NOVEL ASPECTS OF NEUROTRANSMITTERS

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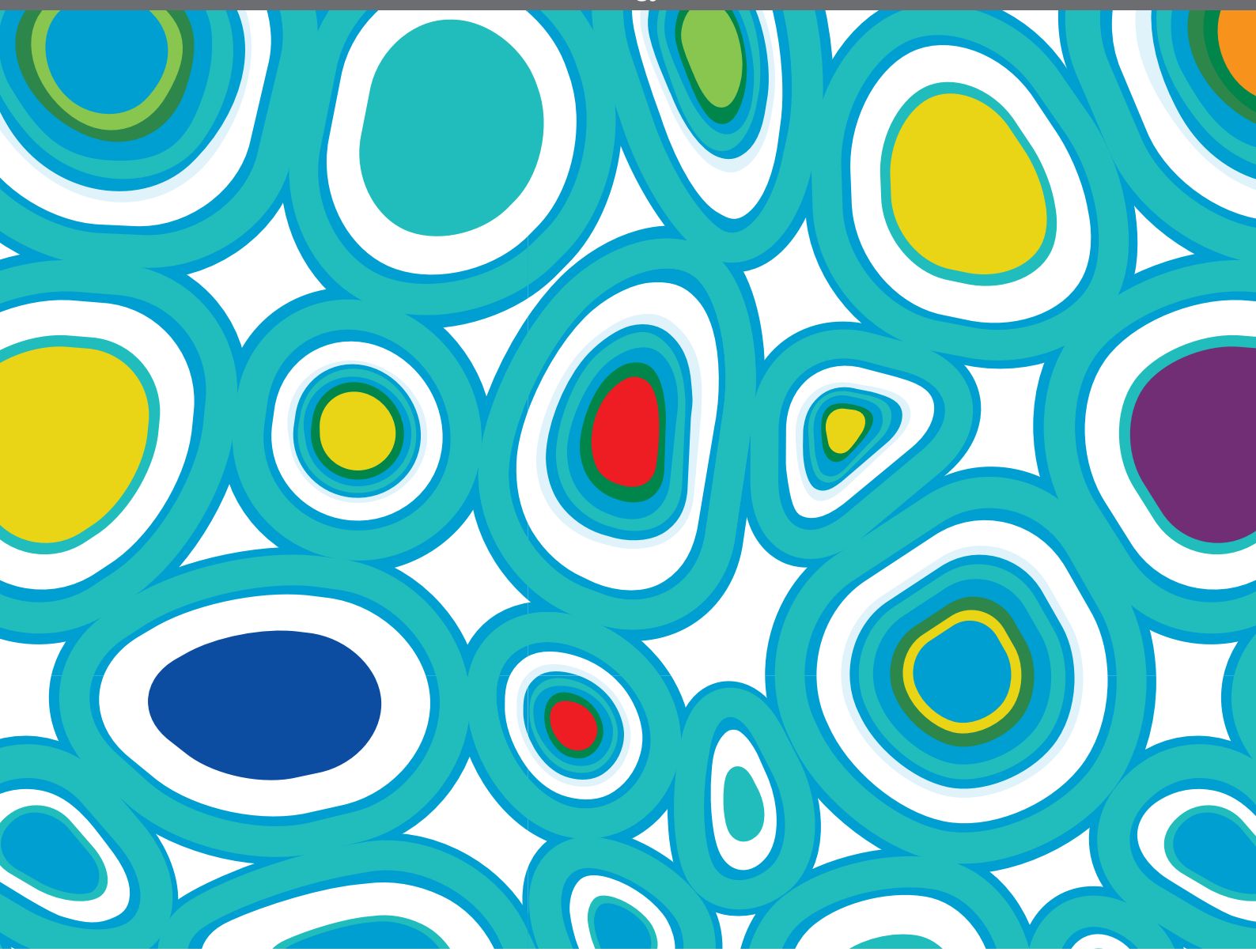

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NOVEL ASPECTS OF NEUROTRANSMITTERS

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EDITORIAL
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Editorial: Novel Aspects of Neurotransmitters

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Keywords: neurotransmitters, cancer, evolution, novel aspects, immunoregulation, asthma, anxiety and depression

Editorial on the Research Topic

Novel Aspects of Neurotransmitters

Neurotransmitters are not the privilege of neural system and they have multiple functions in the peripheral organs. The aim of this special issue is to improve our understanding of non-canonical functions of neurotransmitters, as well as their novel aspects in neural system.

Cancer neuroscience is an emerging field and it reveals a promising future in preclinical and translational research. In this collection, there are several papers focus on the novel roles of neurotransmitters on cancer research.

Perineural invasion is a common phenomenon which indicates a poor prognosis in multiple cancers. Chen et al. made an interesting revision of perineural invasion and stress hormone in gynecological cancers (Chen et al.). More significantly, almost 100% pancreatic cancer has perineural invasion and neurotransmitters play important roles in the innate and adaptive immune responses in the pancreatic cancer microenvironment (Liang et al.).

We believe that cancer is a complicated, flexible, systemic disease encompassing multiple dysregulated processes within the neuro-endocrine-immune system, which might open a wide range of therapeutic options (Jiang et al.).

Besides in cancer, 5-Hydroxytryptamine, Glutamate, and ATP have broad biological functions on multiple types of non-neural cells (Franco et al.). Francelin et al. show in a systematized way about the participation of neurotransmitters in the maturation of T lymphocytes in the thymus (Francelin et al.). Neurotransmitters and neuropeptides are also involved in the pathological processes in asthma (Pavón-Romero et al.). In Anxiety and Depression, brain transmitters could be modulated by intestinal microbiota (Huang and Wu). The locus coeruleus (LC) tyrosine-hydroxylase (TH) neurons and the TH:LC-paraventricular thalamus circuit may be involved in regulating emergence from anesthesia (Ao et al., 2021).

Moroz leads us to explore, in an elegant dissertation, the evolutionary aspects of neurons origin. He presents the most relevant hypotheses on the aspects that have given rise to neuronal types, their organization, way of communication, and the mechanisms underlying these characteristics (Moroz). Moroz and Romanova present an interesting dissertation on the advantages of synapses in evolution in different evolutionary orders, discussing the pros and cons observed in developing this neural communication process, emphasizing the participation of lipid components and the involvement of organelles such as the endoplasmic reticulum and mitochondria (Moroz and Romanova).

Neurotransmitters are ancient molecules, e.g. acetylcholine exists in microorganisms (Whittaker, 1963). Most of neurotransmitters appeared earlier than the neural system. Besides in the neural system, neurotransmitters have a lot of basic functions to be discovered in the future, such as the serotonylation, which is a newly recognized post-translational modification (Muma and Mi, 2015; Bader, 2019) where serotonin is covalently incorporated into proteins via transamidation.

Therefore, we believe that the first step to take advantage of the full potential of the new aspects of neurotransmitters is to make them known and show their clinical, therapeutic, and research potential. With this research topic, we contribute to achieving this goal, and we are convinced

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that the readers of the papers that comprise it will acquire a comprehensive view of the full potential of this knowledge.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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REVIEW
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Systemic Regulation of Cancer Development by Neuro-Endocrine-Immune Signaling Network at Multiple Levels

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The overarching view of current tumor therapies simplifies cancer to a cell-biology problem in which neoplasms are caused solely by malignant cells and the exploration of carcinogenesis and tumor progression largely focuses on somatic mutations and other genetic abnormalities of cancer cells. The limited therapeutic response indicates that cancer is driven not only by endogenous oncogenic factors and reciprocal interactions within the tumor microenvironment, but also by complex systemic processes. Homeostasis is the fundamental premise of health, and is maintained by systemic regulation of neuro-endocrine-immune axis. Cancer is also a systemic disease that manifested by dysfunction of the nervous, endocrine, and immune systems. Multiple axes of regulation exist in cancer, including central-, organ-, and microenvironment-level manipulation. At each specific regulatory level, the tridirectional communication among the nervous, endocrine, and immune factors transmit flexible signaling to induce proliferation, invasion, reprogrammed metabolism, therapeutic resistance, and other malignant phenotypes of cancer cells, resulting in the extremely poor prognosis of this lethal disease. Understanding this coordinated signaling network will enable the development of new approaches for cancer treatment via behavioral and pharmacological interventions.

Keywords: systematic regulation, neurotransmitter, inter-organ communication, immune evasion, chronic stress, perineural invasion

INTRODUCTION

In the past several decades, therapeutic options for cancer have been developed from surgery, chemotherapy and radiotherapy to targeted therapy and immunotherapy. However, the prognosis of cancers remains tremendously unsatisfying and the death rate is still high (Siegel et al., 2017). Herein, the vast investments in cancer research and unsatisfactory status of treatment have highlighted a question: what is the nature of cancer? The traditional view of solid tumors is the autonomous abnormal growth of heterogeneous populations of neoplastic cells in local tissues.

The tumor microenvironment (TME) has emerged as an equally important determinant of malignant tumor behaviors. However, there is still no space to explain how psychosocial and neuroendocrine factors might affect tumor initiation and development.

Host homeostasis is maintained by perfect interactions between the nervous, endocrine and immune systems involving different glands and organs. The neuro-endocrine-immune (NEI) network, first proposed by Besedovsky and Sorkin (1977), acts as an integrated unit to optimize health and develop defense against various complex pathological processes. Reciprocal influences have been eloquently evidenced among the NEI network; neurotransmitters, neuropeptides, and hormones affect immune functions, and immune cell products can affect neuro-endocrine mechanisms. Alteration of any system components or loss of integrated connections appears to be a marker of disease risk, especially for cancers. Psychological factors such as stress, anxiety and depression, endocrine factors and immunological mediators are closely associated with tumor initiation and development. Therefore, the NEI network may be a potentially ideal index reflecting cancer diseases.

In this review, we propose that cancer is a complicated, flexible and systemic disease encompassing multiple dysregulated process within the NEI system, including dysregulation at the central-, organ-, and TME levels. Then, emerging evidence regarding the role of the NEI system at each regulatory level is summarized. Last, given the role of the NEI signaling network in dictating the malignant phenotype, opportunities for developing innovative therapeutic approaches are discussed.

CANCER IS A SYSTEMIC DISEASE

Nervous System and Cancer

The question of whether physical dysfunction and mental disorders are associated with the prevalence, incidence and outcome of cancers has attracted much attention for centuries. External psychosocial processes activate cortical and limbic structures of the central nervous system, which subsequently ultimately activate the hypothalamic–pituitary–adrenal (HPA) axis and sympathetic nervous system (SNS) to trigger defeat/withdrawal responses and fight-or-flight stress responses, respectively (Antoni et al., 2006) (**Figure 1**). In the HPA axis, corticotrophin-releasing hormone is secreted from the paraventricular nucleus of the hypothalamus, stimulating the anterior pituitary to produce adrenocorticotrophic hormone, which in turn induces the downstream release of the glucocorticoid hormone cortisol from the adrenal cortex. Similarly, activation of the SNS induces the secretion of norepinephrine (NE) and epinephrine (E) from the adrenal medulla. There is strong evidence for links between alterations in neuroendocrine dynamics and tumor pathogenesis (Lutgendorf et al., 2010). Neuroendocrine factors, particularly catecholamines and cortisol, have been shown to have modulatory effects on immune processes related to tumor surveillance (Reiche et al., 2004). In preclinical models, chronic stress is sufficient to promote tumor growth and angiogenesis in ovarian carcinoma (Thaker et al., 2006), breast cancer lung colonization (Chen et al., 2018), EGFR inhibitor resistance in non-small cell lung cancer (NSCLC) (Nilsson et al., 2017), and therapy-induced anticancer immunosurveillance (Yang H. et al., 2019). In contrast, positive factors such as physical activity and optimism are known to reduce the risks of many human cancer types and predict longer survival (Allison et al., 2003; Moore et al., 2016). Thus, mental health, a key component of systemic regulation of physical function, is a key element for cancer initiation and progression.

Endocrine and Cancer

Emerging persuasive evidence has revealed that systemic diseases, such as endocrine disorders and metabolic alterations, are involved in the development of non-endocrine tumors. Disrupted endocrine rhythms induced by stress or circadian deregulation are known to favor tumorigenesis (Antoni et al., 2006). Apart from the known stress-related hormones (glucocorticoids, prolactin, oxytocin, and dopamine), abnormalities in thyrotropin-releasing hormone (TRH), thyroid-stimulating hormone (TSH), and growth hormone (GH) have also been found in several cancer types such as colon cancer, gastric cancer, and lung cancer (Kamijo et al., 1987; Mazzoccoli et al., 2010). Moreover, epidemiological data and mechanistic studies have well documented the causal association between obesity and cancers (Renehan et al., 2015; Iyengar et al., 2016). A comparative risk assessment showed that diabetes and high BMI (BMI greater than or equal to 25 kg/m^2) related to approximately 4.5% of all incident cancers. Diabetes and high BMI are associated with 25.8% and 31.9% increases in cancer prevalence respectively as risk factors (Pearson-Stuttard et al., 2018). The global burden from cancers, including liver, breast, endometrial, gallbladder and pancreatic cancer, are attributable to diabetes (Tsilidis et al., 2015). Intriguingly, prevailing concepts have recognized obesity as a spectrum of neuropsychological diseases that, are influenced by stress-related psychosocial factors and addiction-like behavior, rather than just an endocrine disease, suggesting close neuro-endocrine interactions (Jauch-Chara and Oltmanns, 2014; Niccolai et al., 2019).

Immunity and Cancer

The importance of the immune system in cancer development has been greatly appreciated and immunotherapy is one of the most promising strategies for cancer treatment, which has been elegantly reviewed elsewhere (Galluzzi et al., 2018; June et al., 2018). Immune cells receive and respond to signals from neurotransmitters and hormones. Neuroendocrine mediators such as adenosine are profoundly implicated in immunosuppressive mechanisms in cancers (Vijayan et al., 2017). Essentially, neuroendocrine and immune factors fuel one another. Stress exposure causes a purine metabolic disorder in $CD4^+$ T cells and induces anxiety-like behavior (Fan et al., 2019); innate immune cells affect neurotransmitter metabolism, neuronal endocrine function, and neuroplasticity by secreting cytokines (Engler et al., 2017; McKim et al., 2018). Moreover, depression can prime larger cytokine responses to pathogens or stressors; chronic stress, obesity, and sleep disorders

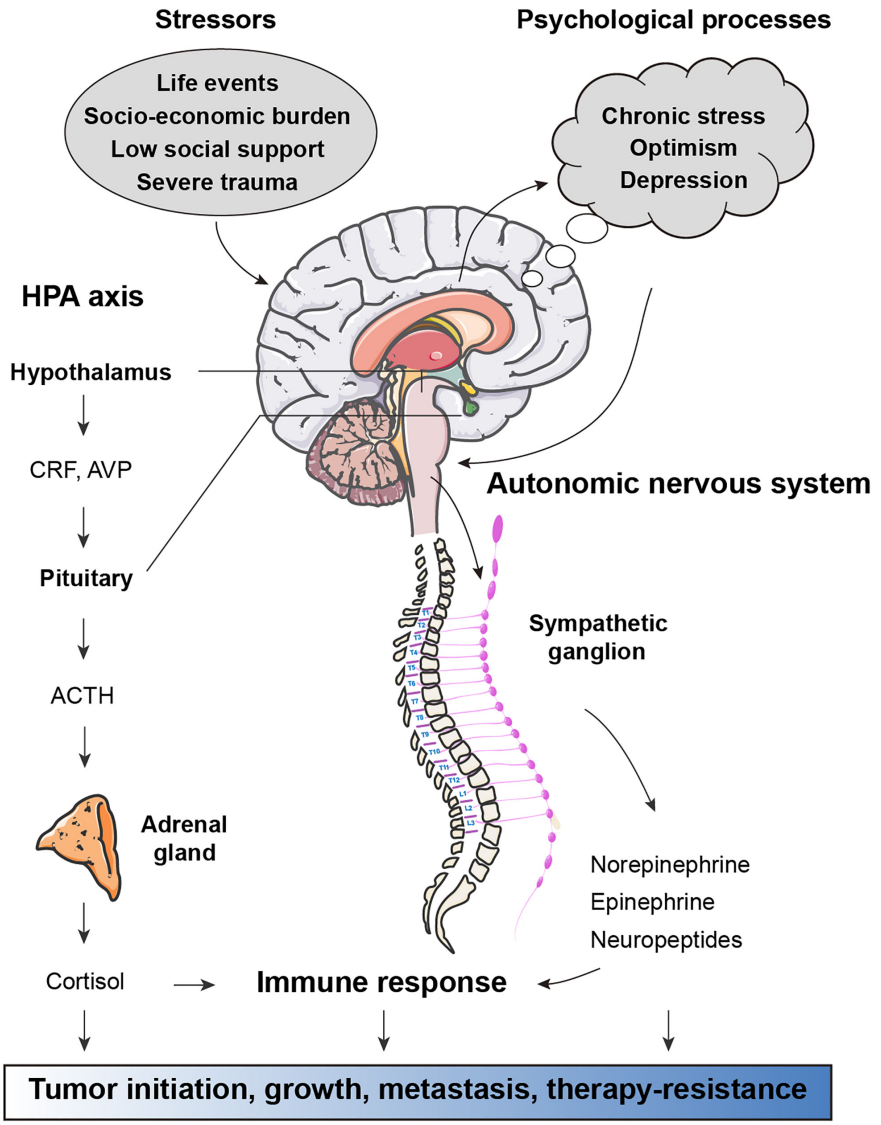

FIGURE 1 | Effects of stress-associated factors on cancer pathogenesis. Stressful events result in activation of cortical and limbic structures of the central nervous system, which in turn activate the hypothalamic–pituitary–adrenal (HPA) axis and autonomic nervous system (ANS). In response to psychological stress, the paraventricular nucleus of the hypothalamus secretes corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), both of which stimulate the anterior pituitary to release adrenocorticotrophic hormone (ACTH). Circulating ACTH activates the adrenal cortex to produce glucocorticoid hormone cortisol. ANS responses to stress are largely mediated by activation of the sympathetic nervous system (SNS) subsequent secretion of epinephrine (E) and norepinephrine (NE). Factors released from HPA and ANS axis have direct effects on cancer pathogenesis. Moreover, glucocorticoids, catecholamines, and other neuroendocrine factors can influence immune response to support tumor initiation, growth, metastasis, and therapy-resistance.

contribute to prolonged and unrestrained inflammatory responses, which further facilitate the development of cancer (Del Rey and Besedovsky, 2017).

Neuro-Endocrine-Immune-Related Genes and Cancer

Molecular evidence supports that the aberrantly expressed genes involved in systemic regulation also contribute to malignancy. To elucidate the function of genes in cancer development and progression, we previously performed a large-scale cDNA transfection screen and found 2,836 genes involved in cell growth and survival (Wan et al., 2004). These genes are categorized into four groups: I, genes related to basic cellular activities for cell survival and growth; II, genes participating in components of the TME; III, genes involved in host-cell systemic regulation and homeostasis; and IV, genes with unknown functions in cell survival. Among them, the third category of genes, which are related to host-cell systemic regulation, attracted our attention. These genes regulate four types of physiological activities: I, response to environmental changes (nutrition and redox); II, immune reaction (for example, cytokine/chemokine

receptors); III, cellular electrophysiology (ion channels, exchange transporters, and small molecule transporters); and IV, nervous and hormonal regulation (neurotransmitter receptors, hormone receptors, insulin receptors, and progesterone receptors).

Collectively, diverse physical or pathological manifestations can result from central nervous system regulation, depending on the interconnected associations among nervous system, hormonal, and immune regulators. Hence, we presume that cancer is a systemic disease characterized by uncontrolled proliferation in local lesions (liver, stomach, pancreas, breast, prostate, etc.). This concept describes a systemic regulation of both "microscopic" and "macroscopic" aspects, including dysregulation of the NEI network at central, organ, and TME levels.

REGULATION AT THE CENTRAL LEVEL

Negative Stress Response and Cancer

The association between psychological and physiological factors of cancer risk and progression has been prevailingly confirmed in the past several decades. Chronic stress, caused by the persistent activation of the HPA system and the SNS, is involved in the development, progression, and mortality of various types of malignant cancer (Russell and Lightman, 2019). A meta-analysis comprehensively evaluating 165 studies referring to multiple kinds of cancer indicated that stress-related psychosocial factors are closely related to higher cancer incidence; meanwhile, poor prognosis and survival of diagnosed patients are showed in 330 studies, and high mortality is noted in 53 studies (Chida et al., 2008). A study of 281,290 individual participants showed that work stress is significantly associated with the risk of colorectal, lung, and esophageal cancers (Yang T. et al., 2019). Moreover, stressful life events, such as threats, death of close family members, and financial difficulties, have been proposed to contribute to the etiology of breast cancer (Lillberg et al., 2003). Studies in preclinical models have provided compelling evidence regarding the effects of chronic stress on tumorigenesis and the underlying molecular mechanisms. For instance, chronic neuropsychological stress promotes Kras-induced pancreatic tumorigenesis, driven by activated β2-adrenergic receptor signaling, nerve growth factor secretion, and upregulation of catecholamines. A successful combination therapy using tropomyosin receptor kinase inhibitor with gemcitabine was described in a pancreatic cancer animal model, but the rationale and mechanistic insight on the successful combination was not provided (Renz et al., 2018a). In ovarian carcinoma, a pioneering study showed that behavioral stress enhances tumor angiogenesis *in vivo* and thereby promotes malignant cell growth, which resulted from aberrant β-adrenergic activation of the cAMP-PKA signaling pathway (Thaker et al., 2006). In addition to tumorigenesis, mental stress has been proven to aggravate drug resistance. The cooperative signal between stress hormones activating β2-adrenergic receptors and mutant EGFR results in inactivation of liver kinase B1 (LKB1), a tumor suppressor, and upregulation of IL-6, which leads to tyrosine kinase inhibitor (TKI) resistance in a T790M-independent manner in the treatment of NSCLC (Nilsson et al., 2017), suggesting that combinations of the well characterized and safely administered β-blockers with EGFR TKIs merit further investigation as a strategy to overcome drug resistance. Chronic stress is known to decrease cellular immunity and immunosurveillance. Recent experimental studies have elucidated that stress-induced glucocorticoid surge and *Tsc22d3* upregulation in dendritic cells subvert chemotherapy or immunotherapy-induced anticancer immunosurveillance; in cancer patients, there is a close correlation among plasma cortisol levels, TSC22D3 expression in circulating leukocytes and negative mood (Yang H. et al., 2019). However, more works are warranted to determine the effects of chronic stress on other immune cells and to constitute actionable therapeutic targets.

Positive Stress Response and Cancer

On the other hand, eustress stimulation and positive factors are efficient to hinder tumor growth. Environmental enrichment (EE) is a component of animal husbandry that aims to enhance sensory, cognitive, motor, and social stimuli, which are considered necessary for the optimal psychological and physiological well-being of animals. Mice living in the enriched housing environment showed less tumor growth and more frequent remission in melanoma and colorectal cancer, caused by elevated serum brain-derived neurotrophic factor (BDNF) and markedly lower leptin concentrations (Cao et al., 2010). Interestingly, EE inhibits mammary tumor growth rate with intact leptin signaling in diet-induced obesity models (Foglesong et al., 2019). In pancreatic cancer, EE was reported to significantly reduce tumor weight in subcutaneous and orthotopic mouse models via down-regulation of the expression of mitochondria-related genes (Li et al., 2015). Mechanistically, the modulation and enhancement of immunosurveillance and immune defense by EE is a robust player in anticancer events. EE exerts its protective effects through elevating the ratio of $CD8^+$ cytotoxic T lymphocytes (CTL); because both propranolol and mifepristone can block the EE-associated modulation of CTLs, suggesting that both SNS and HPA axis are involved (Xiao et al., 2016). In most conditions, natural killer (NK) cells play a predominant role in the tumor inhibition induced by EE exposure (Garofalo et al., 2015; Song et al., 2017; Takai et al., 2019). Consistently, observations from glioma studies revealed that depletion of NK cells reduces the anti-tumor efficacy of EE (Garofalo et al., 2015). Additionally, EE exposure also induces microglia/macrophage (M/Mφ) activation, suggesting the diverse immunomodulatory effects of EE in cancers. Excitingly, EE was recently found to have an additive effect with the immune checkpoint therapy against tumor development (Watanabe et al., 2020).

In humans, a higher degree of optimism is associated with a lower cancer-related mortality. For instance, optimistic individuals have a 16% lower hazard ratio for all cancers compared with pessimistic individuals (Kim et al., 2017). In parallel, leisure-time physical activity is associated with conceivable anti-tumor effects through sympathetic activation, reduced endocrine factors (sex hormones, insulin, and myokines), and improved immune function (Hojman et al., 2018). Therefore, NEI regulation couples exercise to cancer

prevention and treatment. In summary, at the central-regulation level, the interruption of neuro-hormone-immune balance is a vital driver of malignant tumors. It is still an open question whether EE has additional physiological functions apart from its effects on the neuro-hormone-immune networks.

REGULATION AT THE ORGAN LEVEL

Inter-Organ Signaling Communication

Many physiological functions and pathological initiation and progression originate from communication between organs, which occurs via hormones and cytokines, produced by both peripheral organs and the central nervous system (Karsenty and Olson, 2016; Castillo-Armengol et al., 2019). Two excellent examples are inter-organ growth coordination: growth-impaired organs induce the systemic growth inhibition of undamaged organs by producing Dilp8 hormone (Boulan et al., 2019), and endocrine-regulated metabolic homeostasis (Scopelliti et al., 2019). Moreover, the crosstalk between the liver and intestine by FGF19 performs important functions in cholesterol metabolism and energy homeostasis (Castillo-Armengol et al., 2019). Lipid metabolites work as long-range hormones to transmit signals around several organs, including liver, muscle, and adipose tissue, to regulate systemic energy metabolism and immune metabolism (Liu et al., 2014). Intriguingly, the endocrine capability is not restrained to traditional endocrine organs (such as the pancreas, liver, and adrenal gland). Bone has been uncovered to affect the pancreas, testes, and brain by secreting osteocalcin; natriuretic peptides released from the heart stimulate fatty acid oxidation in liver and white adipose tissue (Karsenty and Olson, 2016). Dysregulation of inter-organ interactions can exist as a reason or predictor for certain diseases. For example, hepatic steatosis has a casual role in the progression of insulin resistance in other tissues, such as skeletal muscles; steatotic hepatocytes release a kind of secreted protein, hepatokine, which puts dramatically impairment to liver (by promoting insulin resistance and injuring glucose effectiveness) and distant organs (including skeletal muscles, pancreas, adipose tissue, immune cells, and blood vessels) (Meex and Watt, 2017). Therefore, deciphering the molecular mechanism of these inter-organ communications is helpful for redefining therapeutic strategies to fight metabolic disorders and other related diseases.

