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The Neuro-Psychological Axis of Pancreatic Cancer as a Novel Target for Intervention

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Abstract

This review summarizes experimental and clinical data in support of the hypothesis that the known risk factors for Pancreatic Ductal Adenocarcinoma (PDAC), smoking, psychological stress, alcohol consumption, diabetes and pancreatitis-, create hyperactivity of the neurotransmitters norepinephrine and epinephrine in the pancreas. Smoking, psychological stress and alcohol sensitize the nicotinic acetylcholine receptors (nAChRs) that regulate the synthesis and release of PDAC stimulating stress neurotransmitters norepinephrine and epinephrine by nerves of the symapthicus, the adrenal gland and by pancreatic cancer cells and their epithelial precursor cells while simultaneously desensitizing the nAChRs that govern the synthesis and release of the PDAC inhibiting neurotransmitter g-aminobutyric acid (GABA). Experimental data generated in vitro and in animal models emphasize a key role of G_s-coupled β-adrenergic and PGE2 receptors in the activation of multiple signaling pathways by stress neurotransmitters in PDAC. The clinical behavior of PDAC confirms that sympathetic nerves that release stress neurotransmitters are important mediators of PDAC progression. Emerging experimental evidence suggests that lessons learned from the long-term management of cardiovascular disease, which is governed by sympathicus hyperactivity, can be successfully utilized to improve survival rates of PDAC patients and to prevent the development of PDAC in individuals at risk.

Keywords: Pancreatic Ductal Adenocarcinoma (PDAC); Pancreatic cancer; Nervous system

Introduction

Cancer of the exocrine pancreas is the fourth leading cause of cancer deaths in developed countries, with a mortality >95% within one year of diagnosis [1]. This cancer is classified as pancreatic ductal adenocarcinoma by histopathology and is therefore referred to as "PDAC" in this review. Smoking, chronic pancreatitis and diabetes are established risk factors for PDAC [2]. More recent findings additionally suggest chronic psychological stress as a risk factor for PDAC [3,4] and PDAC patients demonstrate higher levels of psychological stress than individuals with other cancers at the time of diagnosis [5]. Chronic abuse of alcohol has also been suggested to increase the risk for PDAC but epidemiological assessments of this association have yielded less conclusive results [6,7]. The mechanisms how these diverse factors increase the risk for PDAC or may negatively impact intervention strategies are poorly understood.

The regulation of cardiovascular function by the autonomic nervous system and interference with this regulatory network by smoking, alcohol abuse and psychological stress have long been recognized as contributing factors to cardiovascular disease [8]. However, information on a potential regulatory role of a similar neuro-psychological axis for PDAC has only recently emerged [9,10].

The autonomic nervous system with its two branches, the sympathicus and parasympathicus (Figure 1), regulates functions of the mammalian organism that are not under voluntary control. Acetylcholine is the neurotransmitter released by the parasympathicus and initiates organ and cellular responses by binding to acetylcholine receptors. These receptors are comprised of two families distinguished by their different abilities to bind nicotine or muscarine [11]. Nicotine has selective high affinity as an agonist for all nicotinic acetylcholine receptors (nAChRs) whereas muscarine is a selective agonist for muscarinic acetylcholine receptors (mAChRs). All nAChRs are ligand-gated ion channels that are enclosed by alpha subunits (α1-α10) alone ("homomeric" α7nAChR) or together with one or more non alpha subunits (β1-β4). The nAChRs expressed at the neuro-muscular junction (α1nAChR) additionally express gamma and/or delta subunits [12]. The five known mAChRs (M_1-M_5) form G-protein complexes with G_i or G_q in the cell membrane [13] (Figure 1). The catecholamine neurotransmitters norepinephrine and epinephrine are synthesized by neurons and nerves of the sympathicus in response to binding of acetylcholine to nAChRs. They are also synthesized and released by the adrenal medulla in response to cholinergic stimulation by psychological stress [14], a phenomenon that lead to their classification as "stress neurotransmitters". The stress neurotransmitters initiate cell and organ responses by binding as agonists to adrenergic receptors (Figure 1). These receptors are comprised of two families, the alpha (α-ARs) and beta-adrenergic receptors (β-ARs) [11]. Norepinephrine and epinephrine have similar affinities to the β1-AR whereas norepinephrine has little effects on the β2-AR for which epinephrine is a highly potent agonist. Norepinephrine has higher affinity than epinephrine to the α1-AR) [15,16] whereas both catecholamines have similar affinities to the α2-AR [15]. The α-1AR is coupled to the G-protein G_q that activates phospholipase C (PLC), inositol three phosphate (IP_3) and Diacylglycerol (DAG), thereby increasing intracellular Ca^{2+} [17] (Figures 1 and 2). The α2-AR is coupled to the inhibitory G-protein Gi that inhibits the formation of intracellular cAMP by inactivating adenylyl cyclase [17] (Figure 2). All three known β-ARs (β1-β3) are coupled to the stimulatory G-protein G_s while the β2-AR is additionally coupled to G_i [17,18] (Figure 1). The stimulatory G-protein G_s activate adenylyl cyclase [17,18] (Figure 2), the single

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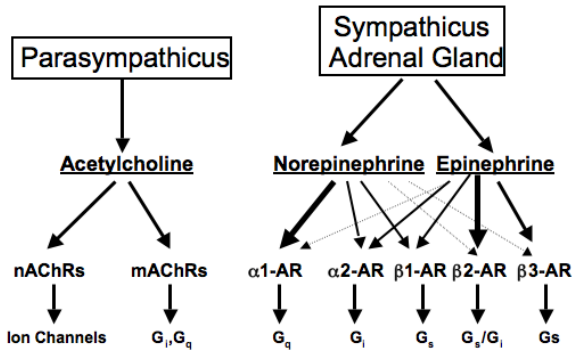

