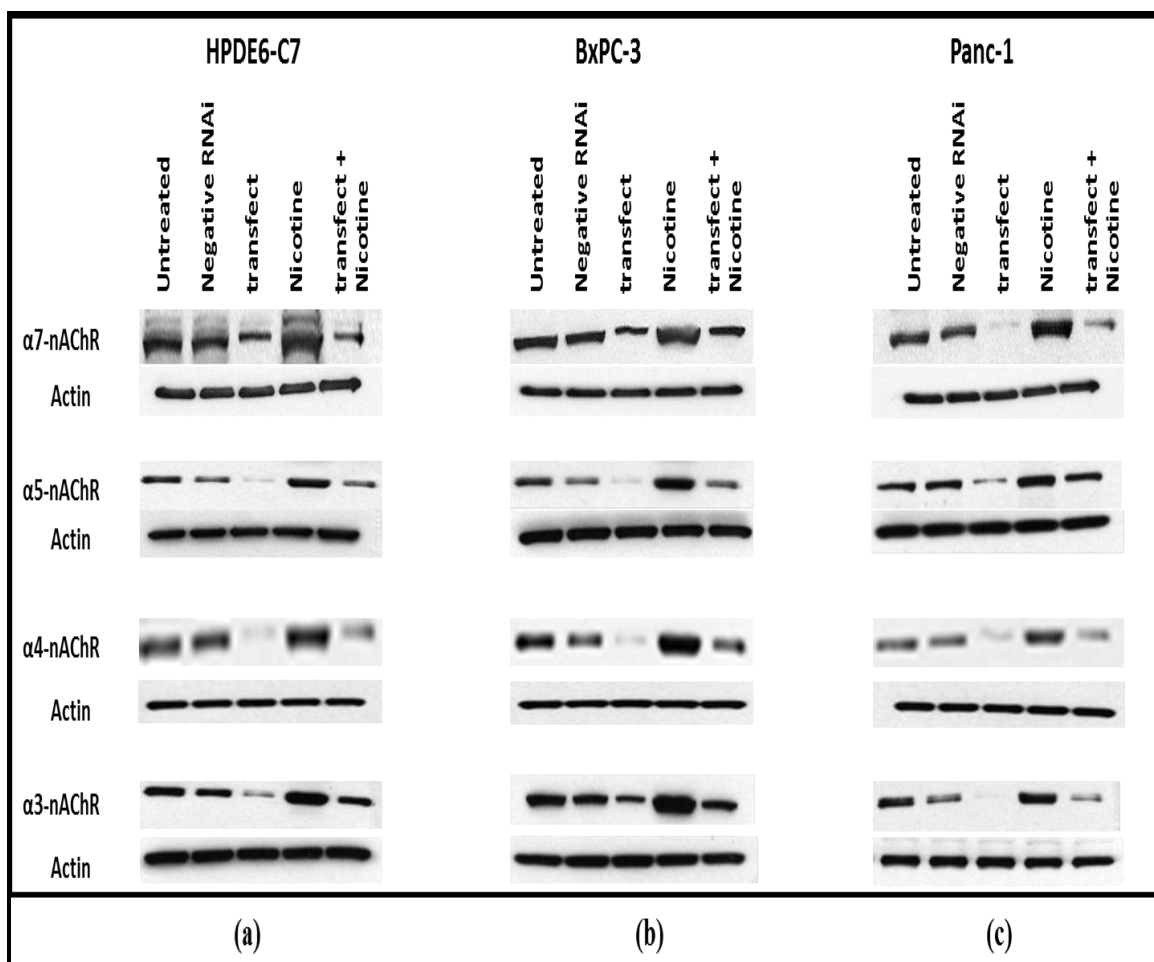


Figure 4A-C. Western blots showing the effects of gene knockdown on protein expression of $\alpha 3$, 4, 5, and 7-nicotinic acetylcholine receptors in HPDE6-C7 (a), BxPC-3 (b) and Panc-1 (c) cells in the presence and absence of nicotine (1 μ M for 30 minutes). The house keeping protein β -actin was used as a control to ensure equal loading of proteins.

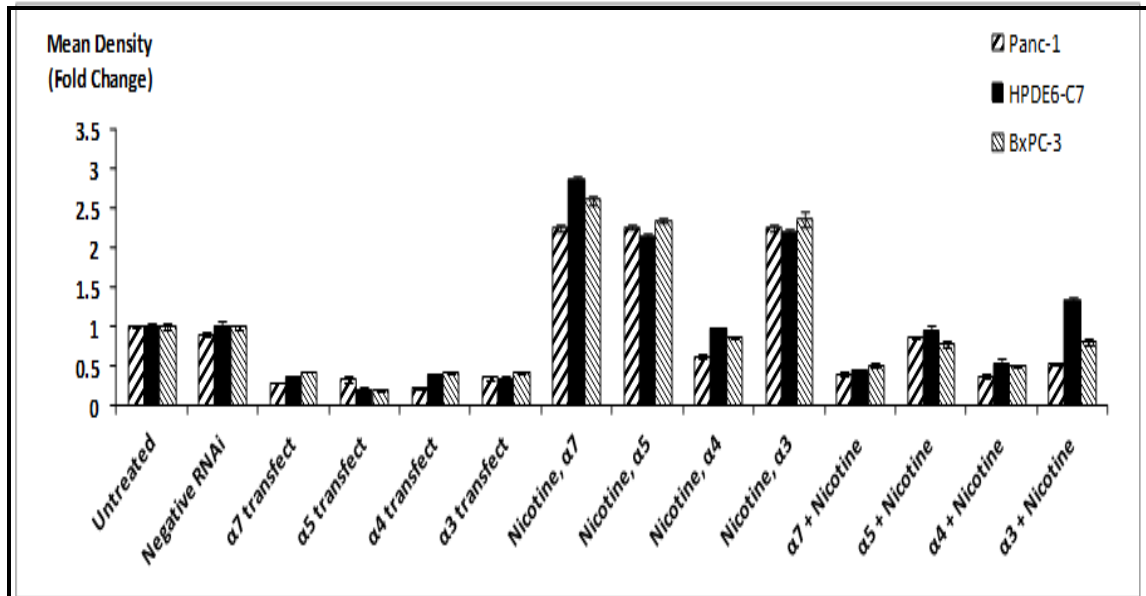


Figure 4D. Densitometric analysis of figures 4a-c. The columns represent means and $\pm$ SD of two mean density readings per band from three independent western blots ($n = 6$) expressed as fold changes in expression of $\alpha 3$, 4, 5 and 7-nAChRs.

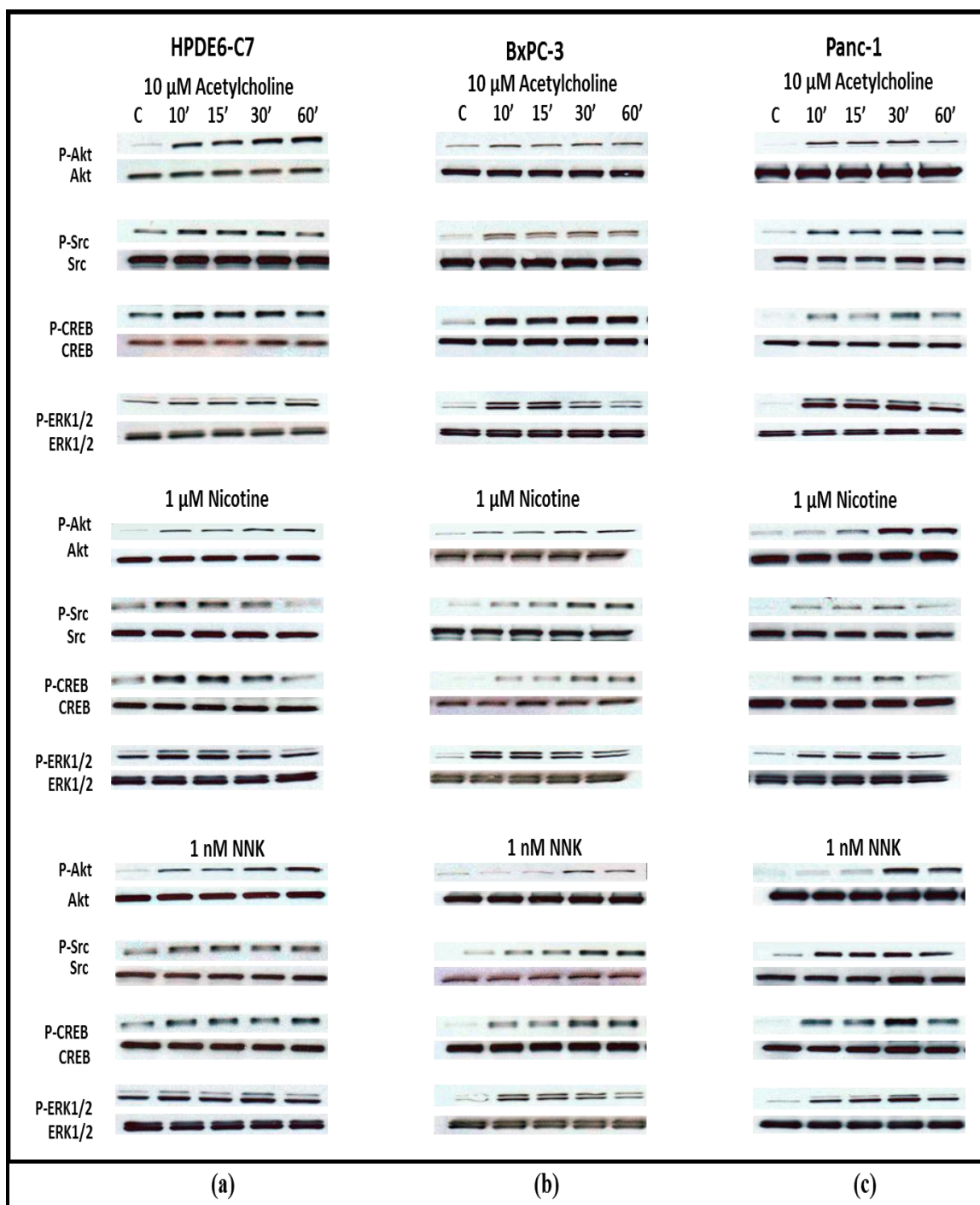


Figure 5. Western blots assessing phosphorylation of Akt, Src, CREB, and ERK1/2 in HPDE6-C7 (a), BxPC-3 (b) and Panc-1 (c) cells treated with 10 μ M acetylcholine, 1 μ M nicotine and 1 nM NNK at different time exposures.

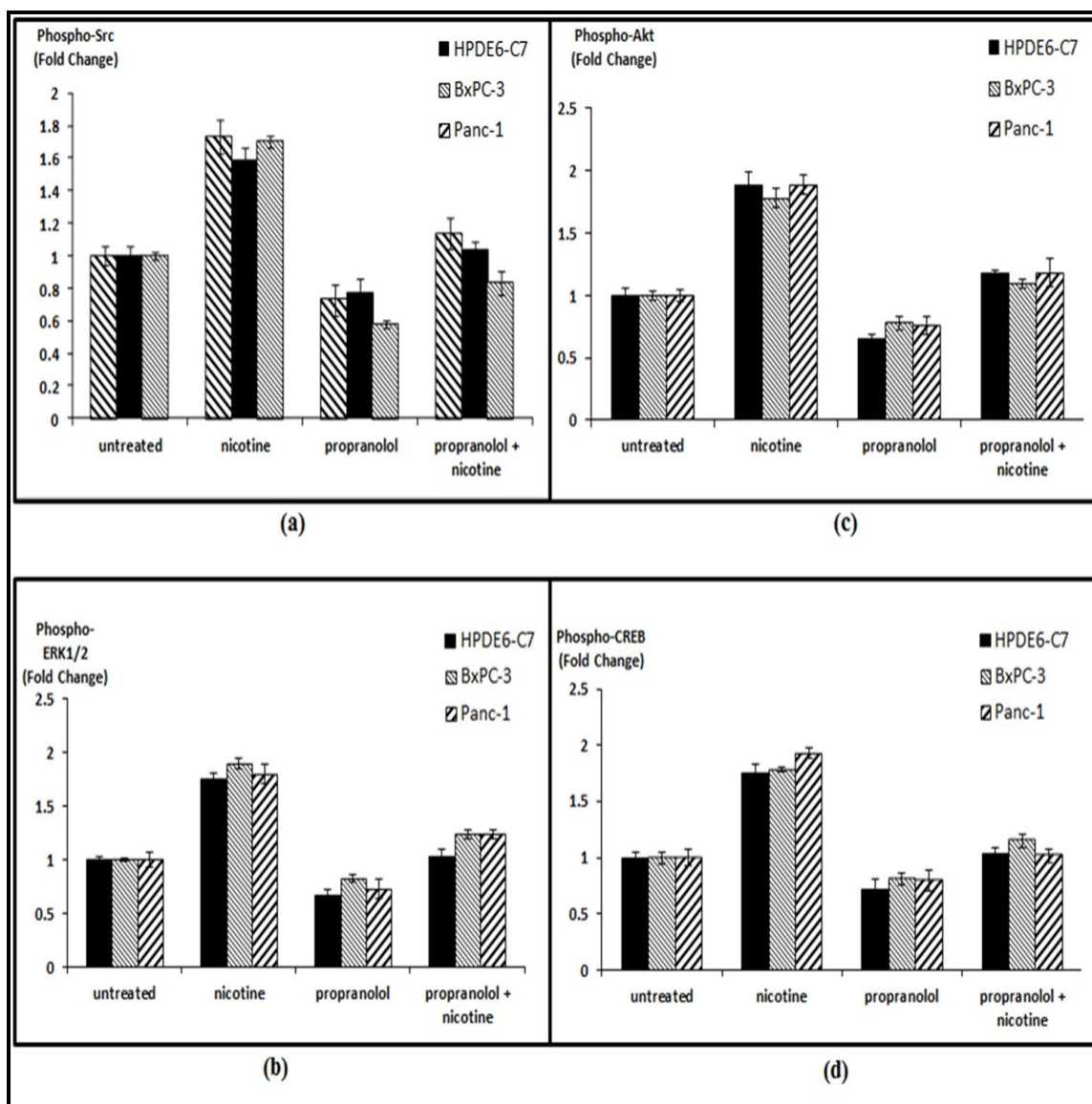


Figure 6. ELISA assays showing phosphorylation levels of Src (a), ERK1/2 (b), Akt (c), and CREB (d) for HPDE6-C7, BxPC-3 and Panc-1. Nicotine significantly ($p < 0.001$) induced the phosphorylation of all signaling proteins investigated. Propranolol, a beta-blocker, significantly ($p < 0.001$) reduced base level and nicotine induced phosphorylation of all four proteins. Representative data points are mean and $\pm$ SD from five samples per treatment group.

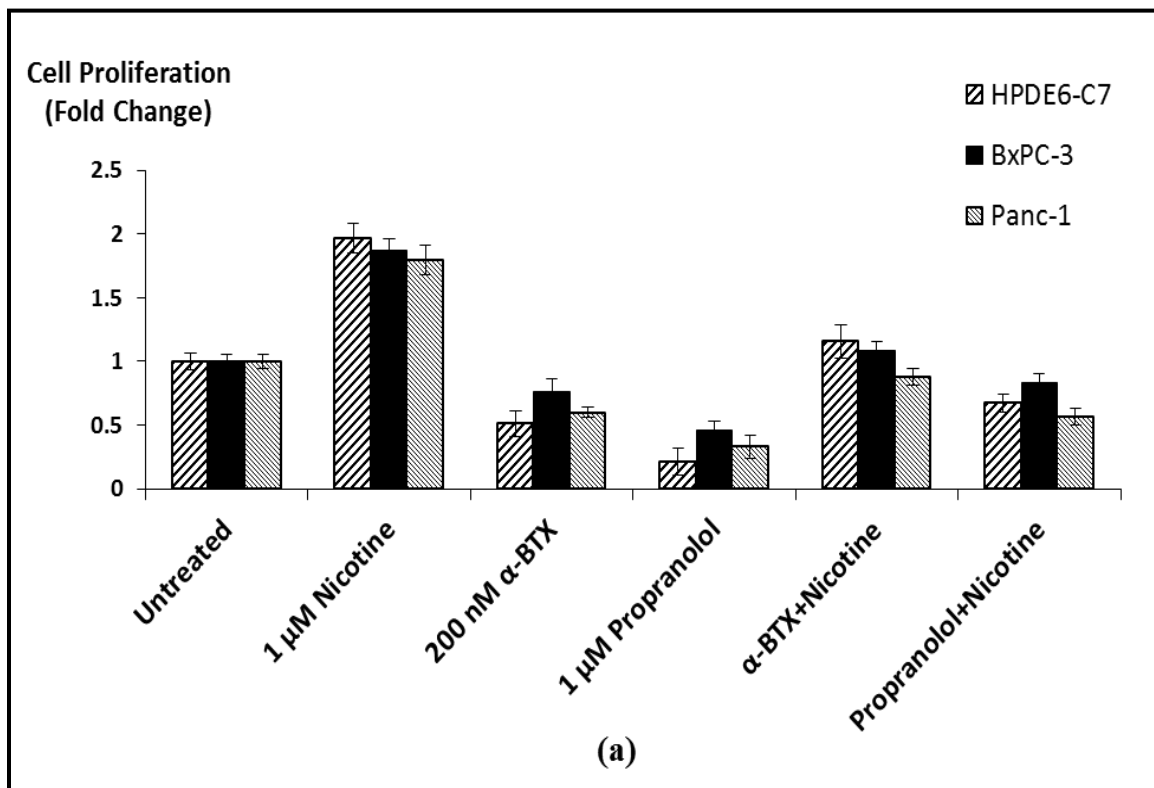


Figure 7A. Cell proliferation of HPDE6-C7, BxPC-3 and Panc-1 cells assessed by MTT assays showing inhibition of nicotine-induced cell proliferation by the site-selective α 7-nAChR antagonist, α -bungarotoxin (α -BTX), and by the beta-blocker propranolol. Columns in the graph represents mean $\pm$ SD of five samples per treatment group.