Inter-Organ Signaling and Cancer

Inter-organ communication is also profoundly implicated in tumorigenesis (**Figure 2A**). For example, cytokines and extracellular vesicles secreted from adipose tissue can be transported to liver tissues and induce changes in hepatokine transcription and autophagy processes, which ultimately contribute to hepatocellular carcinoma development (**Figure 2B**). Growing evidence suggests that obesity has been assigned with specific roles in neural regulation, endocrine regulation, and immune responses in tumor progression. First, obesity-induced changes prompt imbalances in sympathetic-parasympathetic activity, which might be an advantage for tumorigenesis. Second, obesity-related hyperleptinemia and central leptin resistance are promoters of tumor invasiveness and migration. Finally, obesity leads to immune dysfunction, including but not limited to imbalance of macrophages, decreased activity of NK cells, and altered intestinal immunity (Gan et al., 2018). Cachexia is an energy-wasting syndrome that frequently occurs in cancers. In cancer cachexia, many organs, such as bone, brain, liver and gut, as well as tumor tissue, secrete inflammatory factors or pro-cachectic cytokines to promote skeletal muscle wasting (Argiles et al., 2018); whether this process is direct or indirect warrants further investigation. Endocrine and central nervous system perturbations combined with cachexia-related mediators can also elicit catabolic changes in adipose tissues (Baracos et al., 2018).

Recent findings demonstrated system-wide coordination and communication among tissue clocks (Dyar et al., 2018; Koronowski et al., 2019). Peripheral organs harbor their own autonomous circadian rhythms but are synchronized by the hypothalamic suprachiasmatic nucleus via neuro-endocrine pathways (Mohawk et al., 2012). Epidemiological and clinical data confirm a close connection between the disruption of circadian rhythms and hormone-dependent cancers, such as breast cancer and prostate cancer (Masri and Sassone-Corsi, 2018). Cancer can disturb the systemic circadian clock to induce multi-organ chronic inflammation, metabolic disorders, and cachexia by secreting hormones and cytokines (Baracos et al., 2018). A landmark paper showed that lung adenocarcinoma distally rewires circadian transcription and metabolism in the liver but not other organs through altered pro-inflammatory responses via secretion of IL-6, TNF-α, and lactate (Masri et al., 2016) (**Figure 2C**). However, it remains unclear whether dysregulated liver metabolism further boosts tumor progression in the context. More interestingly, a preclinical study has shed light on the molecular link of circadian disruption with tumorigenesis. Circadian disruption promotes cholestasis, peripheral clock disruption, and sympathetic dysfunction, which lead to activation of the well-known liver tumor promoter, constitutive androstane receptor (CAR), resulting in progression of non-alcoholic fatty liver disease (NAFLD)-induced hepatocarcinogenesis (Kettner et al., 2016). This study connects circadian homeostasis of liver metabolism to tumorigenesis and provides promising candidate strategies for prevention of metabolic syndrome-induced hepatocarcinogenesis by restoration of bile acid homeostasis and inhibition of CAR activation.

Perineural Invasion and Cancer

Autonomic and sensory nerves innervate and regulate the physiological functions of various types of peripheral organs. The nervous system has also been postulated to play an important role in tumorigenesis. A reciprocal signaling loop between the pancreas and sensory neurons supports inflammation associated with oncogenic Kras-induced neoplasia (Saloman et al., 2016). Aberrant changes in the nervous system, including increase in the size (neural hypertrophy) and number (increased neural density), are profoundly implicated in the process of tumorous deterioration (Demir et al., 2015). Perineural invasion (PNI) is characterized as a course of neoplastic invasion of nerves, which facilitates the crosstalk between cancer cells and nerve

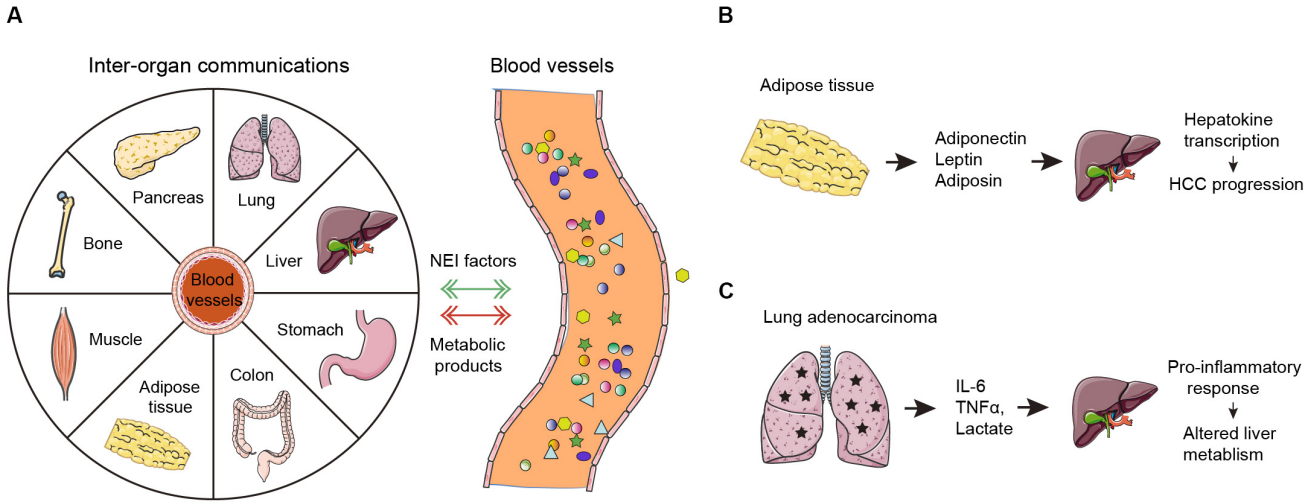

FIGURE 2 | Neuro-endocrine-immune factors mediates tumor-organ communication. **(A)** In normal conditions, inter-organ communications are mediated primarily by neuro-endocrine-immune (NEI) factors. In cancers, the tumor microenvironment-derived endocrine hormones, immune factors (cytokines and chemokines) and metabolic byproducts (such as lactate) are secreted into the blood. These neuro-endocrine-immune factors can connect systemic metabolism and induce metabolic alterations in many human organs including the liver, the pancreas, adipose tissue, skeletal muscle and others as shown. Moreover, peripheral organs further facilitate tumorigenesis by potentially supplying metabolic fuel. **(B)** Adiponectin, leptin, and adiposin are secreted by adipose tissues. Then, they target the liver to increase hepatokine transcription, which ultimately promotes HCC progression. **(C)** Lung adenocarcinoma derived IL-6, TNFα, and lactate target the liver to induce pro-inflammatory response and alter the liver metabolism.

fibers and contributes to unconventional tumorigenesis; cancer cells stimulate nerve infiltration in the tumor by the release of neurotrophic growth factors; conversely, nerve endings release neurotransmitters that can activate tumor growth and metastasis through the stimulation of specific membrane receptors (Boilly et al., 2017). PNI is emerging as an important pathologic feature of some malignancies, including the pancreas, colon and rectum, prostate, head and neck, biliary tract, and stomach (Bapat et al., 2011). Autonomic nerve fibers infiltrated in the prostate gland regulates prostate cancer development and dissemination (Magnon et al., 2013). Similarly, vagal innervation promotes gastric cancer cell stemness via M3 receptor-mediated Wnt signaling and denervation suppresses gastric tumorigenesis (Zhao et al., 2014). Recently, an additional mechanism that links PNI to pancreatic ductal adenocarcinoma (PDAC) progression was outlined; PNI is associated with hyperactivation of cholinergic signaling, which reprograms the immune microenvironment through impairing $CD8^+$ T cell infiltration and favoring Th2 over Th1 differentiation (Yang et al., 2020). While it is appreciated that the communication between the nervous system and cancers is determinant for tumor development, further studies about the origin of tumor-infiltrating nerves and how they are recruited to tumor tissues and their biological functions is required to determine the nerve dependence of cancers.

Hormone and Organ-Specific Tumorigenesis

Other examples of organ-specific tumorigenesis are hormones, which are involved in tumor initiation of endocrine organs, including pancreatic cancer, breast cancer, and prostate cancer (Jasienska et al., 2017). Pancreatic cancer is manifested by severe cachexia, marked insulin resistance and diabetes mellitus. Significant loss and dysregulation of Langerhans Islets are present in pancreatic cancer and are likely caused by diabetogenic substances released from tumor cells (Javeed et al., 2015). Therefore, considerable progress suggests that diabetes is secondary to pancreatic cancer. Since diabetes often precedes pancreatic cancer, it is also identified as a risk factor for malignancy. Despite the nature of this issue awaiting further research, there is an opportunity to initiate screening for diabetes to aid early detection of pancreatic cancer (Singhi et al., 2019). In the prostate, androgen receptor (AR) signaling is critically regulated by the hypothalamic–pituitary–testicular axis, adrenal gland steroidogenesis and prostate cell intrinsic factors. Sustained androgen receptor (AR) signaling is the major driver of castration-resistant prostate cancer (CRPC) and promotes prostate cancer development through the regulation of not only transcription networks but also genomic stability and DNA repair (Mills, 2014; Watson et al., 2015). Similarly, most breast cancers are driven by estrogen receptor (ER). Endocrine-related therapies that target ER dependence by selective estrogen receptor modulators have provided substantial improvements in patient outcomes (Turner et al., 2017).

Collectively, these facts inspire us to conclude that tumorigenesis depends on tissue-specific context, which is, not only determined by particular oncogenic mutations, but also tremendously influenced by environmental factors at the organ-level. Moreover, information exchange among organs through neuro-hormone-immune axis dramatically affects tumor progression.

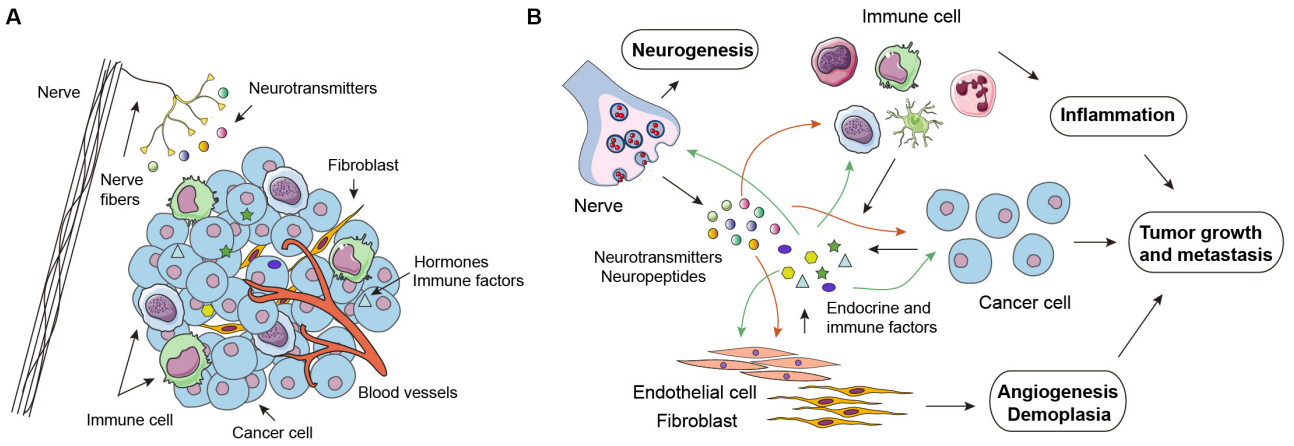

FIGURE 3 | Neuro-endocrine-immune regulations in the tumor microenvironment. **(A)** In addition to cancer cells, the tumor microenvironment (TME) is infiltrated by many other cell types, including cancer-associated fibroblasts (CAFs), endothelial cells (ECs), and various immune cell subsets, such as macrophages, natural-killer cells, dendritic cells, and T cells. Notably, emerging evidence has revealed that infiltrated nerve is an important component of the TME. **(B)** Neurotransmitters (catecholamines and acetylcholine) released by nerve endings and endocrine-immune factors secreted by cancer cells and other cell components in the TME have a stimulatory impact on tumor growth, metastasis, angiogenesis, demoplasia, and inflammation in an autocrine or paracrine manner.

REGULATION AT THE TUMOR MICROENVIRONMENT LEVEL

Tumor Microenvironment

The TME is the primary location for the initiation and progression of cancer, and encompasses multiple cell types and substances (**Figure 3A**). On the one hand, cancer cells receive signals released by their surrounding stroma, thus promoting cell survival, proliferation, mobility, and other malignant phenotypes. Conversely, cells such as cancer-associated fibroblasts, endothelial cells, and immune cells are functionally sculpted by cancer cells through diverse secreted factors. Reciprocal paracrine interactions between cancer cells and the proximal stromal and immune cells ultimately result in a milieu that favors tumor growth, metastasis, angiogenesis, and evading host immunosurveillance (**Figure 3B**). Similar to the central- and organ-level regulation, TME is also under the control of the NEI axis. Neurotransmitters, hormones, cytokines, and chemokines are pivotal signaling molecules that implement the microenvironmental regulation of the NEI axis.

Neural Signaling in Tumor Microenvironment

Similar to the processes of neoangiogenesis and lymphangiogenesis, compelling evidence suggests the possibility of the formation of new nerve fibers within tumor tissues, a phenomenon termed neoneurogenesis, which has been experimentally demonstrated to be important for tumor initiation and progression (Hanoun et al., 2015; Faulkner et al., 2019) (**Figure 3A**). Neurotransmitters or neuropeptides liberated by nerve fibers in the microenvironment are in the position of stimulating both cancer and stromal cells. Adrenergic neurotransmitters, including norepinephrine (NE) and epinephrine (E), play an important role in the fight-or-flight response by increasing blood flow to muscles, output of the heart, pupil dilation, and blood sugar, and perform their functions by binding to α and β receptors. Recent studies suggest that tumor-specific sympathetic denervation inhibits tumor progression (Magnon et al., 2013), contributes to tumor-induced changes in metabolism (Borniger et al., 2018), and reduces the expression of immune checkpoint molecules, such as programmed death-1 (PD-1), programmed death ligand-1 (PD-L1), and FOXP3 (Kamiya et al., 2019). Previously, we found that monoamine oxidase A (MAOA), the major NE/E degrading enzyme, is significantly downregulated in HCC, and conducts an inhibitory function in NE-induced HCC metastasis via blocking β-AR signaling and EGFR transactivation (Li et al., 2014). Notably, activation of the SNS also stimulates macrophage infiltration and inhibits cellular anti-tumor immune responses indicative of its regulatory role in the immune microenvironment (Cole et al., 2015). Apart from the autonomic nervous system, inhibition of cholinergic signaling and muscarinic receptors is sufficient to suppress gastric tumorigenesis and ablation of sensory neurons has been shown to slow the initiation and progression of pancreatic cancer (Zhao et al., 2014; Saloman et al., 2016). Enhanced cholinergic signaling in pancreatic cancer suppresses cancer cell stemness, $CD11b^{+}$ myeloid cells, TNFα levels, and metastatic growth in the liver (Renz et al., 2018b). Our research has proven that acetylcholine (Ach) can promote the migration and invasion of HCC cells, but inhibit their apoptosis through binding of androgen receptor (Nie et al., 2013). Therefore, it seems plausible that cancer cells can exploit locoregional neural plasticity to facilitate their outgrowth or invasive capacity. Moreover, the vagus nerve is proposed to inhibit colorectal tumorigenesis because of its anti-inflammatory properties; splenic denervation or deletion of the spleen-derived anti-inflammatory peptide TFF2 interrupts the anti-inflammatory neural arc, resulting in the expansion of myeloid derived suppressor cells (MDSCs) and

colorectal cancer (Dubeykovskaya et al., 2016). Therefore, it is of great importance to note that there is a tropic relationship between the cancer type and the innervation source. In PDAC, we have also figured out that TME is enriched with tumor-originated 5-hydroxytryptamine (5-HT). More intriguingly, PDAC cells have increased levels of 5-HT receptor HTR2B, which contributes to elevated tumor glycolysis under metabolic stress and promotes the growth of PDAC (Jiang et al., 2017). Pancreatic cancer cells are capable of readily absorbing 5-HT in a transport-mediated manner. The abundant and fast accumulation of intracellular 5-HT greatly activates the small GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1), which is necessary for acinar-to-ductal metaplasia (ADM), a vital process in cancerous transformation (Saponara et al., 2018). 5-HT is also a versatile immunomodulatory molecule in the TME that affects platelet activation and macrophage polarization to accelerate tumor metastasis and dissemination (Jiang et al., 2019). Collectively, the neuro-immune interaction is essential for driving cancer development and progression. The interplay between tumor infiltrating nerves, tumor cells, and non-tumor cells has been largely elucidated. Therefore, the identification of nerve dependence in cancer might prove to be a promising therapeutic opportunity.

Endocrine-Immune Signaling in Tumor Microenvironment

In addition to neurotransmitters, hormones also affect many vicious behaviors of cancer cells. There are no doubts of the direct promotional effect of estrogens and androgens on tumors of reproduction-related organs (prostate, endometrium, breast and ovary). Estrogens may also induce an immunosuppressive TME through shifting the balance in favor of Th2 responses, proliferation of regulatory T cells and MDSCs, increased PD-L1 expression, and inhibition of the anti-tumor effect of $CD8^+$ T cells and NK cells (Svoronos et al., 2017; Welte et al., 2017). Likewise, androgen-mediated suppression of immune reactivity lowers the threshold for cancer and promotes tumor progression (Gubbels Bupp and Jorgensen, 2018). Additionally, multiple known endocrine hormones, such as insulin, prostaglandins, glucocorticoids, prolactin, and progesterone, have been observed to play a role in cancers by modulating malignant phenotypes or anti-tumor immune responses (Klil-Drori et al., 2017; Wang and DuBois, 2018; Dandawate et al., 2019). For instance, inhibition of prolactin receptor signaling by diphenylbutylpiperidine antipsychotic drugs reduces the growth of PDAC (Dandawate et al., 2019); prostaglandin regulates tumor-associated immunosuppression by inducing MDSC and Treg differentiation, macrophage polarization from M1 to M2, and production of PD-L1 (Wang and DuBois, 2018). Our group found that mineralocorticoid receptor expressed in hepatocellular carcinoma suppresses cancer progression by regulating the miR-338-3p-PKLR axis and the Warburg effect (Nie et al., 2015). Notably, an obvious example of an indirect effect is that upregulated thyroid hormones, stimulated by acute or chronic stress, are involved in tumor evolution by alternating T-cell lymphoproliferative responses, implicating the role of the neuron-endocrine-immune cascade in modulating tumor progression. Therefore, targeted inhibition of hormone-related signaling may act as a novel strategy to eliminate tumor cells within the TME and enhance the effects of immunotherapies.

CONCLUSION

In this review, we have focused on the interrelationships between NEI factors and cancer initiation and metastasis and proposed that cancer is a systetic disease influenced by multidimensional regulation: central-level regulation, organ-level regulation, and TME-level regulation. At each level, nervous system, endocrine, and immune factors play a critical role. At the central-level of regulation, emotions play essential roles in the growth, angiogenesis, and metastasis of cancer, as chronic stress promotes cancer progression, while the enriched environment (positive attitude, exercise) may hamper this process. Organ-level modulations range from changes in whole-body homeostasis to alterations in pathological conditions such as cancers. The TME contains various cellular types executing concrete functions, under the regulation of signaling molecules including neurotransmitters, hormones, cytokines/chemokines, and other types of secreted proteins. Although the roles of the nervous, endocrine, and immunes system in tumor initiation and progression have been investigated in recent decades, the knowledge is too detailed and scattered to easily understand the systemic contribution. Hence, our proposal of systemic regulation may provide novel avenues for a better understanding of mechanisms in carcinogenesis and development, digging out sensitive biomarkers for diagnosis and effective therapeutic interventions. Moreover, it provides a certain theoretical basis for studying the impact of social and mental factors on cancer patients, and improving the comprehensive treatment of postoperative rehabilitation of cancer patients including social family care and psychological counseling.

From the preventative and therapeutic points of view, it is also worth noting that a variety of inhibitors targeting the NEI axis have been widely used in clinical practice, such as beta-blockers, Ach receptor antagonists, and 5-HT receptor inhibitors. For example, beta-blockers are currently prescribed for cardiovascular diseases, but many retrospective studies have revealed a beneficial effect in patients with prostate cancer (Grytli et al., 2014), colorectal cancer (Cui et al., 2019), and multiple myeloma (Hwa et al., 2017). It is unclear how beta blockers improve patient survival and impede tumor progression. Further research is warranted to fully uncover the complexity of the mechanisms along with the interplay with endocrine and immune systems. Therefore, our hypothesis further support the repurposing of already approved drugs, which not only minimizes time and costs, but also may prove to be an innovative therapies in oncology.

Finally, we emphasize that the role of systemic regulation of tumors does not negate the importance of local lesions, as local lesions and the whole body are interdependent. For early and mid-term malignant tumors, whether treatment involves surgery, chemotherapy, or local interventional therapy, the removal of

tumor lesions is conducive to the functional repair of the overall regulatory system. Importantly, cancer treatment should be based on the premise of not sacrificing the activity of the body's regulatory system, and more attention should be paid to the detection of systemic markers, including markers of nervous, endocrine, and immune systems.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Brain Neurotransmitter Modulation by Gut Microbiota in Anxiety and Depression

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Anxiety and depression are highly prevalent mental illnesses worldwide and have long been thought to be closely associated to neurotransmitter modulation. There is growing evidence indicating that changes in the composition of the gut microbiota are related to mental health including anxiety and depression. In this review, we focus on combining the intestinal microbiota with serotonergic, dopaminergic, and noradrenergic neurotransmission in brain, with special emphasis on the anxiety- and depression-like behaviors in stress-related rodent models. Therefore, we reviewed studies conducted on germ-free rodents, or in animals subjected to microbiota absence using antibiotics, as well as via the usage of probiotics. All the results strongly support that the brain neurotransmitter modulation by gut microbiota is indispensable to the physiopathology of anxiety and depression. However, a lot of work is needed to determine how gut microbiota mediated neurotransmission in human brain has any physiological significance and, if any, how it can be used in therapy. Overall, the gut microbiota provides a novel way to alter neurotransmitter modulation in the brain and treat gut–brain axis diseases, such as anxiety and depression.

Keywords: serotonin, dopamine, noradrenaline, gut microbiota, anxiety- and depression-like behavior

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INTRODUCTION

Anxiety and depression are heterogeneous and complex diseases that can have devastating effects on the function and quality of life of individuals, and increase the risk of suicide (Arsenault-Lapierre et al., 2004). The overall burden of anxiety and depression is steadily increasing and now exceeds most other major diseases (Tyrovolas et al., 2020). Their onset can occur from childhood to adolescence and last a lifetime (Thapar and Riglin, 2020). In addition, anxiety and depression are often comorbid (Park and Kim, 2020) and relapse-prone conditions (Ali et al., 2017).

Despite the mechanisms of anxiety and depression are still unclear, neurotransmitters such as serotonin [also named 5-hydroxytryptamine (5-HT)], dopamine (DA), and noradrenaline (NE) have explained the pathophysiology of anxiety and depression over several decades (Olivier and Olivier, 2020; Shao and Zhu, 2020). An increasing number of evidence reveals the importance of the gut microbiota in the pathogenesis of anxiety and depression (Rieder et al., 2017). Gut microbiota and its metabolites are at least partially involved in the afferent input of the vagus nerve (Forsythe et al., 2014) and the regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Sudo et al., 2004). Perhaps unsurprisingly, the gut microbiota has also been shown to be related to tryptophan

metabolism and neurotransmitter production (Barrett et al., 2012; O'Mahony et al., 2015). Given the need to elucidate the potential role of gut microbiota in regulating neurotransmitter modulation in anxiety and depression, it seems important to summarize the evidence provided so far regarding the effects of neurotransmitters, in addition to uncovering behavioral alterations specific to these neurotransmitters.

METHODS

We searched for studies including text words related to microbiota (microbiome or flora) and neurotransmitter, and anxiety or depression. The search was carried out using the PubMed, Web of Science, and Embase databases. We included *in vivo* studies investigating gut microbiota in relation to anxiety and depression, and in which neurotransmitters are part of the pathophysiology, to facilitate comparisons. Studies excluded from the scope of search were or contained one or more of the following: did not use rodent species, did not look at the specific microbiota strain or absence condition, were not measuring anxiety- and depression-like behavior outcomes, no brain neurotransmitter was measured, did use genetic model, were not published in English. Additionally, in case there were fewer than three studies on the same neurotransmitter (e.g., gamma aminobutyric acid), these papers were also excluded due to lack of comparability. A total of 15 studies met the criteria for our review at the end of the selection process (**Figure 1**).

NEUROTRANSMITTER MODULATION AND BEHAVIORAL OUTCOMES IDENTIFIED UPON MODIFICATIONS WITH GUT MICROBIOTA

In this section, we summarize neurotransmitter parameter and behavior results identified in *in vivo* studies which used treatment with modifications by gut microbiota in the context of stress-related rodent models (**Supplementary Table 1**).

Serotonin

5-Hydroxytryptamine is a neurotransmitter with important physiological significance in human body, involved in regulating many key processes, including behaviors, mood, gastrointestinal secretion, and peristalsis (Berger et al., 2009; Bamalan and Al Khalili, 2020). Antidepressants that act on 5-HT are utilized as front line drugs for many psychiatric disorders, such as major depressive disorder, post-traumatic stress disorder, anxiety, and bipolar disorder (Masand and Gupta, 1999; Bandelow et al., 2017). Although 5-HT is widely distributed throughout the body, 90–95% of 5-HT exists in the gastrointestinal tract (Gershon and Tack, 2007). Thus, it may not be surprising that the growing literature links the gut microbiota to host levels of 5-HT.

In germ-free (GF) rodents, two studies found no change of 5-HT and/or 5-hydroxyindoleacetic acid (5-HIAA) in the hippocampus and/or frontal cortex as well as in the striatum, but different anxiety-like behavioral alterations with early-life stress in C57BL/6N mice or acute stress in F344 male rats (Crumeyrolle-Arias et al., 2014; De Palma et al., 2015). Another two studies discovered reduced anxiety-like behavior in GF Swiss Webster mice, one in two reported higher 5-HT and 5-HIAA levels in the hippocampus of male mice, but no change of 5-HT and 5-HIAA levels in the hippocampus of female mice (Clarke et al., 2013), while another one found lower 5-HT receptor 1A (HTR1A) in the hippocampal DG rather than in the hippocampal CA1 of GF Swiss Webster female mice (Neufeld et al., 2011). In a model of antibiotic-induced depletion of the gut microbiota, one study revealed a greater display of depression-like behavior in Sprague–Dawley male rats. In tandem with the clear behavioral alteration, they also found lower 5-HT and higher 5-HIAA/5-HT in the hippocampus, and reduced 5-HIAA/5-HT in the hypothalamus (Hoban et al., 2016).