Figure 1: The autonomic nervous system with its two branches, the parasympathicus and the sympathicus, regulates cell and organ functions that are not under voluntary control. Parasympathetic nerves release the neurotransmitter acetylcholine that is an agonist for the ion channel family of nicotinic acetylcholine receptors (nAChRs) and for muscarinic receptors (mAChRs) that are coupled to the inhibitory G-protein Gi or to G_q. Sympathetic nerves and the adrenal medulla release the stress neurotransmitters norepinephrine and epinephrine that are agonists for the G-protein coupled receptor families of a-and b-adrenergic receptors (ARs). Norepinephrine binds preferentially to the G_q-coupled α1-AR while having little activity at the β2-AR. Epinephrine binds preferentially o β2-ARs thus activating both, stimulatory Gs-and inhibitory G_i-proteins. Both stress neurotransmitters bind with similar affinities to the G_i-coupled α2-AR and to the Gs-coupled β1-AR.

rate-limiting step required for the formation of intracellular cyclic adenosine monophosphate (cAMP). In turn, cAMP activates protein kinase A (PKA), which activates a number of different downstream effectors in a cell type-specific manner.

PDAC cells express multiple nAChRs [19], mAChRs [20], α1-, α2- [21], β1- and β2-ARs [22-24]. These cells are therefore highly susceptible to intracellular signals initiated by these neurotransmitter receptors in response to autonomic nervous system-mediated release of their respective agonists and systemic increases in norepinephrine and epinephrine in response to psychological stress (Figure 1). In addition, numerous epithelial cells and cancers with phenotypic features of such epithelia have the ability to synthesize and release acetylcholine [25] as well as norepinephrine and epinephrine [19,26-28] and express the required neurotransmitter receptors, providing them with the ability for self regulation in an autocrine fashion. PDAC cells thus synthesize and release acetylcholine [29] that in turn stimulates the synthesis and release of norepinephrine and epinephrine [19]. The resulting increase in intracellular cAMP downstream of b-ARs, activates PKA, stimulating the Arachidonic Acid (AA) cascade via the release of AA [22] while simultaneously activating the Epidermal Growth Factor Receptor (EGFR) pathway via transactivation of the EGFR [30] and by cAMP-dependent release of its two agonists, EGF [31] and amphiregulin [32] (Figure 2). Activated PKA additionally activates the transcription factor Cyclic Adenosine Response Element Binding Protein (CREB) directly via phosphorylation [19,33] (Figure 2) while reported PKA-dependent activations of Extracellular Signal Regulated Kinases (ERK) AKT and Src [19] were likely triggered via the EGFR pathway (Figure 2). In accord with these in vitro findings, norepinephrine, activated PKA, p-CREB [34], the EGFR [35] and the AA-metabolizing enzyme cyclooxygenase 2 (COX-2) [36] are frequently over- expressed in human PDAC tissues. Angiogenesis is also increased via cAMP-mediated increase in vascular endothelial growth factor downstream of beta-adrenergic signaling in PDAC cells (Figure 2) [37]. In addition to β-ARs, the cAMP/PKA cascade is activated by the two G_s-coupled receptors (EP_2, EP_4) [32] for prostaglandin E_2 (PGE_2; Figure 2) that is formed from AA by COX-2.

Selective pharmacological blockage of β2-ARs synergized with the leading PDAC therapeutic gemictabine to induce apoptosis via inhibition of transcription factor nuclear factor κB (NF-κB) activation and down regulation of Bcl-2 protein in PDAC cell lines BXPC-3 and Mia PaCa-2 *in vitro* while additionally inhibiting cell proliferation and invasion via reduction in CREB, NF-kB and activator protein 1 (AP-1) [38]. A selective β2-AR antagonist also effectively induced apoptosis associated reduction in of caspase3 and caspase 9 expressions in PDAC cell line PC-2 [39]. Another laboratory reported significant stimulation of PDAC proliferation associated with induction of p-ERK in the human PDAC cell line Panc-1 *in vitro* and in mouse xenografts by the selective β-AR agonist isoproterenol and these responses were blocked by the broad-spectrum b-AR antagonist propranolol [24].

PDAC cells and pancreatic duct epithelial cells additionally synthesize and release the amino acid neurotransmitter g-aminobutyric acid (GABA) [40] and they express the G_i-coupled GABA-B receptors, GABA-B-R1 and GABA-B-R2 [34]. Similar to its role as main inhibitory neurotransmitter in the nervous system, GABA inhibits