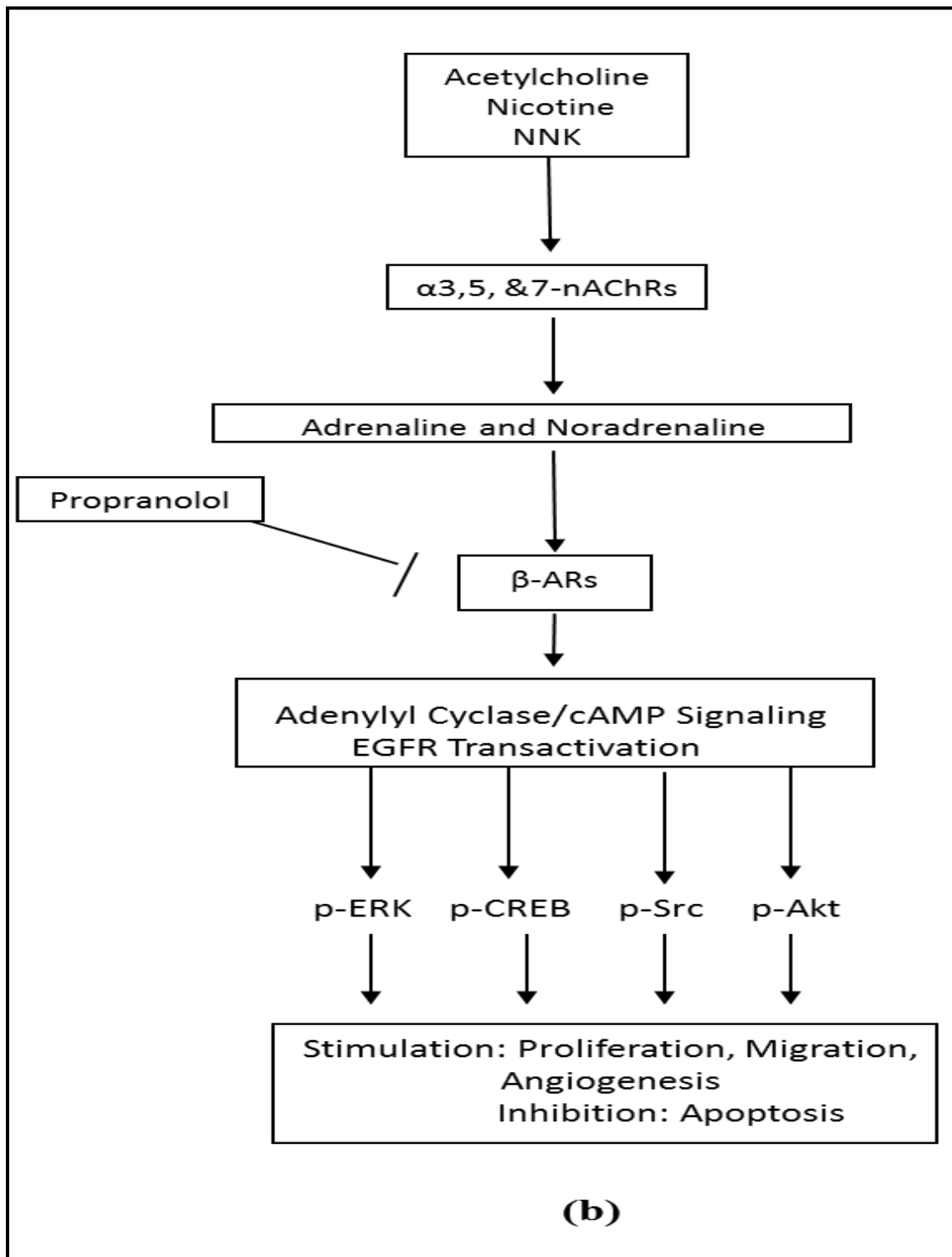


Figure 7B. Schematic diagram of the proposed stimulation of pancreatic cancer cells by the catecholamine loop that is jointly regulated by the $\alpha 3, 5, \text{ \& } 7$ -nAChRs and β -ARs.

CHAPTER IV:

Effects of Chronic Nicotine on the Autocrine Regulation of Pancreatic Cancer Cells and Pancreatic Duct Epithelial Cells by Stimulatory and Inhibitory Neurotransmitters

CHAPTER IV

Brief explanatory statement

This chapter is a slightly revised version of a manuscript that has been accepted for publication in “Carcinogenesis” on July 2012.

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The use of “we” in this chapter refers to the contributions of the co-authors and I in the project. My contributions in this paper include (1) running all experiments relevant to the paper, (2) analysis and interpretation of the data, (3) statistical analysis of all data, (4) photographic and graphic documentation of results, (5) manuscript writing. Co-authors contributions to this paper include experimental design and writing editorial assistance by Dr. Schuller and technical assistance by Dr. Hussein Al-Wadei.

Abstract

Pancreatic ductal adenocarcinoma (PDAC) has a mortality rate near 100 %. Smoking is a documented risk factor. However, the mechanisms of smoking-associated pancreatic carcinogenesis are poorly understood. We have shown that binding of nicotine to nicotinic acetylcholine receptors (nAChRs) expressing subunits $\alpha 7$, $\alpha 3$, and $\alpha 5$ in PDAC and pancreatic duct epithelial cells in vitro triggered the production of the neurotransmitters noradrenaline and adrenaline by these cells. In turn, this autocrine catecholamine loop significantly stimulated cell proliferation via cAMP-dependent signaling downstream of beta-adrenergic receptors. However, the observed responses only represent acute cellular reactions to single doses of nicotine whereas nicotine exposure in smokers is chronic. Using the PDAC cell lines BxPC-3 and Panc-1 and immortalized pancreatic duct epithelial cell line HPDE6-C7, our current experiments reveal a significant sensitization of the nAChR-driven autocrine catecholamine regulatory loop in cells pre-exposed for 7 days to nicotine. The resulting increase in catecholamine production was associated with significant inductions in the phosphorylation of signaling proteins ERK, CREB, Src and AKT, up-regulated protein expression of nAChR subunits $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\alpha 7$ and increased responsiveness to nicotine in MTT and cell migration assays. All three cell lines produced the inhibitory neurotransmitter γ -amino butyric acid (GABA), an activity inhibited by gene knockdown of the $\alpha 4\beta 2$ nAChR and suppressed by chronic nicotine via receptor desensitization. All of the observed adverse effects of chronic nicotine were reversed by treatment of the cells with GABA, suggesting the

potential usefulness of this agent for the improvement of PDAC intervention strategies in smokers.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) comprises over 90 % of all pancreatic cancers and has a mortality rate near 100 % within two years of diagnosis [1,2]. Smoking is a documented risk factor [3-6] and smokers have a twofold risk to develop PDAC [7,8]. However, the mechanisms of smoking-associated pancreatic carcinogenesis are poorly understood. This lack of mechanistic insight may significantly contribute to the poor clinical outcomes of currently available preventive and therapeutic strategies for pancreatic cancer [9].

Among numerous carcinogenic and toxic substances contained in cigarette smoke, nicotine has been widely studied because of its documented addictive properties [10,11]. Most biological effects of nicotine are mediated by nicotinic acetylcholine receptors (nAChRs) which operate as pentameric ion channels enclosed by homomeric alpha subunits or heteromeric alpha and beta-subunits [12]. Classic research on the function of nAChRs has focused on the nervous system. However, discoveries that nAChRs regulate the proliferation [13] and apoptosis [14] of lung cancer cells have triggered numerous investigations on the regulatory role of this receptor family in a variety of cancers. It has thus been shown that binding of nicotine to the homomeric $\alpha 7$ nAChR stimulates the proliferation, angiogenesis, neurogenesis and metastatic potential of the most common human cancers (reviewed in [15]). The majority of these studies have interpreted the

observed cancer stimulating effects of nicotine as direct signaling responses downstream of the $\alpha 7$ nAChR [15]. By contrast, we have recently shown that binding of nicotine to nAChRs expressing subunits $\alpha 7$, $\alpha 3$ and $\alpha 5$ in PDAC and pancreatic duct epithelial cells in vitro triggered the synthesis and release of the stress neurotransmitters noradrenaline and adrenaline by these cells [16]. In turn, this autocrine catecholamine loop significantly stimulated cell proliferation via cAMP-dependent signaling downstream of beta-adrenergic receptors [16]. However, the observed responses only represent acute cellular reactions to single doses of nicotine whereas nicotine exposure in smokers is chronic. Our current experiments reveal significant sensitization of the nAChR-driven autocrine catecholamine regulatory loop by chronic nicotine. In addition, our data show that PDAC and pancreatic duct epithelial cells produce the inhibitory neurotransmitter γ -aminobutyric acid (GABA), an activity regulated by the $\alpha 4\beta 2$ nAChR and desensitized by chronic nicotine. Interestingly, all of these effects of chronic nicotine were reversed by treatment of the cells with GABA.

Materials and Methods

Chemicals, primers and antibodies

Lipofectamine 2000 Reagent, stealth-1973 for the CHRNA4 gene, stealth RNAi Negative Control Low GC Duplex, and Opti-MEM I reduced serum medium 1X were all purchased from Invitrogen Corporation (Carlsbad, CA, USA). The primer used to interfere with the $\alpha 4$ subunit mRNA was sense, GAC CGC AUC UUC CUC UGG AUG UUC A and antisense, UGA ACA UCC AGA GGA AGA UGC GGU C.

The TE Buffer 1X was purchased from Promega Corporation (Madison, WI, USA). The 2-Cat and GABA-Research ELISA Kits were purchased from Rocky Mountain Diagnostics Incorporation (Colorado Springs, CO, USA). ELISA kit for Human Dopamine-Beta Hydroxylase was purchased from MyBioSource (San Diego, CA, USA). ELISA kits for ERK1/2 [pTpY185/187] and CREB [pS133] were purchased from Invitrogen Corporation (Carlsbad, CA, USA). The CytoSelect Cell Migration Assay was purchased from Cell BioLabs, Inc. (San Diego, CA, USA).

The antibodies AKT (60 kDa), p-AKT (60 kDa), Src (60 kDa), p-Src (60 kDa), anti-rabbit and anti-mouse were all purchased from Cell Signaling (Danvers, MA, USA). The primary antibody anti-nicotinic acetylcholine receptor alpha4 (55 kDa) was purchased from Millipore (Billerica, MA, USA). The nicotinic acetylcholine receptor subunits $\alpha 7$ (56 kDa), $\alpha 3$ (57 kDa), $\alpha 5$ (53 kDa), GAD65 (65 kDa), GAD67 (67 kDa) and β -actin (42 kDa) antibodies were purchased from Abcam (Cambridge, MA, USA). Nicotine ((-)-Nicotine hydrogen tartrate salt, minimum 98 % TLC) and γ -aminobutyric acid (GABA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The lysis buffer used to extract proteins along with Pierce ECL western blotting substrate were purchased from Thermo Scientific (Rockford, IL, USA).

Cell culture

The human pancreatic ductal adenocarcinoma cell lines Panc-1 and BxPC-3 were purchased from the American Type Culture Collection (Manassas, VA, USA). The immortalized human pancreatic duct epithelial cell line, HPDE6-C7, was clonally

established after transduction of the HPV16-E6E7 genes into primary cultures of pancreatic duct epithelial cells and was a kind gift from Dr. Tsao (Division of Cellular and Molecular Biology, Department of Pathology, Ontario Cancer Institute/Princess Margaret Hospital, University of Toronto, Toronto, ON, Canada). All cell lines have been authenticated at the beginning of the current study by RADIL (Research Animal Diagnostic Laboratory, Columbia, MO, USA) by species-specific PCR evaluation.

The Panc-1 cell line was maintained in DMEM medium supplemented with 10 % Fetal Bovine Serum (FBS). BxPC-3 cells were maintained in RPMI 1640 medium supplemented with 10 % FBS. HPDE6-C7 cells were maintained in Keratinocyte Serum Free Medium (KSFM) supplemented with 25 mg / 500 ml Bovine Pituitary Extract (BPE) and 2.5 µg / 500 ml Epidermal Growth Factor (EGF) (GIBCO Invitrogen Corporation, Grand Island, NY, USA). All cell lines were grown without antibiotics in an atmosphere of 5 % CO₂, 99 % relative humidity, and 37 °C.

Analysis of intracellular and secreted GABA in response to acute nicotine treatments

All three cell lines were maintained in their respective complete medium until reaching 65 % confluence, at which time they were switched to basal medium for 24 hours starvation. Cells were then switched into fresh basal media and were divided into two groups. The first group of cells was either untreated or treated with 1 µM nicotine for 1, 5, 15, or 30 minutes. The second group of cells was either untreated or treated with 10 pM, 500 pM, 1 nM, 500 nM, 1 µM, or 10 µM nicotine for 30 minutes. The culture media, containing secreted GABA were then collected in 15 ml test tubes. The cells which

contained synthesized intracellular GABA were lysed and harvested into 1.5 ml eppendorf tubes after a one time wash with warm 1X PBS. Quantitative analyses of intracellular and secreted GABA of five samples per treatment group were conducted using GABA-Research ELISA kit following the vendor's recommendations. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Analysis of total GABA, adrenaline and noradrenaline in response to chronic nicotine

Unpretreated cells from each cell line or cells pretreated for 7 days with nicotine (1 μ M) were exposed to a range of nicotine concentrations (10 pM, 500 pM, 1 nM, 500 nM, 1 μ M, or 10 μ M) for 30 minutes prior to harvesting. For the 7 days treatment group, media and treatment was replaced every 24 hours. The culture media, containing secreted catecholamines and GABA were then collected in 15 ml test tubes. The cells which contained synthesized intracellular catecholamines and GABA were lysed and harvested into 1.5 ml eppendorf tubes after a one time wash with warm 1X PBS. Total (secreted plus intracellular) catecholamines and GABA of five samples per treatment group was analyzed using 2-Cat and GABA-Research ELISA kits respectively following the vendor's recommendations. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Gene knockdown of the $\alpha 4nAChR$

Cells from all three cell lines were grown for 24 hours in their respective complete media. Cells were then switched to Opti-MEM I media and were divided into several groups. Groups 1 and 2 from each cell line were left untreated in Opti-MEM I media for 24 hours. Group 3 was transfected for 24 hours with stealth RNAi Negative Control Low GC Duplex. Groups 4 and 5 were transfected with stealth-1973 for the CHRNA4 gene for 24 hours in Opti-MEM I media. Once the 24 hours transfection was complete, all cells were switched into their respective basal media. Groups 1, 3, and 4 were left untreated for 30 minutes in basal media whereas groups 2 and 5 were treated with 1 μ M nicotine for 30 minutes in basal media. All transfections were done using Lipofectamine 2000 reagent following the instructions of the manufacturer. Cell lysates were then harvested and collected in 1.5 ml eppendorf tubes after one time wash with warm 1X PBS for GABA analysis by immunoassays as described above. The transfection efficiency was assessed by western blots using $\alpha 4nAChR$ as the primary antibody and actin as the loading control following the procedure which is outlined below. Following background subtraction, mean densities of 2 rectangular areas of standard size per band from three independent westerns were determined for the calculation of mean values and standard deviations (n = 6) of protein expression.

Assessment of dopamine-beta hydroxylase levels

Cells from the three cell lines were divided into 4 groups. Group 1 was left untreated. Group 2 was treated with 1 μ M nicotine for 30 minutes. Group 3 was treated

with 1 μ M nicotine for 7 days. Group 4 was treated with 1 μ M nicotine for 7 days followed by 1 μ M nicotine 30 minutes before harvesting. Media and treatments were replaced every 24 hours for groups 3 and 4. The cells were then lysed and harvested into 1.5 ml eppendorf tubes after a one time wash with warm 1X PBS. Quantitative analyses of dopamine-beta hydroxylase activity of five samples per treatment group were conducted using human dopamine-beta hydroxylase ELISA kit following the vendor's recommendations. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Protein analyses by Western blotting and ELISA assays

The levels of phosphorylated signaling proteins ERK and CREB were assessed by ELISA assays, using ERK1/2 [pTpY185/187] and CREB [pS133] ELISA kits from Invitrogen. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Protein expression of nAChR subunits $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 7$ as well as GAD65, GAD67 and the phosphorylated and unphosphorylated forms of signaling proteins AKT and Src was determined by Western blotting.

For both types of analysis, cells from HPDE6-C7, BxPC-3, and Panc-1 were either untreated or treated with 1 μ M nicotine, 30 μ M GABA or GABA plus nicotine for 7 days in complete media. Media and treatments were changed every 24 hours for all groups. Protein samples were prepared using lysis buffer (50-mmol / L Tris-HCl, 1 % NP-40, 150-mmol / L NaCl, 1-mmol / L phenylmethanesulfonylfluoride, 1-mmol / L

Na₃VO₄, 1-mmol / L NaF, and 1 µg / mL of aprotinin, leupeptin, and pepstatin). After heat denaturation, protein samples were electrophoresed using 12 % SDS gels (Invitrogen) and blotted onto membranes. The membranes for Western blots were blocked (5 % nonfat dry milk solution) for one hour at room temperature. Membranes of group 1 were then incubated overnight at 4 ° C with the following primary antibodies: nicotinic acetylcholine receptor subunit α3, 4, 5 and 7, GAD65, GAD67, p-AKT and p-Src determine expression or phosphorylation levels of these signaling proteins or receptors. The primary antibodies Src, AKT and β-actin were used as a loading control to ensure equal loading of proteins. All membranes were then washed (0.5 % Tween 20 / TBS) and incubated with their respective fluorescent secondary antibodies for two hours. Protein bands were then visualized with enhanced chemiluminescence reagent (Pierce ECL Western Blotting Detection Substrate). Following background subtraction, mean densities of 2 rectangular areas of standard size per band from three independent westerns were determined and mean values and standard deviation (n = 6) of protein expression were calculated.

Cell proliferation by MTT assay

Cells from the three cell lines were seeded in 6-well plates at a density of 20,000 cells per well in their respective complete media. Cells were then divided into 5 groups (n=5). The first group of cells was left untreated for 7 days and harvested 24 hours later. The second group was left untreated for 7 days, then treated with a single dose of 1 µM nicotine that was removed 30 minutes later and harvested 24 hours later. The third group

was treated with 30 μ M GABA for 7 days and harvested 24 hours later. The fourth group was treated with 1 μ M nicotine for 7 days followed by a single dose of nicotine at the EC₅₀ concentration of nicotine established in the ELISA assays for adrenaline production after chronic nicotine for each cell line (Figures 2d, e, and f: 1.184 nM for HPDE6-C7, 13.96 nM for BxPC-3 and 14.14 nM for Panc-1) that was removed 30 minutes later and harvested 24 hours later. The fifth group was similar to the fourth one except that these cells were additionally pre-treated with 30 μ M GABA for 7 days. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) colorimetric assay (Sigma-Aldrich St Louis, MO, USA) was used to assess cell proliferation following instructions by the vendor. This assay is based on the cleavage of the tetrazolium salt MTT to formazan in metabolically active cells. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at a primary and reference wavelengths of 570 nm and 650 nm respectively.

Cell migration assay

Using 6-well plates that contain polycarbonate membrane filter inserts (8 μ M pore size) provided by a cell migration assay kit (Cell BioLabs, San Diego, CA, USA), cells from each cell line were seeded onto the top chamber above the filter insert at a density of 20,000 cells per well in their respective complete media. Cells were then divided into identical treatment groups (n=5) as described for the MTT assays (above). Migration ability of the cells was assessed following the instructions provided in the kit.

Optical density of samples was read at 560 nm using an uQuant Bio-Tek Instrument ELISA reader.

Statistical analysis of data

GraphPad Instat 3 software (GraphPad Instant biostatistics, San Diego, CA, USA) was used for the transformation of data to fold increase and normalization and to test significant differences among treatment groups. Statistical tests used included parametric One Way ANOVA and Tukey-Kramer multiple comparison test when the data followed a normal distribution. When data failed to pass the normality test of Kolmogorov and Smirnov provide by the GraphPad software, they were instead analyzed by non-parametric Kruskal-Wallis ANOVA followed by the non-parametric Dunn's multiple comparison test. In addition, ImageJ from NIH was used for mean density determination of bands from three independent Westen blots. Data of the immunoassays are expressed as mean and + / - standard deviation of five samples per treatment group. Densitometry data of western blots are expressed as mean values and standard deviations of 2 density determinations per band from three independent westerns per antibody (n = 6). Nonlinear regression analysis of sigmoidal dose-response curves for GABA, noradrenaline and adrenaline was used to determine EC₅₀ values of dose-response curves for nicotine in untreated and 7-day pretreated cells.