Seven studies investigated the antidepressant and/or anxiolytic effects of microbiota-based interventions on 5-HT modulation in anxiety and/or depression (Desbonnet et al., 2010; Liang et al., 2015; Sun et al., 2018; Li et al., 2019; Liao et al., 2019; Tian et al., 2019). Two studies detected live and heat-killed *Lactobacillus paracasei* PS23 in early life stress and corticosterone-treated models, respectively, but showed different results. For instance, both live and heat-killed *L. paracasei* PS23 treatment did not change 5-HT, 5-HIAA and 5-HIAA/5-HT in the hippocampus of early life stress induced model, while live *L. paracasei* PS23 treatment increased the level of 5-HT in the hippocampus and striatum of corticosterone-induced mice model rather than heat-killed PS23 treatment. In addition, both live and heat-killed *L. paracasei* PS23 treatment had no effect on the 5-HIAA in the prefrontal cortex and striatum of corticosterone-treated model. Besides that, probiotic *Bifidobacterium infantis* 35624 treatment resulted in reversal of depression-like behavioral deficits, but unchanged for 5-HIAA/5-HT in the hippocampus of early life stressed rat model. All the other five strains conducted by four studies were able to increase the brain 5-HT in stress-related rodent models. Regarding to specific brain area, *Lactobacillus helveticus* NS8, *Bifidobacterium longum*, and *Lactobacillus rhamnosus* increased the level of 5-HT in the hippocampus of rodents. Administration of *B. longum* subspecies *infantis* CCFM687, *B. longum* and *L. rhamnosus* showed higher 5-HT content in the frontal cortex. In particular, the study of *B. longum* subspecies *infantis* CCFM687 also found higher expression of 5-hydroxytryptophan (5-HPT) and no significant changes of HTR1A mRNA in the prefrontal cortex of male mice. In another study, chronic unpredictable mild stress-induced mice treated with *B. longum* and *L. rhamnosus* had higher tryptophan hydroxylase (TPH) but lower indoleamine 2,3-dioxygenase (IDO) in the prefrontal cortex and hippocampus compared with model group.

In the selected papers, only one study examined the effect of certain microbiota strain in GF mice, the behavior outcome showed live *Lactobacillus plantarum* PS128 had the anxiolytic effect along with higher 5-HT and 5-HIAA in the striatum without anti-depressant effects, rather the 5-HT, 5-HIAA, and 5-HIAA/5-HT in the prefrontal cortex and hippocampus as well as the 5-HIAA/5-HT in the striatum had no changes (Liu et al., 2016). In the same study, outcome of heat-killed

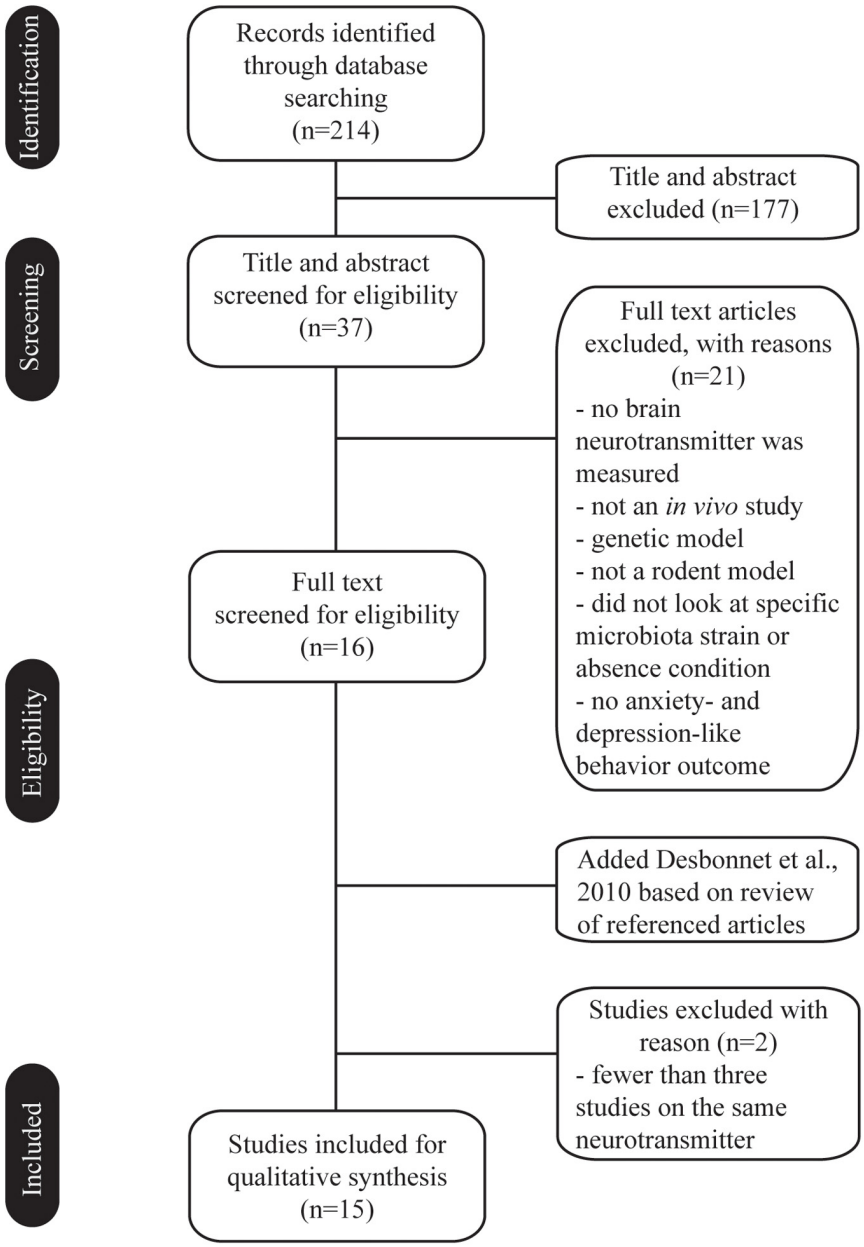

FIGURE 1 | Flow chart of the selection process. Fifteen *in vivo* studies met the criteria and investigated the effect of gut microbiota in anxiety and depression, and in which brain neurotransmitters are part of the pathophysiology.

L. plantarum PS128 tested in parallel has no statistical difference compared with the GF group.

The evidence summarized in this section highlights the role of gut microbiota in regulating 5-HT modulation in anxiety- and depression-like behavior in models of stress.

Dopamine

Dopamine is the main catecholaminergic neurotransmitter, synthesized centrally and peripherally, that plays a pivotal role in multiple physiological processes such as emotion, memory, attention, motivation, reward, and food intake (Klein et al., 2019; Kleinridders and Pothos, 2019). DA system dysregulation has been related to anxiety (Carpenter et al., 2012; Moraga-Amaro et al., 2014), depression (Camardese et al., 2014; Belujon and Grace, 2017), and gut microbes (Gonzalez-Arancibia et al., 2019). In terms of crosstalk between gut and brain, the results (Han et al., 2018) strongly support that the vagus nerve is the key mediator.

Two studies observed the DA level in the hippocampus of GF rodents; both of them did not found significant change in comparison to specific pathogen-free (SPF) controls, although they reported inconsistent anxiety-like behavior under different stress (Crumeyrolle-Arias et al., 2014; De Palma et al., 2015).

In GF F344 male rats, decreased DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and HVA/DA were found in the frontal cortex, while reduced HVA and HVA/DA, an index of DA turnover, were reported in the hippocampus and striatum, but no statistic change of DA and DOPAC in the hippocampus and striatum was found. In a study using antibiotic-induced depletion of the gut microbiota in Sprague–Dawley male rats, they found higher levodopa (LDOPA) and HVA in the prefrontal cortex, lower HVA in the hippocampus, and lower HVA/DA in the amygdala and striatum. DA content had no significant changes in the above three brain regions compared to control group (Hoban et al., 2016).

Out of the four studies investigating the beneficial role of microbiota strain in DA system of depression models, all the three strains had capability to reduce depression- and/or anxiety-like behaviors. With respect to *L. paracasei* PS23 reported by two studies (Liao et al., 2019; Wei et al., 2019), both live and heat-killed *L. paracasei* PS23 administration decreased DOPAC and HVA, but not (DOPAC + HVA)/DA, in the hippocampus of early-life stress-induced male mice model. However, only heat-killed PS23 increased DA in the hippocampus and prefrontal cortex of corticosterone-treated mice model, and both live and heat-killed *L. paracasei* PS23 showed no effect on DOPAC in the prefrontal cortex of corticosterone-induced model. In the other two studies, one showed that DA had not changed significantly in the hippocampus and prefrontal cortex of chronic restraint stress-induced model after *L. helveticus* NS8 administration (Liang et al., 2015), while another study showed that treatment with *Bifidobacterium* CECT 7765 decreased DA level in the hypothalamus of early-life stress-induced male mice model (Moya-Perez et al., 2017).

In one study examined the effect of certain probiotic in GF mice, the behavior outcome showed that live *L. plantarum* PS128 exhibited the anxiolytic effect accompanying with higher DA and HVA in the striatum but not in the prefrontal cortex, hippocampus, the DOPAC and HVA/DA in the prefrontal cortex, hippocampus, and striatum did not change significantly (Liu et al., 2016). In the same study, outcome of heat-killed *L. plantarum* PS128 tested in parallel showed no effect on behavior and brain DA system in GF mice.

Based on the literatures summarized above, the potential of gut microbiota, to alleviate anxiety- and/or depression-like behavior, would take place via DA modulation.

Noradrenaline

Noradrenaline has been known for its role in the pathogenesis of anxiety (Kalk et al., 2011; Zheng et al., 2019) and depression (Seki et al., 2018) for a long time. Interestingly, it appears NE also controls satiation (Asarian and Bachler, 2014). In addition, it has been reported that the microbiota influenced NE level in the gut lumen of mice (Asano et al., 2012), but whether the bacteria produce NE to alter behavior through an indirect path was not determined.

Noradrenaline modulation of GF rodents was measured in two studies, one in the context of increased anxiety-like behavior (Crumeyrolle-Arias et al., 2014) and another in the context of decreased depression-like behavior only in male mice under early-life stress (De Palma et al., 2015); in both conditions, NE was not affected in the brain. However, depletion of the microbiota with non-absorbable antibiotics has been reported to increase depression-like behavior; this effect was related to elevated level of NE in the striatum, but not in the prefrontal cortex, hippocampus, amygdala, or hypothalamus.

Administration of *L. helveticus* NS8, heat-killed *Enterococcus fecalis* (EC-12), and *Bifidobacterium* CECT 7765 improved anxiety-like behavior under different stress-related models (Liang et al., 2015; Moya-Perez et al., 2017; Kambe et al., 2020). NE is released via activation of central adrenoceptor β3 (Adrb3) (Claustre et al., 2008), while heat-killed *E. fecalis* (EC-12)-treated male mice expressed higher Adrb3 in the prefrontal cortex compared with control mice. In an early-life stress-induced model, *Bifidobacterium* CECT 7765 effectively reduced the content of NE in the hypothalamus of C57BL/6J male mice. Antidepressant effect was also demonstrated, in respect of *L. helveticus NS8* treatment showing elevated NE in the striatum but not in the prefrontal cortex of chronic restraint stress-induced male rats (Liang et al., 2015), whereas *B. infantis* 35624 treatment did not alter NE in the amygdaloid cortex of early-life stress-induced male rats (Desbonnet et al., 2010).

The limited evidence available on NE in anxiety- and depression-like behavior suggests that they could be influenced by gut microbiota.

Overall, modifications of gut microbiota can affect brain systems of 5-HT, DA, and NE in various rodent models of stress, as well as their anxiety- and depression-like behaviors.

DISCUSSION

This review summarizes the impacts of gut microbiota on the serotonergic, dopaminergic, and noradrenergic modulation in anxiety and depression. One way to address the effects of gut microbiota on the brain is to destroy the gut microbial ecology. Therefore, GF rodents provide a good tool to test the gut microbial colonization from early to adulthood (Gonzalez-Arancibia et al., 2019). While at first glance similar to the GF model, antibiotics represent another unique model to study the gut microbiome. Since destroying the microbiome can negatively affect the host, supplementation of the microbiome has been used as a strategy to optimize host performance. The introduction of known or suspected beneficial probiotic microorganisms is an intuitive way to study the relationship between the host and the microbiome. In this regard, it has been shown that levels of 5-HT, DA, and NE, and their respective precursor, metabolites, or receptors have significant variations across different brain regions in rodents with altered gut microbiota compared with their controls. However, it should be kept in mind that it may not only be a specific microbiota that is beneficial to microorganisms, but fundamentally provide nutrients that promote the growth of beneficial microorganisms (Duran-Pinedo and Frias-Lopez, 2015). A limitation of this review is that we did not include studies of prebiotics and fecal microbiota transplantation which are also general ways to alter microbiome composition, as there

is little control over which microorganisms will metabolize or proliferate the prebiotics (Ford et al., 2014), and what kinds of fecal microbiota will be transplanted.

When considering how the bacteria may affect the brain neurotransmitters, one possible mechanism is that metabolite produced by the intestinal flora can be used as precursors for the synthesis of neurotransmitters in the central nervous system; for instance, *B. infantis* has been found to increase plasma tryptophan levels, thereby affecting the transmission of brain 5-HT (Desbonnet et al., 2010). Even though bacteria have been shown to have the capability to produce a series of major neurotransmitters including 5-HT, DA, and NE (Strandwitz, 2018), it is unlikely to influence brain directly because they cannot cross the blood–brain barrier. Besides, it must be taken into account that release of neurotransmitters is also regulated by other neural circuits (Russo and Nestler, 2013), and the influence of intestinal microbes on other networks cannot be excluded, which raises an open question: the regulation of brain neurotransmitters by gut microbes is direct, or indirect? Up to this review, this is still an unsolved question; moreover, the potential mechanisms of how the gut microbiota can affect the anxiety- and depression-like behavior via neurotransmitters are required to validate. Nevertheless, the studies reviewed indicated a close connection between intestinal symbionts and neurotransmitters in neuropsychiatric diseases, and it seems to be a possible way to communicate along the gut–brain axis. In addition, since most of the existing work has been done in animals, more well-designed human clinical trials are needed. Finally, more and more evidence supports that treatment for anxiety and depression could take advantage of intervention at the gut microbiota, either through reasonable use of antibiotics or via identification of novel microbial strains that influence the brain serotonergic, dopaminergic, and noradrenergic activity.

AUTHOR CONTRIBUTIONS

FH wrote the manuscript and researched the publication landscape. XW revised the manuscript and devised the idea. Both authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

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Neurotransmitters Modulate Intrathymic T-cell Development

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The existence of a crosstalk between the nervous and immune systems is well established. Neurotransmitters can be produced by immune cells, whereas cytokines can be secreted by cells of nervous tissues. Additionally, cells of both systems express the corresponding receptors. Herein, we discuss the thymus as a paradigm for studies on the neuroimmune network. The thymus is a primary lymphoid organ responsible for the maturation of T lymphocytes. Intrathymic T-cell development is mostly controlled by the thymic microenvironment, formed by thymic epithelial cells (TEC), dendritic cells, macrophages, and fibroblasts. Developing thymocytes and microenvironmental cells can be influenced by exogenous and endogenous stimuli; neurotransmitters are among the endogenous molecules. Norepinephrine is secreted at nerve endings in the thymus, but are also produced by thymic cells, being involved in controlling thymocyte death. Thymocytes and TEC express acetylcholine receptors, but the cognate neurotransmitter seems to be produced and released by lymphoid and microenvironmental cells, not by nerve endings. Evidence indicates that, among others, TECs also produce serotonin and dopamine, as well as somatostatin, substance P, vasoactive intestinal peptide (VIP) and the typical pituitary neurohormones, oxytocin and arg-vasopressin. Although functional data of these molecules in the thymus are scarce, they are likely involved in intrathymic T cell development, as exemplified by somatostatin, which inhibits thymocyte proliferation, differentiation, migration and cytokine production. Overall, intrathymic neuroimmune interactions include various neurotransmitters, most of them of non-neuronal origin, and that should be placed as further physiological players in the general process of T-cell development.

Keywords: neurotransmitters, thymus, thymic epithelial cells, T-cell development, thymocytes

INTRODUCTION

The concept of neuroimmune crosstalk was established several decades ago. One key factor in determining such communication is the fact that both the nervous and immune systems use similar molecular moieties, and therefore apply a common syntax to communicate with each other. Accordingly, classic types of neurotransmitters can be produced by immune cells, whereas

cytokines can be secreted by cells of nervous tissues. In the same vein, cells from the two systems express the correlated receptors, although signal transduction may be specific for a given cell type. Herein, we discuss the thymus as a paradigm for the expression and role of different kinds of neurotransmitters, particularly of non-neuronal origin.

The thymus is a primary lymphoid organ responsible for the generation of T lymphocytes in vertebrates, from fish to mammals (Geenen and Savino, 2019). This process is dependent on interactions controlled by the thymic tridimensional network, composed of thymic epithelial cells (TEC), thymic dendritic cells (TDC), macrophages, fibroblasts; as well as the extracellular matrix, cytokines, chemokines, hormones; and components of the nervous system. During thymocyte differentiation, bone marrow-derived early T-cell precursors (ETP) enter the organ by the corticomedullary region of the thymic lobules and subsequently migrate to the subcapsular region. From this region, immature thymocytes, including those bearing the phenotypes $TCR^{-}CD3^{-}CD4^{-}CD8^{-}$ (double-negative or DN, standing for the membrane expression of CD4 and CD8) and $TCR^{low}CD3^{low}CD4^{+}CD8^{+}$ (double-positive for the same markers, or DP) migrate to the inner cortex, and then to the cortico-medullary region. Developing cells next migrate to the medullary region, where they become mature $TCR^{high}CD3^{high}CD4^{+}CD8^{-}$ (single-positive for CD4 or CD4SP) or $TCR^{high}CD3^{high}CD4^{-}CD8^{+}$ (single-positive for CD8 or CD8SP) cells (summarized in **Figure 1A**). Those cells ultimately migrate to the cortico-medullary region and leave the thymus to specific regions in peripheral lymphoid organs (Francelin and Verinaud, 2011; Savino et al., 2016).

The migration facilitates the encounter and the interactions of thymocytes with different components of the thymic microenvironment. For example, the TCR/CD3 complex, expressed by thymocytes, interacts with self-antigens presented by molecules of the major histocompatibility complex (MHC), expressed by microenvironmental cells, including TEC and TDC. Thymocytes bearing TCR/CD3 complexes that interact with low or medium affinity with MHC-presented self-antigens are positively selected and continue their maturation process. Conversely, thymocytes bearing TCR/CD3 complexes that interact with high affinity with MHC-presented self-antigens are negatively selected and die by apoptosis. Those selective processes prevent the exit of dysfunctional mature T cells or cells with autoimmune potential (Francelin and Verinaud, 2011; Savino et al., 2016).

As for components of the nervous system that could impact thymus physiology, nerve fibers and neurotransmitters are spread through the parenchyma, and both thymocytes and TECs express neurotransmitter receptors. This can be exemplified by the intrathymic presence of autonomic nervous system (ANS) fibers, providing noradrenergic fibers within the thymus parenchyma (Leposavić et al., 2008; Godinho-Silva et al., 2019). The main source of these nerves is the postganglionic neurons in the upper paravertebral ganglion of the sympathetic chain, predominantly from the upper cervical and stellate ganglia (Nance and Sanders, 2007; Leposavić et al., 2008). Most of the nerves are composed of noradrenergic fibers that enter the thymus through the capsule and are distributed along with the capsule and septa as fibers or following the vasculature of the connective tissue that forms the organ. Neuronal-derived norepinephrine release is modulated by presynaptic receptors of noradrenergic axonal terminals, which express the α2 adrenergic presynaptic receptors, muscarinic and nicotinic acetylcholine receptors, as well as purinergic receptors and receptors for prostaglandins (PGE2) (Haskó et al., 1995), thus illustrating a complex intrathymic control of norepinephrine release at the nerve endings.

Peptidergic innervation can also be seen within the organ, and comprise, for example, Neuropeptide Y, neurotensin, vasoactive intestinal peptide (VIP) among others (Mignini et al., 2011, 2014). Nevertheless, it is important to point out that neurotransmitters of non-neuronal origin can also be found in the thymus, and that parenchymal lymphoid and non-lymphoid cells can express the respective cognate receptors, as seen below.

INTRATHYMIC EXPRESSION OF NON-NEURONAL NEUROTRANSMITTERS AND THEIR CORRESPONDING RECEPTORS

As mentioned above, neurotransmitters are constitutively produced in the thymus by both autonomic innervation as well as thymic cells (Leposavić et al., 2008; Mignini et al., 2014). They comprise amino acids, monoamines, and peptides, which, as a whole, participate in the general process of T cell differentiation. Accordingly, both thymic microenvironmental cells and thymocytes express specific receptors (see **Table 1**).

For example, there is an intrathymic cholinergic system represented by acetylcholine (ACh), which is synthesized and released by TECs as well as thymocytes (Rinner et al., 1999; Kawashima and Fujii, 2004; Panneck et al., 2014). These same cell types also express the corresponding cholinergic receptors, thus pointing to a non-neuronal autocrine/paracrine cholinergic circuit acting within the organ (Kawashima and Fujii, 2004). In fact, intrathymic ACh impacts thymocyte development directly by acting upon thymocyte via cholinergic receptors; or indirectly, through microenvironmental cells, with consequences on developing thymocytes (Rinner et al., 1999; Kawashima and Fujii, 2004). Actually, when thymocytes were co-cultured with TECs in the presence of ACh, a pro-apoptotic effect was observed. This effect was reversed after the treatment with a nicotinic receptor antagonist, indicating that thymic cells are responsive to cholinergic modulation (Rinner et al., 1999). Additionally, multiple nicotinic receptor subunits have been described as being expressed by thymocytes and TECs, including α2, α3, α5, α7, β4, ε in TECs and α3, α5, α7, β2, β4 in thymocytes (Mihovilovic et al., 1997; Kawashima and Fujii, 2004). The fact that both cell types express nicotinic receptor subunits reinforces the idea that ACh may be an important player in TEC-thymocyte interactions (Rinner et al., 1999; Kawashima and Fujii, 2004). The subunit expression also varies according to the cell maturation profile, as it has been shown that especially α3 and β4 are decreased in more mature single-positive thymocytes, indicating that ACh may also

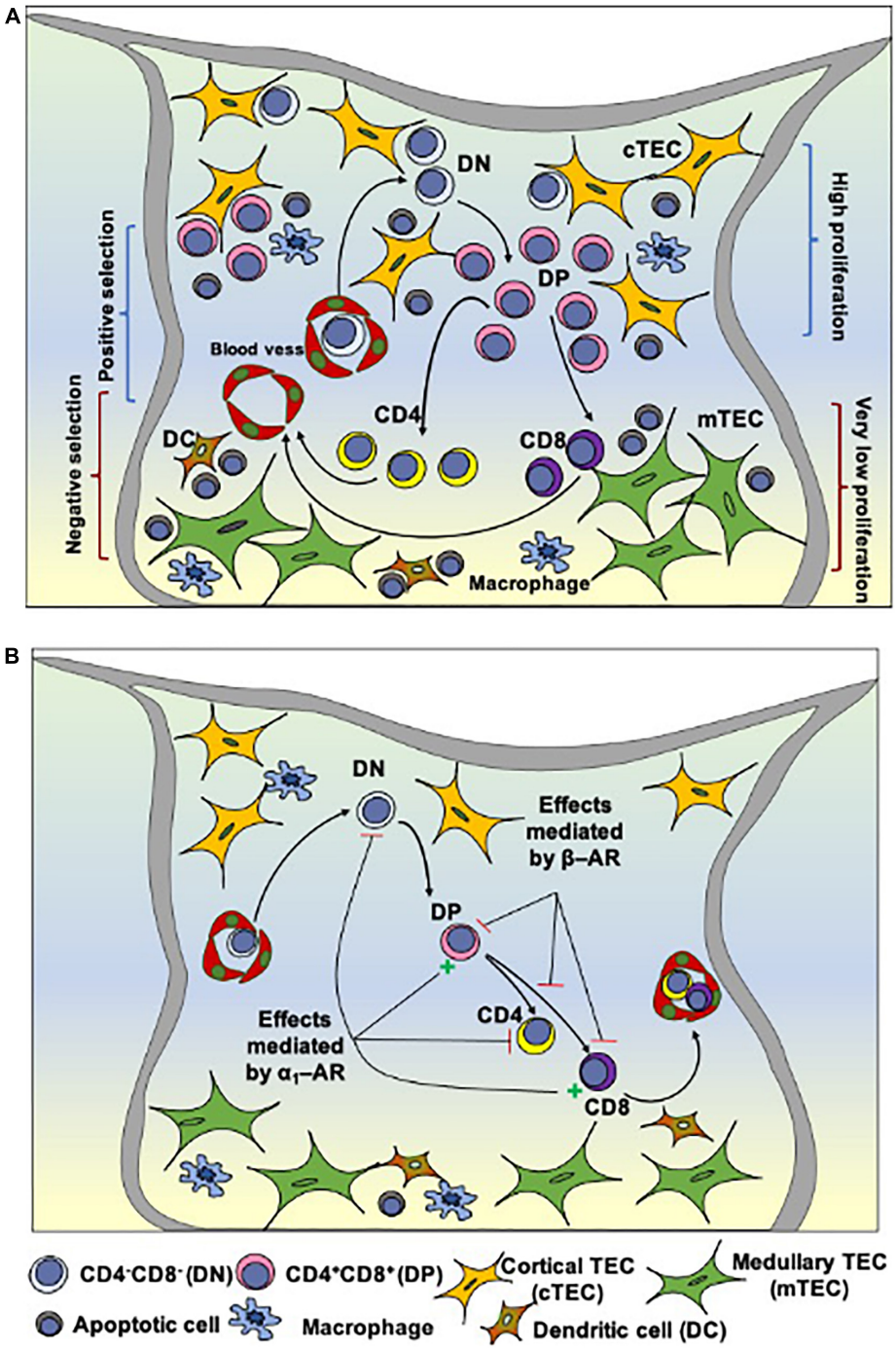

FIGURE 1 | Intrathymic T cell differentiation within the thymic microenvironment and stages that can be modulated by norepinephrine. **(A)** Shows schematically the general process of thymocyte differentiation, in the context of the thymic microenvironment. Bone marrow-derived precursors enter the organ through capillaries at the corticomedullary junction and migrate toward the outer cortex where they proliferate, but do not express the CD3/TCR complexes as well as the accessory molecules CD4 and CD8. There CD4/CD8 double-negative cells (DN) evolve to express TCR as well as CD4 and CD8 (becoming double-positive cells, DP) and, under the control of the thymic microenvironment, undergo positive selection, with positively-selected thymocytes migrating toward the medulla, where some of them will die by negative selection. Mature CD4 or CD8 single-positive thymocytes will eventually leave the thymus. **(B)** Depicts the influence of α and β-adrenergic receptors (AR) along with thymocyte differentiation in the rat thymus. Activation of α1-AR reduces the frequency of DN cells and increases DP cells. It is hypothesized that signaling through β-AR (via regulation of Thy-1 expression) modulates the selection process, reducing positive and/or increasing negative selection, and detains thymocyte differentiation, while α1-AR signaling may prevent the differentiation of positively selected cells toward the $CD4^+$SP phenotype. Stellate yellow cells: cortical TECs; stellate green cells: medullary TECs; round cells with a purple nucleus: thymocytes in different stages of development; blue cells: macrophages; brown cells: dendritic cells (DCs). Reduction = – and increase = + Adapted from Leposavić et al. (2008) and Savino et al. (2015).