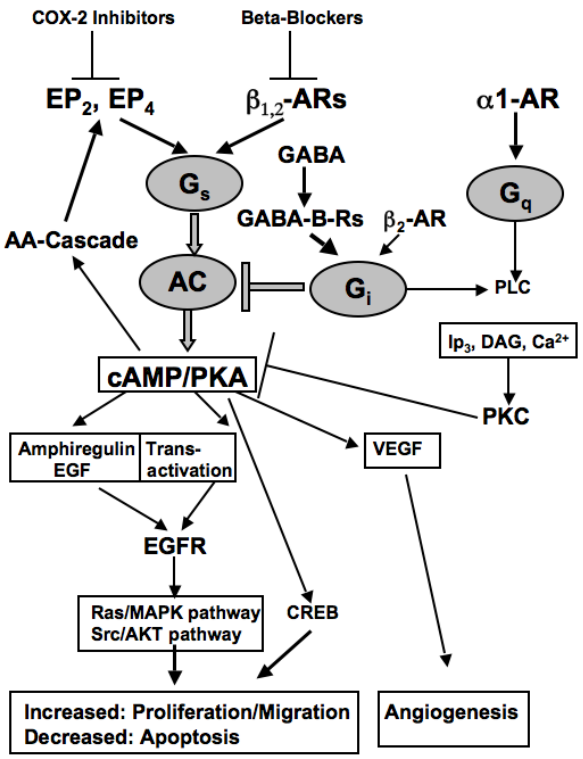

Figure 2: Simplified cartoon of G_s-dependent regulatory cascades in pancreatic cancer cells and their normal epithelial precursor cells. Activated Adenylyl Cyclase (AC) increases intracellular cAMP and activated PKA, which activate the EGFR pathway via transactivation and release of EGF and amphiregulin while at the same time increasing the production of Vascular Endothelial Growth Factor (VEGF) and arachidonic acid (AA). COX-2 inhibitors reduce Gs-signaling by blocking the G_s-coupled receptors (EP2 and EP4) for prostaglandin E2 while beta-blockers inhibit G_s-signaling coupled to beta-adrenergic receptors (β-ARs). GABA inhibits signaling downstream of all G_s-coupled receptors by binding to the G_i-coupled GABA-B receptors (GABA-B-Rs) that inhibit the activation of AC. The β2-AR which is coupled to both, G_s and G_i, inhibits excessive AC-signaling via G_i activation. Receptors coupled to G_q, such as the α_1-AR, can inhibit cAMP-dependent signaling by PKC-induced inhibition of PKA activation.

the β-adrenergic cancer-stimulating signaling cascades in PDAC cells by binding to GABA-B-Rs, causing G_i-mediated inhibition of cAMP formation (Figure 2) [34,40]. *In vitro* and *in vivo* experiments have shown that GABA has powerful anti-cancer effects on PDAC by inhibiting cell proliferation, migration and angiogenesis via this mechanism [10,34,37]. Immunohistochemical investigations of human PDAC tissue microarrays have shown that GABA is frequently suppressed whereas norepinephrine is overexpressed [34], suggesting that the absence of cAMP-inhibition via this neurotransmitter in PDAC may significantly contribute to the development and progression of this cancer.

The current review summarizes experimental evidence in support of the hypothesis that all known risk factors for PDAC modulate important components of the neuro-psychological regulatory axis of this cancer illustrated in Figure 3 and that PDAC intervention strategies need to address this issue in order to become more successful.

Effects of Tobacco Constituents on Pancreatic Cancer

Nicotine is generally thought to be responsible for the addictive properties of cigarette smoke and other tobacco products. Nicotine addiction is mediated by nicotine-induced changes in the expression and function of brain nAChRs. Chronic exposure to nicotine increases

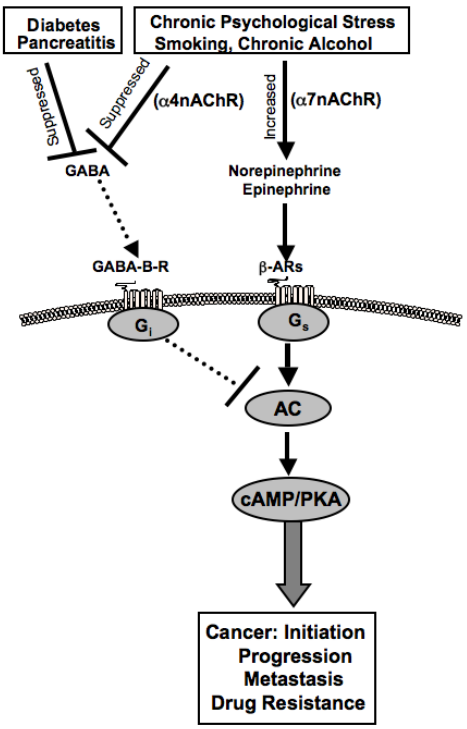

Figure 3: Working model illustrating how all known risk factors for pancreatic cancer increase PDAC-stimulating regulatory cascades downstream of activated Adenylyl Cyclase (AC). Chronic psychological stress, smoking and chronic alcohol ingestion sensitize the α7 nicotinic acetylcholine receptor (α7nAChR), thereby increasing the synthesis and release of norepinephrine and epinephrine by sympathetic nerves, the adrenal gland and by pancreatic cancer cells and their normal precursor cells. At the same time, each of these three risk factors desensitizes the α4β2nAChR, resulting in the suppression of cancer inhibiting GABA. Diabetes suppresses pancreatic GABA levels by reducing the number of functional beta cells in pancreatic islets that are a major source of pancreatic GABA. Pancreatitis suppresses pancreatic GABA by replacing GABA-producing pancreatic epithelial cells by fibro-inflammatory tissues.

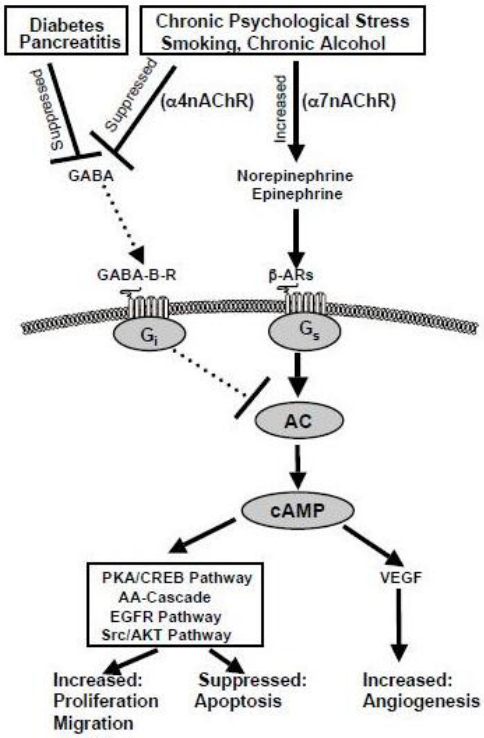

Figure 4: Working model illustrating how all known risk factors for pancreatic cancer increase PDAC-stimulating regulatory cascades downstream of activated Adenylyl Cyclase (AC). Chronic psychological stress, smoking and chronicalcohol ingestion sensitize the α7 nicotinic acetylcholine receptor (α7nAChR), thereby increasing the synthesis and release of norepinephrine and epinephrine by sympathetic nerves, the adrenal gland and by pancreatic cancer cells and their normal precursor cells. At the same time, each of these three risk factors desensitizes the α4β2nAChR, resulting in the suppression of cancer inhibiting GABA. Diabetes suppresses pancreatic GABA levels by reducing the number of 38 functional beta cells in pancreatic islets that are a major source of pancreatic GABA. Pancreatitis suppresses pancreatic GABA by replacing GABA-producing pancreatic epithelial cells by fibro-inflammatory tissues.