Results

Effects of acute and chronic nicotine on GABA levels

Studies by our laboratory have shown that treatment of PDAC cells with GABA in vitro [17] and in mouse xenografts [18] inhibits tumor growth. Contrary to widely held belief that the production and release of pancreatic GABA is restricted to the endocrine cells of pancreatic islets [19], our data show that both PDAC and the pancreatic duct epithelial cells synthesize and release GABA in vitro (Figures 1a-b and Figures 2a-c). Exposure of the cells to single doses of nicotine (1 μ M) over time revealed a time-dependent decrease ($p < 0.0001$) in synthesis and release of GABA (Figures 1a-b). Gene knock-down of the $\alpha 4$ nAChR subunit significantly ($p < 0.001$) reduced GABA levels in untreated cells while additionally decreasing the nicotine-induced suppression of GABA (Figure 1c). Moreover, gene knockdown of the $\alpha 4$ -nAChR showed a significant ($p < 0.0001$) reduction in its protein expression and significantly reduced nicotine induced expression of the receptor (Figure 1d). These findings identify nAChRs expressing the $\alpha 4$ subunit as important regulators of GABA production in the three investigated cell lines.

Acute exposure of cells for 30 minutes to single doses of nicotine at concentrations from 10 pM through 10 μ M revealed concentration-dependent decrease in total (intracellular plus secreted) GABA in all three cell lines (Figures 2a-c). The suppression of total GABA by identical concentrations of nicotine was enhanced even further in cells pretreated for 7 days with nicotine (Figures 2a-c) and the EC_{50} values of nicotine that caused these responses were significantly ($p < 0.0001$) lower after chronic nicotine than in the un-pretreated cells.

Effects of chronic nicotine on production of noradrenaline and adrenaline

We have previously shown that exposure of the three investigated cell lines to a single dose of nicotine causes synthesis and release of the catecholamine neurotransmitters noradrenaline and adrenaline, both of which stimulated cell proliferation [16]. In accord with these findings, our current results show a significant and concentration dependent increase ($p < 0.0001$) in total adrenaline (Figures 2d-f) and noradrenaline (Figures 3a-c) in unpretreated cells exposed to concentrations of nicotine from 10 pM to 10 μ M for 30 minutes. The production of both catecholamines was markedly enhanced ($p < 0.0001$) and EC_{50} values were significantly reduced when the cells were pretreated for 7 days with nicotine and then exposed for 30 minutes to identical concentrations of nicotine (Figures 2d-f and Figures 3a-c). In accord with these findings, the levels of the enzyme dopamine-beta-hydroxylase, which catalyzes the formation of noradrenaline from dopamine, were significantly ($p < 0.0001$) increased after 30 minutes of exposure to 1 μ M nicotine in all three cell lines (Figure 3d). In turn, chronic exposure to nicotine for 7 days further upregulated the expression of this enzyme (Figure 3d).

Effects of chronic nicotine in the presence and absence of GABA treatment on protein expression of nAChRs

We have previously shown that nAChRs with subunits $\alpha 3$, 5 and 7 nAChRs jointly regulated the production of noradrenaline and adrenaline in the three investigated cell

lines [16] while our current data identified the $\alpha 4nAChR$ as the regulator of GABA production. We therefore assessed the effects of chronic nicotine in the presence and absence of GABA on the protein expression of these receptors using western blots. As shown in Figures 4a and b, chronic nicotine significantly induced ($p < 0.0001$) the protein expression of all 4 receptors in each of the three cell lines. GABA treatment alone did not significantly change the expression of these receptors as compared with the control groups (Figure 4b). However, the protein induction of all 4 receptors in response to chronic nicotine was significantly ($p < 0.001$) reduced by simultaneous chronic exposure of each cell line to GABA (Figure 4b).

Effects of chronic nicotine in the presence and absence of GABA on phosphorylated signaling proteins

Binding of agonists to β -ARs activates adenylyl cyclase, leading to the formation of cAMP and phosphorylation of the transcription factor CREB by activated protein kinase A [20]. In addition, activated protein kinase A transactivates the EGFR pathway in pancreatic cancer cells and pancreatic duct epithelia, leading to the phosphorylation of the extracellular signal regulated kinases ERK1/2 [21] and activation of the Src and AKT pathways [16]. We therefore assessed the phosphorylation of AKT and Src by semi-quantitative western blotting in our three cell lines after exposures to nicotine, GABA or the combination of both agents for 7 days. ELISA assays were used to monitor the phosphorylation of CREB and ERK. As figures 5a and b show, chronic 1 μM nicotine significantly ($p < 0.0001$) induced phosphorylation of AKT and Src signaling proteins in

both pancreatic cancer cell lines and immortalized pancreatic duct epithelial cells. Simultaneous chronic exposure of the cells to GABA significantly ($p < 0.0001$) reduced the induction of both phosphorylated proteins by chronic nicotine while chronic GABA treatment of unpretreated cells suppressed the activation of these proteins to near background levels (Figures 5a-b). Analysis of p-ERK and p-CREB by ELISA assays revealed a similar trend with significant ($p < 0.0001$) inductions by chronic nicotine and significant ($p < 0.001$) reversal of these effects by treatment with GABA (Figure 6a).

Effects of chronic nicotine in the presence and absence of GABA on the GABA synthesizing enzymes GAD65 and GAD67

The isozymes GAD65 and GAD67 catalyze the formation of GABA from glutamate [22]. Having shown that our investigated cell lines produce GABA in vitro, we therefore investigated the levels of GAD65 and GAD67 by Western blots in cells exposed to 1 μ M nicotine for 7 days in the presence and absence of simultaneous treatment with GABA. Our data show a significant nicotine-induced reduction ($p < 0.0001$) in the expression of both isozymes as compared with the controls. These responses were significantly ($p < 0.0001$) reversed by simultaneous chronic treatment with GABA while GABA treatment alone significantly ($p < 0.001$) increased GAD levels in unpretreated cells (Figures 5a, c).

Effects of chronic nicotine in the presence and absence of GABA on cell proliferation and migration

We have previously shown that blocking $\alpha 7$ -nAChR by its selective antagonist, alpha-bungarotoxin, or of β -adrenergic receptors by propranolol significantly reduced cell proliferation induced by a single dose of nicotine in untreated PDAC cells [16]. In the current study, we assessed cell proliferation as an indicator of tumor growth and cell migration as an indicator of metastatic potential. Both assays revealed a significant ($p < 0.0001$) increase in cellular responsiveness to a single dose of nicotine in the cells pretreated for 7 days with nicotine in comparison to untreated cells (Figures 6b, c). When the pretreatment with nicotine was accompanied by 7 days of pretreatment with GABA, the observed increased sensitivity to nicotine was significantly reduced ($p < 0.0001$; Figures 6b-c).

Discussion

Smoking is an important risk factor for pancreatic cancer [1]. However, the mechanisms of smoking-associated pancreatic carcinogenesis are far from understood. Recent studies by our laboratory have shown that the proliferation of the three currently investigated cell lines is significantly stimulated by the activation of an autocrine catecholamine loop regulated by $\alpha 3$, 5 and 7nAChRs upon acute exposure to single doses of nicotine [16]. Single doses of nicotine thus activated multiple signaling proteins commonly overexpressed in pancreatic cancer via noradrenaline/adrenaline-induced cAMP-dependent signaling downstream of beta-adrenergic receptors [16]. Our current

study extends these findings to show that all three cell lines additionally synthesize and secrete GABA, a neurotransmitter previously identified by us as a potent tumor suppressor for PDCA in vitro [17] and in xenograft models [18]. This novel finding contrasts the widely held belief that endocrine beta cells in the pancreatic islets are the sole source of pancreatic GABA that is transported to the exocrine pancreas via the pancreatic blood circulation [22]. The observed significant reduction of GABA production in untreated cells and inhibition of nicotine-induced GABA suppression by gene knockdown of the $\alpha 4$ nAChR additionally identifies this nAChR as the upstream regulator of GABA in all three cell lines. Normal pancreatic duct epithelial cells as well as PDAC cells thus express the complete machinery for the regulation of their own growth stimulation and inhibition by neurotransmitters.

The observed chronic nicotine-induced increase in receptor protein of nAChRs $\alpha 3$, $\alpha 5$, and $\alpha 7$ in conjunction with significantly reduced EC_{50} values yielding significantly higher levels of catecholamine production indicate sensitization of these receptors by chronic nicotine. By contrast, the significantly reduced EC_{50} values for nicotine-induced GABA production in conjunction with significant suppression of GAD and GABA levels in cells chronically exposed to nicotine suggest that the observed increase in protein of the GABA regulating $\alpha 4$ nAChR was a reaction to desensitization of the receptor. This interpretation gains strong support from the significant increases in sensitivity to nicotine in 7 day nicotine pretreated cells observed in cell proliferation and cell migration assays. Our findings are in accord with changes of these receptors in the nicotine addicted brain [23] and imply a shift of neurotransmitter-mediated autocrine

regulation of pancreatic duct epithelial cells and PDAC cells from a balanced state to selective prevalence of cancer stimulating catecholamine neurotransmitters accompanied by suppression of the inhibitory GABA system by chronic nicotine. This effect is further exacerbated by the observed significant increase in dopamine-beta-hydroxylase levels by chronic nicotine as the induction of this enzyme leads to increased synthesis of noradrenaline.

The observed participation of three different nAChRs ($\alpha 3$, $\alpha 5$, $\alpha 7$) in the regulation of catecholamine production is in accord with the reported redundancy of these cancer stimulating receptors in other epithelial cells [24]. In fact sequential investigations have shown, that chronic exposure of oral keratinocytes to nicotine initially induce the protein expression of nAChRs containing subunit complexes comprised of $\alpha 3$, $\alpha 5$, $\beta 2$ and $\beta 4$ followed by induction of the homomeric $\alpha 7$ nAChR protein [25]. In turn, this sequence in nAChR upregulation may be a reflection of the differences in the magnitude of Ca^{2+} influx in response to agonist among nAChRs, with the $\alpha 7$ nAChR demonstrating the greatest permeability to Ca^{2+} . The regulatory role of nAChRs expressing the $\alpha 4$ subunit for GABA synthesis and release observed in our current experiments is in accord with similar findings in human small airway epithelial cells and lung adenocarcinomas with phenotypic features of these cells [26]. However, contrary to the $\alpha 7$ nAChR whose stimulatory role on numerous cancers has been widely published [15], current knowledge on the role of the $\alpha 4$ nAChR in the regulation of cancer is rudimentary at best. The desensitization of this receptors and associated suppression of GABA by chronic nicotine in the brain is considered a major cause of nicotine addiction [23]. Our current data imply

that analogous events in pancreatic duct epithelial and PDAC cells significantly increase their propensity for cancerous growth and metastasis.

The signaling proteins ERK, Src and AKT are commonly overexpressed in PDAC [27-29] and are current targets of pancreatic cancer therapy albeit with little success [9]. Our recently published investigations on the cellular responses of PDAC cells to a single dose of nicotine have shown that all three of these signaling proteins as well as CREB are phosphorylated when the nAChR triggered increase in noradrenaline/adrenaline activates beta-adrenergic receptor signaling [16]. Our current data show additional significant increases over single dose acute nicotine treatments in the activated forms of these proteins in cells chronically exposed to nicotine. In conjunction with the observed changes in nAChRs, catecholamine and GABA production, chronic nicotine thus has deleterious effects at several levels on the complex network that governs the growth regulation of PDAC and pancreatic duct epithelial cells.

The significant reduction of all investigated effects of chronic nicotine in the current experiments by simultaneous chronic treatment of our three cell lines with GABA provides a mechanistic explanation for the reported reversal of nicotine-induced progression of PDAC xenografts by GABA treatment [18]. We have previously established that the nicotine-induced phosphorylation of CREB, ERK, Src and AKT are cAMP-dependent events in the signaling pathway downstream of beta-adrenergic receptors [21,30] and that GABA inhibits the formation of cAMP via the G α i-coupled GABA-B receptor [17]. The strong reversal by GABA treatment of increased levels in these phosphorylated signaling proteins as well as cellular responsiveness in proliferation

and migration assays in response to chronic nicotine was therefore an expected outcome of the current study. However, it is noteworthy that GABA also significantly reduced the chronic nicotine-induced modulations in the expression and function of all investigated nAChRs as well as the induction of dopamine-beta-hydroxylase and suppression of GAD. These findings are in accord with observations in the nervous system that some of the posttranscriptional mechanisms that cause the nicotine-induced upregulation of nAChR protein are caused by increases in cAMP [31]. On the other hand, mechanisms involved in the observed effects of GABA on nicotine-induced changes in dopamine-beta-hydroxylase and GAD expression need yet to be elucidated.

The beneficial effects of GABA on chronic nicotine-induced changes in the auto regulation of PDCA cells and pancreatic duct epithelial cells of our current experiments are in accord with previous reports that GABA inhibits the norepinephrine-induced migration of cell lines from cancer of the colon [32] and mammary gland [33] and has strong tumor suppressor function in lung adenocarcinoma in vitro [34] and in PDAC xenografts [17]. However, in PDAC cell lines that overexpressed the $\alpha 5$ subunit of GABA-A receptors, GABA stimulated cell growth, an effect thought to involve reversal of receptor function from hyperpolarizing to depolarizing, leading to an increase in intracellular calcium levels [35].

Nicotine is generally classified as a non-carcinogenic agent because it does not cause cancer in healthy experimental animals. However, it has been shown that sensitization of lung nAChRs by pathologically increased CO_2 levels that increase Ca^{2+} influx rendered nicotine a lung carcinogen in hamsters [36]. Our current data provide

strong evidence for a key role of nicotine in the development and progression of pancreatic cancer of individuals with modulated nAChR functions due to chronic nicotine exposure. While blocking upstream regulatory receptors such as the $\alpha 3$, 4, 5 and 7nAChRs and β -adrenergic receptors may inhibit nicotine induced cell proliferation, angiogenesis, and migration of pancreatic cells [16], the use of such agents for cancer intervention is problematic due to the important functions of these receptors in the regulation of the nervous system, cardiovascular functions and immune responses. In addition, the presence of beta-adrenergic agonists and stimulators of cAMP in numerous widely used over the counter drugs would effectively nullify any beneficial effects of nAChR blockage. GABA, on the other hand, offers the promise to significantly improve clinical outcomes of pancreatic cancers that do not overexpress the GABA-A receptor $\alpha 1$ subunit and may be suitable for the prevention of this malignancy in smokers.

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Appendix

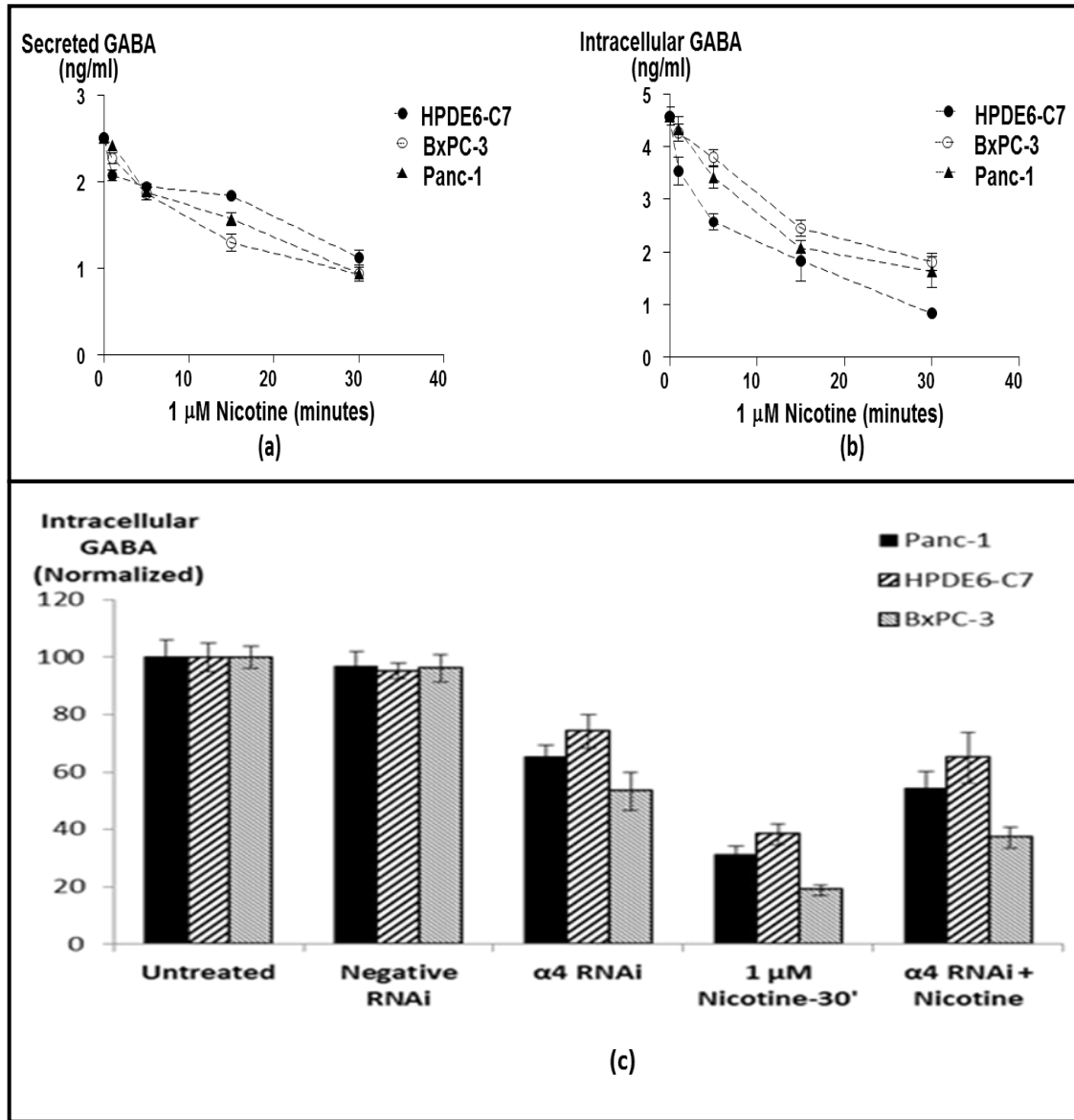


Figure 1A-C. Secreted (a) and intracellular (b) GABA levels in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 1 μ M nicotine from 1 to 30 minutes. (c) ELISA assays showing intracellular GABA levels in cells in the presence and absence of gene knockdown of the $\alpha 4$ -nAChR in the presence and absence of 1 μ M nicotine for 30 minutes. Data points are mean and $\pm$ SD from five samples per treatment group.