TABLE 1 | Intrathymic expression of non-neuronal neurotransmitters and their corresponding receptors.

Non-neuronal neurotransmitter	Cell type producer		Cell types expressing the corresponding receptors		References
	TEC	Thymocytes	TEC	Thymocytes	
Acetylcholine	+	+	α2, α3, α5, α7, β4, ε*	α3, α5, α7, β2, β4*	Rinner et al., 1999; Kawashima and Fujii, 2004; Panneck et al., 2014
Dopamine	+	Lowlevels detected	ND	D1, D2, D3, D4, D5	Pilipović et al., 2008; Mignini et al., 2009, 2013; Lifantseva et al., 2016
Norepinephrine	+	+	α1, β1, β2**	α1, β2**	Marchetti et al., 1994; Leposavić et al., 2008; Pešić et al., 2009; Roggero et al., 2011
Serotonin	ND	+	ND	5-HT1B, 5-HT1F, 5-HT2A, 5-HT2B, 5-HT6, 5-HT7	Stefulj et al., 2000; Lifantseva et al., 2017
Vasoactive intestinal peptide	+	ND	ND	VIPR, VIP_1R	Delgado et al., 1996a,b; Silva et al., 2006
Neuropeptide Y	+	ND	ND	Y_1,Y_2,Y_3 (possibly)	Medina et al., 2000; Silva et al., 2006
Calcitonin gene related peptide	+	ND	ND	$CGPR_1$	Kurz et al., 1995; Silva et al., 2006
Substance P	+	+	ND	NK-1R	Santoni et al., 2002; Silva et al., 2006
Oxytocin	+	ND	ND	OTR	Robert et al., 1991; Hansenne et al., 2005; Savino et al., 2016
Arg-Vasopressin	+	ND	ND	V1bR	Robert et al., 1991; Hansenne et al., 2005; Savino et al., 2016
Somatostatin	+	ND	sst1, sst2A	sst2A, sst3, sst4	Ferone et al., 2002; Silva et al., 2006

*ND, Not determined; TEC, thymic epithelial cells; * α 2-7, β2-4, and ε, Nicotinic receptor subunits; D1-5, Doparminergic receptor; ** α1, and β1-2, Norepinephrine receptor; 5-TH1-7, Serotoninergic receptors; VIPR, Vasoactive Intestinal Peptide Receptor; Y, neuropeptide receptor; CGPR, Calcitonin Gene Related Peptide Receptor, NK-1R, Neurokinin 1-Receptor, OTR, Oxytocin Receptor, V1bR, Vasopressin V1b Receptor, sst 2-4, Somatostatin Receptor.*

play a role in intrathymic T-cell differentiation (Mihovilovic et al., 1997). Moreover, an autocrine loop may take place in endothelial cells, since Ach is produced by these cells, which also express cholinergic receptors (Kawashima and Fujii, 2004). A further non-excludent hypothesis is that Ach produced by endothelial cells in the thymus act through a paracrine way, to modulate the interactions between thymocytes and endothelial cells and also TEC-thymocyte interaction.

In a second vein, the stimulation of the Ach receptor modulates nitric oxide which in turn acts by inducing vasodilation controlling permeability and blood circulation (Kawashima and Fujii, 2004).

Regarding monoamines neurotransmitters, the catecholamine system seems to be a major player in terms of the roles of neurotransmitters in thymocyte development. Norepinephrine (NE) is the main catecholamine of the ANS within the thymus. Interestingly, in addition to the neuronal input, the thymus presents a non-neuronal catecholaminergic network; thymocytes and TECs express catecholaminergic receptors and enzymes necessary for NE production, such as tyrosine hydroxylase (Leposavić et al., 2008). In this respect, inhibition of this enzyme evoked reduction in NE contents in rat thymocytes and adult thymus (Radojevíc et al., 2014).

Catecholamines secreted and released intrathymically engage with α and β adrenergic receptors expressed by thymocytes and TECs, shown in **Table 1** and **Figure 1B** (Leposavić et al., 2008; Roggero et al., 2011). Briefly, thymocytes of adult mice and fetuses express β-AR binding sites (Singh et al., 1979). Interestingly, in the rat thymus, β2-AR, which is the most expressed adrenergic receptor in the organ, can be modulated by sexual hormones, as it was demonstrated that β2 –AR density can vary according to the marked alterations of the sex steroid hormone milieu in female rats (Marchetti et al., 1994). In a second vein, immature thymocytes in the mouse thymus express lower amounts of β-AR than their mature counterparts (Radojcic et al., 1991), suggesting that the adrenergic system participates in early T cell development through the α-AR induced signaling pathway (Kavelaars, 2002; Pešić et al., 2009). The α-AR receptors detected in the rat and human thymuses are not observed in peripheral blood mononuclear cells, suggesting that the expression of these receptors is strictly regulated during the T-cell development (Kavelaars, 2002). It is also interesting to note that non-lymphoid cells represent the major populations expressing α1-AR, even though distinct thymocyte populations could also express this receptor, mainly in immature thymocytes (Pešić et al., 2009).

Of note, phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts norepinephrine to epinephrine, was also detected in the cortex and medulla of the rodent thymus, with higher density in the cortex (Warthan et al., 2002). Although the specific cell type expressing this enzyme was not detected, thymocytes would be an appropriate candidate due to tyrosine hydroxylase expression in these cells (Warthan et al., 2002; Leposavić et al., 2008). The presence of this enzyme in the thymus suggests *de novo* synthesis of epinephrine in the thymus, and that thymocytes could uptake NE and convert to epinephrine (Warthan et al., 2002; Leposavić et al., 2008). Taking into account that the affinity of epinephrine for β2-AR is much higher than NE and that β2-AR is the major β-AR subtype in the thymus

(Marchetti et al., 1994), it is conceivable to think that such a conversion could enhance β2-AR driven effects upon thymocyte differentiation (Leposavić et al., 2008).

Dopamine (DA) levels were also detected in rat thymocytes, although in low levels (Pilipović et al., 2008). On the other hand, DA stores were reported in the rat thymus, located mainly in the corticomedullary and medulla of the thymic lobules, alongside vesicular monoamine transporters and thymic cells that express dopaminergic receptors (Mignini et al., 2009). This indicates that catecholamines, and especially DA, of thymic origin, can modulate the final stages of intrathymic T cell differentiation (Mignini et al., 2009, 2013; Lifantseva et al., 2016).

Immunoreactivity for the five dopamine receptor subtypes (D1, D2, D3, D4, and D5) were detected in the thymic tissue, with higher expression in cortico-medullary junction and medulla (Mignini et al., 2009, 2013). Since D1 and D3 expression is detected in the rat thymus as early as day 16 of fetal development (E16) and D5 appeared 1 day later, both DA stimulatory (D2, D3, and D4) and inhibitory (D1 and D5) pathways may be involved in the early thymus organogenesis and upon the initial events of thymocyte differentiation and migration (Lifantseva et al., 2016). Accordingly, DA receptors expression pattern is modified as thymocytes differentiate into the CD4SP or CD8SP subpopulations, and between them, suggesting that the intrathymic dopaminergic system plays a role on thymocyte lineage progression within the organ (Mignini et al., 2013).

Encompassing all monoamine neurotransmitters, thymic cells express components of the serotonergic system, as exemplified by the production of serotonin by thymocytes. Moreover, serotonergic receptors are detected in both embryonic and adult rat thymocytes (**Table 1**; Stefulj et al., 2000; Lifantseva et al., 2017) and their signaling seems to be related to the control of cytokine production (IL-4 and IL-2) by developing thymocytes (Shenoy et al., 2013).

The intrathymic peptidergic neurotransmitter complex, produced by thymocytes and microenvironmental cells (in addition to nerve fibers) comprises neuropeptide Y (NPY), somatostatin (SOM), substance P (SP), calcitonin-gene-related-peptide (CGRP), neurotensin, vasoactive intestinal polypeptide (VIP), PACAP (pituitary adenylate cyclase-activating peptide) as well as the oxytocin and Arg-vasopressin, which play a role in the thymus microenvironment as well as lymphocyte maturation (Silva et al., 2006; Mignini et al., 2011). In the human thymus, NPY and VIP are the major peptidergic components modulating T cell development (Medina et al., 2000; Silva et al., 2006; Mignini et al., 2011). The intracellular signaling mediated by NPY through different functional NPY receptors exerts an inhibitory chemotactic and proliferative effect on the developing thymocytes, suggesting a role for NPY in intrathymic T cell migration and emigration, which can be modified by the aging process (Medina et al., 2000). The presence of NPY^{+} fibers in the thymus associated with mast cells and macrophages, in addition to thymocytes, suggests a role for NPY in the maintenance of thymic non-lymphoid cell populations, which, in turn, contributes to thymocyte development (Mignini et al., 2011, 2014).

As regards VIP, it has been shown that it induces the differentiation of DP into CD4-SP cells, as ascertained by T cell lines (Pankhaniya et al., 1998). In a second vein, it has been demonstrated that DP and SP, but not DN thymocytes, express VIP and VIP 1 receptor (VIP1-R) genes, indicating a possible autocrine/paracrine role for VIP in thymocyte differentiation, proliferation, and survival (Delgado et al., 1996a,b). Interestingly, *in vitro* thymocyte proliferation and cytokine production were inhibited after the TCR/CD3 complex stimulation in the presence of VIP (Xin et al., 1994), although *in vivo,* effects on thymocyte proliferation were not observed following VIP treatment. However, rat thymic cells that received VIP antagonists (VIP-A1) had enhanced mitotic activity (Trejter et al., 2001), indicating that VIP effects are dependent on the thymic microenvironmental components. VIP also seems to inhibit cell death as it has also been demonstrated that both VIP, as well as PACAP27 and PACAP38 peptides, can inhibit spontaneous thymocyte death and dexamethasone-induced death through a single receptor, VIP-R (Delgado et al., 1996a). Of note is also the fact that NPY, VIP, and PACAP inhibit thymocyte chemotactic response to N-Formyl-methionyl-leucyl-phenylalanine, a well-known lymphocyte chemoattractant (Schubert and Müller, 1989).

Yet, human thymocytes express different subtypes of somatostatin receptor (SSRs) during the developing process, which are activated upon binding with their ligands, the somatostatin (SOM) produced by TECs. The intracellular pathways mediating the SOM-dependent activities are involved in the maturation and selection of T cell repertoire through regulating the proliferation and maturation process of immature thymocytes, which include their migration through thymus stroma, cytokine production, and thymic export (Ferone et al., 2002; Silva et al., 2006).

Substance P is endogenously produced in the thymus from different species by thymocytes and TECs, being mainly involved in the thymocytes rescue from spontaneous and NK-1R antagonist (SR140333)-induced apoptosis (Santoni et al., 2002; Silva et al., 2006).

Calcitonin gene-related peptide (CGRP) and its cognate receptor are also constitutively expressed in the thymus (Kurz et al., 1995). Both ligand and receptor are expressed by cells in the medulla and corticomedullary junction of the thymic lobules, suggesting the involvement of CGRP upon thymocyte proliferation during positive selection (Kurz et al., 1995; Mignini et al., 2014), although definitive functional studies are still needed.

Lastly, oxytocin and vasopressin are present in the human thymus and TECs can constitutively produce both neurohormones (Moll et al., 1988; Robert et al., 1991; Savino et al., 2016). Their function in thymocyte development is still not clear, but they seem to be involved in the control of CD8-SP cells cycle (Robert et al., 1991; Hansenne et al., 2005; Savino et al., 2016), as well as in the expression of tissue-specific antigens by the thymic epithelium, with consequent effect upon thymocyte selection process, particularly negative selection events (Geenen and Savino, 2019).

Overall, neurotransmitters exert a complex role in thymic microenvironment and, as consequence, in thymocyte

differentiation. Since a diverse number of thymic cells are able to secrete and respond to those molecules, neurotransmitters can act directly on thymocytes or on stromal and endothelial cells. Hence, this data can lead us to the hypothesis that neurotransmitters affect the release of immunocompetent T-cells which will alter peripheral immune responses.

CONCLUSION

Taken together, the data discussed herein unravel the complex range of interactions in the thymus that are mediated by neurotransmitters and can modulate the thymus physiology by the induction and/or inhibition of cell survival, proliferation, differentiation, and migration. Accordingly, neurotransmitters seem to provide a complex network of interactions, involving microenvironmental cells, endothelial cells and developing thymocytes ultimately modulating the intrathymic T-cell development. Conceptually, these findings lead to the notion that neurotransmitters, of both neuronal and non-neuronal origin, have distinct functions in the thymus, which impact T-cell development. In this respect, it is plausible that thymic homeostasis is partially dependent on neurotransmitters, and therefore, pathological alterations in the neurotransmitters mediated circuits in the thymus may have consequences upon normal thymocyte development. Yet, the molecular mechanisms of action and control of intrathymic production and release of these neurotransmitters remain an open space for further investigation.

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All authors equally contributed to the production of this manuscript.

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5-Hydroxytryptamine, Glutamate, and ATP: Much More Than Neurotransmitters

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5-hydroxytryptamine (5-HT) is derived from the essential amino acid L-tryptophan. Although the compound has been studied extensively for its neuronal handling and synaptic actions, serotonin 5-HT receptors can be found extra-synaptically and not only in neurons but in many types of mammalian cells, inside and outside the central nervous system (CNS). In sharp contrast, glutamate (Glu) and ATP are better known as metabolism-related molecules, but they also are neurotransmitters, and their receptors are expressed on almost any type of cell inside and outside the nervous system. Whereas 5-hydroxytryptamine and Glu are key regulators of the immune system, ATP actions are more general. 5-hydroxytryptamine, ATP and Glu act through both G protein-coupled receptors (GPCRs), and ionotropic receptors, i.e., ligand gated ion channels. These are the three examples of neurotransmitters whose actions as holistic regulatory molecules are briefly put into perspective here.

Keywords: serotonin, adenosine receptors, purinergic signaling, P2 receptors, immune system, heart, COVID-19, inflammatory bowel disease

INTRODUCTION

Neurotransmitters like dopamine or serotonin are more known for their actions in the central nervous system (CNS). However, they act both in the CNS and in the periphery as regulators of multiple functions and are key in maintaining whole body homeostasis. Recent studies suggest a dopamine link between the gut and the CNS that is likely mediated by cells of the immune system (see Franco et al., 2021 for review). Lymphocytes and other cells of the myeloid lineage express many receptors for neurotransmitters: dopamine, serotonin, glutamate, etc. It seems that the immune system has evolved in parallel to the nervous system and that the same molecules regulate their functioning. Even the immunological synapse, constituted by the dendritic/antigen-presenting cells and the lymphocyte, is very similar to the canonical synapse established between two neurons or between a neuron and a myocyte (neuromuscular junction). A detailed account of the novel aspects of every neurotransmitter is out of the scope of the present article. We have decided to put into perspective three examples with their analogies and differences, namely 5-hydroxytryptamine (5-HT), glutamate (Glu) and ATP. Glu and ATP share their involvement in cell's metabolism. 5-HT and Glu share their ability to regulate events of the immune system: from

antigen presentation and T-cell activation, to inflammation. 5-HT, Glu and ATP, taken as regulatory molecules, share being the endogenous agonists of two types of cell surface receptors: G protein-coupled receptors (GPCRs) and ionotropic receptors. These examples provide, in our opinion, a perspective on the novel opportunities for better understanding the interrelationships between different organs of the mammalian body and for better knowledge of the physiopathological mechanisms of disease. The extracellular effective concentration of each of these 3 molecules depends on release and uptake, that may vary from tissue to tissue (and from cell to cell). Addressing the mechanisms and proteins involved in release/uptake is out of the scope of the present perspective article. However, it is worth noting that the therapeutic possibilities of receptors for ATP, Glu or 5-HT are limited due to the huge variety of receptors, i.e., by the need of finding highly selective drugs. An alternative approach is to target the transporters. This approach was successful in the case of the Prozac^{R} antidepressant drug, that was approved in 1988 and whose active principle, fluoxetine, acts as an inhibitor of 5-HT reuptake, mainly at the level of the presynaptic neuron (Benfield et al., 1986; Fuller, 1986; Sommi et al., 1987).

GLUTAMATE

As pointed out by Hertz (2006), acceptance by the neurosciences community of glutamate as the major excitatory transmitter in the CNS did not come until 1984 (Fonnum, 1984; **Figure 1**). Apart from being one of the 20 amino acids in mammalian proteins, Glu is, directly or indirectly, involved in the metabolism of all cells in the human body. Transamination between Glu and alpha-ketoglutarate is the link to the Krebs cycle, which operates in every cell (erythrocytes are an exception). Its effects as regulator are mediated by metabotropic receptors, which are members of the GPCR superfamily and 3 types of ligand-gated ionotropic channels known as: N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate receptors[1] (Watkins and Jane, 2006). The function as neurotransmitter is mainly mediated by ionotropic receptors that, as metabotropic receptors, are not only expressed in the postsynaptic membrane but also extrasynaptically and in neural cells other than neurons (Borroto-Escuela et al., 2018). Among many other possible examples within the CNS, astrocytes use Glu and glutamine for glia-neuron communication and it is estimated that 20% of cerebral glucose consumption is used to provide energy for Glu metabolism and Glu-mediated actions (Hertz, 2006).

The number of Glu receptors and of receptor combinations is enormous. Ionotropic receptors are constituted by various subunits that form homomers or heteromers. The number of combinations to form functional ligand-gated ion channels is very high as there are, as of today, 16 human genes coding for subunits: 7 of NMDA, 4 of AMPA and 5 of kainate receptors. On top of that, the number of human metabotropic receptors is 8 ($mGlu_1$ to $mGlu_8$, Alexander et al., 2019), and they can assemble to form homo- or heterodimers, further increasing the number of possible combinations (Duthey et al., 2002; Doumazane et al., 2011; Gregory and Goudet, 2021). Consistent with early regulatory role in Evolution these receptors are not only found in the nervous system but in cells of every any mammalian organ/tissue (O'Rourke and Boeckx, 2020). Glu effects in the periphery are difficult to ascertain due to the doubts on whether the effects are receptor mediated or metabolic. The use of pharmacological tools has, however, circumvented this issue in some cases, for example in the immune system. On the one hand, metabotropic receptors are key modulators of events taking place in the so-called immunological synapse. Dendritic cells release Glu in a non-vesicular manner and using the X_c^- cystine/glutamate antiporter. In the absence of antigens the expressed Glu receptors impede activation, whereas after productive antigen presentation, different mGlu receptors are expressed to enhance T cell proliferation and secretion of proinflammatory cytokines (Pacheco et al., 2006). On the other hand, circulating cells of the white lineage express Glu receptors thus showing that they may respond to the amino acid; resting and activated lymphocytes plus human cell lines of lymphoid origin express, at least, $mGLu_1$ and $mGlu_5$ receptors (Pacheco et al., 2004). It is an open question whether blood Glu levels are important in the control of immune function or it is required a particular environment of cell-to-cell communication in which Glu is released to act locally. Thus, it is unlikely that Glu acts in endocrine fashion. Reviews on Glu role of the physiology of dendritic and T cells are found elsewhere (Pacheco et al., 2007, 2009). Nicoletti et al. (2007) provided an authoritative review on Glu receptor involvement in other non-neural systems and in cancer. In fact, recent work has focused on the facilitation in cancer progression by ionotropic Glu receptors, that arise as targets to manage and refine anti-cancer management (Stepulak et al., 2014; Ribeiro et al., 2017; Ma et al., 2020).

Glu receptor-based therapeutic strategies will require, first, identifying the most convenient target in each disease and, second, the development of highly selective drugs or of allosteric modulators (Nicoletti et al., 2011; Ahmed et al., 2020; Qu et al., 2021).

ATP

This part of the article is dedicated to the discoverer of purinergic nerves, Prof. Geoffrey Burnstock, who was a fine and inspiring scientist (Hoffmann et al., 2020; Abbracchio, 2021; Franco, 2021). ATP may be released by different cells, including peripheral and central neurons (see Burnstock, 2007 and references therein). Neither identification of ATP as neurotransmitter nor convincing colleagues of this fact (storage in vesicles, release to the synaptic cleft after challenge, etc.) was an easy task. An historical perspective from discovery to the state of the art in 2014 is found in Burnstock (2014). Similar to Glu, ATP is involved in metabolism; actually, ATP is key for life on Earth. Probably for this reason Evolution selected ATP levels as a snitch for homeostasis. The drop in ATP levels would

[1] https://www.guidetopharmacology.org/

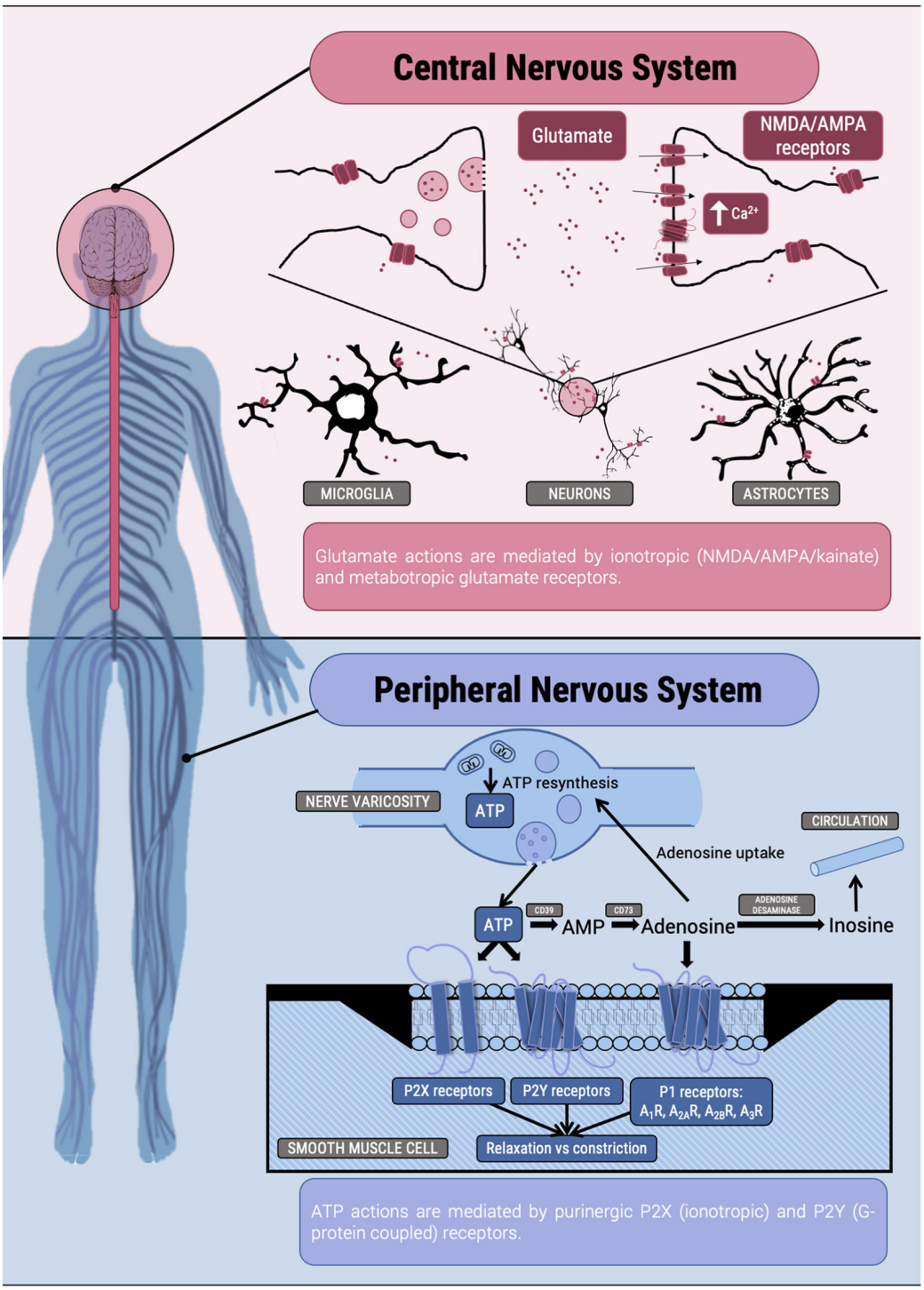

FIGURE 1 | Scheme of some actions of Glu and ATP in the nervous system.

be considered an emergency signal. As for Glu, ATP was first detected as a neurotransmitter to later being found in many systems and is now considered a holistic regulator of whole-body homeostasis. As a neurotransmitter it has a key role in the peripheral nervous system and cumulative evidence points to a key role in visceral pain; Burnstock wrote in 2016 a review that "*describes purinergic mechanosensory transduction involved in visceral, cutaneous, and musculoskeletal nociception and on the roles played by receptor subtypes in neuropathic and inflammatory pain*" (Burnstock, 2016). ATP is also involved in

pain derived from cancer via P2X receptors (Wen-Jun et al., 2020; Zhang et al., 2020).

Not as high as for Glu receptors but the number of so-called purinergic receptors, that respond to ATP but also to ADP and pyrimidine nucleotides, is very high. Ionotropic receptors are known as P2X and GPCRs are denoted as P2Y (**Figure 1**). From X-ray crystallography it is deduced that ionotropic receptors are either homotrimers or heterotrimers, and 7 are the subunits so far discovered ($P2X_1$ to $P2X_7$). Soon after the cloning of the genes for P2Y receptors and of subunits of P2X receptors, it was clear that extracellular ATP was not only acting as neurotransmitter (Illes et al., 2020; Jacobson et al., 2020). Actually, much of its action is mediated by receptors located in different cells and in different cell locations, e.g., not only in the myocyte membrane part of the neuromuscular junction and not only in smooth muscle cells but also in lymphocytes (see below), the kidney (Vallon et al., 2020), the lung (Wirsching et al., 2020), etc.