the protein expression of all nAChRs via several post-transcriptional and post-translational mechanisms without changes in expression levels of mRNA [41]. In the case of the homomeric (comprised only of alpha subunits) α7nAChR, this protein up regulation is accompanied by sensitization of the receptor whereas the heteromeric α4b2nAChR is desensitized [12]. These different responses to chronic nicotine are generally attributed to the high affinity of nicotine for the α4b2nAChR as opposed to a comparatively lower affinity for the α7nAChR. The brain homomeric α7nAChR regulates the synthesis and release of the excitatory neurotransmitters norepinephrine, epinephrine, serotonin, dopamine and glutamate [42] whereas the heteromeric (comprised of alpha plus non-alpha subunits) α4b2nAChR regulates the synthesis and release of the inhibitory neurotransmitter GABA [42]. With the α7nAChR sensitized and α4β2nAChRs desensitized, excitatory brain neurotransmitters predominate while inhibitory GABA is suppressed, resulting in nicotine addiction and craving.

It has long been recognized that metabolites of the two nicotine-derived carcinogenic nitrosamines N-nitroso-nicotine-ketone (NNK) and N-nitroso-nornicotine (NNN) cause activate point mutations in K-*ras* and inactivating mutations in the tumor suppressor gene *p53*, with NNK being the more potent mutagen [43]. In light of the key role of *ras* in the EGFR pathway (Figure 2) which is often mutated in human

PDACs [44], it was therefore initially believed that these mutations are the sole driving forces of smoking-associated cancers, including PDAC. However, receptor-binding assays have additionally shown that NNK and NNN are nAChR agonists, with NNK having a 1000-fold higher affinity than nicotine to the α7nAChR and NNN having a 4000-fold higher affinity than nicotine to the α4β2nAChR [45,46]. These direct interactions of NNK and NNN with nAChRs therefore significantly contribute to both, smoking-associated carcinogenesis and addiction. NNK is additionally an agonist for β-ARs with significantly higher affinity than norepinephrine for β1-ARs and significantly higher affinity than epinephrine for β2-ARs [47], thus further intensifying its PDAC stimulating effects via the regulatory cascade shown in Figure 3 and 4.

While nAChRs were initially thought to be specific receptors expressed only in the central and peripheral nervous system and at neuro-muscular junctions, it is now understood that they are ubiquitously present in all mammalian cells where they regulate a host of different functions in a cell type-specific manner [48]. *In vitro* studies have shown that binding of acetylcholine, nicotine or NNK to nAChRs with subunits α3, α5, and α7 jointly stimulated cell proliferation and migration of PDAC cells and immortalized pancreatic duct epithelial cells by activating the synthesis and release of norepinephrine and epinephrine [19]. The two stress neurotransmitters then triggered the activation of a complex signaling cascade downstream of G_s-coupled β-ARs in a cAMP-dependent manner [19] (Figure 2). Under physiological conditions, the signaling responses to stress neurotransmitters in the pancreas are counterbalanced by α4β2nAChR -mediated synthesis and release of GABA that in turn binds to the G_i-coupled GABA-B-Rs, thus blocking the activation of adenylyl cyclase (Figure 2). However, even single-dose exposures to nicotine or NNK suppressed GABA production by desensitizing the α4β2nAChR [40]. Chronic (7 days) exposure of the cells to acetylcholine, nicotine or NNK significantly increased the protein expression of all nAChRs in PDAC and pancreatic duct epithelial cells [40]. Compared with cells exposed only to a single dose of these nAChR agonists, production of stress neurotransmitters was significantly increased and the cells additionally responded to lower concentrations of agonist, indicative of a sensitized α7nAChR. By contrast, GABA production was significantly suppressed below the levels observed with single doses of agonist [40]. Phosphorylated signaling proteins ERK, Src, AKT and CREB downstream of β-ARs as well as vascular endothelial growth factor and COX-2, all of which are frequently over expressed in human PDAC tissues, were significantly induced by the observed changes in nAChR expression and function [37,40]. It hence appears that the observed *in vitro* responses of PDAC cells to chronic nicotine or NNK were the equivalent of molecular events in the brain responsible for nicotine addiction. While such nAChR modulations in the brain affect cognition and mood, they significantly increase proliferation, migration and angiogenesis in PDAC. Addition of GABA to the culture media completely abrogated all nAChR agonist-induced modulations in the protein expression and function of nAChRs α3, α4, α5 and α7, reducing cancer-stimulating signaling cascades below base levels observed in control cells [40]. Studies in mouse PDAC xenograft models fully supported these *in vitro* findings by showing strong promotion of PDAC growth associated with increased expression of the same signaling proteins induced by nicotine or NNK *in vitro* when the mice were given chronic nicotine (200 mg/ml for 4 weeks) in the drinking water. Treatment of the mice by GABA injections completely blocked all of these responses to nicotine [49].

In support of these experimental findings, it has been shown that smoking suppresses the mammalian GABA system as a whole [50] while pancreatitis [51] and diabetes [52] both reduce GABA production locally in the pancreas. These three risk factors for PDAC thus share adverse effects on PDAC inhibiting GABA signaling, a fact that may significantly contribute to their positive etiological association with PDAC. In addition, smoking increases the systemic levels of stress neurotransmitters via nAChR-mediated release from the adrenal gland and nerves of the sympathicus [8], which helps explain why smoking is the strongest known risk factor for PDAC.