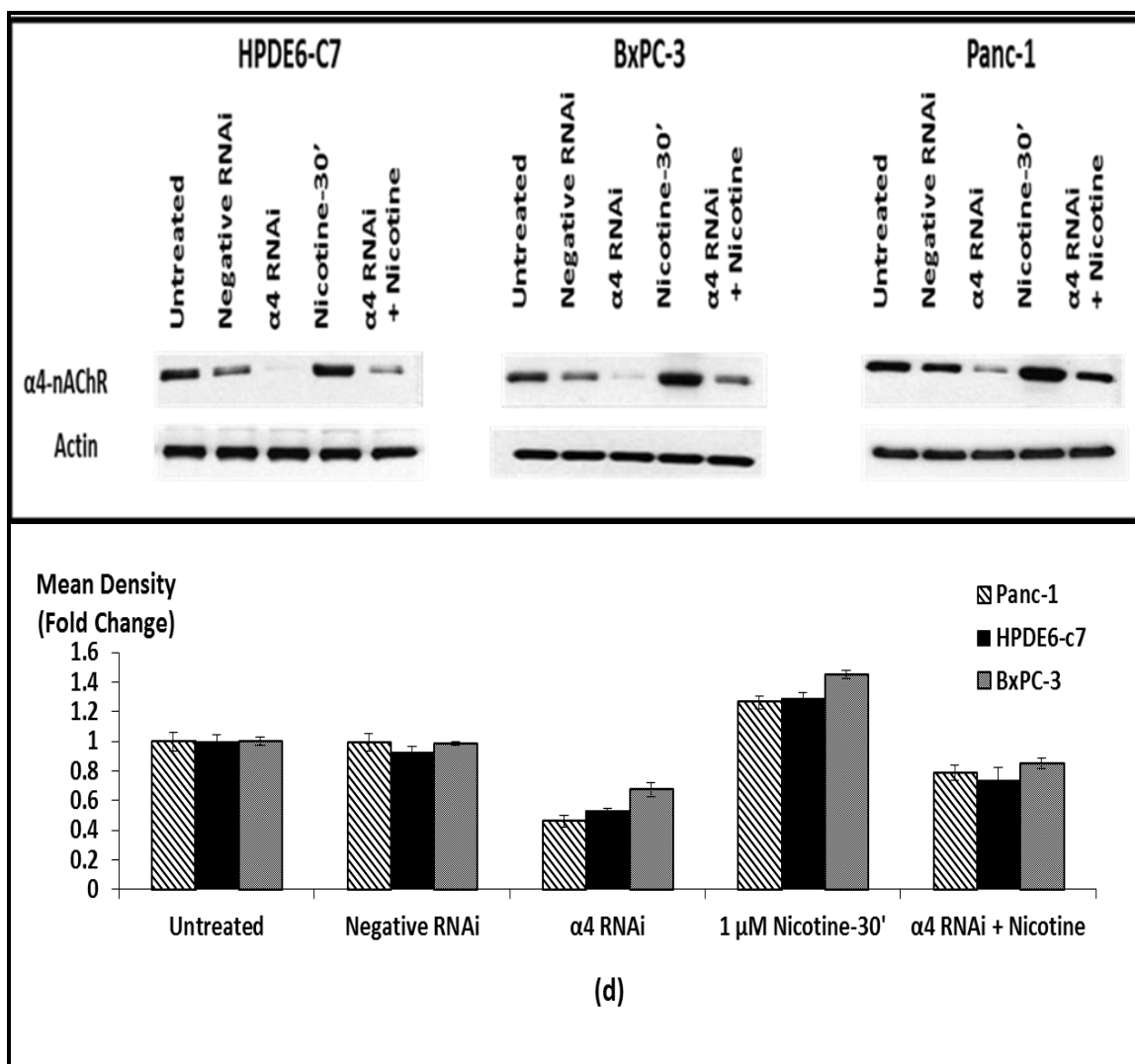


Figure 1D. Western blots showing the effects of gene knockdown on protein expression of the $\alpha 4$ -nAChR in the presence and absence of nicotine (1 μ M for 30 minutes). The house keeping protein β -actin was used as a control to ensure equal loading of proteins. The columns in the graph represent means and $\pm$ SD of two mean density readings per band from three independent western blots ($n = 6$) expressed as fold changes in expression of $\alpha 4$ -nAChR.

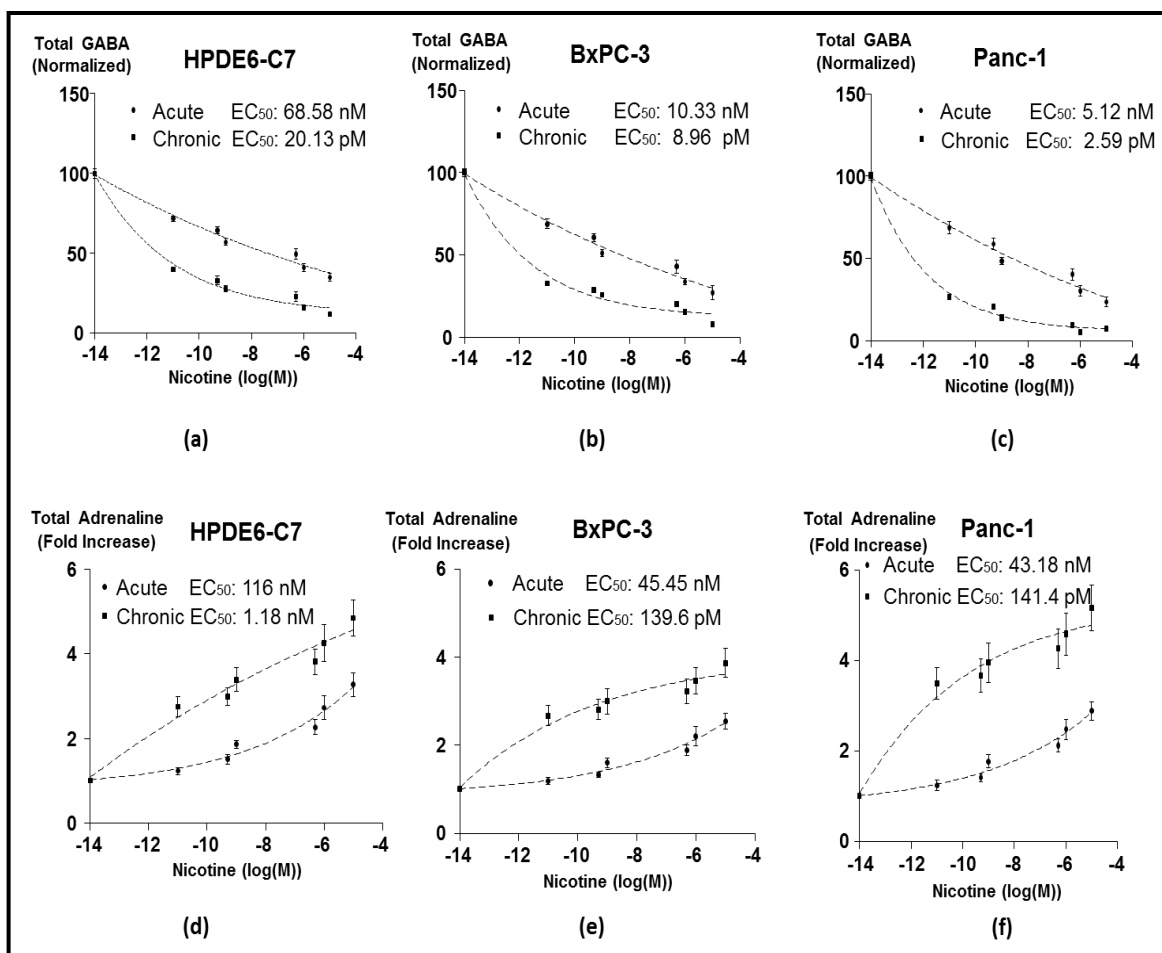


Figure 2. Total (secreted plus intracellular) GABA levels (a-c) and total (secreted plus intracellular) adrenaline levels (d-f) in HPDE6-C7, BxPC-3 and Panc-1 cells treated with single doses of nicotine from 10 pM through 10 μ M for 30 minutes or pretreated with 1 μ M nicotine for 7 days and then exposed to identical concentrations of nicotine. Data points are mean and $\pm$ SD from five samples per treatment group.

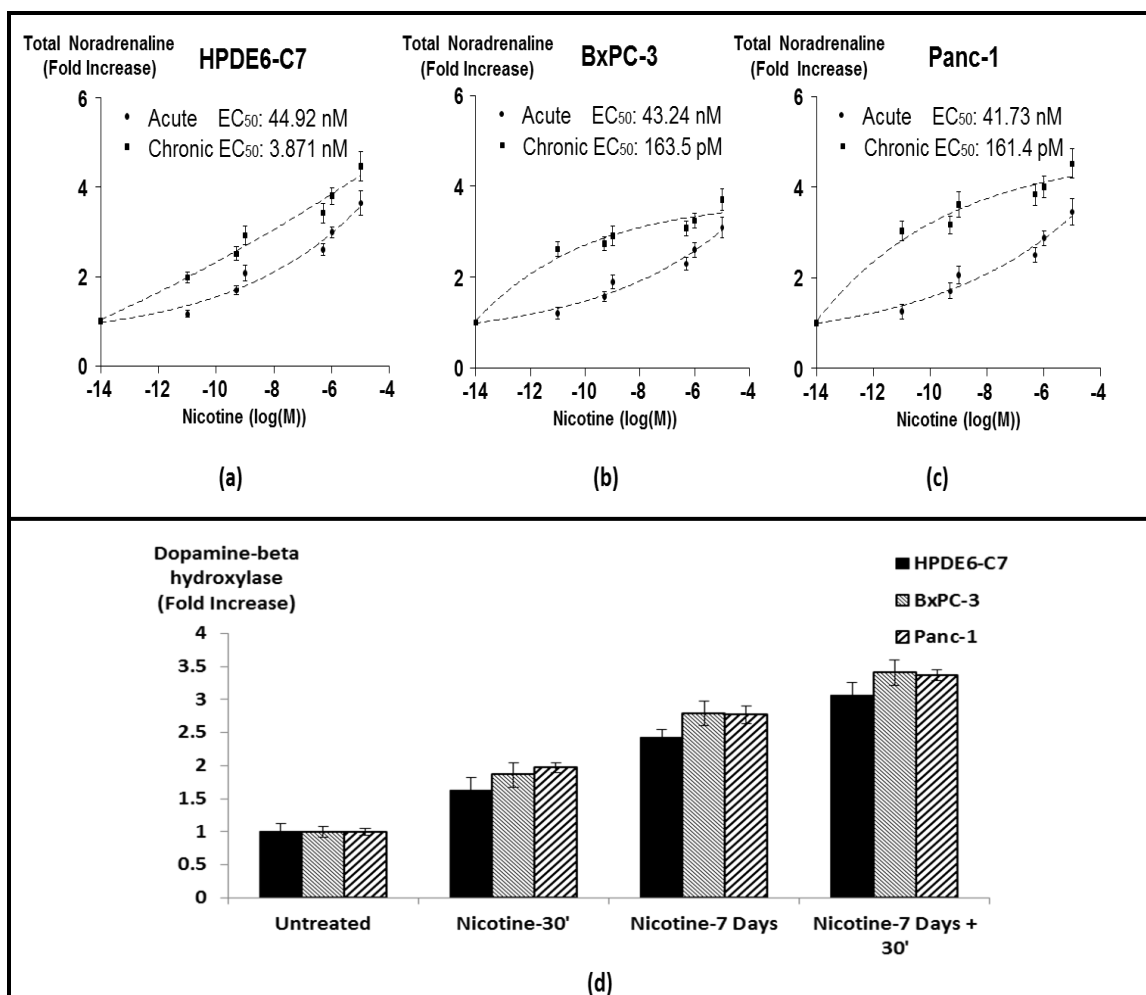


Figure 3. Total (secreted plus intracellular) noradrenaline levels (a-c) in HPDE6-C7, BxPC-3 and Panc-1 cells treated with single doses of nicotine from 10 pM through 10 μ M for 30 minutes or pretreated with 1 μ M nicotine for 7 days and then exposed to identical concentrations of nicotine. Levels of dopamine-beta hydroxylase (d) in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 1 μ M nicotine for 30 minutes, pretreated with 1 μ M nicotine for 7 days, or pretreated with 1 μ M nicotine for 7 days followed by 30 minute nicotine treatment prior to harvesting. Data points are mean and $\pm$ SD from five samples per treatment group.

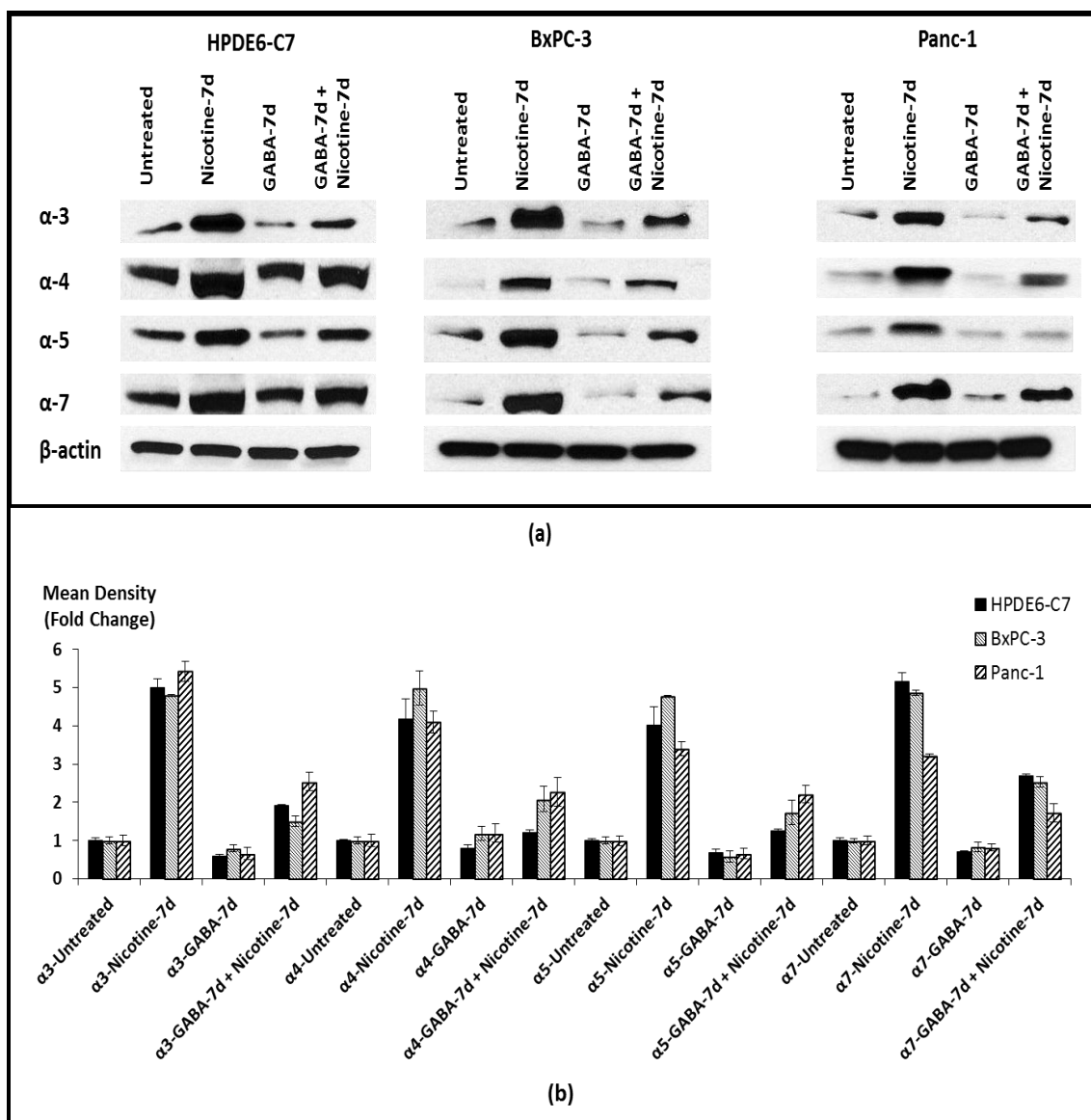


Figure 4. Western blots assessing protein expression of nAChR subunits α 3, α 4, α 5 and α 7 in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 1 μ M nicotine, 30 μ M GABA or GABA + nicotine for 7 days. The house keeping protein β -actin was used as a control to ensure equal loading of proteins (a). The columns in graph (b) represent means and + / - SD of two mean density readings per band from three independent western blots (n = 6) expressed as fold changes in expression of α 3, 4, 5 and 7-nAChRs.

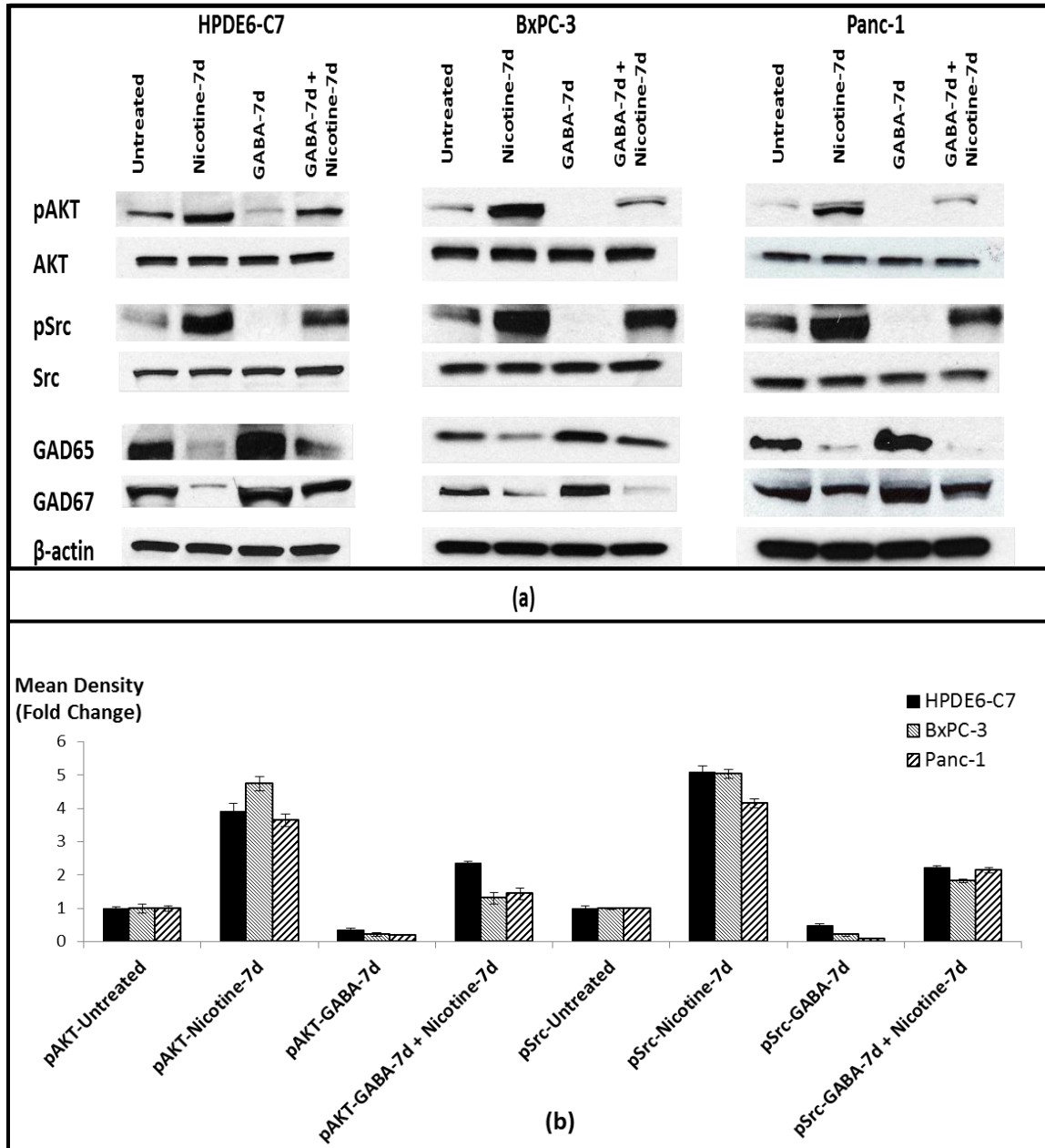


Figure 5A-B. Western blots assessing phosphorylation of AKT, Src, GAD65 and GAD67 in cells treated with 1 μ M nicotine, 30 μ M GABA and GABA + nicotine for 7 days (a). The columns in graph (b) represent means and $\pm$ SD of two mean density readings per band from three independent western blots ($n = 6$). The un-phosphorylated proteins AKT, Src and β -actin were used as controls to ensure equal loading of proteins.