As for Glu, and for similar reasons, it is considered that ATP as regulator acts in microenvironments in which close cell-to-cell communication occurs. ATP action in the CNS is important, not necessarily due to neurotransmission mechanisms but via activation by ATP on the multiple P2X and P2Y receptors that are expressed in neural cells. An excellent account of the purinergic action in the brain was probably one of the last papers of Burnstock (2020). Again, the immune system appears as the target of many studies devoted to the role of nucleotides acting via P2X and P2Y receptors. The laboratory of F. di Virgilio has contributed to significantly increase the knowledge on the role of P2 receptors in the control of the immune system function; indeed other laboratories have also contributed (see Rayah et al., 2012; Di Virgilio et al., 2017, and references therein). The $P2X_7$ receptor is attracting attention in the last decade for its potential as target in some diseases. Surely, $P2X_7$ receptor is involved in regulating infection and inflammation but there is controversy as how to pharmacologically address its potential to make it provide antiinflammatory actions (Di Virgilio et al., 2017; Savio et al., 2018). We would like to add that purinergic signaling is relevant in a prevalent disease for which there are few therapeutic opportunities, namely intestinal inflammation (Longhi et al., 2017).

The section cannot be complete without presenting P1 receptors; there are four (A_1, A_{2A}, A_{2B}, and A_3) all belonging to the GPCR superfamily (**Figure 1**). Virtually all extracellular ATP is broken down by various nucleotidases to produce adenosine, which is the endogenous agonist of purinergic P1 receptors. Thus, all the regulatory action triggered by ATP is later followed by adenosine receptor-mediated signaling. Adenosine is not considered a neurotransmitter but a neuromodulator. P1 receptors are found in almost any neural cell and also in virtually any peripheral cell in the human body. Importantly, cardiovascular actions of the compound were detected in the twenties (past century) and about 30 years later it was suggested as potential drug to combat tachycardias. Adenosine in bolus administration was approved for the treatment administered with great success to critically ill patients suffering from paroxysmal tachycardia (Drury and Szent-Györgyi, 1929; Wolf and Berne, 1956; Buckley et al., 1959). Further drugs targeting P1 receptors have not been approved as quickly as those targeting adrenergic (beta-blockers) and histamine (H2 antagonists) receptors. P1 receptor antagonist are considered as generally safe. Then natural compounds, caffeine and theophylline, which are non-selective antagonists of these receptors, are approved for human use (Franco, 2009; Franco et al., 2013; Oñatibia-Astibia et al., 2016, 2017), and more recently, an antagonist of the A_{2A} receptor, istradefylline (Mizuno and Kondo, 2013; Kondo et al., 2015), has been approved (in Japan and United States) for adjuvant therapy in Parkinson's disease. In addition, there are good prospects for P1 receptor ligands to make more effective the immune-based anti-cancer therapy (Ohta and Sitkovsky, 2011; Hatfield and Sitkovsky, 2016; Fong et al., 2020; Sitkovsky, 2020; Willingham et al., 2020).

Finally, we would like to end this section with the very interesting discovery of purinergic (both P1 and P2) receptor involvement in acupuncture-mediated health benefits. To our knowledge the first ATP/acupuncture link was established by Smith and Kenyon (1973) and the first adenosine/acupuncture links were established by Liu et al. (1994a,b). Acupuncture leads to the release of ATP that acts on P2 receptors to produce, among other, analgesia (Burnstock, 2013; Tang et al., 2016, 2019; Tang and Illes, 2020). The subsequent conversion of ATP to adenosine leads to the participation of proximally located P1 receptors (Trento et al., 2021). In summary, there is consensus in that a significant number of acupuncture interventions leads to ATP release to the extracellular milieu and P2 (and P1) engagement.

SEROTONIN

5-HT (or serotonin) is known for being one of the main neurotransmitters in the nervous system of mammals; it plays a crucial role as modulator of essential elements of our daily life such as mood, sleep, social behaviors, learning and appetite (Barnes and Sharp, 1999; Gingrich and Hen, 2001; Canli and Lesch, 2007). Although serotonin is best known for regulating higher functions, it is also key in maintaining whole body homeostasis.

While there is a tendency to describe 5-HT as a brain-based and derived molecule, the 95% of the 5-HT is synthetized, stored and released into the gut. This is mainly accomplished by the enterochromaffin cells (Gershon et al., 1965; Costedio et al., 2007; Gershon and Tack, 2007), which are capable of synthetizing it from L-tryptophan by the action of the tryptophan hydroxylase (TPH, Yu et al., 1999). The role of 5-HT in the correct functionality of the gut is a controverted theme, as it is reported that 5-HT modulates gut peristalsis, secretion and motility (Gershon and Tack, 2007), whereas other studies using TPH1 knockout mice, which are unable to synthetize 5-HT, show no alteration of intestinal motility (Li et al., 2011). Nonetheless, it is suggested that serotonin receptors may be targets to combat irritable bowel syndrome (Gershon and Tack, 2007).

There is evidence of a link between the gut and the brain that is somehow mediated by 5-HT (Hooper et al., 2012; Carabotti et al., 2015). This has also been demonstrated for dopamine and it is a hot topic in relationship with Parkinson's disease

(see Franco et al., 2021 for recent review). Among the most likely mechanisms of intercommunication, cells of the immune system may be mediators that respond to 5-HT due to the expression of some of its receptors. It is known that different cell types of the innate immune system, including dendritic cells (O'Connell et al., 2006), monocytes (Finocchiaro et al., 1988) and mastocytes (Tamir et al., 1982) express components of the serotoninergic machinery, e.g., TPH, serotonin transporters and serotonin receptors. Accordingly, it is possible to generate 5-HT and/or to respond to it. A similar trend is found in the adaptive immune system in cells such as T and B lymphocytes (Gordon and Barnes, 2003). Actually, almost any blood cell, except erythrocytes, expresses enzymes related to 5-HT handling and transport, and serotonin receptors. Overall it is assumed that the compound regulates from inflammation to chemotaxis (Pfeiffer et al., 1985; Konig et al., 1994).

5-HT action depends on the cell type and, eventually, in the activation state, for instance in resting versus activated blood cells. On the one hand, 5-HT enhances the production of IFN-γ by human NK cells (Hellstrand et al., 1993) and alters the amount of cytokines released by dendritic cells in such a way that the release of TNF-α and IL-6 is decreased while that of IL-1β and IL-8 is increased (Idzko et al., 2004). On the other hand, in macrophages, 5-HT reduces LPS-induced release of pro-inflammatory cytokines also skewing macrophages to the anti-inflammatory M2 phenotype. The involved receptors are 5-HT_{2B} and 5-HT_7 (Quintero-Villegas and Valdés-Ferrer, 2019). Furthermore, the use of a 5-HT_7 receptor agonist in experimental sepsis reduces the plasma levels of IL-6, IL-1β, and of lung NFκB, thus reducing the death rate (Cadirci et al., 2013). However, there are scenarios in which 5-HT potentiates inflammation via other receptors, namely serotonin 5-HT_3 and 5-HT_4 receptors (Salaga et al., 2019). The different capacity to modulate cytokine production in opposite outcomes shows that serotoninergic action is receptor and tissue-specific.

Probably the first hints of the regulatory role of 5-HT in inflammation came from evidence of alterations on both immune function and serotoninergic signaling in some psychiatric disorders. Although the 5-HT blood levels have not real diagnostic value, they serve to establish some interesting correlations. Onore et al. (2012) documented alterations in immune function in subjects with autism spectrum disorder that correlated with high blood 5-HT levels (Veenstra-Vanderweele and Blakely, 2012). Correlations between depression and alterations in both immune and serotonergic systems are widely reported in the literature. Levels of pro-inflammatory cytokines such as IL-6 and IFN-γ from T-cells and IL-β1 and TNF-α from the cells of the innate immune system correlate with depression (Raison et al., 2009; Rybka et al., 2016). Also, there is a correlation between risk of depressive moods and single nucleotide polymorphisms found in inflammatory genes crucial for T-cell function. There is even evidence postulating that antidepressant medication can have anti-inflammatory action. For example selective 5-HT reuptake inhibitors, which are used as antidepressants, are reported to reduce and normalize cytokine levels in depressed patients (Basterzi et al., 2005).

SARS-CoV-2 infection leads to COVID-19 that consists of 3 phases that are not manifested in all patients: asymptomatic or low-asymptomatic phase, mildly asymptomatic phase and severe phase (Ayres, 2020). In addition, neurological post-COVID-19 alterations are unexpected side effects of unknown causes and coursing without any defined trend. In psychiatric patients these potential neurological manifestations may aggravate their status. Stressful and anxious conditions may be responsible, at least in part, of post-COVID-19 side effects (Salleh, 2008; Jones and Thomsen, 2013); an increase of IL-6 may also impact in the severity of depression (Zorrilla et al., 2001). In the most severe cases, one of the most harmful events is a robust inflammation with a cytokine release syndrome that may lead to 10-, 100-, and even 1,000-fold increase in the reference blood levels of IL-1β, IL-6, and TNF-α (Chatenoud, 1989). About 40% of the patients treated in the critical care unit display an acute respiratory distress syndrome associated with pneumonia (Guan et al., 2020). In summary, COVID-19 may lead to overstimulation of the immune system (Giamarellos-Bourboulis et al., 2020) and it has been suggested that inhibition of serotonin reuptake may reduce exacerbated inflammation (Hamed and Hagag, 2020). Remarkably, this hypothesis has been supported by a recent study reporting less risk of intubation or death in patients taking antidepressant medication (Hoertel et al., 2021). Inhibitors of 5-HT reuptake could be a novel and effective treatment for severe COVID-19 cases as they could ameliorate the cytokine release syndrome thanks to their capacity to reduce the levels of pro-inflammatory cytokines. For example, these compounds significantly increase oxygen saturation in patients with severe chronic obstructive pulmonary disease (Perna et al., 2004). At the level of speculation, the compounds may act by both reducing inflammation and the levels of anxiety and stress of COVID-19 patients. Data from research with other viruses show that inhibiting serotonin uptake has antiviral properties by downregulating (in lymphocytes) the expression of HIV receptor and coreceptor (Greeson et al., 2016) or by reducing viral replication of Coxsackevirus B4 (Alidjinou et al., 2015). Indeed pancreatic infection with this virus is completely abolished by the widely used 5-HT reuptake inhibitor, fluoxetine (Alidjinou et al., 2015).

Although 5-HT is normally known as a neurotransmitter, it is undeniable that its acts as a holistic modulator in virtually all organs and cell types. It is assumed that every cell in the mammalian body expresses one (or more) of the reported serotonin receptors (14 in total): 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, 5-HT_4, 5-HT_{5A}, 5-HT_{5B}, 5-HT_6, and 5-HT_7 (Barnes et al., 2021). Except the 5-HT_3, which is an ionotropic receptor, the others belong to the GPCR superfamily; Gi is the canonical G protein of the 1 and 5A types, Gs is the canonical G protein of 4 and 6 receptor types, and members of the 2 type preferentially couple to Gq. There are many unknowns due to the large number of receptors and the potentially different signaling pathways triggered by serotonin receptor activation, but any new scientific achievement will be extremely useful to understand 5-HT action and to identify further serotonin-related targets (transporters or receptors) to combat a variety of diseases.

DEDICATION

This article is dedicated to Prof. Geoffrey Burnstock, discoverer or purinergic nerves and founder of a new field of research, who left us in 2020. *Que la tierra te sea leve profesor.*

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

RR-S, JL, JC, GN, and IR-R compiled information and manuscript from the literature. IR-R and RF did the conceptualization and designed this perspective manuscript. RF and JC wrote the first draft. All authors edited the manuscript and agreed in submitting the final version.

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Locus Coeruleus to Paraventricular Thalamus Projections Facilitate Emergence From Isoflurane Anesthesia in Mice

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Locus coeruleus (LC) sends widespread outputs to many brain regions to modulate diverse functions, including sleep/wake states, attention, and the general anesthetic state. The paraventricular thalamus (PVT) is a critical thalamic area for arousal and receives dense tyrosine-hydroxylase (TH) inputs from the LC. Although anesthesia and sleep may share a common pathway, it is important to understand the processes underlying emergence from anesthesia. In this study, we hypothesize that LC TH neurons and the TH:LC-PVT circuit may be involved in regulating emergence from anesthesia. Only male mice are used in this study. Here, using c-Fos as a marker of neural activity, we identify LC TH expressing neurons are active during anesthesia emergence. Remarkably, chemogenetic activation of LC TH neurons shortens emergence time from anesthesia and promotes cortical arousal. Moreover, enhanced c-Fos expression is observed in the PVT after LC TH neurons activation. Optogenetic activation of the TH:LC-PVT projections accelerates emergence from anesthesia, whereas, chemogenetic inhibition of the TH:LC-PVT circuit prolongs time to wakefulness. Furthermore, optogenetic activation of the TH:LC-PVT projections produces electrophysiological evidence of arousal. Together, these results demonstrate that activation of the TH:LC-PVT projections is helpful in facilitating the transition from isoflurane anesthesia to an arousal state, which may provide a new strategy in shortening the emergence time after general anesthesia.

Keywords: locus coeruleus, paraventricular thalamus, general anesthesia, emergence, optogenetics and DREADDs

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INTRODUCTION

General anesthesia (GA) is a reversible state of unconsciousness induced by various kinds of general anesthetics (Brown et al., 2010; Brown et al., 2011). Achieving a controllable and smooth emergence from general anesthesia is of particular importance for surgical patients. Traditionally, emergence from general anesthesia has been treated as a purely passive process. However, accumulating evidence suggest that emergence from anesthesia is also an active and controllable process (Kelz et al.,

Abbreviations: LC, Locus coeruleus; AVV, adeno-associated virus; EEG, electroencephalogram; TH, tyrosine hydroxylase; Cre, Cre recombinase; GA, general anesthesia; DA, dopamine; NE, norepinephrine; LORR, loss of the righting reflex; RORR, return of the righting reflex; CNO; clozapine N-oxide, BSR, burst suppression ratio; ChR2; channelrhodopsin-2; EYFP, enhanced yellow fluorescent protein; NAc, nucleus accumbens; BNST, bed nucleus of the stria terminalis.

2019). Rencently, there has been more interests on actively and rapidly inducing emergence from GA (Tarnal et al., 2016; Kelz et al., 2019).

The locus coeruleus (LC), a brainstem pontine nucleus of noradrenergic neurons, plays a critical role in core behavioural and physiological processes (Chandler et al., 2019; Poe et al., 2020). Recent studies have illustrated how the complex efferent system of LC neurons selectively mediates specific behaviours (Berridge and Waterhouse, 2003; Reyes et al., 2008; Li et al., 2018). It has been reported that LC TH population promotes immediate sleep-to-wake transitions (Carter et al., 2010). However, it remains to be defined whether LC TH neurons participate in anesthesia arousal.

The paraventricular thalamus (PVT), the main component of the dorsal thalamic midline, has been implicated in chronic stress, emotion and arousal (Colavito et al., 2015; Hua et al., 2018; Ren et al., 2018). PVT neurons are primarily excitatory neurons (Frassoni et al., 1997). A recent study has shown that lesion of the PVT causes fragmentation of wakefulness and that the optical stimulation of PVT glutamatergic neurons during sleep induces transitions to wakefulness (Ren et al., 2018). This study also shows that activation of PVT neurons induce an acceleration of emergence from general anesthesia. Considered together, both LC and PVT are involved in the anesthesia arousal. These data thus support that both LC and PVT might be involved in the anesthesia arousal, a circumstance that has not been experimentally demonstrated yet.

Given that retrograde neural tracing confirmed the anatomical TH:LC-PVT projection (Beas et al., 2018), we hypothesized that TH:LC-PVT projections played an important role in anesthesia arousal. In this study, we found that LC TH neurons were active during passive emergence from isoflurane anesthesia. Chemogenetic activation of LC TH neurons elicited behavioral and cortical arousal from isoflurane anesthesia. Furthermore, c-Fos expression increased in the PVT after LC TH neurons activation. Optogenetic excitation of the TH:LC-PVT projections promoted cortical arousal and emergence from anesthesia. In addition, chemogenetic inhibition of this circuit prolonged emergence process. Thus, these results reveal that the TH:LC-PVT projections play a critical role in accelerating anesthesia emergence in rodents.

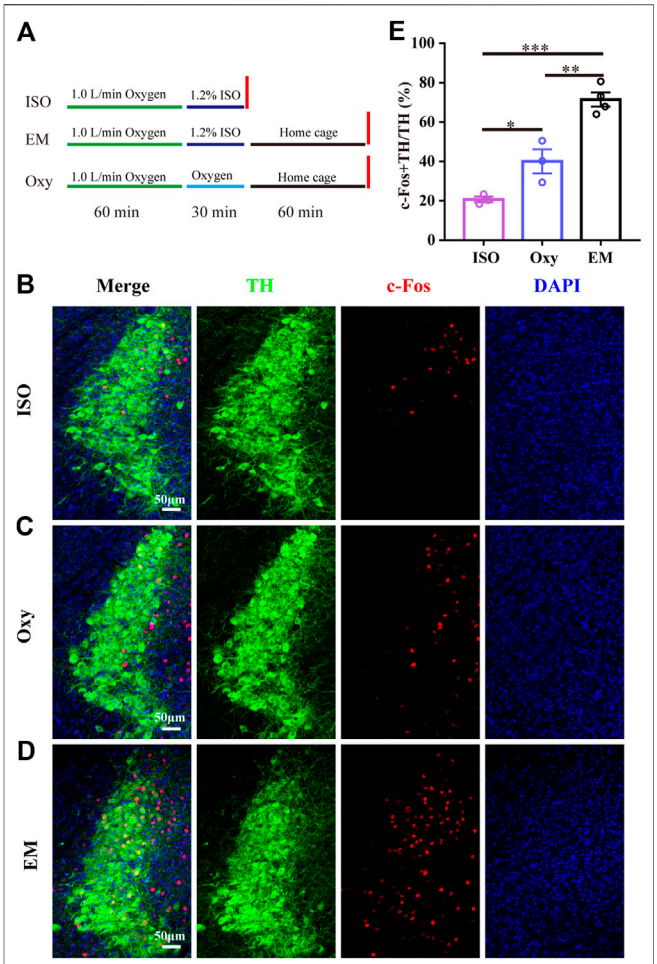

FIGURE 1 | LC TH neurons are active during passive emergence from isoflurane anesthesia. **(A)** The experimental protocol for the c-Fos quantification experiments in three groups of mice. First group (ISO) received 1.2% isoflurane anesthesia and was euthanized before awakening. The second group (EM) received the same isoflurane anesthesia treatment and underwent passive emergence from anesthesia. The third group (Oxy) did not underwent isoflurane anesthesia and received oxygen. **(B–D)** Representative photomicrographs showing the co-localization of c-Fos (red) and TH neurons (green) in LC for ISO group, Oxy group and EM group, respectively. **(E)** The percentage of c-Fos + TH/TH in ISO group (n = 3), Oxy group (n = 3) and EM group (n = 4). Data were analyzed by one-way ANOVA with post hoc Bonferroni multiple-comparison test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Data shown as means ± SEM.

MATERIALS AND METHODS

Animals

The guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals were strictly followed throughout the experiment. Wild type C57BL/6J male mice and tyrosine hydroxylase (TH)-internal ribosome entry site (IRES)-Cre in heterozygous male mice (8–12 w) weighing 25–30 g were used for all experiments and randomly assigned to groups. All mice were given food and water *ad libitum* with a controlled temperature (22 ± 2°C) under a 12 h/12 h light/dark cycle (lights on between 7:00 and 19:00). All efforts were made to minimize the number of mice used. All mice were acclimated to the animal facility at least 3 days before the initiation of the experiments. To minimize the influence of the diurnal rhythm of sleep-wakefulness on results, all experiments were performed between 10:00 and 19:00. We chose to perform the experiment between 10:00 and 19:00 because PVT neurons display state-dependent activity patterns with the highest activity shown during the hours of darkness (Ren et al., 2018). Thus, we conducted the experiments when the activity of PVT neurons is relatively low. All animal experiments were conducted according to protocols approved by the Institute of Animal Care Committee at Zhongnan Hospital of Wuhan University.

Surgery

Mice were anesthetized with pentobarbital sodium and placed in a stereotaxic apparatus (Narishige, Japan). Adeno-associated viruses (AAVs, BrainVTA Technology Co., Ltd., China) were injected bilaterally into the LC (coordinates, bregma: AP = −5.26 mm; ML = ± 1.05 mm; DV = −3.75 mm, 200 nl for each site). After each injection, the glass micropipette was kept in place for 5 min to ensure sufficient virus diffusion. All viruses were sufficiently expressed for at least 3 w before the experiments. Optical fibers (diameter: 200 μm, Coocore Inc., China) were implanted above the PVT (coordinates, bregma: AP = −1.1 mm; ML = +0.55 mm; DV = −2.8 mm, with a 10° angle towards the midline). For chemogenetic inhibition experiments, a guide cannula (OD, 0.48 mm; ID, 0.34 mm; length, 3.5 mm; RWD) was implanted above the PVT (coordinates, bregma: AP = −1.1 mm; ML = +0.55 mm; DV = −2.75 mm, with a 10° angle towards the midline). Two stainless steel screw electrodes was implanted on the skull surface (coordinates, recording electrode, bregma: AP = +1.75 mm; ML = −0.4 mm; reference electrode: cerebellum). The coordinates were measured from bregma according to The Mouse Brain in Stereotaxic Coordinates (Paxinos and Franklin, 2004). The EEG electrodes were affixed to the skull with Super-Bond C&B and dental acrylic. After surgery, mice were allowed to recover for at least 1 w before the experiments.

Experiment Protocol

c-Fos Expression in the LC After Exposure to Isoflurane With or Without Emergence

C-Fos immunohistochemistry was used to determine the activity of the LC TH neurons during emergence from isoflurane anesthesia. The time course of the c-Fos expression experiment is schematized in **Figure 1A**. Briefly, one group of mice was exposed to 30 min 1.2% isoflurane (ISO) anesthesia protocol and sacrificed immediately at the end of the anesthesia. Another group of mice was also exposed to the anesthesia protocol for 30 min and then allowed to emerge (EM) in their home cage, 60 min later mice were sacrificed. The last group of mice was exposed only to 100% oxygen (Oxy) for 30 min and then sacrificed 60 min later. The isoflurane concentration was monitored with Drager Vamos Plus Anesthesia Monitor (Dräger, Germany) and maintained constant through the experiment.

Chemogenetic Activation of LC TH Neurons Experiment

AAV-hSyn-DIO-hM3Dq-mCherry or AAV-EF1a-DIO-EYFP was injected bilaterally into the LC of TH-Cre mice. Clozapine N-oxide (CNO, dissolved in saline, Enzo Life Science Inc., United States) was administered intraperitoneally (i.p.) to activate the hM3Dq receptors 1 h before isoflurane infusion. Saline treatment was used as control. We employed the c-Fos, a neural activity marker (Carter et al., 2017), to verify that the LC TH neurons could be activated by CNO. Briefly, LC TH-hM3Dq mice were injected i. p. with CNO (1 mg/kg) or saline and then killed 90 min later for c-Fos immunohistochemistry.

In the induction and emergence test, mice were placed in a cylindrical chamber filled with 1.2% isoflurane in oxygen at a flow rate of 1 L/min. The time interval from isoflurane exposure to loss of righting reflex (LORR) was recorded as induction time. The cylindrical chamber was gently rotated by 90° every 15 s until the mouse on its back and loss the ability to right itself (Luo et al., 2018; Wang et al., 2019). In addition, the time interval between mice removed from the chamber and return righting reflex (RORR) with all four paws on the ground was recorded as emergence time. LORR and RORR are well-established surrogate measure of rodent animals for determining the onset and recovery of general anesthesia, respectively (Franks, 2008). The investigator was blinded to the allocated groups. In electroencephalogram (EEG)

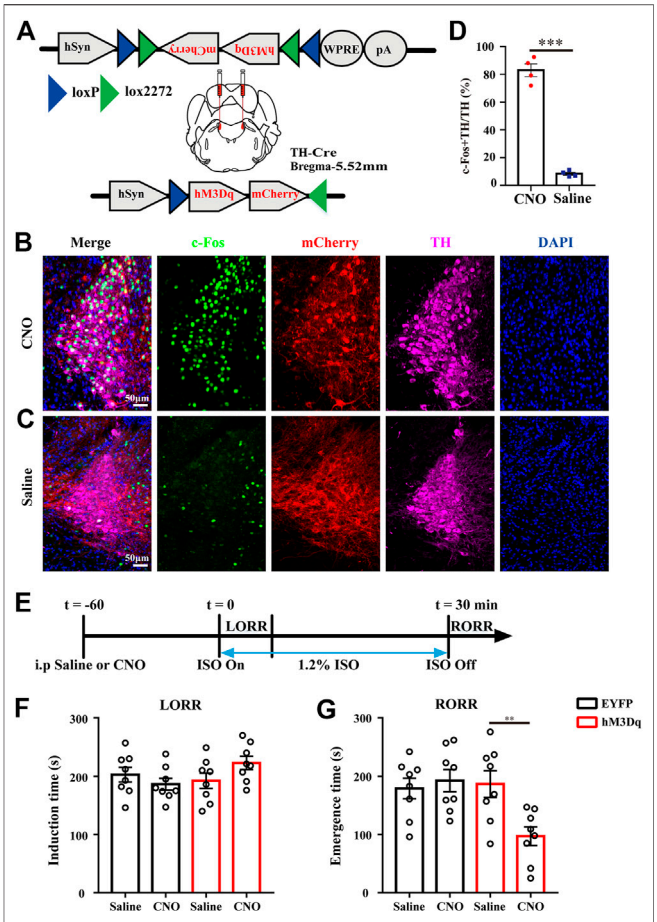

FIGURE 2 | LC TH neurons activation facilitates emergence from anesthesia. **(A)**Schematic diagram showing the injection target of LC in TH-Cre mice and representation of hM3Dq vectors injected under control of the hSyn promoter. **(B,C)** Representative images showing the co-localization of c-Fos (green), TH (purple) and mCherry (red) in LC of hM3Dq mice treated with CNO **(B)** or saline **(C)**. **(D)** The percentage of c-Fos + TH/TH in the LC of hM3Dq mice injected i. p. with CNO or saline (n = 4 in each group, the two-tailed unpaired t-test, ***p < 0.001). **(E)**Timeline for measuring induction and emergence time in hM3Dq mice injected i. p. with CNO or saline. **(F)** Induction time with exposure to 1.2% isoflurane after injected i. p. with saline or CNO of EYFP (black) and hM3Dq (red) mice, respectively (n = 8 in each group, two-way RM ANOVA with Tukey's multiple comparisons test). **(G)** Emergence time with exposure to 1.2% isoflurane after treatment with saline or CNO of EYFP (black) and hM3Dq (red) mice, respectively (n = 8 in each group, two-way RM ANOVA with Tukey's multiple comparisons test, **p < 0.01). Data shown as means ± SEM.