NNK caused PDAC development in hamsters with ethanol-induced pancreatitis [53] and the tumor tissues showed increased protein expression of the α7nACHR, activated PKA, p-CREB, p-ERK, COX-2 and VEGF whereas the general β-AR antagonist (beta-blocker) propranolol prevented the development of PDAC in this animal model [54]. These experimental *in vivo* findings further emphasize the key role of signaling downstream of β-ARs in the development and progression of PDAC. Studies with this hamster model of PDAC additionally showed that the non-steroidal anti-inflammatory agent ibuprofen, that blocks the formation of COX-2-dependent metabolites from arachidonic acid, partially prevented the development of PDAC [55]. As illustrated in Figure 3 and 4, β-ARs are upstream regulators of the AA-cascade but additionally regulate the EGFR pathway, the PKA/CREB pathway and angiogenesis in PDAC, providing an explanation why the beta-blocker propranolol more effectively prevented PDAC than ibuprofen.

Recent investigations in a transgenic mouse model of PDAC with constitutively activated K-*ras* have shown that chronic treatment of the mice with low dose aspirin significantly prevented the progression of intraepithelial lesions to overt PDAC, a response associated with reduction in COX-2 expression [56]. This was a landmark finding because it documents that the mutated K-*ras*, although a downstream effector in the AA cascade and EGFR cascade of PDAC, can nevertheless be controlled by the upstream inhibition of COX-2-dependent AA metabolites. Beta-blockers or G_i-mediated inhibition of adenylyl cyclase would likely have even greater PDAC preventive potency in this mouse model and in human PDACs that express mutated K-*ras*.

Animal experiments typically use nicotine in the drinking water at a dose of 200 mg/ml (432 mM/L) to approximate the blood nicotine concentrations observed in heavy smokers. This dose of nicotine significantly increased the growth of PDAC xenografts in mice when administered to the animals over a 4-week period [49]. By contrast, the descending doses of nicotine contained in Nicotine Replacement (NRT) products yield significantly lower systemic nicotine concentrations. These products were therefore initially considered as "safe" alternatives to smoking and are in fact utilized by many nicotine addicts as long-term tools to satisfy their habit. In addition, PDAC therapy in smokers is often accompanied by treatment with NRT products to eventually eliminate the causative factor smoking. However, the sensitization of the α7nAChR with concomitant desensitization of the α4β2nAChR observed in PDAC cells exposed for 7 days to 1 mM nicotine *in vitro* [40] suggest that nicotine concentrations significantly below the blood levels observed in heavy smokers may have adverse effects on the clinical outcomes of PDAC therapy. In support of this hypothesis, a recent study with mouse xenografts from PDAC cell lines showed that low dose nicotine (1 mM/L in the drinking water) significantly increased the resistance of PDAC to gemcitabine when administered to the animals for 4 weeks [57]. This effect of nicotine was associated with a reduction in gemcitabine-induced caspase 3 and apoptosis while additionally inhibiting the gemcitabine-induced reduction in the levels of multiple phosphorylated signaling proteins, including ERK, Src and AKT [57]. These findings suggest that even moderate smoking or NRT may negatively impact clinical outcomes of gemcitabine therapy in

PDAC patients. In light of the fact that gemcitabine is the leading PDAC therapeutic and is often accompanied by NRT, the observed induction of gemcitabine resistance by low dose nicotine may significantly contribute to the documented poor prognosis of this cancer.

Numerous publications have reported PDAC stimulating effects of nicotine *in vitro* and in animal models. It has thus been shown that nicotine induced PDAC cell migration *in vitro* via α7nAChR-mediated upregulation of the MUC4 mucin associated with activation of JAK2/STAT3, ERK and Src [58]. In accord with these findings, exposure to cigarette smoke significantly increased the size of orthotopically implanted PDAC xenografts in mice accompanied by increased expression of MUC4, α7nAChR and p-STAT3 in the tumor tissue [58]. Another study reported induction of Src-dependent differentiation-1 inhibitor transcription factor by nicotine in PDAC cells *in vitro* and inhibition of orthotopically implanted PDAC xenografts by gene knockdown of this transcription factor [59]. Another laboratory reported α7nAChR-mediated induction of the secreted phosphoprotein osteopontin that is associated with cell migration, by nicotine in PDAC cells *in vitro* and showed expression of osteopontin in the majority of investigated PDAC tissue samples from smokers [60,61]. Moreover, it was shown that NNK caused dose-and time-dependent increases in the proliferation and migration of PDAC cells BXPC-3 and MIAPACA-2 accompanied by increases in FAK and ERK activation and inhibited by the beta-blocker propranolol or the cruciferous vegetable flavone Apigenin [62]. In addition, immortalized pancreatic duct epithelial cells exposed *in vitro* to cigarette smoke or NNK responded with AKT-dependent reduction in apoptosis while autophagy was stimulated [63]. The modulation of signaling proteins and transcription factors in response to nicotine in the cited publications were frequently interpreted as effects immediately downstream of nAChRs. However, the discovery that PDAC cells and pancreatic duct epithelial cells produce norepinephrine and epinephrine in response to nicotine [19] indicates that most of these responses were indirectly caused by binding of nicotine-induced stress neurotransmitters to b-ARs (Figure 4).