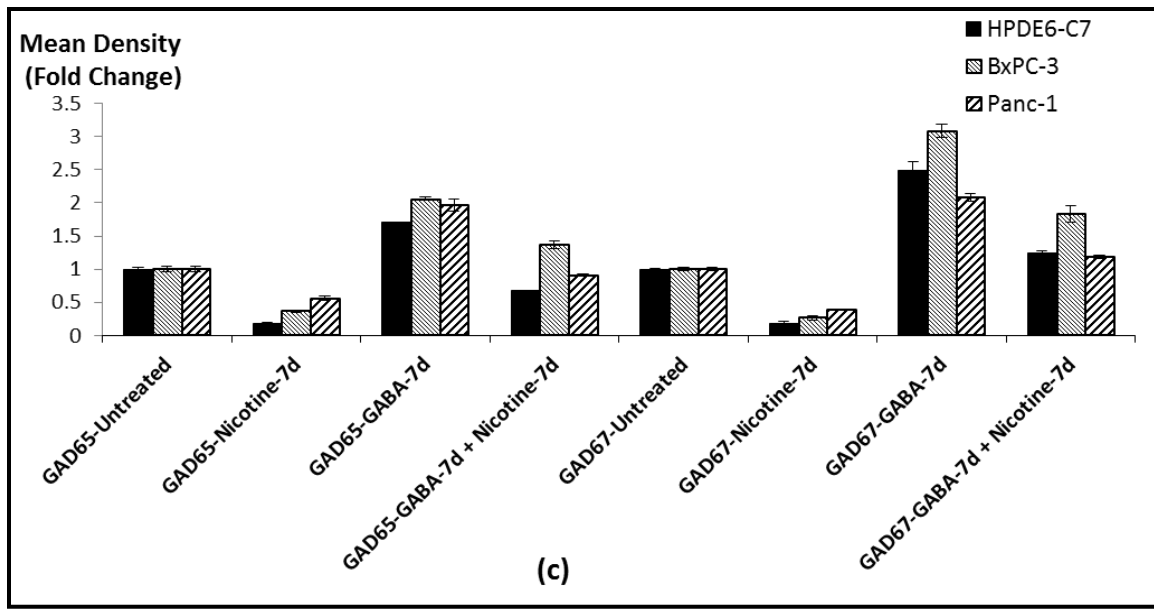


Figure 5C. Densitometric analysis of GAD65 and GAD67 western bands presented in figure 5a. The columns represent means and $\pm$ SD of two mean density readings per band from three independent western blots ($n = 6$) expressed as fold changes in protein phosphorylation.

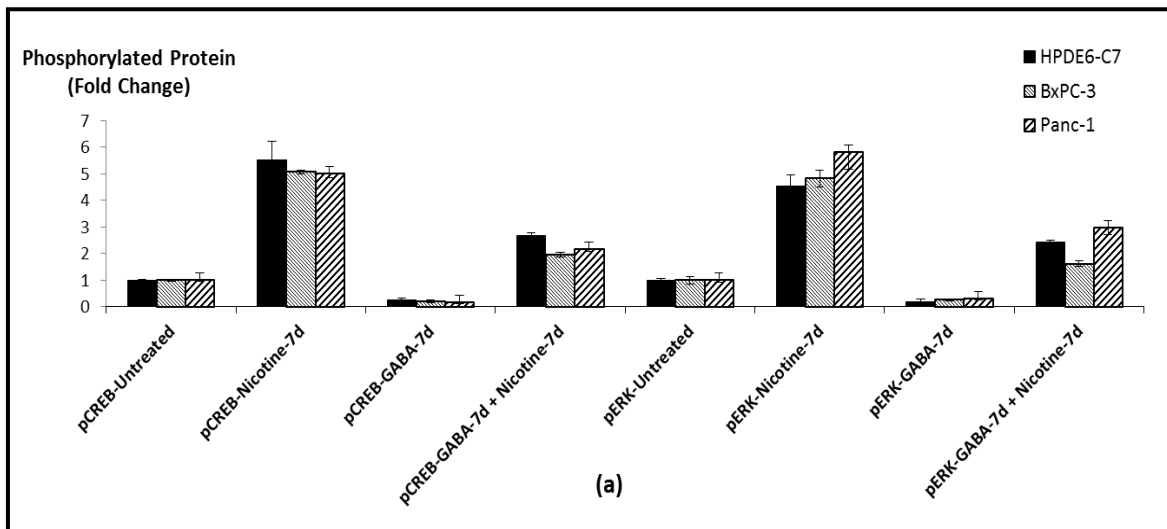


Figure 6A. Phosphorylation of CREB and ERK assessed by ELISA assays in HPDE6-C7, BxPC-3 and Panc-1 cells showing inhibition of nicotine-induced phosphorylation of these signaling proteins by GABA. The columns represent means and $\pm$ SD of 5 samples per treatment group.

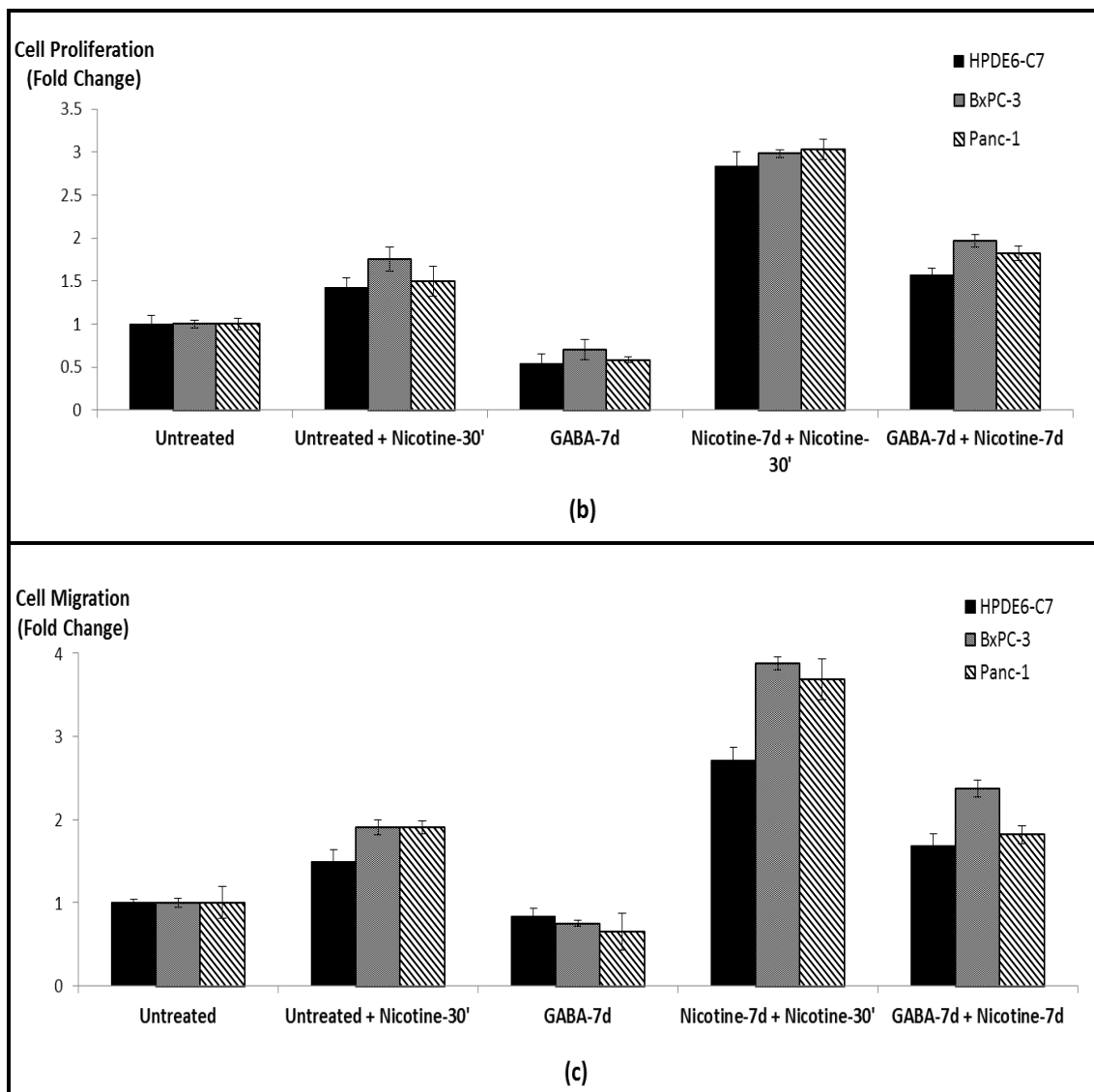


Figure 6B-C.Cell proliferation (b) assessed by MTT assays and metastatic potential (c) assessed by cell migration assays in HPDE6-C7, BxPC-3 and Panc-1 cells showing inhibition of nicotine-induced cell proliferation and migration by GABA. The columns represent means and + / - SD of 5 samples per treatment group.

CHAPTER V:

Interaction of Chronic Ethanol with Nicotinic Receptor-Mediated Regulation of Pancreatic Cancer Cells and Pancreatic Duct Epithelia

CHAPTER V

Brief explanatory statement

This chapter is a slightly revised version of a manuscript that has been submitted for publication in “Neoplasia” on July 2012.

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The use of “we” in this chapter refers to the contributions of the co-authors and I in the project. My contributions in this paper include (1) running all experiments relevant to the paper, (2) analysis and interpretation of the data, (3) statistical analysis of all data, (4) photographic and graphic documentation of results, (5) manuscript writing. Co-authors contributions to this paper include experimental design and writing editorial assistance by Dr. Schuller and technical assistance by Dr. Hussein Al-Wadei.

Abstract

Pancreatic cancer has a high mortality rate and smoking and alcoholism are risk factors. We have shown that nicotinic acetylcholine receptors (nAChRs) regulate pancreatic ductal epithelia and pancreatic ductal adenocarcinoma (PDAC) cells in an autocrine fashion by stimulating their production of noradrenaline and adrenaline while suppressing γ -amino butyric acid (GABA). Our current study has investigated the effects of chronic ethanol on the expression and regulatory function of these receptors and explored potential cooperative effects of chronic ethanol with chronic nicotine. Using MTT assays, cell migration assays, Western blotting and immunoassays in two PDAC cell lines and in immortalized human pancreatic duct epithelial cells, our data show that chronic ethanol increases cell proliferation and migration via increased catecholamine synthesis and suppression of inhibitory GABA by modulating the protein expression and responsiveness of nAChRs $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 7$. GABA reversed all effects of ethanol whereas chronic nicotine enhanced the responses to ethanol. Our findings suggest that alcoholism and smoking cooperatively induce multiple nAChR-dependent cancer stimulating signaling pathways while suppressing cancer inhibitory GABA. Our data identify GABA as a promising agent for the prevention of PDAC in individuals at risk due to chronic alcohol consumption and smoking.

Introduction

Pancreatic ductal adenocarcinoma (PDAC), which has phenotypic and functional features of pancreatic duct epithelial cells, is one of the most aggressive neoplastic

diseases with a mortality rate near 100 % within one year of diagnosis [1,2]. The poor prognosis of PDAC is caused by its unresponsiveness to available therapeutics and its propensity to grow asymptotically until it has reached an advanced stage [3,4]. Smoking, chronic alcohol consumption and alcoholism-induced pancreatitis are documented risk factors for PDAC [5-8]. Numerous publications have focused on the mechanisms how nicotine, its nitrosated carcinogenic derivative NNK and other tobacco related carcinogens stimulate pancreatic cancer development and progression [9]. We have recently identified an important role of nicotinic acetylcholine receptors (nAChRs) and neurotransmitters controlled by these receptors in the regulation of PDAC proliferation and migration in vitro [10]. Binding of nicotine or its nitrosated carcinogenic derivative 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to the $\alpha 3$, $\alpha 5$ and $\alpha 7$ -nAChRs resulted in the synthesis and release of adrenaline and noradrenaline [10], which are β -adrenergic receptor agonists that activate adenylyl cyclase [11,12]. In turn, this reaction increased cAMP synthesis, leading to induction of cell proliferation, migration and angiogenesis via cAMP-dependent signaling [10,13-15].

While the $\alpha 4$ -nAChR was not involved in the regulation of noradrenaline and adrenaline in these cells, it regulated the synthesis and release of the inhibitory neurotransmitter GABA and chronic nicotine desensitized this receptor, thereby reducing GABA production [10,14,16]. These findings identified the suppression of GABA as an important event in facilitating PDAC development and progression and suggested that nutritional supplementation with GABA may have anti-cancer activity on PDAC via the

GABA-B receptor-mediated inhibition of adenylyl cyclase activation observed in vitro [14].

In the current experiments, we have investigated the effects of chronic ethanol on the protein expression and responsiveness of nAChRs $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 7$ in immortalized pancreatic duct epithelial cells and two PDAC cell lines and analyzed the effects of these changes on neurotransmitter production, cell proliferation, cell migration and activation of key signaling proteins. Moreover, we have tested the potential inhibition of these cellular responses by GABA and explored potential cooperative effects of chronic ethanol and chronic nicotine on these nAChRs and their downstream effectors.

Materials and Methods

Chemicals, antibodies and assay kits

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) colorimetric assay kit was purchased from Sigma-Aldrich (St Louis, MO, USA). The CytoSelect Cell Migration Assay kit was purchased from Cell BioLabs, Inc. (San Diego, CA, USA).

The 2-Cat and GABA Research ELISA Kits were purchased from Rocky Mountain Diagnostics Incorporation (Colorado Springs, CO, USA). ELISA kits for Akt [pS473], ERK1/2 [pTpY185/187], and CREB [pS133] were purchased from Invitrogen Corporation (Carlsbad, CA, USA). The c-Src Kinase assay kit was purchased from MBL International (Woburn, MA, USA). Both the cAMP and PKA activity ELISA assays were purchased from Enzo Life Sciences (Farmingdale, NY, USA).

The antibodies anti-rabbit and anti-mouse were purchased from Cell Signaling (Danvers, MA, USA). The primary antibody anti-nicotinic acetylcholine receptor $\alpha 4$ (55 kDa) was purchased from Millipore (Billerica, MA, USA). The nicotinic acetylcholine receptor subunits $\alpha 7$ (56 kDa), $\alpha 3$ (57 kDa) and $\alpha 5$ (53 kDa) antibodies as well as GAD65 (65 kDa), GAD67 (67 kDa) and β -actin (42 kDa) antibodies were all purchased from Abcam (Cambridge, MA, USA). Nicotine ((-)-Nicotine hydrogen tartrate salt, minimum 98 % TLC), γ -amino butyric acid (GABA) and isobutylmethylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol was purchased from Fisher Scientific International (Fair Lawn, NJ, USA). The lysis buffer used to extract proteins along with Pierce ECL western blotting substrate were purchased from Thermo Scientific (Rockford, IL, USA).

Maintenance and chronic treatments of cultured cells

The immortalized human pancreatic duct epithelial cell line, HPDE6-C7, was kindly provided to us by Dr. Tsao (Division of Cellular and Molecular Biology, Department of Pathology, Ontario Cancer Institute/Princess Margaret Hospital, University of Toronto, Toronto, ON, Canada). This cell line was clonally established after transduction of the HPV16-E6E7 genes into primary cultures of pancreatic duct epithelial cells. The human PDAC cell lines Panc-1 and BxPC-3 were purchased from the American Type Culture Collection (Manassas, VA, USA). All cell lines were authenticated at the beginning of the study by Research Animal Diagnostic Laboratory (RADIL, Columbia, MO, USA) by species-specific PCR evaluation.

Cell lines Panc-1 and BxPC-3 were maintained in DMEM and RPMI media respectively and were both supplemented with 10 % Fetal Bovine Serum (FBS). HPDE6-C7 cells were maintained in Keratinocyte Serum Free Medium (KSFM) supplemented with 25 mg / 500 ml Bovine Pituitary Extract (BPE) and 2.5 µg / 500 ml Epidermal Growth Factor (EGF) (GIBCO Invitrogen Corporation, Grand Island, NY, USA). All cell lines were grown without any antibiotics in an atmosphere of 5 % CO₂, 99 % relative humidity and 37 °C.

Chronic treatment of cells in complete medium with ethanol (17 mM), GABA (30 µM) or nicotine (1 µM) was for 7 days. Fresh medium containing each drug was replaced every 24 hours. In the untreated control cells, the medium was replaced every 24 hours. Treatment groups were as follows:

Control: cells maintained for 7 days in complete medium without drugs;

Ethanol: cells treated with ethanol for 7 days;

Ethanol + GABA: cells treated simultaneously for 7 days with ethanol and GABA;

GABA: cells treated with GABA for 7 days;

Nicotine: cells treated with nicotine for 7 days;

Nicotine + ethanol: cells treated simultaneously with nicotine and ethanol.

The concentration (17 mM) of ethanol used is the equivalent of the legal blood alcohol limit of 0.08 % in the USA. The concentration (30 µM) is the equivalent of 21.7 mg/person/day and is within the recommended range of GABA as a nutritional supplement. The concentration (1 µM) of nicotine used is within the range of systemic nicotine concentrations in moderate smokers.

Assessment of cell proliferation by MTT assay

All three cell lines were seeded into 6-well plates at a density of 20,000 cells per well (n= 5) in their respective complete media. Cells were then left untreated or treated with ethanol, GABA or both for 7 days. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) colorimetric assay (Sigma-Aldrich St Louis, MO, USA) was then used for the assessment of cell proliferation following instructions provided by the vendor. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at a primary and reference wavelengths of 570 nm and 650 nm respectively.