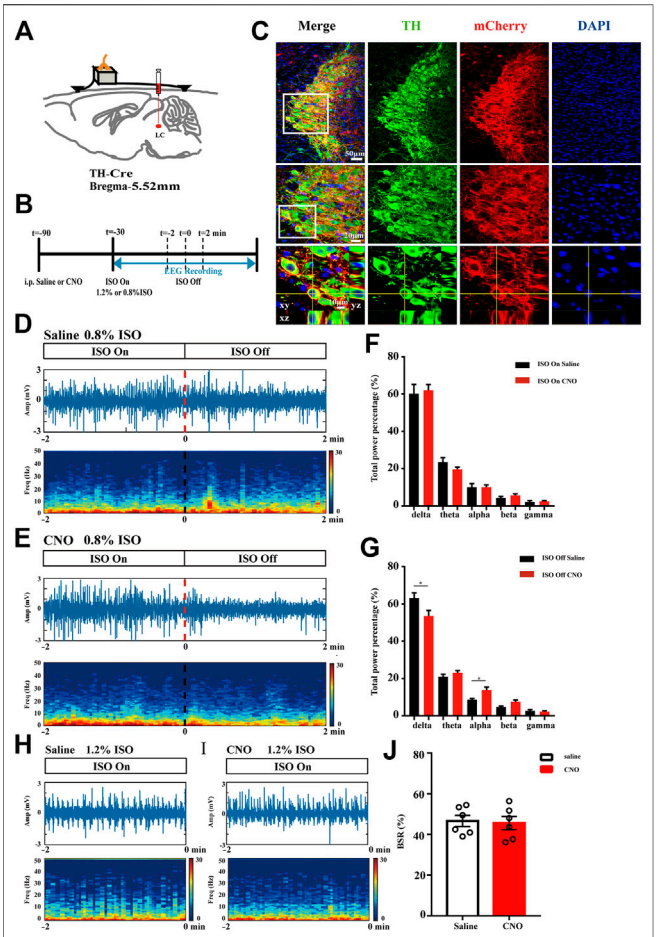

FIGURE 3 | LC TH neurons activation drives cortical arousal. **(A)** Schematic diagram showing bilateral virus injection and EEG recording. **(B)** Timeline for EEG recording during isoflurane anesthesia and emergence in hM3Dq mice injected i. p. with CNO or saline. **(C)** Specific expression of hM3Dq-mCherry (red) in LC TH (green) neurons. Insets were amplified in below. **(D,E)** A representative raw EEG trace (top) and the corresponding power spectrogram (bottom) during 0.8% isoflurane anesthesia and emergence period in hM3Dq-saline **(D)** and hM3Dq-CNO **(E)** mice. 0 on the time axis indicates the cession of isoflurane. **(F)** Power spectrum analysis was conducted on 2 min window before cession of isoflurane in hM3Dq-saline and hM3Dq-CNO mice (n = 6 in each group, the two-tailed unpaired t-test). **(G)** In the emergence period, power spectrum analysis was conducted from 0.8% isoflurane inhalation stop to 2 min window (n = 6 in each group, the two-tailed unpaired t-test, $^*p < 0.05$). **(H,I)** A representative raw EEG trace (top) and the corresponding power spectrogram (bottom) during 1.2% isoflurane anesthesia in hM3Dq-saline **(H)** and hM3Dq-CNO **(I)** mice. **(J)** In the anesthesia period, BSR was calculated at 2 min window before cession of isoflurane in hM3Dq-saline and hM3Dq-CNO mice (n = 6 in each group, the two-tailed unpaired t-test). Data shown as means ± SEM.

test, after 1 h of CNO (1 mg/kg, i. p.) or saline injection, mice were anesthetized (1.2% or 0.8% isoflurane), and EEG were recorded continuously for 30 min (**Figure 3B**). The burst suppression ratio (BSR) was calculated as the percentage of EEG suppression in the 2 min interval before cessation of 1.2% isoflurane. Power spectrum analysis was conducted on the 2 min interval before cessation of isoflurane and the 2 min interval after cessation of isoflurane.

c-Fos Expression in the PVT After Chemogenetic Activation of LC TH Neurons

In order to weaken the unfamiliar stimuli and new environment stimuli, mice were placed into the cylindrical chamber individually for at least 1 h with 100% oxygen (1 L/min) flowing starting at least 3 days before the experiment. On the day of the experiment, CNO (1 mg/kg) or saline were administered i. p. in hM3Dq mice 1 h before isoflurane application. After 1 h conditioning, 1.2% isoflurane was administered to the chamber for 30 min. At the end of isoflurane anesthesia, mice were killed for c-Fos immunohistochemical experiments. There was no other operation for mice during this time, and all experiments were conducted in the light phase.

Optogenetic Activation Experiments

For optogenetic activation experiments, AAV-EF1a-DIO-ChR2(H134R)-EYFP or AAV-EF1a-DIO-EYFP was injected bilaterally into the LC of TH-Cre mice. After at least 3 w, optical fibers were implanted above the PVT. During test, optical stimulation was performed with blue light (473 nm) from a laser (Shanghai Dream Lasers Technology Co., Ltd., China). The power of the 473 nm laser at the tip of the fiber was 12–15 mW measured using an optical power meter (PM100D, Thorlabs, United States). The 473 nm blue light pulse trains (10 Hz, 10 ms duration) were applied to excite the TH:LC-PVT projections (Ren et al., 2018). In the optogenetic experiments, EEG were recorded continuously for 40 min. Optical manipulation (10 Hz, 1 min) was applied after constant 30 min 1.2% or 0.8% isoflurane administration (**Figure 5B**). BSR and power spectrum analysis were calculated from 1 min interval before and during the optical stimulation. In the induction test (**Figure 5I**), optical stimulation was administered at the start of 1.2% isoflurane delivery until mice lost righting reflex in the cylindrical chamber. In the emergence test (**Figure 5J**), mice were anesthetized for 30 min, once the isoflurane delivery turned off, sustained optical stimulation was delivered until the mice returned righting reflex.

Chemogenetic Inhibition Experiments

For chemogenetic inhibition experiments, AAV-hSyn-DIO-hM4Di-mCherry or AAV-EF1a-DIO-EYFP was injected bilaterally into the LC of TH-Cre mice. After at least 3 w, guide cannulae were implanted above the PVT. CNO (5 μM, 500 nl) or the same volume saline was locally infused into PVT at a rate of 250 nl/min by a microinjection pump (KD Scientific, United States) in each mouse 20 min before anesthesia. After injection, the infusion cannula was left for 2 min to allow for infusion. Each mouse was administered with CNO or saline separated by at least 3 days washout period. The experimental protocol was shown in **Figure 6B**. Induction time and emergence time were recorded as described above.

Electroencephalogram Analysis

EEG signals were amplified and collected by a RM-6240EC device (Chengdu Instrument Factory, China) at a sampling rate of 800 Hz. The signals were filtered between 0.1 and 100 Hz. The total power spectrum, BSR, and power distribution in each

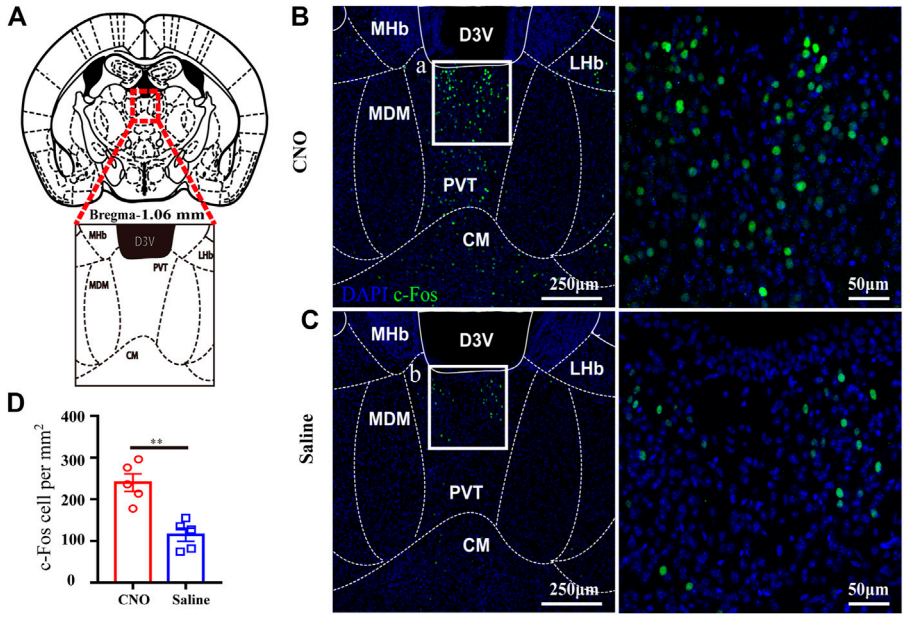

FIGURE 4 | LC TH neurons activation enhances neuronal activity in PVT. **(A)** Schematic diagram showing the location of the PVT and local structures in mouse. **(B,C)** Representative photomicrographs of c-Fos expression in the PVT in hM3Dq- CNO and hM3Dq-saline mice. Insets were amplified on the right. **(D)** Quantification of c-Fos positive nuclei in the PVT of hM3Dq-saline and hM3Dq-CNO mice (n = 5 in each group, two-tailed unpaired t-test, $^{**}p < 0.01$). Data shown as means ± SEM.

frequency band were completed by MATLAB 2016a (The MathWorks, United States). The BSR was calculated by using previously established methods (Vijn and Sneyd, 1998; Vazey and Aston-Jones, 2014). Power spectrum analysis was conducted as previously described (Wang et al., 2019). Delta, theta, alpha, beta, gamma and total spectral powers were calculated using the frequency bands 1–4 Hz, 4–8 Hz, 8–15 Hz, 15–30 Hz and 30–50 Hz, respectively. Relative powers were calculated by dividing the averaged signal power across each band's frequency range by the total power of 1–50 Hz.

Immunohistochemistry

Mice were deeply anesthetized and transcardially perfused with ice-cold 0.9% saline, followed by 4% paraformaldehyde. The brains were harvested and post-fixed overnight at 4°C. Brain was continuously sectioned into 40 μm coronal slices using a vibratome (Leica VT1000S, Leica Biosystems, Germany). Brain sections were incubated in 1 × PBS solution containing 0.3% Triton X-100 and 10% normal goat serum (Bosterbio, United States). Sections were incubated with primary antibody diluted in PBS plus 10% normal goat serum overnight at 4°C for 24 or 48 h. Incubated slices were then washed by PBS three times (10 min each), then incubated for 90 min with secondary antibody in PBS, and subsequently washed three times in PBS (10 min each) at room temperature. Primary antibodies used: rabbit polyclonal anti-c-Fos (1:3000, Synaptic Systems Cat# 226003, RRID:AB_2231974, Germany) and mouse monoclonal anti-TH (1:1000, Millipore Cat# MAB318, RRID: AB_2201528, United States). Secondary antibodies were Alexa Fluor 488 goat anti-rabbit (Thermo Fisher Scientific Cat# A-11008, RRID:AB_143165, United States), Alexa Fluor 488 goat anti-mouse (Thermo Fisher Scientific Cat# A-11029, RRID: AB_2534088, United States), Alexa Fluor 594 goat anti-rabbit (Thermo Fisher Scientific Cat# A-11012, RRID:AB_2534079, United States), Alexa Fluor 594 goat anti-mouse (Thermo Fisher Scientific Cat# A-11032, RRID:AB_2534091, United States) and Alexa Flour 647 donkey anti-mouse (Thermo Fisher Scientific Cat# A-31571, RRID:AB_162542, United States). Secondary antibodies were diluted with 1:600 upon use. After staining DAPI (5 μg/ml, Cat#: 10236276001, Roche, Switzerland) and washing three times with PBS, sections were mounted on glass microscope slides, dried and covered with mounting media.

Imaging and Quantification

Images were captured using a laser confocal fluorescent microscope (Lecia sp8, Germany). Quantification of c-Fos within the PVT was performed as described previously (Ren et al., 2018; Ao et al., 2020). Briefly, the number of c-Fos positive neurons in the PVT was counted at alternate sections from approximately Bregma −0.94 and to −1.46 mm (five sections per mouse) along the rostral-caudal axis. The investigator blinded to the group information manually counted the c-Fos-positive cells in a ROI (a square area with a side length of 500 μm) located within PVT in each section using ImageJ (National Institutes of Health) software. Colocalization of c-Fos and TH immunofluorescence in the LC was quantified on adjacent sections from approximately bregma −5.3 to −5.7 mm (five sections per mouse) using ImageJ. The location of PVT or LC was based on mouse brain atlas (Paxinos and Franklin, 2004). If the viral transfection was poor, or the optical fiber and guide cannula site was incorrect, the mouse's associated data was excluded.

Statistical Analysis

All statistical analyses were performed using the Graphpad Prism 7.0. All data were presented as mean ± SEM. Sample sizes were determined based on previous publications on general anesthesia regulation using chemogenetic and optogenetic approaches (Taylor et al., 2016; Zhou et al., 2018; Yin et al., 2019; Wang et al., 2020). We first performed the normality test on each dataset using the Shapiro-Wilk test. Otherwise, non-parametric tests were used. Two-tailed paired and unpaired t tests, one-way ANOVA and two-way ANOVA followed by the Tukey's multiple comparisons test were used. Statistical significance was set at $p < 0.05$.

RESULTS

The LC TH Neurons Are Active During Passive Emergence From Isoflurane Anesthesia

Given that LC plays an important role in changing the arousal level (Carter et al., 2010; Benarroch, 2018), it is possible that LC participates in emergence from anesthesia. Thus, we subjected the wild type mice to 100% oxygen exposure alone (Oxy group), 1.2% isoflurane anesthesia (ISO group) and 1.2% isoflurane anesthesia with emergence (EM group) followed by examining c-Fos expression in LC. The experimental protocol was shown in **Figure 1A**. We calculated the percentage of c-Fos + TH/TH, and found that the percentage in EM group (71.5 ± 3.6%, $n = 4$) was significantly higher than that in the ISO group (20.7 ± 1.5%, $n = 3$) and Oxy group (40.1 ± 6.1%, $n = 3$) (**Figures 1B–E**) [F (2, 7) = 40.82, $p = 0.0001$]. The massive increase in c-Fos activity in the EM group suggests that the LC TH neurons may participate in passive emergence from isoflurane anesthesia.

LC TH Neurons Activation Facilitates Emergence From Anesthesia

The higher activity in LC TH neurons during anesthesia emergence suggests that the LC may be important for recovery from anesthesia. To express the DREADD receptor hM3Dq in LC TH neurons, AAV-hSyn-DIO-hM3Dq-mCherry were stereotaxically injected in LC of TH-cre mice. This AAV construct contains a FLEX switch, which includes loxP and lox2272, to ensure stable expression of hM3Dq in Cre-expressing cells (**Figure 2A**). To confirm hM3Dq receptor excitation lead to increased TH neuronal activity *in vivo*, the LC TH-hM3Dq mice were injected i. p. with CNO (1 mg/kg) or saline and killed 90 min later for c-Fos immunohistochemistry (**Figures 2B,C**). We calculated the percentage of c-Fos + TH/TH and found that CNO increased the percentage in the LC, it was 83.0 ± 4.6% in the CNO group ($n = 4$) and 8.5 ± 1.1% in the saline group ($n = 4$)[t (6) = 15.79, $p = 0.0003$] (**Figure 2D**).

To test whether activation of LC TH neurons could modulate the speed of induction and emergence from anesthesia, 60 min after CNO or saline injection, mice were subjected to the induction and emergence test. **Figure 2E** showed the

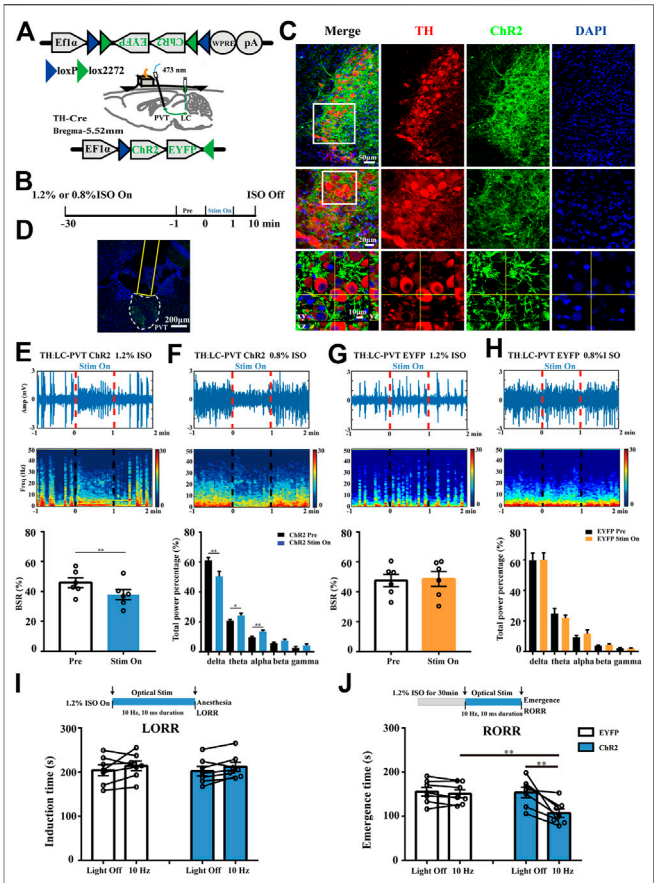

FIGURE 5 | Optogenetic activation of TH:LC-PVT terminals promotes arousal from isoflurane anesthesia. **(A)** Schematic of bilateral LC injection, optic fiber implantation and EEG recording. **(B)** Experimental protocol for optical stimulation and EEG recording during isoflurane anesthesia. After 30 min inhalation of isoflurane, the mice were optically stimulated for 1 min (stim on). **(C)** Specific expression of ChR2-EYFP (green) in LC TH (red) neurons. Insets were amplified in below **(D)** Representative ChR2-EYFP terminals and the location of optical fiber in the PVT. **(E,F)** A representative raw EEG trace (top) and the corresponding power spectrum (middle) at 1 min before, 1 min during and 1 min after optical stimulation in ChR2 mouse during 1.2% **(E)** or 0.8% **(F)** isoflurane anesthesia. BSR was calculated at 1 min before and 1 min during optical stimulation in ChR2 mice (bottom, $n = 6$, two-tailed paired t-test, $^{**}p < 0.01$). EEG power percentage of different frequency bands at 1 min before (black) and 1 min during (blue) optical stimulation in ChR2 mice (bottom, $n = 6$, the two-tailed paired t-test, $^{*}p < 0.05$, $^{**}p < 0.01$). **(G,H)** A representative raw EEG trace **(top)** and corresponding power spectrum **(middle)** at 1 min before, 1 min during and 1 min after optical stimulation in EYFP control mouse during 1.2% **(G)** or 0.8% **(H)** isoflurane anesthesia. BSR was calculated at 1 min before and 1 min during optical stimulation in control mouse (bottom, $n = 6$, the two-tailed paired t-test). EEG power percentage of different frequency bands at 1 min before (black) and 1 min during (blue) optical stimulation in control mice (bottom, $n = 6$, two-tailed paired t-test) **(I)** Experimental protocol for optical stimulation during 1.2% isoflurane anesthesia induction **(top)**. Effects of activating TH:LC-PVT terminals on the induction time in EYFP (control) and ChR2 mice (bottom, $n = 7$ in each group, two-tailed paired t-test). **(J)** Experimental protocol for optical stimulation during 1.2% isoflurane anesthesia emergence **(top)**. Effects of activating TH:LC-PVT terminals on the emergence time in EYFP (control) and ChR2 mice (bottom, $n = 7$ in each group, two-tailed paired t-test or two-tailed unpaired t-test, $^{**}p < 0.01$). Data shown as means ± SEM.

experimental protocol. We set EYFP infected mice treated with either saline or CNO and hM3Dq infected mice treated with saline as control groups. Compared with control groups, activation of LC TH neurons did not obviously change induction time by two-way ANOVA multiple comparisons [$F(1, 7) = 3.884$, $p = 0.0894$] (**Figure 2F**). However, activation of LC TH neurons shortened the emergence time (**Figure 2G**). The hM3Dq mice injected with CNO (97.3 ± 15.9 s, $n = 8$) showed a significant decrease in emergence time relative to the hM3Dq-saline group (186.8 ± 22.8 s, $n = 8$), the EYFP-CNO group (192.5 ± 19.0 s, $n = 8$) and the EYFP-saline group (179.1 ± 17.7 s, $n = 8$) [$F(1, 7) = 17.54$, $p = 0.004$] (**Figure 2G**). Taken together, these results suggest that LC TH neurons activation facilitates emergence from isoflurane anesthesia.

LC TH Neurons Activation Drives Cortical Arousal

Having performed behavior tests to reveal facilitated anesthesia recovery with LC TH neurons excitation, we next evaluated the impact of LC TH neurons activation on the brain activity under anesthesia by EEG recording. **Figure 3A** showed schematic of bilateral LC injection and EEG recording. Timeline for EEG recording during isoflurane anesthesia and emergence was shown in **Figure 3B**. Specific expression of hM3Dq-mCherry in LC TH neurons were shown in **Figure 3C**. Power spectrum analysis showed that the percent distributions of the five frequency bands were similar between the CNO group ($n = 6$) and the saline group ($n = 6$) while the mice were exposed to 0.8% isoflurane (**Figures 3D–F**). However, upon switching off the 0.8% isoflurane, the CNO group showed a significant decrease of delta power [$t(10) = 2.325$, $p = 0.0424$] and increase of alpha power [$U = 3.000$, $p = 0.0152$] in the first 2 min (**Figures 3D,E,G**). However, during the 1.2% anesthesia period, there was no significant difference in BSR between the CNO group ($n = 6$) and the saline group ($n = 6$) [$t(10) = 0.2392$, $p = 0.8157$] (**Figures 3H–J**). Therefore, the results demonstrate that the LC TH neurons excitation promotes cortical arousal during anesthesia emergence.

LC TH Neurons Activation Enhances Neuronal Activity in Paraventricular Thalamus

Given that the PVT is a central node for wakefulness and it heavily receives projections from the LC (Beas et al., 2018; Ren et al., 2018), we evaluated PVT neurons activity after LC TH neuronal excitation in hM3Dq mice. **Figure 4A** showed the location of the PVT and local structures. The representative photomicrographs of c-Fos expression in the PVT of hM3Dq mice treated with CNO or saline were shown in **Figures 4B,C**. After CNO or saline injection in LC TH-hM3Dq mice respectively, the number of c-Fos positive nuclei in PVT was 239.8 ± 21.3% in the CNO group ($n = 4$) and 114.7 ± 15.7% in the saline group ($n = 4$) [$t(10) = 4.733$, $p = 0.0019$] (**Figure 4D**), suggesting that LC TH neurons excitation enhances the PVT activity, and the PVT may be a critical target of LC to drive emergence from anesthesia.

Optogenetic Activation of TH:LC-PVT Terminals Promotes Arousal From Isoflurane Anesthesia

To test whether the TH:LC-PVT projections could promote arousal from general anesthesia, we placed optical fibers above the PVT of LC TH-ChR2-EYFP mice to stimulate the LC TH terminals (**Figure 5A**). The timeline for optogenetic activation and EEG recording during isoflurane anesthesia was shown in **Figure 5B**. After 3–4 w virus expression, specific expression of ChR2-EYFP were observed in LC TH neurons (**Figure 5C**). In addition, dense EYFP fibers were observed in the PVT (**Figure 5D**). Optically stimulating LC TH fibers in the PVT reduced BSR from 45.8 ± 3.3% to 37.9 ± 3.4% during 1.2% isoflurane anaesthesia in ChR2 mice ($n = 6$) [$t(5) = 5.305$, $p = 0.0032$] (**Figure 5E**), but not in control mice ($n = 6$) [$t(11) = 0.7344$, $p = 0.4957$] (**Figure 5G**). Besides, optogenetic activation of LC TH fibers produced a decrease in delta power ($n = 6$) [$t(5) = 4.668$, $p = 0.0055$], whereas the power in theta ($n = 6$) [$t(5) = 2.719$, $p = 0.0418$] and alpha ($n = 6$) [$t(5) = 5.098$, $p = 0.0038$] was statistically increased. However, there was no difference in the EEG power spectrum in control mice with stimulation ($n = 6$) (**Figure 5H**). In addition, optical excitation of LC TH fibers in the PVT altered EEG activity in the ChR2 mice from an anesthesia state to an awake-like state and returned to an anesthesia state after cessation of stimulation (**Figures 5E,F**).

To further investigate whether activation of TH:LC-PVT projections were sufficient to influence induction and emergence from isoflurane anesthesia, we designed the experiment as shown in **Figures 5I,J**. Optical stimulation of LC TH terminals in the PVT did not significantly alter the induction time under 1.2% isoflurane in ChR2 ($n = 7$) [$t(6) = 1.197$, $p = 0.2763$] or control mice ($n = 7$) [$t(6) = 1.211$, $p = 0.2714$] (**Figure 5I**). Notably, photostimulation of TH:LC-PVT projections in ChR2 mice facilitated emergence from isoflurane anaesthesia. The emergence time was shortened from 153.6 ± 11.9 s to 106.9 ± 9.9 s in ChR2 mice ($n = 7$) [$t(6) = 3.712$, $p = 0.0099$], but not in control mice ($n = 7$) [$t(6) = 1.465$, $p = 0.1933$] (**Figure 5J**). Taken together, these findings indicate that TH:LC-PVT projections are sufficient for anesthesia emergence.