Effects of Psychological Stress on PDAC

High levels of psychological stress are associated with increased cancer mortality and PDAC patients showed higher levels of stress than patients with cancers at other organ sites [3-5]. As pointed out in the introduction, the stress neurotransmitters norepinephrine and epinephrine are agonists for alpha and beta-adrenergic receptors. The important regulatory function of b-ARs in PDAC cells (Figure 3 and 4) therefore suggests that psychological stress may promote the development and progression of this cancer. In support of this hypothesis, the growth of subcutaneous mouse xenografts from PDAC cell lines Panc-1 (with activating point mutations of K-*ras*) and BXPC-3 (without ras mutations) was significantly increased by exposure of the mice to social stress for 4 weeks [10]. The stress-induced xenograft progression was accompanied by significant increases in the protein expression of nAChRs α3, α4, α5, α6 and α7, p-ERK, p-AKT, p-Src and p-CREB in the xenografts as well as increases in plasma and tumor tissues of cortisol, norepipephrine, epinephrine, and VEGF and of cAMP in the cellular fraction of blood [10]. By contrast, the protein expressions of the two glutamate decarboxylase isozymes GAD 65 and GAD 67, which catalyze the formation of GABA from glutamate, were significantly reduced [10]. In accord with this finding, the levels of GABA in plasma and in xenograft tissues were suppressed. Treatment of the mice with GABA abrogated stress-induced tumor growth while blocking stress-induced increases in cAMP, VEGF, and in the protein induction of all investigated signaling proteins. In addition, GABA significantly reduced tumor growth and the levels of all of these effectors in mice not exposed to stress [10]. A follow-up study with the same mouse models revealed that the observed PDAC stimulating effects of social stress on xenograft growth, levels of neurotransmitters, cAMP and VEGF and on expression of signaling proteins was highly reproducible [37]. In addition, these experiments showed that the levels of the AA metabolite PGE2 were significantly increased by social stress in plasma and xenograft tissues, a response accompanied by significant increases in COX-2 in the xenografts [37]. Treatment of the mice with the selective COX-2 inhibitor celecoxib significantly decreased all of these responses to social stress while combination treatment with celecoxib and GABA further enhanced the inhibition of all investigated PDAC stimulating effects of social stress [37]. *In vitro* experiments with Panc-1 and BXPC-3 cells corroborated these findings by showing that exposure of the cells for 7 days to epinephrine at the concentration (15 nM) measured in xenografts of social stress-exposed mice increased cell proliferation more than 2-fold in both cell lines while additionally increasing cell migration more than 4-fold [37]. Celecoxib (1 μM) completely abrogated both effects of epinephrine while additionally significantly reducing base level proliferation and migration of cells not exposed to epinephrine [37]. Collectively, these data strongly support the hypothesis that psychological stress promotes the development and progression of PDAC via cAMP-dependent signaling downstream of β-ARs and PGE2 receptors (Figure 2 and 3) and is in accord with *in vitro* findings on the regulatory role of β-ARs in PDAC cells [22-24,30].

Several laboratories have investigated stimulating effects of the stress neurotransmitter norepinephrine on PDAC cell proliferation and migration in vitro. It has thus been shown that norpepinephrine significantly induced the proliferation of Panc-1 cells in a concentration-dependent manner while increasing S-phase population and decreasing G1 and G2-phase populations accompanied by significant increases in cell migration and invasion in scratch wound healing and in transwell Matrigel assays [64]. These responses were accompanied by induction of p38/MAPK. All reported effects of norepinephrine were completely blocked by the beta-blocker propranolol [64]. These findings are in accord with a report from another laboratory that norepinephrine-induced invasion (tested by Matrigel assay) accompanied by increases in MMP-2, MMP-9 and VEGF and inhibited by propranolol in PDAC cell lines BXPC-3 and MiaPaCa-2 [65]. One of the putative precursor cells of PDAC, pancreatic duct epithelial cell, also responded to norepinephrine *in vitro* by cell proliferation associated with increases in interleukin-6 (IL-6) and VEGF [66]. Interestingly, the effects of norepinephrine on cell proliferation and IL-6 were significantly inhibited by the dietary agent sulforaphane from cruciferous vegetables whereas the increase in VEGF remained unchanged. By contrast, a recent publication has reported that a high concentration (10 μM) of norepinephrine inhibited migration in PDAC cell lines Panc-1, MiaPaCa2 and CFPAC1 in a three-dimensional collagen-based migration assay that monitors the locomotion of cells without the influence of cell proliferation [67]. Cell proliferation was also inhibited by this concentration of norepinephrine and both responses were significantly reduced by the selective β1-AR antagonist, atenolol or by propranolol [67]. The observed inhibitory actions of norepinephrine were accompanied by excessive increases in intracellular cAMP (up to 4,000-fold) and activation of phospolipase C gamma and protein kinase C alpha [67]. Recent findings from another laboratory provide a potential explanation for these controversial findings by showing concentration-depended stimulation of proliferation and migration by low concentrations of norepinephrine in PDAC cell linesBXPC-3 and MiaPaCa-2 whereas concentrations of 1 μM and above inhibited both responses [68]. According to the National Library of Medicine normal

levels of norepinephrine in tests of human blood samples range from 0 to 600 pg/ml equivalent to a maximum concentration of 2.9 nM [69]. The plasma and xenograft levels of norepinephrine in control mice were in that range and increased to a 10 nM concentration when the animals were exposed for 4 weeks to social stress that significantly promoted the growth of PDAC xenografts [10,37]. The blood levels of norepinephrine are increased about 50-fold in patients with severe heart failure [70]. By contrast, the 10 μM concentration of norepinephrine that inhibited PDAC cell proliferation and migration *in vitro* [67] represents a 3,500-fold increase of this neurotransmitter above maximum normal blood levels. This high norepinephrine concentration and associated excessive cAMP levels may have switched signaling downstream of the β2-AR from its stimulatory G-protein G_s to its inhibitory G-protein G_i, a phenomenon that has been described in the myocard in response to high circulating epinephrine levels [71]. The classic function of the G_i-protein coupled to the β2-AR is the preservation of cAMP homeostasis by inhibiting the excessive activation of adenylyl cyclase downstream of simultaneously expressed β1- and β2-ARs [72,73]. Moreover, G_i coupled to the β2-AR activates several cAMP-independent signaling pathways, including PLC/PKC [73] which can reduce cAMP-dependent signaling by inhibiting its effector PKA [74] (Figure 2). Alternatively, the presence of α1 and α2-ARs coupled to G_q and G_i, respectively, in PDAC cells (Figure 1) for which norepinephrine has a high affinity, may have contributed to the observed signaling through PLC and PKC (Figure 2). Maintenance of the PDAC cells in antibiotics may have further contributed to aberrant signaling as antibiotics modulate the proteome of cells in culture [75].