Assessment of Cell migration by CytoSelect cell migration assay

The three cell lines were seeded onto the top chamber of polycarbonate membrane filter inserts (8 μ M pore size) in 6-well plates at a density of 20,000 cells per well in their respective complete media. Using identical treatment groups as for the MTT assay (above), a cell migration assay kit (Cell BioLabs, San Diego, CA, USA) was then used to assess the migratory ability of these cells following the vendor's instructions. Optical density of cell samples was read at 560 nm using an uQuant Bio-Tek Instrument ELISA reader.

Protein analyses of nAChR subunits and GAD isozymes by western blotting

The protein expression of nAChR subunits α 3, α 4, α 5, and α 7 and of GAD65 and GAD67 was determined in cells from identical treatment groups as for the MTT and cell migration assays to determine the effects of chronic ethanol and GABA. Expression

levels of the 4 investigated nAChR subunits were additionally assessed in cells treated with chronic nicotine or chronic nicotine plus ethanol.

Protein samples were prepared using lysis buffer (50-mmol / L Tris-HCl, 1 % NP-40, 150-mmol / L NaCl, 1-mmol / L phenylmethanesulfonylfluoride, 1-mmol / L Na_3VO_4 , 1-mmol / L NaF, and 1 μg / mL of aprotinin, leupeptin, and pepstatin). After heat denaturation, protein samples were electrophoresed, using 12 % SDS gels (Invitrogen) and blotted onto membranes. The membranes were then blocked (5 % nonfat dry milk solution) for one hour at room temperature. Membranes were then incubated overnight at 4 °C with the following primary antibodies: nAChR subunit $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 7$, GAD65 and GAD67. The primary antibody β -actin was used as a loading control to ensure equal loading of proteins. All membranes were then washed (0.5 % Tween 20 / TBS) and incubated with their respective fluorescent secondary antibodies for two hours. Protein bands were then visualized with enhanced chemiluminescence reagent (Pierce ECL Western Blotting Detection Substrate). Following background subtraction, mean densities of 2 rectangular areas of standard size per band from three independent westerns were determined and mean values and standard deviation ($n = 6$) of protein expression were calculated.

Assessment of responsiveness to ethanol by immunoassays for the detection of neurotransmitters

Unpretreated cells from each cell line or cells pretreated for 7 days with 17 mM ethanol were exposed to a range of ethanol concentrations (1 μM , 50 μM , 500 μM , 1

mM, 17 mM and 35 mM) for 30 minutes prior to harvesting. The culture media, containing the secreted stress neurotransmitters noradrenaline and adrenaline as well as inhibitory GABA were then collected in 15 ml test tubes. The cells which contained synthesized intracellular catecholamines and GABA were lysed and harvested into 1.5 ml eppendorf tubes after a one time wash with warm 1X PBS. Total (secreted plus intracellular) stress neurotransmitters and GABA of five samples per treatment group was analyzed using 2-Cat and GABA-Research ELISA kits respectively following the vendors' recommendations. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Quantitative assessment of accumulated intracellular cAMP levels

Chronic treatment groups for this assay were identical to those of the MTT and cell migration assays (above). On the sixth day of treatment, cells were washed twice with warm 1X PBS then incubated for 30 minutes with 1 mM of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) to prevent enzymatic breakdown of the cAMP formed. Once the 30 minutes incubation was over, cells were washed with 1X PBS twice then treated with 17 mM ethanol for 24 hours. The cells were then lysed and harvested into 1.5 ml eppendorf tubes following the cAMP ELISA kit's instructions. Quantitative analyses of cAMP levels from five samples per treatment group were conducted using the cAMP ELISA kit following the vendor's recommendations. Absorbance of samples was

read using an uQuant Bio-Tek Instrument ELISA reader at 405 nm primary wavelength with a 550 nm reference wavelength.

Quantitative assessment of PKA activation and phosphorylation levels of signaling proteins by immunoassay

Following identical chronic treatments as outlined for the MTT and cell migration assays, the cells were lysed and harvested into 1.5 ml eppendorf tubes following ELISA kits' instructions. Quantitative analyses of PKA activity, Akt, CREB, Src, and ERK1/2 phosphorylation from five samples per treatment group were conducted using PKA activity, Akt, CREB, c-Src, and ERK1/2 ELISA kits respectively following the vendors' recommendations. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Analysis of neurotransmitter synthesis in response to chronic ethanol and nicotine

Cells from HPDE6-C7, BxPC-3 and Panc-1 were either untreated or treated with 1 μ M nicotine, 17 mM ethanol or the combination of the two for 7 days in complete media. Cells were then lysed and harvested into 1.5 ml eppendorf tubes after a one time wash with warm 1X PBS. Quantitative analyses of intracellular GABA, adrenaline and noradrenaline of five samples per treatment group were conducted using GABA Research and 2-Cat ELISA kits respectively following the vendors' recommendations. Absorbance of samples was read using an uQuant Bio-Tek Instrument ELISA reader at 450 nm primary wavelength with a 630 nm reference wavelength.

Statistical analysis of data

Using GraphPad InStat software (GraphPad, San Diego, CA, USA), all data presented in the figures as bar graphs were analyzed for statistically significant differences among treatment groups by One Way ANOVA and Tukey-Kramer multiple comparison test when data followed a normal distribution. Data that did not pass normality tests were tested for significant differences by non-parametric Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test. GraphPad Prism 5 software (GraphPad, San Diego, CA, USA) was used to establish sigmoidal dose-response curves and to calculate EC₅₀ values by nonlinear regression analysis. Data of the immunoassays are expressed as mean and + / - standard deviation of five samples per treatment group. Using NIH ImageJ, two densitometric determinations per band were assessed in three independent western blots (n=6) per protein and expressed as mean values and standard deviations.

Results

Effects of chronic ethanol in the presence and absence of GABA on cell proliferation and migration

Using an ethanol concentration (17 mM) equivalent to the legal blood alcohol limit of 0.08 % in the USA, we found that all three investigated cell lines responded with more than 2 fold increases ($p < 0.0001$) in the number of viable cells when exposed for 7 days to ethanol (Figure 1a). This response was significantly ($p < 0.001$) inhibited by simultaneous exposure of the cells to GABA (Figure 1a) at a concentration (30 μ M)

within the recommended daily dose of GABA as a nutritional supplement. In addition, 7-day exposure to GABA significantly ($p < 0.0001$) decreased the number of viable cells in the three cell lines not pre-treated with ethanol (Figure 1a). These findings were mirrored by the results of cell migration assays that used identical treatment groups (Figure 1b), with more than 2.5 fold increase in migrated cells after 7-day treatment of the three cell lines with ethanol ($p < 0.0001$) and significant ($p < 0.0001$) inhibition of this effect by GABA.

Effects of chronic ethanol and GABA on the protein expression of nAChRs

We have previously shown that nAChRs expressing the subunits $\alpha 3$, $\alpha 5$ and $\alpha 7$ regulate the proliferation of PDAC cell lines Panc-1 and BxPC-3 as well as immortalized pancreatic duct epithelial cells HPDE6-C7 by activating an autocrine stress neurotransmitter loop [10]. In addition, we have shown that the $\alpha 4$ -nAChR regulates the synthesis and release of GABA in these cells and that GABA inhibits cell proliferation and migration [10,14]. In order to further our understanding on the mechanisms involved in the observed effects of chronic ethanol on cell proliferation and migration, we therefore investigated the effects of chronic ethanol and GABA on the protein expression of these nAChRs. Our data show that chronic exposure to ethanol alone significantly induced ($p < 0.0001$) protein expression of nAChR subunits $\alpha 3$ (4.5 fold), $\alpha 4$ (4 fold), $\alpha 5$ (5 fold) and $\alpha 7$ (5 fold). Simultaneous treatment of cells with GABA significantly ($p < 0.0001$) reduced these effects of ethanol (Figures 2a, b).

Effects of chronic ethanol and GABA on the protein expression of glutamate decarboxylase isozymes

The isozymes glutamate decarboxylase 65 (GAD65) and glutamate decarboxylase 67 (GAD67) catalyze the synthesis of GABA from glutamate [17]. We have previously shown that the protein expression of these enzymes is suppressed resulting in reduced synthesis of GABA when the $\alpha 4$ -nAChR in PDAC cells or pancreatic duct epithelial cells undergoes protein upregulation in response to desensitization by a single dose of nicotine [10]. In analogy to these findings, our current data show significant reductions ($p < 0.0001$) in the protein expression of both GAD isozymes upon chronic ethanol exposure and a reversal of this effect by treatment with GABA (Figures 3a, b).

Concentration-dependent effects of chronic ethanol on total GABA, adrenaline and noradrenaline

In order to assess the functional significance of the observed changes in chronic ethanol-induced protein expression of nAChRs and GADs on their effectors, we measured total (intracellular plus secreted) levels of the inhibitory neurotransmitter GABA and of the stress neurotransmitters noradrenaline and adrenaline in 7-day ethanol pretreated versus non-pretreated cells exposed to a range of concentrations of ethanol (1 μ M - 35 mM). As shown in Figures 4a-c, chronic ethanol exposure reduced total GABA at all concentrations tested and significantly ($p < 0.0001$) decreased the EC_{50} concentrations for the reduction of GABA production in all three cell lines.

By contrast, the concentration-dependent increases in total levels of the stress neurotransmitters noradrenaline and adrenaline were significantly ($p < 0.0001$) enhanced by 7-day pre-exposure to ethanol in all three cell lines (Figures 4d-i) and EC_{50} values of ethanol for these increases were significantly ($p < 0.0001$) reduced.

Effects of chronic ethanol and GABA on intracellular accumulation of cAMP and PKA activation

We have previously shown that nAChRs expressing subunits $\alpha 3$, $\alpha 5$ and $\alpha 7$ jointly regulate the synthesis and release of the stress neurotransmitters noradrenaline and adrenaline in PDAC cells and pancreatic duct epithelial cells [10]. In turn, noradrenaline and adrenaline are agonists for beta-adrenergic receptors [11,12] and increased intracellular cAMP levels via activation of the stimulatory G-protein $G\alpha_s$ in these cells, resulted in the activation of PKA. [10]. We therefore measured intracellular cAMP levels in the current experiments in order to assess the potential modulation of cAMP levels and PKA activation by chronic ethanol. As Figures 5a and b show, chronic treatment of cells with ethanol significantly ($p < 0.0001$) increased intracellular cAMP levels (3-4 folds) in the three investigated cell lines while also significantly ($p < 0.0001$) increasing the levels of activated PKA (2.8-3.4 folds). Simultaneous exposure of the cells to GABA during the 7-day ethanol pretreatment period significantly ($p < 0.0001$) reduced both of these responses to ethanol (Figures 5a, b). In addition, chronic exposure of the cells to GABA alone significantly ($p < 0.0001$) suppressed base levels of cAMP and activated PKA in cells not pretreated with ethanol (Figures 5a, b).

Effects of chronic ethanol in the presence and absence of GABA on the phosphorylation of multiple signaling proteins

We have previously shown that activation of cAMP-dependent PKA results in the phosphorylation of multiple signaling proteins involved in the regulation of proliferation, angiogenesis, metastatic potential and apoptosis in PDAC cells [10,13-15]. We therefore assessed the potential modulation in phosphorylation levels of these signaling proteins by chronic ethanol in the presence and absence of simultaneous treatment with GABA. Our results show that chronic exposure of normal pancreatic duct epithelia and both PDAC cell lines to ethanol significantly induced ($p < 0.0001$) phosphorylation of ERK, CREB, Akt and Src (Figures 6a, b). In analogy to our findings with cAMP and PKA, simultaneous exposure of the cells to GABA during the ethanol pretreatment period significantly ($p < 0.0001$) reduced each of these responses (Figures 6a, b).

Effects of chronic nicotine and ethanol on protein expression of nAChRs

Our laboratory has previously shown that nicotine stimulates the synthesis and release of noradrenaline and adrenaline in PDAC cells and pancreatic duct epithelial cells by binding to nAChRs expressing subunits $\alpha 3$, $\alpha 5$ and $\alpha 7$ [10] while suppressing the synthesis and release of GABA by binding to the $\alpha 4$ -nAChR [14]. We therefore assessed potential cooperative effects of chronic ethanol with chronic nicotine on the protein expression of the $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 7$ -nAChRs. Our data identify distinct cooperative inductions of all four nAChRs when the three investigated cell lines were simultaneously exposed for 7 days to nicotine and ethanol (Figures 7a, b), with significantly ($p < 0.0001$)

higher levels of protein induction ($p < 0.0001$) in cells exposed to both agents than to either agent alone.

Effects of ethanol and nicotine on the synthesis of GABA and stress neurotransmitters

In order to assess the functional significance of the cooperative protein induction of nAChRs observed in the current study by chronic ethanol and nicotine (above), we compared the intracellular levels of GABA, noradrenaline and adrenaline in cells exposed to chronic nicotine, ethanol or both agents for 7 days. Our data show clear cooperative effects ($p < 0.0001$) of nicotine and ethanol on the suppression of GABA observed with either agent alone (Figure 8a). In addition, the stimulation of stress neurotransmitter synthesis by nicotine or ethanol alone was significantly ($p < 0.001$) enhanced by combination treatments with both agents (Figures 8b, c).

Discussion

Smoking and chronic alcohol consumption are established risk factors for pancreatic cancer [1,18,19]. Heavy alcohol consumption is thought to facilitate pancreatic cancer development by increasing the risk for diabetes and chronic pancreatitis which are independent risk factors for this malignancy [8]. However, the mechanisms of how smoking and chronic consumption of moderate amounts of alcohol jointly induce pancreatic cancer are poorly understood.

It has been shown that nicotine addiction and alcohol dependence are highly correlated [20,21] and that chronic nicotine-induced upregulation of nAChR proteins in

brain cells is significantly enhanced by the simultaneous exposure to chronic ethanol [21]. While nAChRs were initially thought to be uniquely expressed in the nervous system, more recent studies have identified these receptors in the majority of mammalian cells, including epithelia [22]. In accord with these reports, we have recently shown that the stress neurotransmitters noradrenaline and adrenaline are synthesized and released by normal pancreatic duct epithelia and PDAC cells in vitro when nicotine binds to nAChRs expressing the subunits $\alpha 3$, $\alpha 5$, or $\alpha 7$ in these cells [10]. In accord with the established function of noradrenaline and adrenaline as beta-adrenergic receptor agonists [11,12], a single dose of nicotine thus increased cAMP and activated PKA, causing the phosphorylation of multiple signaling proteins (p-ERK, p-CREB, p-Akt, p-Src) frequently overexpressed in pancreatic cancer while also stimulating cell proliferation [10]. Earlier studies by our laboratory have shown that exposure of immortalized pancreatic duct epithelial cells to a single dose of ethanol at a concentration (17 mM) equivalent to the legal blood alcohol limit (0.08 %) in the United States stimulates cell proliferation by increasing intracellular cAMP levels, resulting in a significant induction of activated PKA and PKA-dependent phosphorylation of CREB and ERK1/2 [23]. Our current study shows, for the first time, that chronic exposure to this moderate ethanol concentration significantly upregulates the protein expression of nAChRs $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 7$ in pancreatic duct epithelial cells and PDAC cells. In analogy to observations in brain cells [21], the induction of nAChR proteins was further enhanced by simultaneous exposure of the cells to chronic nicotine. The observed significant increases in total noradrenaline and adrenaline after chronic ethanol and associated significant decrease in

EC₅₀ values of ethanol required to stimulate the production of these neurotransmitters indicate functional sensitization of nAChRs $\alpha 3$, $\alpha 5$ and $\alpha 7$. The resulting significant inductions of activated PKA, CREB, ERK Akt and Src after chronic ethanol alone additionally identifies these signaling proteins as the driving forces of chronic ethanol-induced cell proliferation and migration in the three investigated cell lines. While our findings are in accord with reports from other laboratories that alcohol activates signaling proteins such as ERK, JNK, PKC and Stat3 in liver cells [24-29], they suggest that these proteins are downstream effectors of noradrenaline and adrenaline-induced beta-adrenergic signaling.

By contrast, the observed upregulation of $\alpha 4$ -nAChR protein upon chronic exposure to ethanol represents a reaction to desensitization of this receptor as indicated by the concentration-dependent suppression of GABA and associated reduction in GAD65 and GAD67.

These findings are analogous to reports that chronic nicotine desensitizes the $\alpha 4$ -nAChR in the brain [30,31], and that the inhibitory effects of GABA on acute nicotine-induced proliferation of PDAC cells is mediated by the $G\alpha_i$ -coupled GABA-B receptor which inhibits the activation of adenylyl cyclase in these cells [14]. Similar to our findings after a single dose of nicotine [10], chronic exposure to 30 μ M GABA reversed all chronic ethanol-induced cellular changes in the three investigated cell lines, including the observed modulations in nAChRs expression and function, changes in stress neurotransmitter and GABA levels and the resulting changes in signal transduction cascades.

In summary, our findings suggest that changes in nAChRs expression and function in the brain that are associated with nicotine addiction and alcohol dependence are also induced by chronic ethanol and chronic nicotine in PDAC cells and pancreatic duct epithelial cells where they cooperate to stimulate proliferation and migration. Moreover, the strong inhibitory effects of GABA on the observed cellular responses to chronic ethanol identify this neurotransmitter, which is widely used as a nutritional supplement, as a promising agent for the prevention of PDAC in individuals at increased risk due to chronic consumption of alcohol. In light of the fact that the GABA-B receptor agonist baclofen has been identified as an effective therapeutic for alcohol dependence [32,33], nutritional supplementation with GABA may not only have protective effects against PDAC development but simultaneously reduce the craving of alcohol withdrawal in dependent patients.