Chemogenetic Inhibition of TH:LC-PVT Terminals Delays Emergence From Anesthesia

To verify the necessity of this specific pathway in regulating the arousal from anesthesia, we placed cannulae above the PVT of LC TH-hM4Di-mCherry mice to suppress the LC TH terminals by local CNO microinjection (**Figure 6A**). The timeline for chemogenetic inhibition and behavioral tests was shown in **Figure 6B**. After 3–4 w virus expression, specific expression of hM4Di-mCherry were observed in LC TH neurons (**Figure 6C**). The positions of the cannulae were verified (**Figure 6D**). Application of CNO or saline in hM4Di mice ($n = 7$) [$t(6) = 0.4561$, $p = 0.6643$] showed no significant difference in induction time, as well as in EYFP mice ($n = 7$) [$t(6) = 0.4375$, $p = 0.6771$] (**Figure 6E**). However, chemogenetic inhibition of TH:LC-PVT projections significantly prolonged emergence time in hM4Di mice

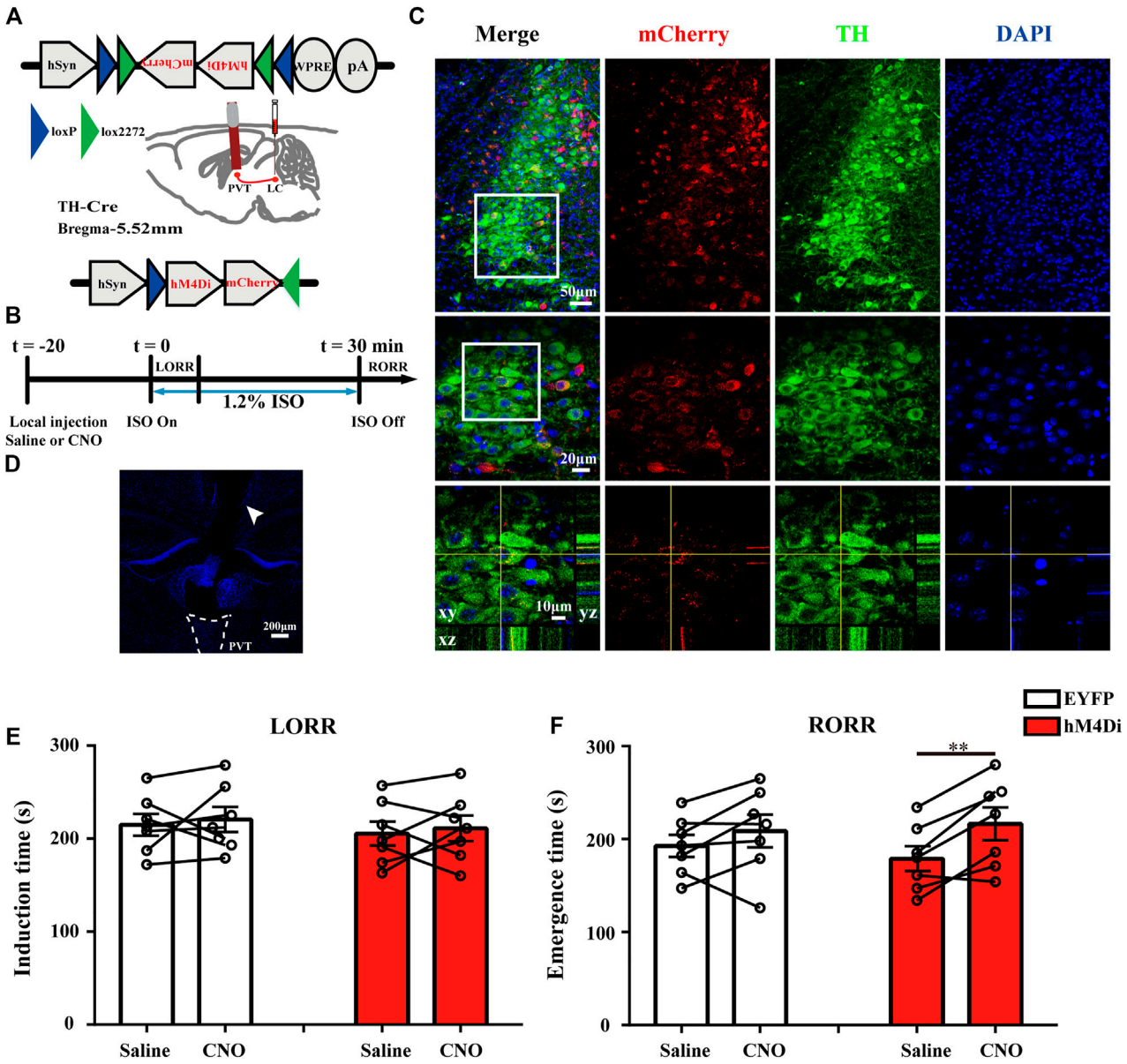

FIGURE 6 | Chemogenetic inhibition of TH: LC-PVT terminals delays emergence from isoflurane anesthesia. **(A)** Schematic of bilateral LC injection and guide cannula implantation. **(B)** Experimental protocol for chemogenetic inhibition of LC TH terminals in the PVT during 1.2% isoflurane anesthesia. **(C)** Specific expression of hM4Di-mCherry (red) in LC TH (green) neurons. Insets were amplified in below. **(D)** DAPI stained coronal brain slice showing the track of the guide cannula (white arrow head) above the PVT. **(E)** Inhibition of TH:LC-PVT terminals showed no significant changes of induction time in hM4Di mice ($n = 7$ in each group, two-tailed paired t-test). **(F)** Inhibition of TH:LC-PVT terminals significantly delayed emergence time in hM4Di mice ($n = 7$ in each group, two-tailed paired t-test, $^{**}p < 0.01$). Data shown as means ± SEM.

treated with CNO (216.4 ± 17.7 s) ($n = 7$), which compared with same mice treated with saline (178.9 ± 13.3s) ($n = 7$) [t (6) = 4.205, $p = 0.0057$] (**Figure 6F**), but not in EYFP mice ($n = 7$) [t (6) = 1.436, $p = 0.2010$] (**Figure 6F**). Thus, these findings indicate that TH:LC-PVT projections are vital for anesthesia emergence.

DISCUSSION

In the current study, we identified that LC TH neurons were active during passive emergence from isoflurane anesthesia and chemogenetic activation of LC TH neurons facilitated emergence from anesthesia. In addition, excitation of LC TH neurons promoted cortical arousal and enhanced PVT activity. Furthermore, optogenetic activation of the TH:LC-PVT projections facilitated arousal, whereas selective inhibition of this circuit delayed arousal from isoflurane anesthesia. Our findings indicate that TH:LC-PVT pathway is involved in emergence from anesthesia.

It has been demonstrated that brain regions that regulate arousal become active during anesthesia recovery process (Muindi et al., 2016; Luo et al., 2018; Luo et al., 2020).

Additionally, the histaminergic (Luo and Leung, 2009), cholinergic (Irmak and de Lecea, 2014), noradrenergic (Berridge et al., 2012; Fu et al., 2017), dopaminergic (Eban-Rothschild et al., 2016; Taylor et al., 2016), and orexinergic pathways (Sakurai, 2007) have been implicated in linking sleep-wake regulation and the emergence from GA. Our c-Fos immunostaining data reveals that LC TH neurons are active during emergence from isoflurane anesthesia. A previous study has documented that LC noradrenergic neurons promote the emergence from general anesthesia (Vazey and Aston-Jones, 2014). Thus, our study extends previous findings on the role of LC noradrenergic system in anesthetic arousal. Specifically, activation of LC TH neurons drove cortical arousal, accelerated behavioral emergence from isoflurane anesthesia and enhanced the PVT activity. Thus, the results indicate that LC TH neurons probably serve as a key element to induce the recovery from GA and this effect may be mediated by activation of the PVT. It is worth noting that LC TH neurons excitation shortened anesthetic emergence but had no singnificant impact on anesthetic induction. The present work adds to the growing body of evidence that the induction and emergence processes are not merely the mirror image of each other, and emergence is an active process characterized by a distinct neurobiology (Kelz et al., 2008; Tarnal et al., 2016).

The anesthesia emergence is of critical importance (Hight et al., 2014). For example, the strategies for safer and active reversal of anesthesia can be designed to bring consciousness recovery as quickly as possible. The PVT in the thalamus's paramedian region is essential for wakefulness (Colavito et al., 2015; Ren et al., 2018; Shao et al., 2019). Furthermore, the PVT receives profuse innervation from LC TH neurons, but receives little innervation by midbrain dopaminergic neurons (Li et al., 2014; Beas et al., 2018). In the present study, optogenetic activation of the TH:LC-PVT projections drove cortical arousal and facilitated emergence, whereas inhibition of TH:LC-PVT projections prolonged emergence. The measures of increased cortical arousal, including a reduction in BSR, decreases in delta power, and increases in theta power and alpha power, were seen after optical stimulation of TH:LC-PVT projections. Thus, LC-PVT projections have a potential use to facilitate the emergence from anesthesia. In this study, we have confirmed the role of the TH:LC-PVT projections in facilitating emergence from anesthesia, however the downstream of the TH:LC-PVT pathway is not clear. The nucleus accumbens (NAc) and bed nucleus of the stria terminalis (BNST) are innervated by the PVT and play roles in sleep-wake control (Ren et al., 2018) (Hua et al., 2018), thus the TH:LC-PVT projections may activate the NAc or BNST to facilitate the emergence from anesthesia (Kirouac, 2015).

Despite the evidence supporting that LC is a noradrenergic center, accumulating data suggest that LC neurons' terminals may co-release dopamine (DA) and norepinephrine (NE) (Devoto et al., 2005; Kempadoo et al., 2016; Takeuchi et al., 2016; Beas et al., 2018). DA and NE have been studied as two different systems, however, they have many similarities such as shared biosynthetic pathway, co-release from TH terminals, innervation of similar regions, and shared intracellular signaling pathways (Ranjbar-Slamloo and Fazlali, 2019). Both NE and DA are well-known to contribute to arousal (Monti and Monti, 2007; Fu et al., 2017). Accordingly, it has been shown that methylphenidate, which is a potent inhibitor of DA and NE transporters, induces the active emergence from anesthesia, indicating that both DA and NE may have synergistic effects (Solt et al., 2011; Chemali et al., 2012).

The current study has several limitations. First, we used only male mice in the experiment. Whether this circuit also promotes anesthesia arousal in female mice remains unclear. Second, we did not measure body fat, liver function and metabolites. The slower emergence in patients under general anesthesia may be due to obesity (Casati and Putzu, 2005). However, we used mice in the normal weight range, so the difference of emergence time was less likely to be resulted from obesity in our study. Nevertheless, the obesity-induced arousal rate change needs further exploration. In addition, volatile anesthetics may be metabolized to reactive and potentially toxic intermediates (Martin, 2005). These metabolites may lead to hepatotoxicity (Singhal et al., 2010; Rajan et al., 2019). In turn, abnormal liver function and metabolism may lead to slow metabolism of anesthetics. Thus, liver function and metabolic abnormalities need to be monitored in the future study to rule out potential factors affecting arousal rate.

CONCLUSION

In summary, stimulation of the TH:LC-PVT terminals is sufficient to accelerate the transition from general anesthesia to an arousal state. A clear understanding of the neural basis is fundamental to research in clinical and basic neuroscience disciplines and anesthesia.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the **Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institute of Animal Care Committee at Zhongnan Hospital of Wuhan University.

AUTHOR CONTRIBUTIONS

YA, BY, and CZ performed the animal studies. YA, BY, CZ, and XZ performed the behaviour test. YA, CZ, BY, and XZ performed the immunohistochemistry experiments. YA, BY, CZ, BW, XZ, DX, and HX designed the research, analysed the data, wrote and edited the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.643172/full#supplementary-material

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REVIEW
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Neuroimmune Pathophysiology in Asthma

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Asthma is a chronic inflammation of lower airway disease, characterized by bronchial hyperresponsiveness. Type I hypersensitivity underlies all atopic diseases including allergic asthma. However, the role of neurotransmitters (NT) and neuropeptides (NP) in this disease has been less explored in comparison with inflammatory mechanisms. Indeed, the airway epithelium contains pulmonary neuroendocrine cells filled with neurotransmitters (serotonin and GABA) and neuropeptides (substance P[SP], neurokinin A [NKA], vasoactive intestinal peptide [VIP], Calcitonin-gene related peptide [CGRP], and orphanins-[N/OFQ]), which are released after allergen exposure. Likewise, the autonomic airway fibers produce acetylcholine (ACh) and the neuropeptide Y(NPY). These NT/NP differ in their effects; SP, NKA, and serotonin exert pro-inflammatory effects, whereas VIP, N/OFQ, and GABA show anti-inflammatory activity. However, CGPR and ACh have dual effects. For example, the ACh-M3 axis induces goblet cell metaplasia, extracellular matrix deposition, and bronchoconstriction; the CGRP-RAMP1 axis enhances Th2 and Th9 responses; and the SP-NK1R axis promotes the synthesis of chemokines in eosinophils, mast cells, and neutrophils. In contrast, the ACh-α7nAChR axis in ILC2 diminishes the synthesis of TNF-α, IL-1, and IL-6, attenuating lung inflammation whereas, VIP-VPAC1, N/OFQ-NOP axes cause bronchodilation and anti-inflammatory effects. Some NT/NP as 5-HT and NKA could be used as biomarkers to monitor asthma patients. In fact, the asthma treatment based on inhaled corticosteroids and anticholinergics blocks M3 and TRPV1 receptors. Moreover, the administration of experimental agents such as NK1R/NK2R antagonists and exogenous VIP decrease inflammatory mediators, suggesting that regulating the effects of NT/NP represents a potential novel approach for the treatment of asthma.

Keywords: asthma, neurotransmitters, neuropeptides, allergy, immunology

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INTRODUCTION

Asthma is a disease characterized by chronic airway inflammation, leading to intermittent symptoms including wheezing, dyspnea, cough, and chest tightness, in combination with variable expiratory airway obstruction. It is estimated that 334 million people suffer from this disease worldwide (Enilari and Sinha, 2019). Asthma is caused by complex interactions between the environment and genetic factors, resulting in heterogeneity in clinical presentation, inflammation,

and a possible remodeling of the airways (Tyler and Bunyavanich, 2019). Type I hypersensitivity (TIHS) is responsible for the greatest part of its pathophysiology (Kubo, 2017). However, the role of neurotransmitters (NT) and/or neuropeptides (NP) in this disease has been less explored than its inflammatory mechanisms.

Recently, anticholinergic drugs prescribed in chronic obstructive pulmonary disease (COPD) (Global Initiative for Asthma, 2020) have shown clinical efficacy in asthma when they are used as adjuvants (Novelli et al., 2012). Likewise, some experimental drugs that modulate the NT and NP response (Milara et al., 2013) have been proposed as therapeutic targets due to their physio-pathological actions in asthma. In this general review, we explain the interaction of both NT and NP with the immune system and bronchial environment in asthma, and their potential use as biomarkers and diagnostic tools, as well as their therapeutic use in patients with this disease in the future.

ASTHMA PATHOPHYSIOLOGY

A strategy used to classify asthma pathophysiology, taking into account its immunological heterogeneity, is the identification of inflammatory cellularity in fluids extracted from the airway (sputum or Bronchoalveolar lavage fluid -BALF-). There are four different groups according to the evidence provided by the cytology of the local samples: (Enilari and Sinha, 2019) eosinophilic, (Tyler and Bunyavanich, 2019) neutrophilic, (Kubo, 2017) mixed, and (Global Initiative for Asthma, 2020) paucigranulocytic. Eosinophilic inflammation is the main type of cellularity identified and is a consequence of allergic and non-allergic processes (Simpson et al., 2006). Allergy is associated with almost 60% of childhood and adult asthma, but is not the only condition that causes eosinophilic inflammation (Pearce et al., 1999; Simpson et al., 2006).

In allergic eosinophilic asthma, atopic subjects are predisposed to develop IgE-mediated allergic sensitization (atopy). Dendritic cells (DCs) take up the allergens (pollens, dust, and mold, among others), which are processed by endosomes and presented to T helper (Th) 2 lymphocytes (Th2), inducing the synthesis of a Th2 profile of interleukins (IL), such as IL-5, IL-4, and IL-13. IL-5 induces the maturation and survival of eosinophils. These cells migrate to the bronchial epithelium via chemoattractant factors, such as eotaxins (CCL11, CCL24, CCL26, and CCL5) (Teran, 2000; Rojas-Ramos et al., 2003) coupled to the CCR3 receptor, while IL-4 and IL-3 favor the change of immunoglobulin (Ig) isotype in B cells, with the subsequent production of IgE (**Figure 1**).

This immunoglobulin recognizes two types of receptors: high-affinity receptors (FcεRI) and low-affinity receptors (FcεRII or CD23). FcεRI receptors are expressed in mast cells (MCs), basophils, DCs, and eosinophils, but are also present in other cells, such as airway smooth muscle cells (ASM), epithelial, and endothelial cells. The coupling between IgE and FCεRI receptors in DCs amplifies their ability to present antigens, and in turn, the activation of allergen-specific Th2 cells is associated with the amplification of allergen-specific IgE production in a vicious cycle of the pathogenic mechanisms of allergic asthma. IgE acts in airway epithelial cells through the CD23 receptor, which is involved in the transport of IgE-allergen complexes across the polarized airway mucosal barrier (Matucci et al., 2018).

Activated eosinophils release mediators, such as major basic protein (MBP), reactive oxygen species (ROS), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8, lipid mediators (cystenil leukotrienes -LTs), and histamine. MBP can mediate epithelial cell damage, while cysLTs contribute to airway remodeling. Neutrophil recruitment is induced by IL-8, whose expression is upregulated in the airways of patients with severe asthma and mixed cellularity (Nakagome and Nagata, 2018). Histamine is also released by basophils and MCs, and is associated with the induction of bronchial smooth muscle contraction, epithelial barrier dysfunction, and the increased secretion of mucus via the H1 receptor, while its coupling with the H2 receptor increases the capillary permeability (Yamauchi and Ogasawara, 2019). Eosinophils and MCs also play a relevant role, producing cysLTS and prostaglandin D2 (PGD2). This last mediator induces eosinophil chemotaxis through a chemoattractant receptor-homologous molecule expressed on TH2 cells (CRTH2), expressed in eosinophils, basophils, Th2 cells, and innate lymphoid cell type 2 (ILC2) (Yamauchi and Ogasawara, 2019). In non-allergic eosinophilic asthma, when the epithelium is injured by any factor, it synthesizes alarmines (IL-25, IL-33, and thymic stromal lymphopoietin-TSLP), which stimulate ILC2, leading to the synthesis of a Th2 profile without allergen-specific IgE involvement, which does not require antigen processing (**Figure 1**) (Kaur and Chupp, 2019).

The pathophysiology of non-eosinophilic asthma is not yet understood. Neutrophil cellularity is associated with both Th1 and Th17 interleukin profiles, as well as with the subsequent activation of macrophages and the release of neutrophil chemokines, such as IL-8 (**Figure 1**; Paplińska-Goryca et al., 2018). Evidence shows that the interaction between specific allergens and IgE/FcεRI on the neutrophil surface enhances functional responses by increasing the secretion of neutrophil products, such as matrix metalloproteinase 9 (MMP-9), neutrophil elastase (NE), myeloperoxidase, IL-8, and ROS (Radermecker et al., 2018).

Asthma treatment is based on corticosteroids (inhaled or oral), leukotriene antagonists and/or β-adrenergic agonists. Recently, the use of anticholinergics and biological antibodies (anti-IgE/anti-IL5) was approved depending on the severity of this disease; the use of any combination reduces inflammatory biomarkers and improves the symptoms (Global Initiative for Asthma, 2020).

BRONCHIAL AIRWAY

Airway Epithelium and PNEC

The bronchial epithelium is a pseudostratified ciliated columnar epithelium with goblet cells (Rock et al., 2010). However, there are other cells, such as pulmonary neuroendocrine cells (PNECs) that constitute approximately 1% of the airway mucosa. PNECs are short pyramid cells with cytoplasmic projections to the lumen grouped in mini-clusters (five

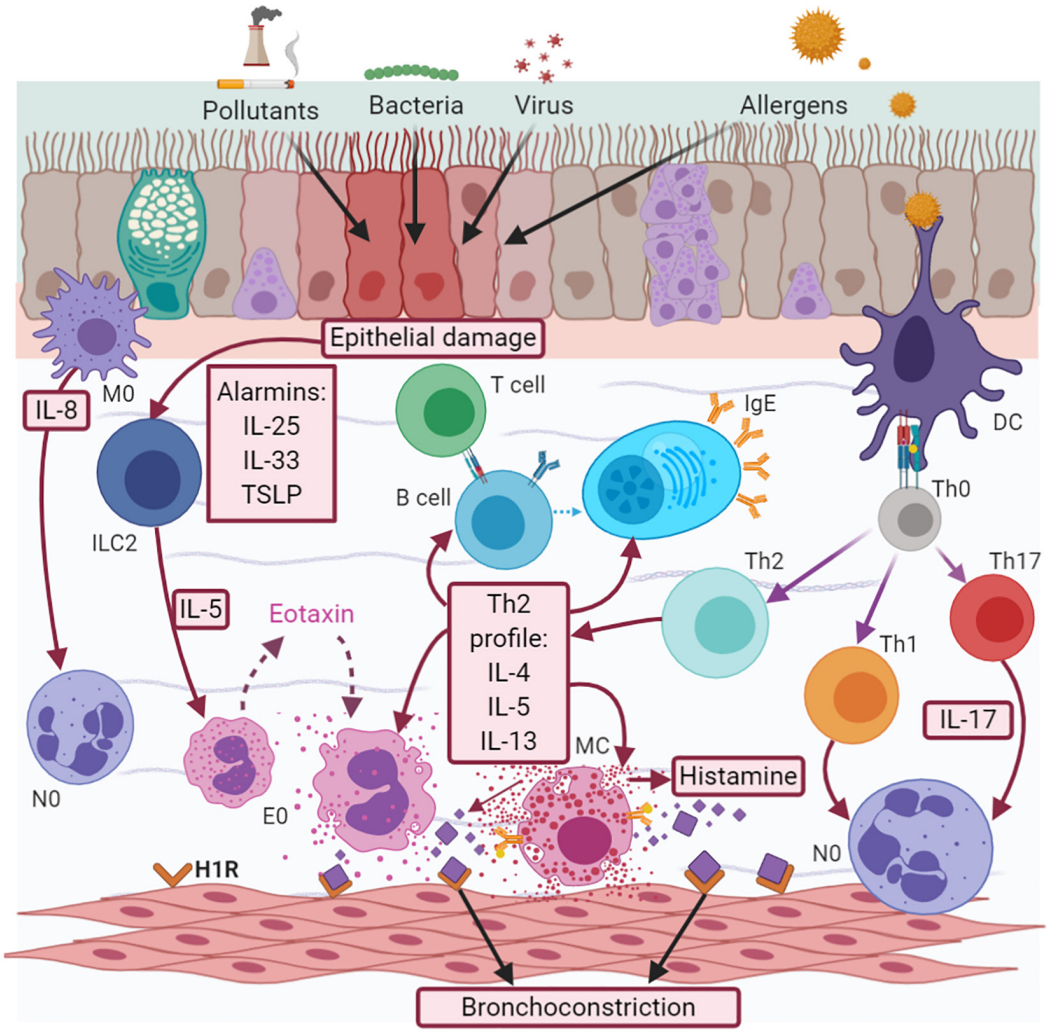

FIGURE 1 | Asthma pathophysiology. Asthma is a complex interaction between cells, cytokines and chemokines. There are two effector cell responses, neutrophilic, and eosinophilic. The eosinophilic response is the most frequent, mediated by the synthesis of a Th2 cytokine profile (IL-5, IL-4, and IL-13) and histamine released when there is an IgE-mediated allergic response; or by alarmins (TSLP, IL-25, and IL-33) in the case of a non-allergic one. Neutrophilic response is less understood and maybe a transition from early Th2 or be a consequence of early Th1/Th17 secondary to macrophage activation and IL-8 release.

cells) or neuroepithelial bodies (NEB) (>20 cells), located at branch junctions (Kuo and Krasnow, 2015). These cells contain NP, NT, and amines stored in dense-core vesicles (DCV) (Branchfield et al., 2016). The vagal fibers of the autonomic nervous system (ANS) comprise the majority of bronchial airway innervation (Kistemaker and Prakash, 2019). However, the mechanism underlying the interaction between PNEC and ANS has yet to be well described. Indeed, some studies have reported that only the NEB are innervated, and not the PNEC (**Figure 2**; Brouns et al., 2006; Kuo and Krasnow, 2015).

Innervation

Two types of nerve fibers (A and C) provide afferent innervation. Type A fibers are myelinated axons classified according to their diameter and conduction impulse. For example, Aδ fibers have a smaller diameter and a slower conduction than Aα fibers. They are considered as mechanoreceptors, and their activation depends on the deep and breathing rate (Nassenstein et al., 2018). In contrast, type C fibers are thin unmyelinic axons with slower conduction velocities than A fibers (Feldman et al., 2017), located in the glands, microvasculature, ASM, and NEB (Drake et al., 2018), and are classified as chemoreceptors and nociceptors (Feldman et al., 2017), transmitting afferent impulses when reacting with stimuli, such as changes in temperature, pH, and mediators released by tissue damage and inflammation (Narula et al., 2014), inducing reflex responses that include mucus discharge, bronchoconstriction, and cough (Undem and Carr, 2002). C fibers have specific receptors for NP, such as Substance P (SP), Neurokinin A (NKA), and Calcitonin Gene-Related Peptide (CGRP), which are involved in ASM contraction (Nassenstein et al., 2018).

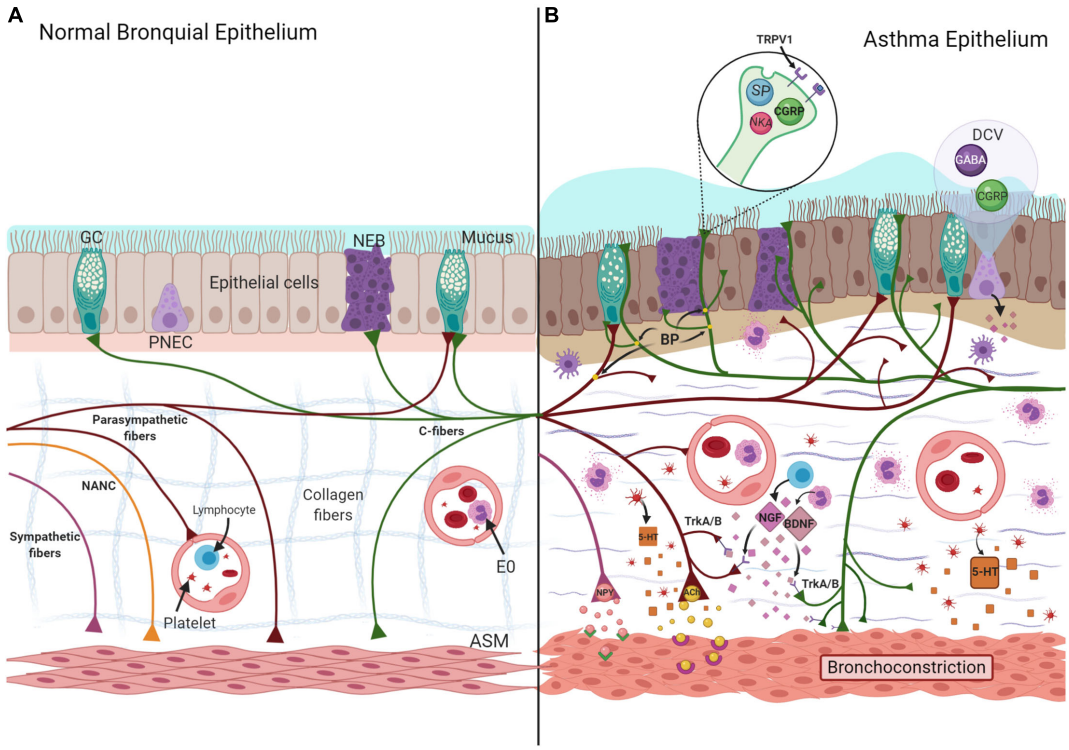

FIGURE 2 | Normal **(A)** and asthma-induced epithelial damage **(B)**. Neurotrophins (NGF- Nerve Growth Factor and BDNF-Brain-Derived Neurotrophic Factor) are synthesized by eosinophils (E0) and lymphocytes, the union to their receptor (TrkA/B), localized in nerves induce a greater length of nerves, increasing branch points (BP), and exposition of nerve endings to the lumen. NT and NP (SP, CGRP, NKA, ACh, 5-HT) are stored and released from these TRPV1 + fibers, increasing mucus secretion, collagenous deposition, and ASM hyperplasia, characteristic findings associated to asthma. Likewise, 5-HT and NPY increase the bronchoconstriction induced by ACh. ASM, airway smooth muscle; DVC, dense core vesicles; GC, Goblet cell; NANC, non-adrenergic/non-cholinergic; NEB, neuroepithelial bodies; PNEC, pulmonary neuroendocrine cell; TRPV1, transient receptor potential vanilloid 1.