Clinical evidence for an important role of norepinephrine and epinephrine in the progression of PDAC comes from observations that nerves of the sympathicus mediate enhanced PDAC cell chemo attraction and motility via release of these neurotransmitters, resulting in neural invasion and the associated neuropathic pain syndrome [76]. The underlining mechanisms of this clinical phenomenon have recently been investigated by co-culture of mouse dorsal root ganglia with PDAC cells (maintained without antibiotics) *in vitro* and in a mouse model of perineural invasion [77]. The investigators showed that PDAC cells MiaPaCa-2 and BXPC-3 invaded the dorsal root ganglia in vitro, an effect enhanced in a concentration-dependent manner by exogenous addition of norepinephrine, with a steep increase in response from 0.1-1 μM/L, reaching near saturation at 10 μM/L. These responses were associated with activation of Stat3, AKT, CREB, MMP2 and MMP9 and were inhibited by propranolol. Mice with perineural invasion of the sciatic nerve by surgically implanted PDAC cells had higher norepinephrine levels than mice with sham operations and the perineuroal invasions were significantly inhibited by treatment of the mice with a Stat-3 blocker [77].

Effects of Alcohol on PDAC

The etiological association of chronic alcohol intake with the risk for PDAC is less conclusive than the epidemiological data on smoking as a risk factor [6,7], possibly because many smokers also drink. However, investigations on the association of alcohol intake with PDAC in never smokers showed a significant increase in PDAC risk in never smokers. This effect was limited to the chronic consumption of liquor but was not seen with beer or wine [78].

It is well established that pancreatitis from any etiology, including alcoholism, is an independent risk factor for PDAC [2]. Apart from inflammatory mediators of the AA-cascade involved in this disease, the reduction in systemic GABA levels reported in patients with pancreatitis [51] may significantly contribute to the development of PDAC (Figure 4).

Only few investigations have addressed potential direct effects of ethanol on PDAC cells or their putative cells of origin. It has thus been shown that immortalized pancreatic duct epithelial cells *in vitro* respond to a single dose of ethanol at a concentration of 17mM (equivalent to the legal blood alcohol limit in the U.S.) with an increase in intracellular cAMP and increased cell proliferation associated with a significant induction of p-ERK1/2 [33]. This ethanol treatment also significantly enhanced these responses to the nicotine-derived tobacco carcinogen NNK [33]. A recent study using the same ethanol concentration over a 7- day exposure period in PDAC cells Panc-1 and BXPC-3 and in immortalized pancreatic duct epithelial cells reported significant induction in the protein expression of nAChRs with subunits α3, α5, and α7. This response was accompanied by significant increases in the synthesis and release of norepinephrine and epinephrine by the cells, increased levels of intracellular cAMP and activated PKA associated with increases in phosphorylated ERK, AKT, Src and CREB and enhanced proliferation and migration [79]. Gene knockdown of nAChR subunits α3, α5, or α7 each significantly reduced these effects of chronic ethanol whereas simultaneous exposure for 7 days to GABA (30 μM) and ethanol inhibited all of these responses to ethanol. These findings are in accord with the observation that low concentrations (100 μM – 10 mM) of ethanol potentate the response of multiple neuronal nAChRs to their physiological agonist acetylcholine [80], a phenomenon thought to contribute to alcohol dependence [81]. Using a higher concentration (217 mM) of ethanol, another laboratory showed increased cell invasion associated with significant up regulation of the transcription factor Snail in immortalized pancreatic duct epithelial cells *in vitro* whereas identical exposure of PDAC cell line Panc-1 only yielded minimal induction of Snail and failed to increase cell migration [82].

The mechanistic aspects of alcohol in the development of PDAC suggested by the *in vitro* investigations summarized above is supported by findings that exposure of pregnant hamsters to ethanol (10%) in the drinking water and NNK (50 mg/Kg as a single subcutaneous injection on the last day of pregnancy) caused the development of PDAC in 60% of the offspring and that treatment of the offspring with the beta-blocker propranolol effectively prevented PDAC development [53,54]. NNK is a weak pancreatic carcinogen when administered as a single agent to rats [83] but does not by itself induce the development of this cancer in hamsters, suggesting significant PDAC promoting effects of alcohol ingestion in smokers.

Conclusions and Future Directions

The data compiled in this review support the hypothesis that adaptive changes of the autonomic nervous system induced by smoking, chronic psychological stress and habitual ingestion of liquor-strength alcohol play a key role in the development, progression and resistance to therapy of PDAC and occur mostly via protein induction associated with sensitization (α7nAChR) or desensitization (α4nAChR) of nAChRs that regulate the synthesis and release of stress neurotransmitters and GABA, resulting in hyperactivity of G_s-mediated cAMP signaling. These PDAC promoting effects are further intensified by identical changes occurring in nAChRs expressed in PDAC cells and their normal epithelial precursor cells. Activating point mutations in k-*ras* and inactivating mutations in *p53* caused by tobacco carcinogens further enhance the cancer-causing potency of these changes because these mutated genes are downstream components of multiple signaling pathways activated by cAMP/PKA. The resulting systemic and cellular

predominance of PDAC-stimulating stress neurotransmitters with simultaneous suppression of inhibitory GABA destroys the homeostasis of cellular signaling pathways required to maintain a healthy pancreas (Figure 4). Diabetes and pancreatitis also negatively impact the balance between PDAC stimulating and inhibiting neurotransmitters by suppressing the production of pancreatic GABA, thus facilitating the development and progression of PDAC.