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Appendix

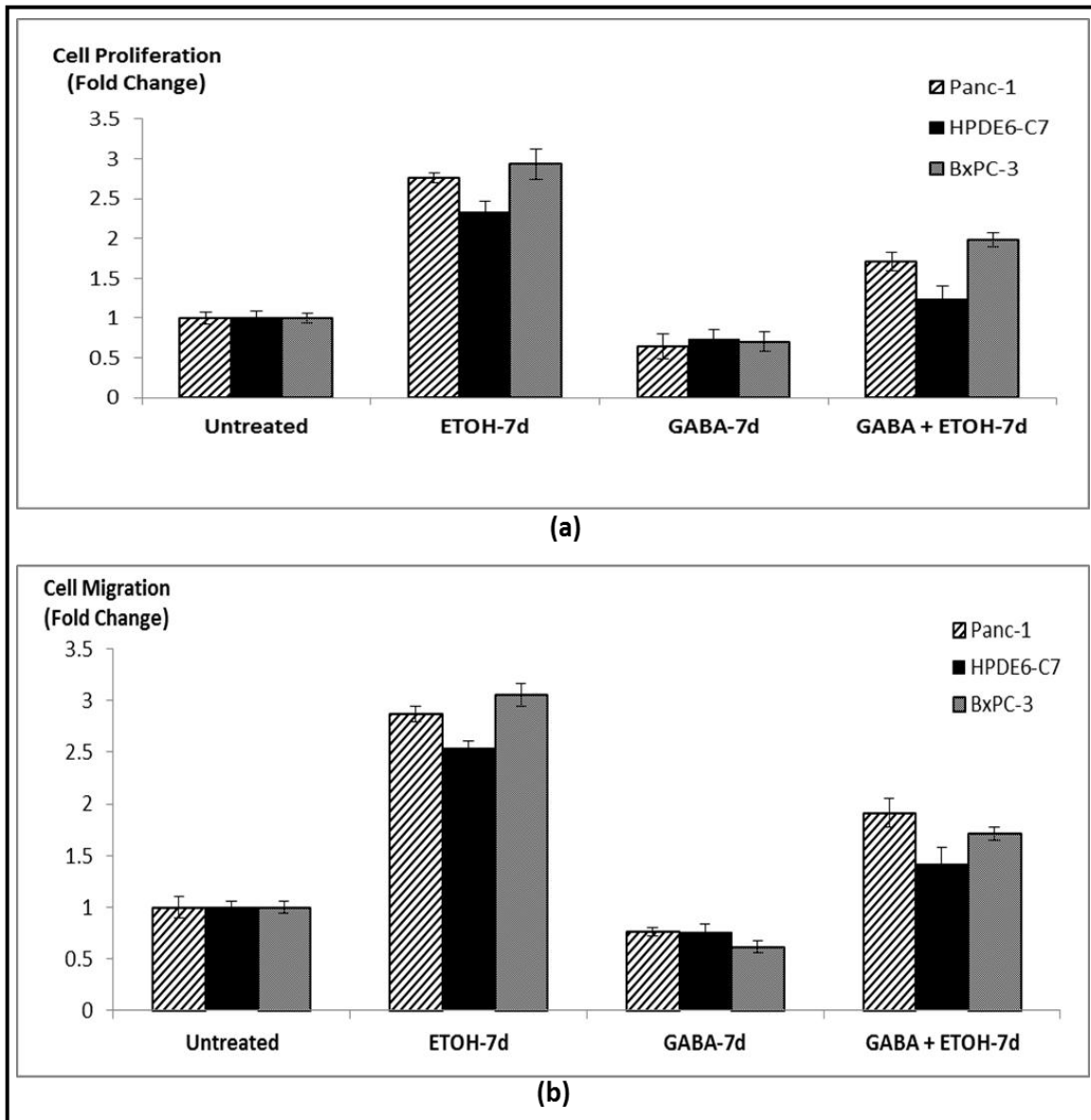


Figure 1. Cell proliferation (a) assessed by MTT assays and metastatic potential (b) assessed by cell migration assays in HPDE6-C7, BxPC-3 and Panc-1 cells showing stimulation of proliferation and migration upon ethanol exposure and a reversal of these effects upon exposure to GABA. The columns represent means and $\pm$ SD of 5 samples per treatment group.

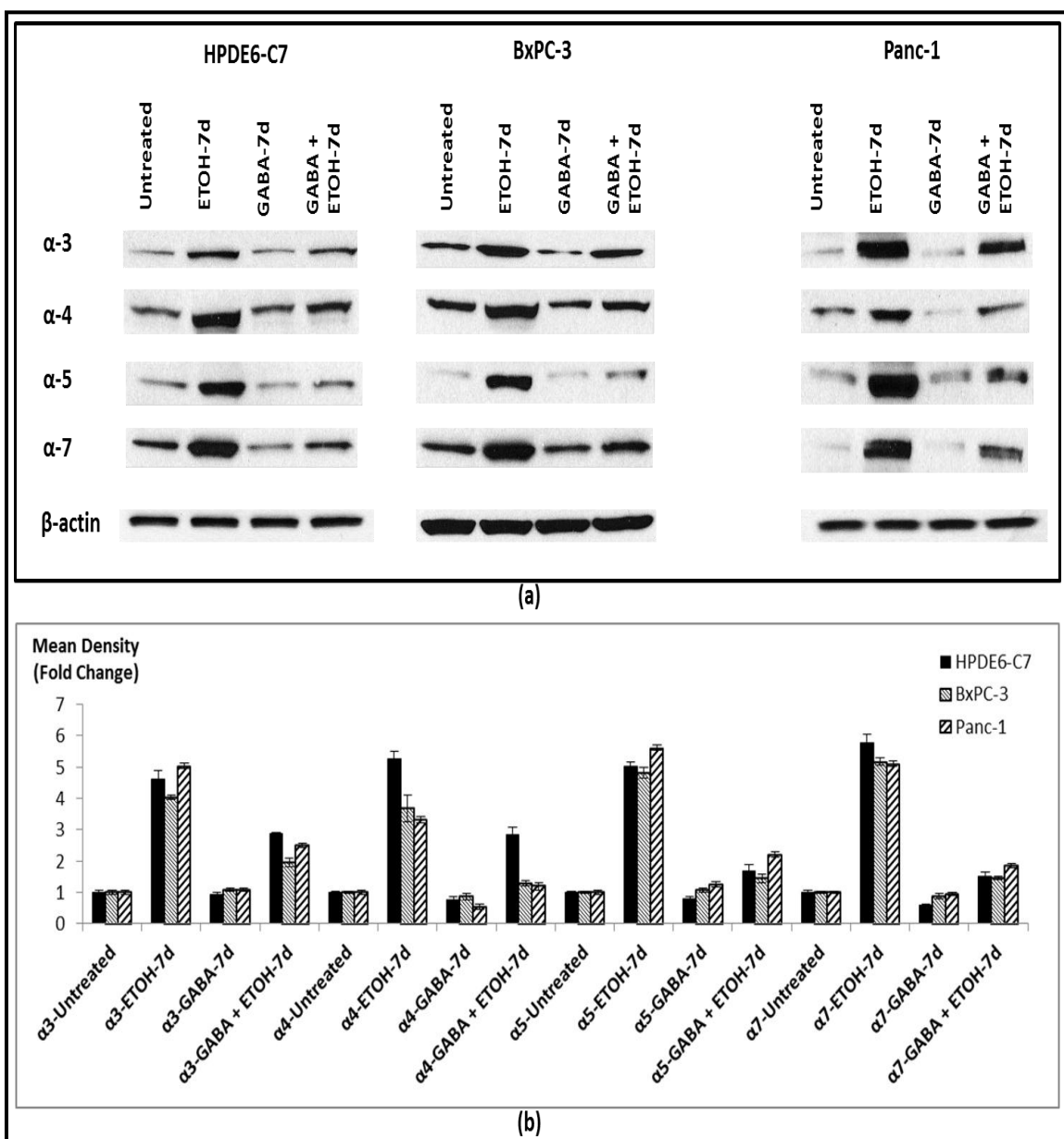


Figure 2. Western blots assessing protein expression of $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 7$ -nAChRs in cells treated with 17 mM ethanol, 30 μ M GABA or both for 7 days. β -actin was used as a control to ensure equal loading of proteins (a). The columns in figure (b) represent means and $\pm$ SD of two mean density readings per band from three independent western blots ($n = 6$) expressed as fold changes in the expression of the nAChR subunits.

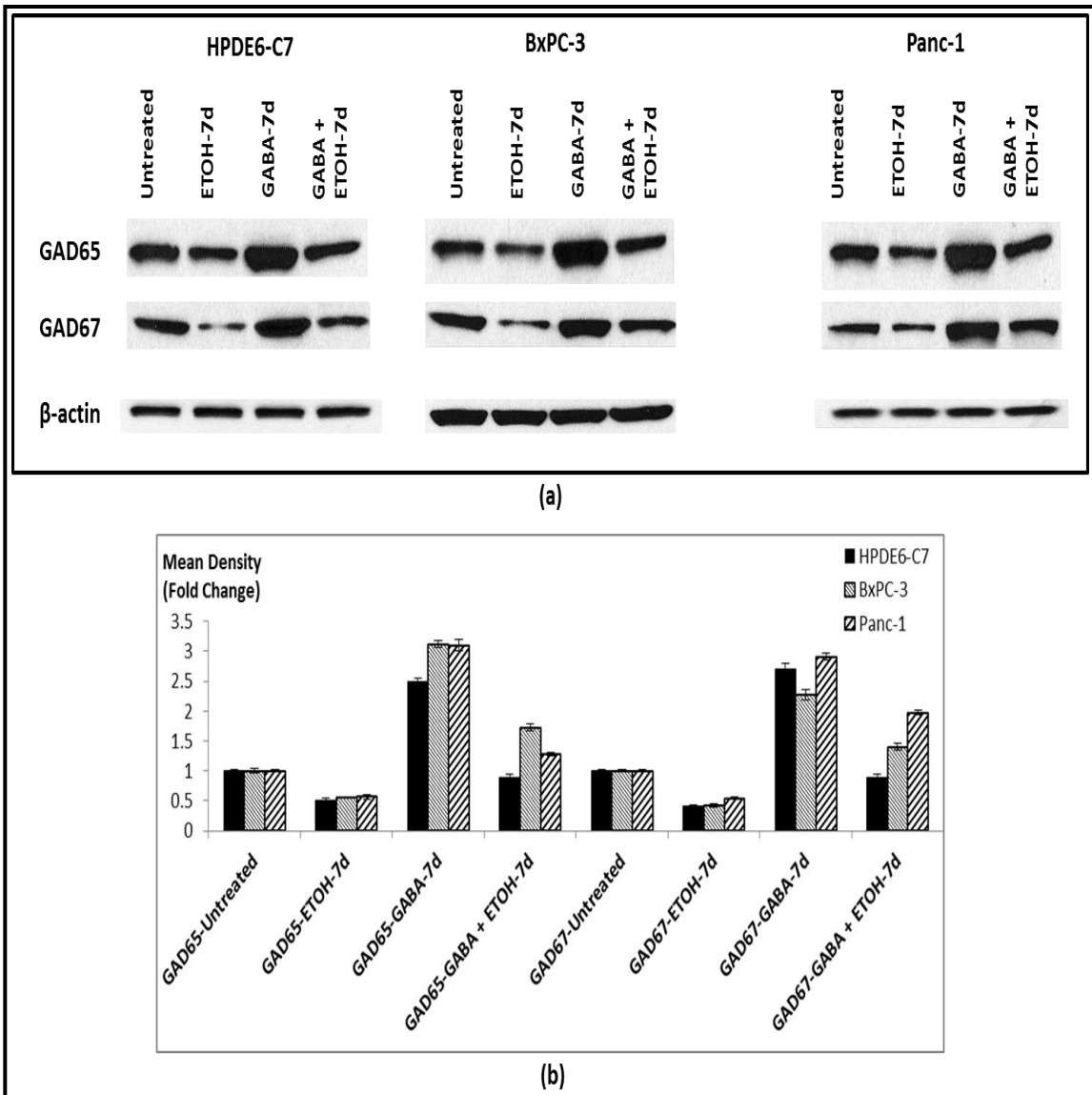


Figure 3. Western blots assessing phosphorylation of GAD65 and GAD67 in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 17 mM ethanol, 30 μ M GABA or the combination of the two for 7 days. The house keeping protein β -actin was used as a control to ensure equal loading of proteins (a). The columns in figure (b) represent means and $\pm$ SD of two mean density readings per band from three independent western blots ($n = 6$) expressed as fold changes in protein phosphorylation.

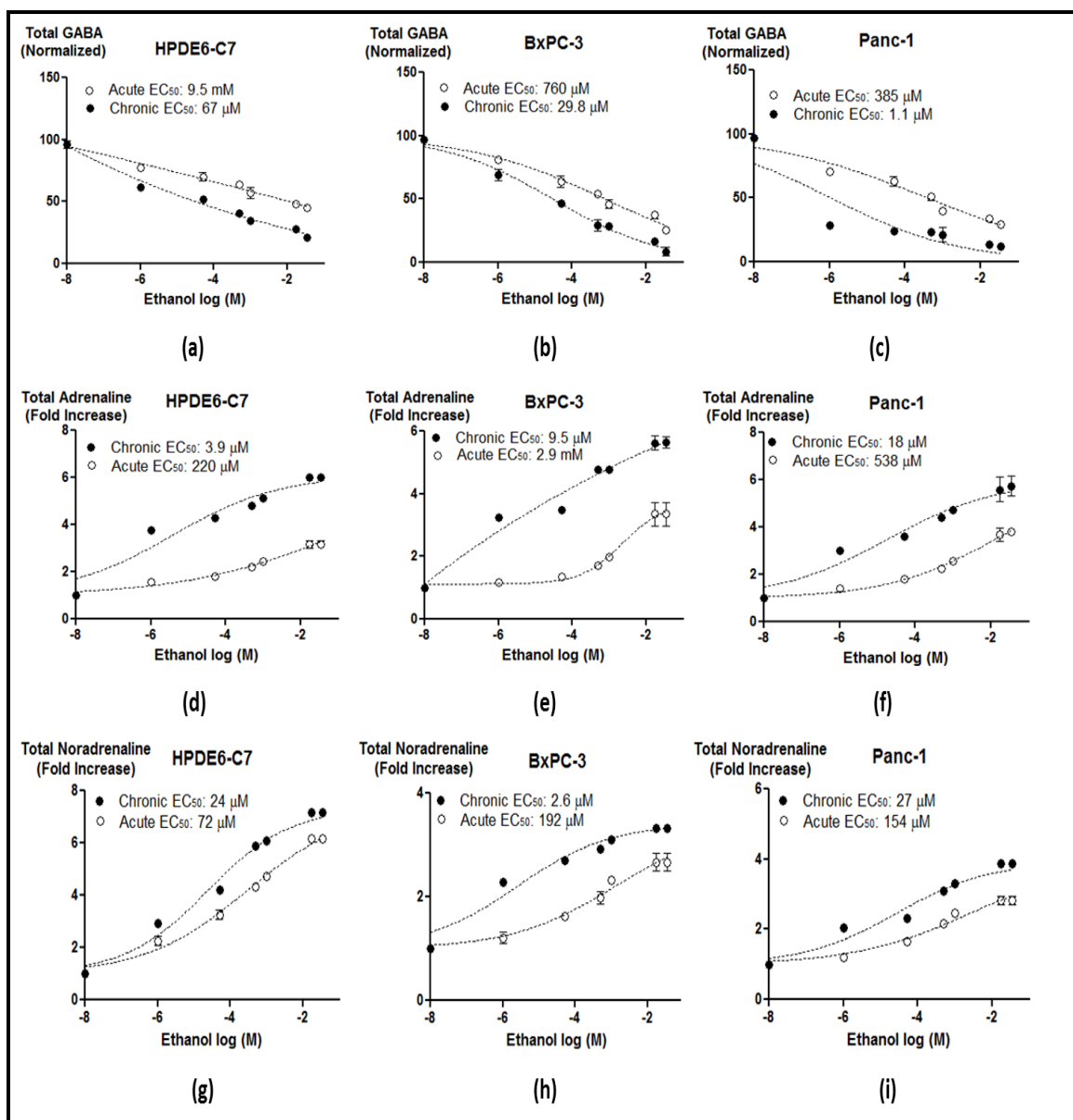


Figure 4. Total (secreted and intracellular) GABA (a-c), adrenaline (d-f) and noradrenaline (g-i) levels in HPDE6-C7, BxPC-3 and Panc-1 cells treated with single doses of ethanol from 1 μM to 35 mM for 30 minutes or pretreated with 17 mM ethanol for 7 days and then exposed to identical concentrations of ethanol. Data points are mean and + / - SD from five samples per treatment group.

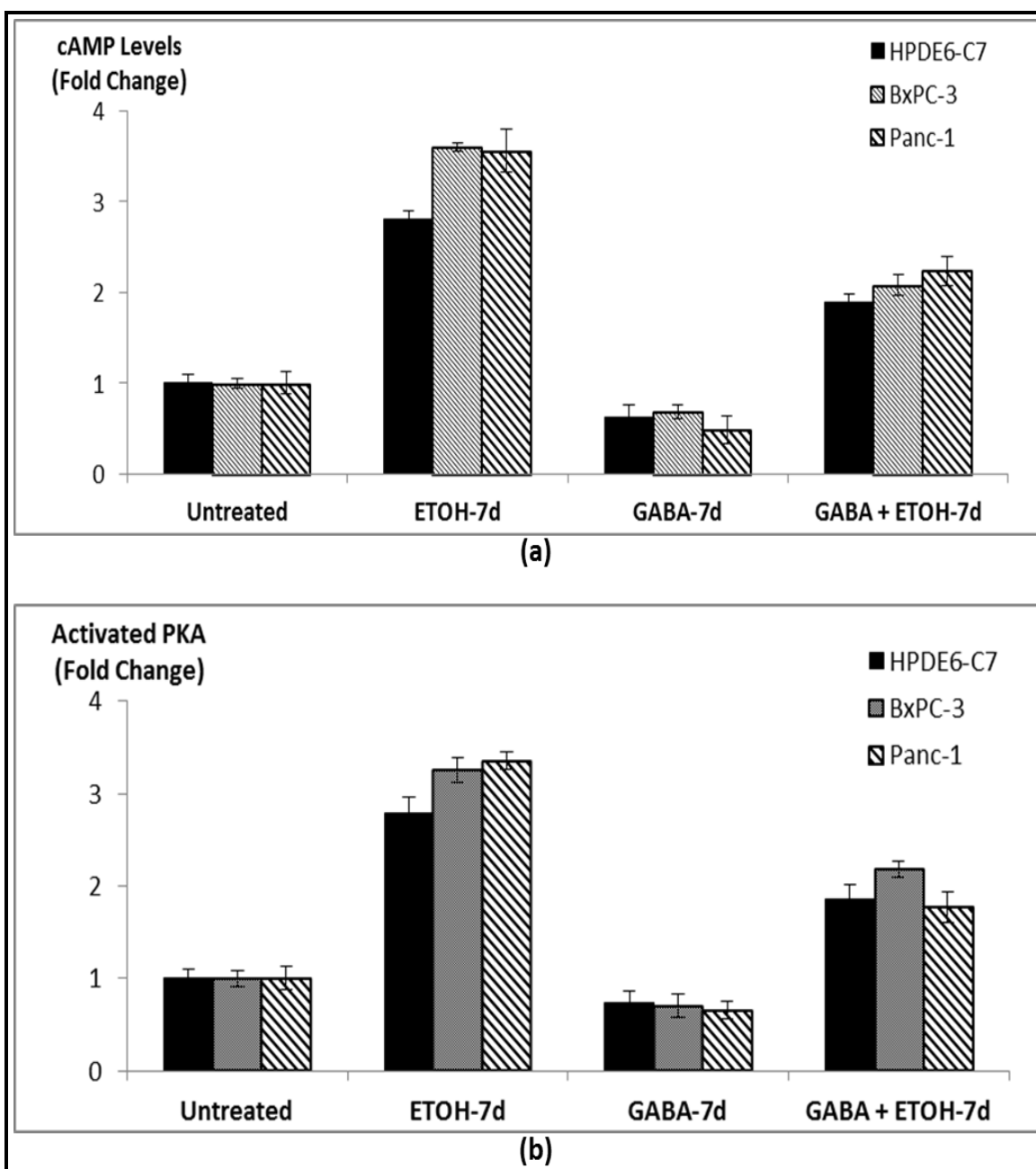


Figure 5. ELISA assays showing cAMP levels (a) and PKA activity (b) in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 17 mM ethanol, 30 μ M GABA or the combination of the two for 7 days. Data points are mean and $\pm$ SD from five samples per treatment group.