The sympathetic branch of the autonomic nervous system and the associated signaling of stress neurotransmitters via β-ARs are increasingly being recognized as an important therapeutic target for several epithelial cancers [9,77,84-86]. There is however conflicting clinical evidence on the effects of beta-blockers on cancer survival. Beneficial effects of beta-blocker therapy has been reported in patients with non-small-cell lung cancer [87], breast cancer [88,89], prostate cancer [90] and melanoma [91]. On the other hand, the use of beta-blockers was associated with a significantly reduced chance of death in patients with ovarian cancer in one study [92] whereas another report showed no significant effects [93]. A recent investigation by Dr. Shah's group that compared the effects of beta-blockers (primarily selective β1-AR antagonists) with that of other anti-hypertensive agents on clinical outcomes in several different types of cancer reported no significant benefits of beta-blockers on survival of patients with lung cancer and breast cancer while individuals with cancer of the prostate or pancreas even showed significantly poorer survivals than patients treated with non beta-blocker antihypertensive agents [94]. Much of this ongoing controversy is caused by the complex long-term effects of beta-blockers that differ greatly with duration and dose of treatment and additionally vary among agents that selectively block only the β1-AR versus a general beta-blocker or antagonists with partial agonist function. However, when critically reviewing these data, it is important to remember that sympathetic nerve hyperactivity with the associated increased plasma levels of norepinephrine is a major driving force of cardiovascular disease [95]. Accordingly, many antihypertensive agents other than beta-blockers inhibit sympathetic nerve activity. While beta-blockers inhibit sympathetic nerve activity by inhibiting the binding of its released neurotransmitters norepinephrine and epinephrine to β-ARs, inhibitors of the angiotensin/renin system as well as imidazoline receptor agonists inhibit norepinephrine release from sympathetic nerves [95]. Moreover, Ca^{2+}-channel blockers, that are also frequently used for the therapy of hypertension, have strong desensitizing effects on the α7 nAChRs expressed in epithelial cancer cells because they inhibit the opening of voltage-gated Ca^{2+} channels that mediate downstream responses to this receptor after membrane depolarization [9,96]. This class of agents therefore has the ability to decrease α7nAChR-mediated synthesis and release of norepinephrine and epinephrine in PDAC cells and their normal epithelial precursor cells. It is thus not surprising, that beta-blockers did not have significantly different effects on a variety of cancers than other antihypertensive agents [94]. Moreover, the chronic use of selective b1-AR antagonists can lead to compensatory hyperactivity of the β2-AR [97,98], a phenomenon that was predicted to have potentially deleterious effects on PDAC that is predominantly regulated by β2-ARs [99]. Significant reductions in survival from prostate cancer and PDAC (both of which are primarily regulated by β2-ARs) in a study with 75% of patients using β1-AR antagonists as compared to other hypertensive agents (none of which selectively blocks β1-ARs) unfortunately support this hypothesis [94].

All of the cited clinical investigations on the usefulness of beta-blockers in cancer patients are hampered by the fact that they were conducted in individuals receiving incidental antihypertensive therapy and therefore suffered from pre-existing sympathetic nerve hyperactivity. Clinical trials need to be conducted to test each antihypertensive agent with known sympathicus-inhibiting potency as well as Ca^{2+}-channel blockers that desensitize the α7nAChR in non-hypertensive PDAC patients receiving standard cancer therapy to assess the potential usefulness of this class of agents on survival and responsiveness to therapy. Such studies should be accompanied by regular measurements of systemic stress neurotransmitters, GABA and cAMP levels and should also include testing for single nucleotide polymorphisms of the β2-AR that have recently been identified as a biomarker for PDAC progression and survival [100].

The reported strong anti-cancer effects of GABA in PDAC cells *in vitro* and in mouse xenografts in the presence and absence of nicotine or psychological stress are highly encouraging. Currently used non-cytotoxic cancer therapeutics such as EGFR tyrosine kinase inhibitors, Src inhibitors or COX-2 inhibitors as well as potentially to be used beta-blockers, aim to block defined components of cancer-stimulating signaling pathways. By contrast, treatment with GABA raises the plasma and tumor levels of this physiological inhibitor of cAMP-dependent signaling so that cAMP homeostasis is restored despite of increased levels of stress neurotransmitters, PGE2 or any other agents that activate adenylyl cyclase. This approach is reminiscent of the strategy to compensate for high blood levels of bad cholesterol by dietary increase in good cholesterol and is particularly appealing because GABA has safely been used for many years as a nutritional supplement. However, there appears to be a subset of PDAC cases that over express the p-subunit of the GABA-A receptor, there by converting GABA from an inhibitory into an excitatory neurotransmitter with PDAC promoting effects [101]. GABA should therefore only be used in individuals with normal expression levels of GABA-A receptor subunits.

Findings that celecoxib significantly inhibited stress-induced promotion of PDAC xenografts with and without activating mutations in k-*ras* [37] while low dose aspirin prevented the progression of preneoplastic lesions to PDAC in a transgenic mouse model of mutated k-*ras* [56] strongly suggest inhibition of COX-2 as a promising strategy for the improvement of clinical outcomes in PDAC undergoing standard cancer therapy. In light of the documented cardiotoxicity of selective COX-2 inhibitors [102], low dose aspirin would be the agent of choice and should be tested alone and in combination with GABA. Low dose aspirin has been safely used for the long-term prevention of cardiovascular disease. Similarly, nutritional supplementation with GABA has served for many years to reduce anxiety and relieve muscle spasms. Both of these agents are therefore also suitable for the prevention of PDAC in individuals at risk.

In summary, sympathicus hyperactivity that drives cardiovascular disease also contributes significantly to PDAC development, progression and resistance to therapy and is enhanced by the additional predominance of stress neurotransmitters produced by PDAC cells and their epithelial precursor cells. While neither disease can be cured, emerging experimental evidence suggests that lessons learned from the long-term management of cardiovascular disease can be successfully utilized to improve survival rates of PDAC patients and to prevent the development of PDAC in individuals at risk.

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