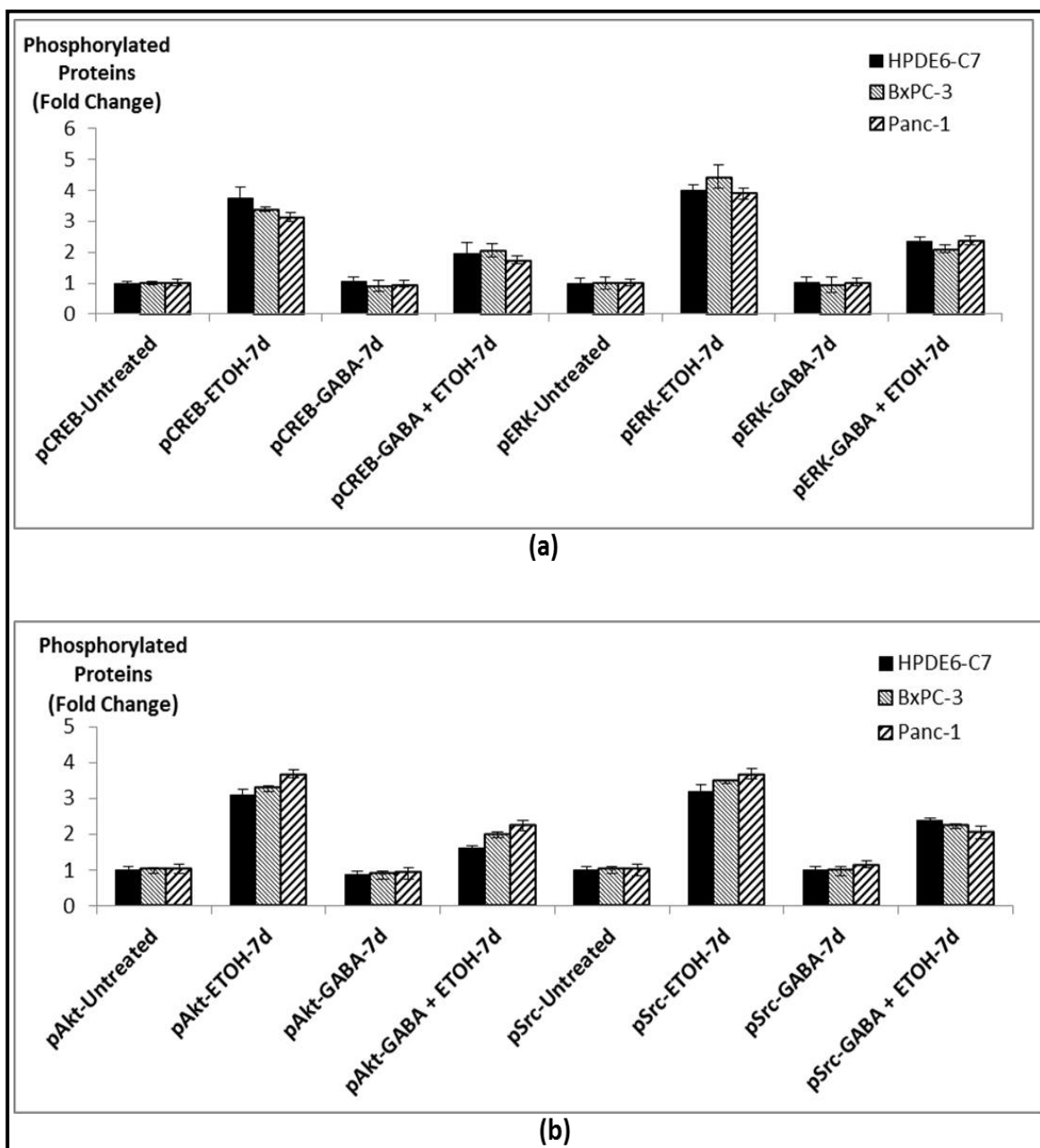


Figure 6. ELISA assays showing phosphorylation of CREB and ERK (a) and Src and Akt (b) in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 17 mM ethanol, 30 μ M GABA or the combination of the two for 7 days. Data points are mean and $\pm$ SD from five samples per treatment group.

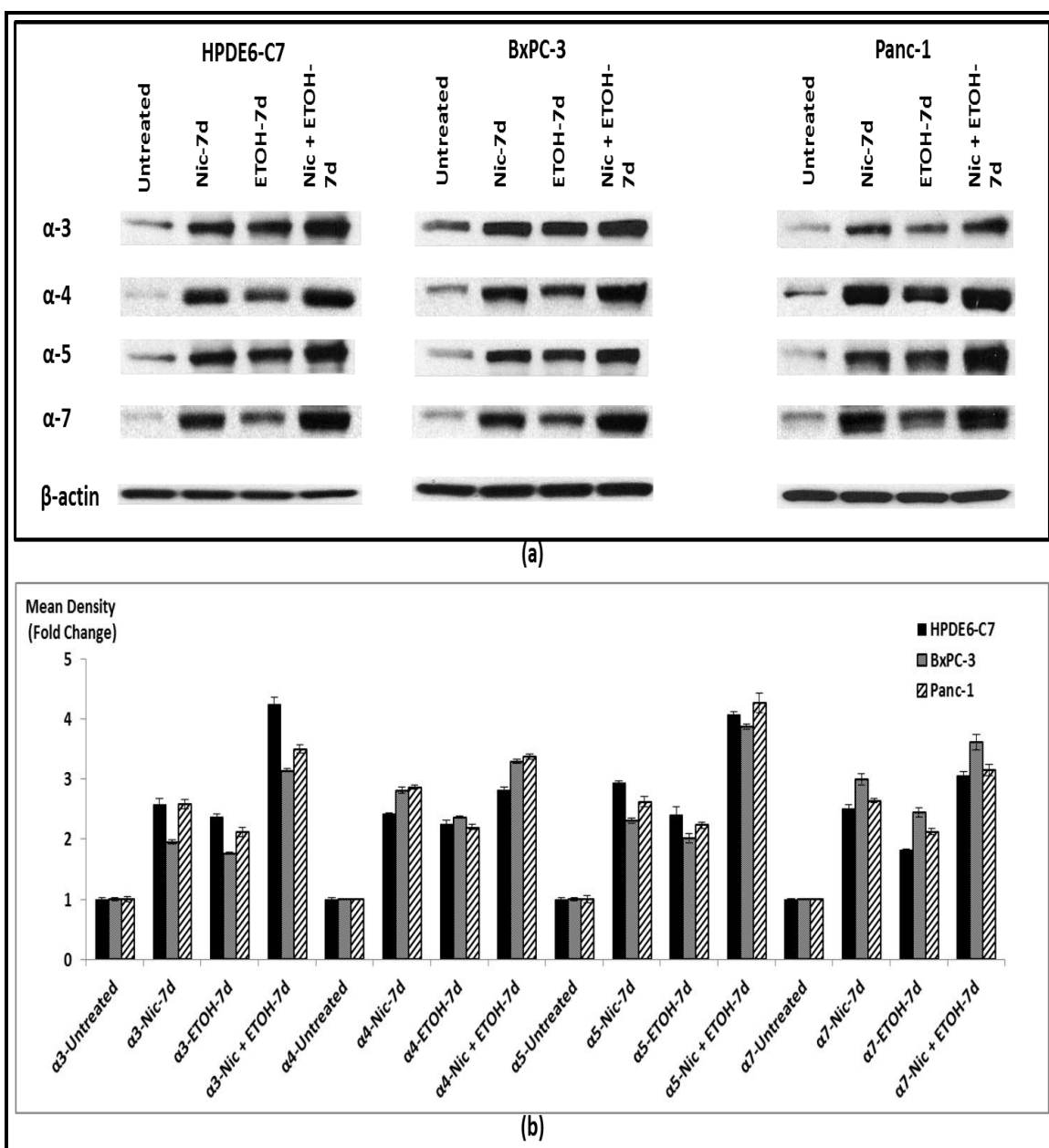


Figure 7. Western blots assessing protein expression of $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 7$ -nAChRs in cells treated with 1 μ M nicotine, 17 mM ethanol or both for 7 days. β -actin was used as a control to ensure equal loading of proteins (a). The columns in figure (b) represent means and $\pm$ SD of two mean density readings per band from three independent western blots ($n = 6$) expressed as fold changes in the expression of the nAChR subunits.

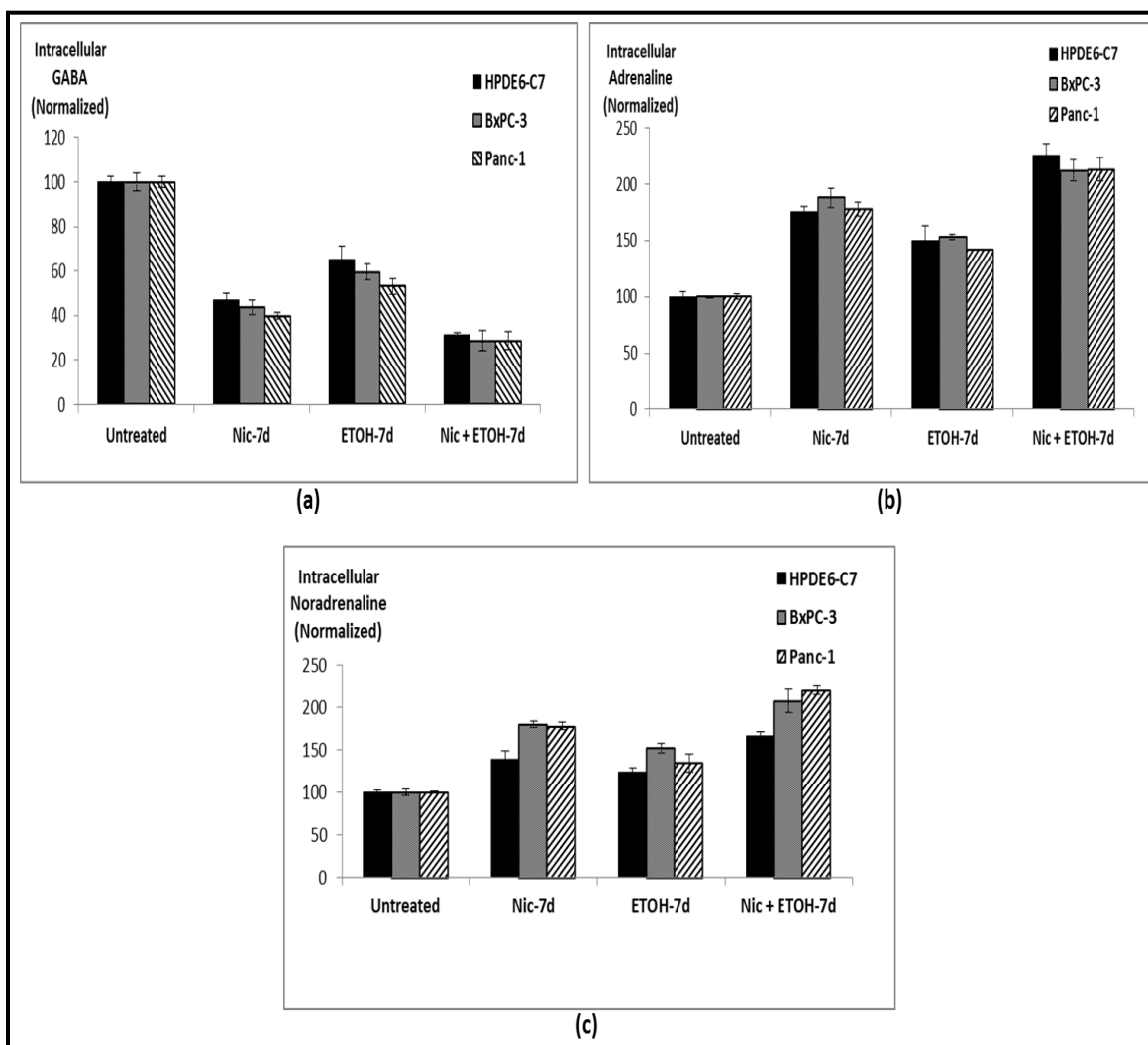


Figure 8. ELISA assays showing intracellular GABA (a), adrenaline (b) and noradrenaline (c) levels in HPDE6-C7, BxPC-3 and Panc-1 cells treated with 1 μ M nicotine, 17 mM ethanol or the combination of both for 7 days. Data points are mean and + / - SD from five samples per treatment group.

CHAPTER VI:

Conclusion

Conclusion

Pancreatic cancer is the fourth leading cause of cancer deaths in western countries with a five year survival rate of less than 5 % and a mortality rate near 100 % within the first two years of diagnosis [1-4]. Epidemiological studies have shown that mortality from pancreatic cancer has been increasing in developed countries between 1950s and 80. Moreover, while mortality rate has been declining in men since 1970s, it has been on the rise for women [5-8]. Several risk factors have been linked to pancreatic cancer development including smoking, diabetes, chronic pancreatitis, chronic alcohol consumption and family history [5,9-11]. Reports show that age, gender and race also play a role in determining risk factor for this malignancy too. The risk of pancreatic cancer increases with age with reports showing near 90 % of all patients being over the age of 55 [10]. Moreover, the risk of pancreatic cancer is slightly higher in men than women; and African Americans are more likely to have the disease than whites [10].

As with all other cancers, pancreatic cancer's development and progression involves the activation of oncogenes, the inactivation of tumor suppressor genes and the deregulation of signaling proteins and their downstream pathways. Some of these signaling proteins include EGFR, Akt, and NF κ B [12]. It is the accumulation of several induced and or inherited mutations in these genes that results in the development and progression of pancreatic cancer and not just one mutation. While most of research in the past focused on these signaling proteins and mutations that are involved in PDAC development; little is done on receptors upstream of these signaling proteins and how they may be involved in the regulation of pancreatic cancer.

Recent studies by our laboratory have shown that upstream receptors such as nicotinic acetylcholine receptors (nAChRs) and β -adrenergic receptors play vital role in regulating pancreatic and lung cancer growth in vitro [13-16]. Activation of the ionotropic nAChRs cause cellular changes that lead to neurotransmitter production regulation. This nAChRs activation results in a systemic increase in the levels of adrenaline and noradrenaline and a reduction in GABA synthesis. These neurotransmitters in turn act on G-protein coupled receptors such as β -adrenergic and GABA-B receptors to regulate pancreatic cancer cells growth and migration via cAMP dependent pathways [13,17-21].

In this project, we focused on studying the role of $\alpha 3$, 4, 5 and 7-nAChRs in regulating growth and migration of normal pancreatic duct epithelial cells (HPDE6-C7) and PDAC cells (BxPC-3 and Panc-1). We tested the effects of exposing these cells to ethanol, nicotine and GABA and whether or not these agents modulate the sensitivity of the nAChRs under investigation. Using western blots and assays for cAMP, GABA, adrenaline, noradrenaline, PKA, CREB, ERK, Akt, Src, cell proliferation and migration, our data show that these cells express an autocrine catecholamine loop that is jointly regulated via $\alpha 3$, 5 and 7-nAChRs [15]. Moreover, our results show that these cells express $\alpha 4$ -nAChR which is involved in the regulation of the inhibitory neurotransmitter GABA production and secretion [11,14,15,22-24].

For the first time, our data show that exposing these cells to nicotine or ethanol results in the up-regulation of protein expression of $\alpha 3$, 4, 5 and 7-nAChRs in a concentration dependent manner [15]. However, while this up-regulation in protein

expression is associated with activation and sensitization of the $\alpha 3$, 5 and 7-nAChRs, the $\alpha 4$ -nAChR is desensitized [unpublished data]. Activation of $\alpha 3$, 5 and 7-nAChRs results in cellular changes that lead to an increase in the synthesis and release of adrenaline and noradrenaline. Both catecholamine act as agonist to β -ARs thereby activating these G-protein coupled receptors. Activation of β -ARs then results in the activation of adenylyl cyclase which leads to an increase in intracellular cAMP levels. This rise in cAMP level results in the activation of PKA and other signaling pathways which ultimately increase the phosphorylation of CREB, ERK, Akt and Src. All of these cellular changes associated with adrenaline and noradrenaline ultimately stimulated cell growth and migration and inhibited apoptosis [11,14,15,22-24].

The $\alpha 4$ -nAChR on the other hand is involved in the regulation of GABA synthesis and release. While binding of nicotine or ethanol to this receptor up-regulate its protein expression, this binding results in desensitization of the receptor thereby reducing GABA synthesis and release. This reduction in GABA level is critical to allow pancreatic cancer progression for it acts as an inhibitory neurotransmitter inhibiting the signaling pathway. Our data show that exposure of HPDE6-C7, BxPC-3 and Panc-1 cells to GABA causes a significant reversal of nicotine- and ethanol- induced cell proliferation and migration [24,25]. Binding of GABA to its G-protein coupled receptor GABA-B results in the inhibition of adenylyl cyclase via the inhibitory G protein subunit of GABA-B receptor. Inhibition of adenylyl cyclase therefore results in a reduction in cAMP levels thus preventing cancer growth and migration [11,25].

In summary, our project provides evidence for the first time that pancreatic cancer development and progression is much more than just gene mutations. Receptors such as nAChRs and neurotransmitters associated with these receptors play vital role in regulating pancreatic cancer growth. Moreover, while our data show promising results of GABA as a potential therapeutic option in vitro, much more research is needed to study its effects in vivo. Statistics of pancreatic cancer are still on the rise and we can only hope for research to help bring those stats down.

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VITA

Mohammed Hussein Abdulhadi Al-Wadei was born on March 10th, 1989 in Al-Ahsa city, Saudi Arabia. Four years later, he moved to Sana'a, Yemen where he received his elementary and part of his secondary (middle) education. On August 2011, he moved to the U.S along with his family where his father would obtain his doctoral degree in Comparative and Experimental Medicine. There, he finished his secondary education and obtained a diploma in science from West High School of Knoxville in December 2005 ranking in the top 1 % of his class.

In January 2006, Mohammed enrolled at the University of Tennessee where he majored in Biochemistry Cellular and Molecular Biology. Besides receiving several honors and awards, his name was listed in the Dean's list of top undergraduate students every year during his undergraduate education. In May 2010, he graduated with a bachelor degree in science with an excellent grade point average ranking in the top 5 %.

In the summer of 2010, Mohammed was admitted as a Ph.D. student in Comparative and Experimental Medicine at the University of Tennessee under the supervision of Dr. Hildegard M. Schuller. Besides participating in several local and national symposiums and conferences, he received an award of research excellence in the graduate students' category in summer of 2011 in the Comparative and Experimental Medicine and Public Health Symposium at the University of Tennessee. On August 2012, he received his doctoral degree with a dissertation entitled "Growth Regulation of Pancreatic Cancer Cells and Their Normal Cells of Origin by Nicotinic Acetylcholine Receptors."