Afferent vagus fibers transmit impulses from the airway to the jugular ganglia (branch of the superior laryngeal nerve) and nodose ganglia (branch of the recurrent laryngeal nerve) (Undem et al., 2004), subsequently traveling to the caudal nucleus of the solitary tract (Freeman et al., 2017). By contrast, efferent innervation is comprised by ANS and non-adrenergic non-cholinergic nerves (NANC). The NANC system shares the parasympathetic nerves derived from the dorsal motor nucleus of the vagus (Kistemaker and Prakash, 2019). On the other hand, sympathetic fibers come from the intermediolateral nucleus (T2 to T7 segments). Acetylcholine (ACh) mediates the physiological actions of the parasympathetic system, which induces bronchoconstriction. However, NANC modulates these actions using diverse NP. For example, vasoactive intestinal peptide (VIP) induces bronchodilation. Likewise, sympathetic fibers interact with the airway through epinephrine, exerting the same effect.

Neuronal Remodeling in the Epithelium

Biopsies from moderate-intermittent asthma patients have a greater length, nerve branching, and more branch points than patients with mild asthma and controls (Drake et al., 2018), exposing the nerve endings to the bronchial lumen, leading to neuronal remodeling (Ollerenshaw et al., 1991). This process has two phases: a regenerative phase, during which axons undergo regrowth and dendrites become new connections, and a degenerative phase, characterized by the incorporation of neurites and synapses (**Figure 2**; Alyagor et al., 2018).

Neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are synthesized by neurons of the central nervous system (CNS) and ANS as small active peptides that, upon coupling with their receptors (tropomyosin receptor kinase A and B), play a substantial role in neuronal remodeling (Keefe et al., 2017). However, bronchial epithelial cells, ASM (Ricci et al., 2004), immune cells, such as lymphocytes (Ehrhard et al., 1993), or eosinophils (Kobayashi et al., 2002), and PNEC can synthesize them. In fact, early life allergen exposure appears to elevate the level of neurotrophins and cause PNEC hyper-innervation and nodose neuron hyperactivity, inducing mucin secretion (Barrios et al., 2017).

With regards to airway remodeling, TNF-α increases the synthesis of BDNF from ASM and enhances the production and deposition of collagen-1, collagen-3, and fibronectin, as well as the activity of MMP-2 and MMP-9 (Freeman et al., 2017), and is involved in muscle cell proliferation (Aravamudan et al., 2012). It has been described that collagen-I favors the expression of CCL5, GM-CSF, and exotoxin (Peng et al., 2005; Chan et al., 2006), contributing to persistent inflammation (Burgess, 2009). NGF has

been found to exert similar actions on the components of the extracellular matrix (Huang et al., 2015).

Chronic inflammation caused by allergen sensitization induces the synthesis of new receptors at the nerve fibers. For example, type A fibers can express another receptor as transient receptor potential vanilloid 1 (TRPV1) (Bron et al., 2003), an ionic channel of transient release potential mainly localized in C fibers (Nassenstein et al., 2018), whose expression is modulated by NGF and BDNF (Bron et al., 2003; **Figure 2**).

TRPV1 is activated by a wide range of stimuli, such as high temperature, protons, voltage (Banner et al., 2011), or endogenous inflammatory factors, such as arachidonic acid metabolites (Hwang et al., 2000). The activation of TRPV1 induces a reflex response, such as cough and bronchoconstriction (Bhattacharya et al., 2007). This receptor is increased in patients with asthma compared to controls and patients with mild asthma (McGarvey et al., 2014).

Capsaicin, a molecule with pungent properties contained in some foods, such as chili, has been used to evaluate the functions of TRPV1 (Groneberg et al., 2004). It is a simple, safe, and reproducible cough provocation test. This challenge is applied in the algorithm of idiopathic chronic cough (Morice et al., 2007) and is a useful tool to evaluate the efficacy of asthma treatment. For example, the use of inhaled corticosteroids (ICS) for at least 3 months reduces cough induced by capsaicin (Di Franco et al., 2001; Ekstrand et al., 2011). Besides, in cough variant asthma, the capsaicin challenge predicts the ICS treatment response better than the methacholine challenge (Park et al., 2007).

Some reports have indicated that the use of anticholinergic agents, such as tiotropium, improves refractory cough in asthma patients and augments the threshold to this substance during the challenge. This suggests that tiotropium suppresses the neuronal activity of TRPV1, a mechanism independent of the muscarinic type 3 (M3) receptor blockade (Fukumitsu et al., 2018).

ACETYLCHOLINE

Acetylcholine (ACh) is one of the main neurotransmitters both in CNS and peripheral nervous system (PNS) (Vogt, 2018). Its release via exocytosis from the parasympathetic nerve endings to the intercellular space. In 1963, ACh was found to be produced in non-nerve cells (Wessler and Kirkpatrick, 2001) including immune cells (Kawashima et al., 1998; Fujii et al., 2012) giving rise to different responses depending on the stimulated receptor (Pedersen et al., 2018). One of the principal receptors where it exerts its function is the muscarinic ACh receptors (mAChRs), belonging to the family of G protein-coupled receptors (GPCRs), with which they share a high degree of homology. Five types have been described (M1–M5) (Caulfield and Birdsall, 1998), three of which exert physiological effects in the airways, namely M1, M2, and M3. M1 is localized over the alveolar walls, M2 in ASM, and M3 in airway epithelium, ASM, and submucosal glands (Mak et al., 1992).

In murine models of allergic asthma (MMAA), ACh contributes to allergen-induced remodeling mainly through the M3 receptor, but not through the M1 or M2 receptors, increasing the mass of ASM (Kistemaker et al., 2014). Likewise, mAChRs are involved in IL-8 synthesis by these cells, enhancing inflammation (Oenema et al., 2010). The agonists of ACh are related to the modulation of a specific type of mucin known as MUC5AC (Kistemaker and Gosens, 2015), the main mucin glycoprotein responsible for mucus viscoelasticity in asthma (Kirkham et al., 2002; Morcillo and Cortijo, 2006). Additionally, ACh induces collagen synthesis (Haag et al., 2008) via M2 and M3 localized in fibroblasts (Matthiesen et al., 2006) and increases its thickness upon stimulation with TGF-β (Grainge et al., 2011). This mechanism plays a role in the process of pro-fibrotic airway remodeling (Haag et al., 2008). However, the use of anticholinergic drugs, such as tiotropium bromide in chronic models of asthma, reduces M3 expression in bronchia, the Th2 profile, and airway hyperresponsiveness (AHR) (Kang et al., 2012; Kurai et al., 2018).

There is evidence that ACh induces a range of effects on immune cells. For example, lung macrophages express all the components from ACh synthesis, including M1-M5 receptors. The ACh agonist stimulates the production of *de novo* mediators, such as leukotriene B4 (LTB4) via M2 and M3, where the antagonist for the latter receptor inhibits this process (Koarai et al., 2012). Likewise, the content of eosinophilic granules, such as eosinophil peroxidase (EPO), increases the expression of *ChAT* and *VAChT* genes (necessary for the synthesis and storage of ACh) in fibroblasts. However, other eosinophilic mediators, such as MBP or eosinophil-derived neurotoxin (EDN), do not have this effect (Akasheh et al., 2014). In DCs treated with ACh, this NT stimulates the expression of the Th2−promoter OX40L, the production of the Th2−chemokines, such as CCL22 or CCL17, and a Th2 profile with reduced IFN-γ synthesis, suggesting that ACh can further promote a Th2 response even in the presence of a strong Th2 inducer, such as TSLP (Gori et al., 2017).

Inflammation mediated by lipopolysaccharide (LPS) and IFN-γ induces M3 expression and fibroblast proliferation (Español et al., 2014). COPD treatment based on anticholinergic drugs, such as Aclidinium, blocks the transduction of M1, M2, and M3 receptors (Milara et al., 2013), inhibiting the development of these cells and collagenous deposition (Milara et al., 2012), similar to the effect that occurs in asthma.

Other receptors stimulated by ACh include nicotinic receptors (nAChR), which are proteins that are ligand-gated ion channels and localized near to the parasympathetic ganglia, where they facilitate neurotransmission (Racké et al., 2006). Specifically, the alpha 7 nicotinic acetylcholine receptor (α7nAChR) is expressed on macrophages and neutrophils, playing an essential role in attenuating the inflammatory response by stimulating the vagus nerve during systemic inflammation (Wang et al., 2003). Its activation induces the suppression of NF-kB with the subsequent inhibition of pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) and chemokines from inflammatory cells in alveolar macrophages, resulting in the attenuation of lung inflammation and injury (Wang et al., 2003; Li J. et al., 2011). ILC2 express α7nAChR, which attenuates the expression of NF-kB and GATA-3, reducing the cytokine production of IL-5 and IL-13. Likewise, it modulates IL-33, which is necessary for activating this kind of lymphocyte (Galle-Treger et al., 2016). Additionally, the high

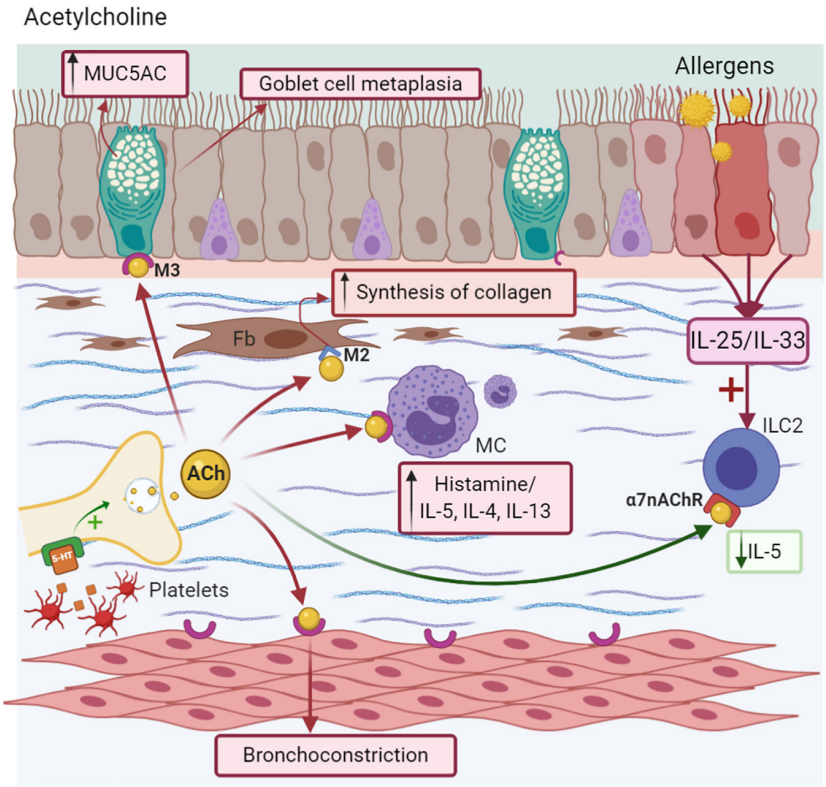

FIGURE 3 | Acetylcholine-ACh. Nerve endings release ACh after depolarization or 5-HT stimuli, causing goblet cell metaplasia, MUC5AC secretion, and bronchoconstriction. It induces Mast cells (MC) degranulation via the M3 receptor and collagen deposition in fibroblast (Fb) via the M2 receptor. Anti-inflammatory effects as decreasing of IL-5 synthesis are due to the α7nAChR activation in ICL2.

expression of α7nAChR in the adrenal medulla is associated with the release of endogenous epinephrine in MMAA, helping to resolve AHR (**Figure 3**; Chen et al., 2017).

The ACh agonist, methacholine, has been used for the diagnosis of asthma (methacholine challenge). Among the main indications are staging the degree of severity AHR and evaluating the effectiveness of the medication in acute and chronic states, or any change in the therapeutic modality (Crapo et al., 2000; Global Initiative for Asthma, 2020). Among all the possible therapies involving both NT and NP, the use of an ACh antagonist is the only therapy approved for the control of asthma (Global Initiative for Asthma, 2020). The effects of muscarinic antagonists include the prevention of both mucous gland hypertrophy and allergen-induced goblet cell hyperplasia, an effect similar to ICS, and partially, the reduction of eosinophilia in the submucosal compartments of cartilaginous and non-cartilaginous airway areas in animals challenged with ovalbumin (OVA) (Bos et al., 2007). Moreover, in a chronic model murine of asthma (MMA) (Kistemaker et al., 2016), muscarinic antagonists were found to decrease smooth muscle mass in addition to ICS, low cell counts (macrophages, eosinophils, and lymphocytes), and decreased IL-5 levels in BALF than other Th2-profile cytokines. Likewise, mice treated with tiotropium had a smaller area of expression of collagen type I and III and significantly reduced M3 receptor expression (Kang et al., 2012). The addition of tiotropium and other antagonizes of M2 and M3 used in COPD as Aclidinium or Glycopyrronium, improves the lung function, reduced the need of oral steroids, and provides beneficial effects on symptom control in patients of all ages with severe asthma, not controlled with convectional therapies (Matera et al., 2020).

SUBSTANCE P

Substance P (SP) is a member of the Tachykinins (TAC) family. This NP is present in both the CNS and PNS (Lai et al., 2008), and in conjunction with CGRP and VIP, mediates the NANC system. Voedisch et al. (2012) TRPV1 + sensory nerves produce and store SP in the large-DCV. This NP is not only released from these neurons upon allergen stimulus (He et al., 2019; Perner et al., 2020), it can also be synthesized by non-neuronal cells, such as lymphocytes (Morelli et al., 2020), DC, eosinophils (Lambrecht et al., 1999), and macrophages (Ho et al., 1997). Once exocytosed from the neuronal soma or axonal terminals, it couples to its specific receptor (Neurokinin receptors -NKRs-), belongs to the GPCR family (Badri and Smith, 2019), expressed either on the same cell or on the neighboring cells (epithelial, endothelial, ASM cells, fibroblasts, and immune cells). NK1R and its isoforms, namely NK1R-F and NK1R-T (Blum et al., 2008), have a higher affinity than NK2R and NK3R (Schelfhout et al., 2006). Some

immune cells, such as Th1, Th17, DC, and neutrophils, express NK1R (Serra et al., 1988; Marriott and Bost, 2001; Morelli et al., 2020). However, eosinophils have NK2R (Raap et al., 2015). Specifically, NK1R-F is expressed in the human brain, while NK1R-T is expressed in the CNS and peripheral tissues, such as bronchial vessels, epithelium, submucosal glands, or endothelium, and is related to inflammation (Mapp et al., 2000; Caberlotto et al., 2003). Both NK1Rs and NK2Rs are found in bronchial ASM cells (Nederpelt et al., 2016) and may mediate bronchoconstriction (Maghni et al., 2003).

SP is present in the serum and BALF of allergic asthma patients (Nieber et al., 1993). In fact, the bronchial branch points are associated with greater SP expression in patients with moderate persistent asthma (Drake et al., 2018). SP induces chemokine synthesis, such as CCL4, CCL5, and IL-8 (Spitsin et al., 2017) specifically, and modulates the chemotaxis of neutrophils, inducing the expression of CXCL2 and CCL3 (Sun et al., 2007). It is also involved in the migration of basophils and eosinophils, an effect comparable to other chemotactic agents, such as LPS (Cima et al., 2010) or C5a, respectively, in an IL-3 microenvironment (Raap et al., 2015). The use of a selective NK1R antagonist (*L733,060*) interferes with this mechanism (Morelli et al., 2020). In immune cells, SP induces T-lymphocyte proliferation *in vitro* by IL-2/IL-2Ra synthesis (Kulka et al., 2008), while in MC, it releases IL-1, GM-CSF, chemokines, oxygen radicals, and LTB4 (Kulka et al., 2008; Li et al., 2018). In addition to IL-33, SP secretes IL-31, TNF-α, and vascular endothelial-derived growth factor (**Figure 4**; Taracanova et al., 2017; Petra et al., 2018). Likewise, SP activates neutrophils through the expression of adhesion molecules, such as CD11b integrin (Sun et al., 2007), in DC, enhancing their survival, which is indispensable for maintaining the eosinophilic airway inflammation perpetuating the Th2 response characteristic of asthma (Voedisch et al., 2012). On the other hand, it downregulates the FcεRI expression in MC (McCary et al., 2010). In turn, interleukins modulate the effects of this NP. For example, IL-12 and IL-18 induce NK1R expression in T cells (Weinstock et al., 2003), but IL-12 and IL-23 enhance *TAC1* expression in macrophages (Blum et al., 2008). However, IL-10 and TGF-β play a relevant role in downregulating these effects (Blum et al., 2008).

SP it has been identified in asthma patients, even is more frequent than in patients with gastroesophageal reflux (GER) (Emilsson et al., 2016). During the bronchoconstriction process, SP increases intracellular Ca^{2+} in ASM cells by decreasing the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and Na^{+}/Ca^{2+} exchanger (NCX) proteins, augmenting the availability of calcium for contraction (Mahn et al., 2009; Li and Shang, 2019). The administration of NK1R antagonists, such as *WIN62577* or *GR304050*, increases SERCA protein with a subsequent decrease in Ca^{2+} concentration, a similar effect of *KB-R7943*, an NCX-specific inhibitor (Li M. et al., 2011; Li and Shang, 2019). In a similar context, the use of an experimental antagonist of NK1R (*CP96345*) (Yaraee and Ghazanfari, 2009) was found to reduce the TGF-β levels, favor ASM relaxation, and reduce the impact of fibrosis on airway remodeling (Li and Shang, 2019).

Additionally, experimental antagonists for this NP (Aprepitant) have shown to improve cough, a cardinal symptom of asthma, in cancer patients, so they can be considered to treat conditions such as asthma (Noronha et al., 2021). However, the drugs designed to block SP in asthma have had limited efficacy in clinical trials, possibly due to unanticipated changes in SP signaling occurred in asthma or changes in its metabolism (Drake et al., 2018).

TACHYKININS

The TAC family comprises NKA, neurokinin B (NKB), Hemokinins (HKs) 1–4, Endokinins (EKs) A-D (Helyes et al., 2010), Neuropeptide K, and Neuropeptide γ, in addition to SP. TACs receptors (NK1R, NK2R, and NKR3) belong to the GPCR family. NKA and NKB bind specifically to NK2R and NK3R, respectively (Nederpelt et al., 2016), whereas HK-1, EKs, and SP bind to NK1R (Kurtz et al., 2002; Grassin-Delyle et al., 2010). However, SP, NKA, and NKB are able to couple to all receptors (Schelfhout et al., 2006; Nederpelt et al., 2016). The modulation of TAC is mediated by NEP (Neprilysin or Enkephalinase). Its deficiency is related to mucus hypersecretion, vascular hyperpermeability, and inflammation in human lung biopsies (Baraniuk et al., 1995).

In OVA-sensitized mice, sensory nerve endings release NKA and SP, followed by an increased temperature, enhancing the percentage and diameter of TAC-immunoreactive neurons, identified as TRPV1 + sensory neurons (Hsu et al., 2013; Le et al., 2014). A positive correlation between reflux and SP/NKA sputum levels was observed in asthma patients with gastroesophageal reflux disease (GERD), suggesting that the thermal or chemical mechanisms involved in GERD allows for the release these NPs (Patterson et al., 2007).

NKA and SP mainly modulate NANC excitatory responses in the airway (Kajekar and Myers, 2008). In human lung biopsies, the three receptors are localized in ASM (Mizuta et al., 2008a), suggesting their role in ASM contraction. In OVA-sensitized guinea pig models, bronchoconstriction induced by NKA, NKB, and SP in this order, are likely to be induced by ACh (Daoui et al., 2000). In the same context, HK-1, EKA, EKB, and the agonist of NKB (*[MePhe^7]-NKB*) also induce bronchoconstriction *in vitro* in human lung biopsies, in contrast to EKC and EKD (Grassin-Delyle et al., 2010; Corboz et al., 2012).

On the other hand, in MMA mediated by the Th1 response, IFN-γ increases NK2R expression in ASM and the NKA levels in BALF, as well as inducing AHR in a dose-dependent manner. In deficient-STAT1 mice, these responses were absent (Kobayashi et al., 2012). These effects have been described in human DCs localized in lung and macrophages from asthma patients (Ohtake et al., 2015). The NKA-NK2R axis stimulates the synthesis of IFN-α and IFN-β in human DCs (Kitamura et al., 2012).

There is scarce evidence of asthma about other TAC. Exists recent reports about the activation of HK-1 by Mas-related G-protein coupled receptor member X2 (MRGPRX2) (Manorak et al., 2018; Thapaliya et al., 2021). SP (Gaudenzio et al., 2016) and other ligands as β-defensins, a type of antimicrobial peptides

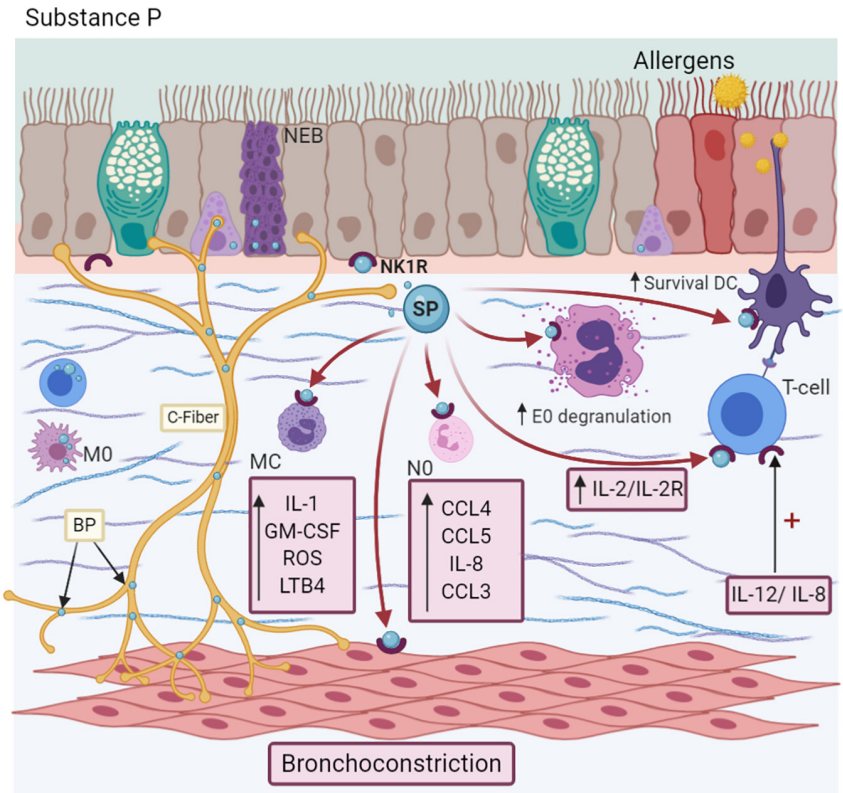

FIGURE 4 | Substance P-SP. C-fibers, NEBs, T cells, and macrophages (M0) synthesize SP. SP induces the synthesis of IL-1, GM-CSF, ROS, and LTB4 synthesis in Mast cells (MC); CCL4, CCL5, IL-8, and CCL3 production in neutrophils (N0); degranulation of eosinophils (E0), dendritic cells (DCs) survival and the promotion of Th2 differentiation. Besides, it causes bronchoconstriction by decreasing SERCA/NCX expression. Its NK1R receptor is localized in many cells and is upregulated in an IL-12/IL-8 microenvironment.

(Guaní-Guerra et al., 2011) released after epithelial injuries (Subramanian et al., 2013), also activate this receptor. This fact was confirmed after a selective NK1R antagonist did not inhibit these effects (Manorak et al., 2018). MRGPRX2 could be also a promising serum biomarker in allergic asthma for monitoring treatment outcomes and determining personalized ICS dose. However, more studies are needed to establish this role (An et al., 2020). HK-1 is also involved in mouse pre-B cell survival and proliferation by increasing IL-7 levels, whereas the NK1R antagonist (*L732138)* increases apoptosis in these cells (Zhang et al., 2000; **Figure 5**).

Many antagonists of TAC receptors have been evaluated for their potential as biomarkers or pharmacological targets in asthma (Ramalho et al., 2011). In the first case, NKA levels in sputum were found to be correlated with asthma exacerbations in children, showing high levels of both NKA and eosinophil count even after remission, compared to the control group (Mostafa et al., 2008). On the other hand, the use of CS-003, a triple NKRs antagonist, administered by inhalation in patients with mild-to-moderate asthma, showed less bronchoconstriction in methacholine challenge. This effect had a duration of ∼8 h without any adverse effects (Schelfhout et al., 2006). Likewise, it was found to inhibit NKA/NKB-induced bronchoconstriction and SP-induced vascular hyperpermeability in guinea pigs (Nishi et al., 2000). The use of NK2R antagonists, such as *MEN-10376* and *SR48968,* reduced the lung insufflation pressure and abolished the effect of HK-1-induced bronchoconstriction, respectively (Krishnakumar et al., 2002), while the antagonists for NK3R (*SB223412* and *SR 142801)* reduced NKB-induced AHR and pulmonary inflation pressure (Corboz et al., 2012). The blockage of these effects by experimental drugs and others as concludes the role of TAC (NKA) as a necessary mediator in the bronchospasm (Joos et al., 2004).

CALCITONIN GENE-RELATED PEPTIDE

Calcitonin-gene related peptide is a NP present in two isoforms both in humans (I/II) and rats (α/β), which have similar homology (>90%) and biological activity (Russell et al., 2014). αCGRP is localized in both the CNS and PNS, whereas βCGRP is present in the enteric nervous system (Muddhrry et al., 1988) and immune cells (Xing et al., 2000), and is specifically synthesized in airways by PNEC (Sui et al., 2018). CGRP is co-stored with SP at the nerve ending of sensory neuron C fibers into the airways (Kajekar and Myers, 2008). Its receptor is a heterodimeric complex called Receptor Activity–Modifying Protein 1 (RAMP1) (McLatchie et al., 1998), which is expressed by airway epithelial

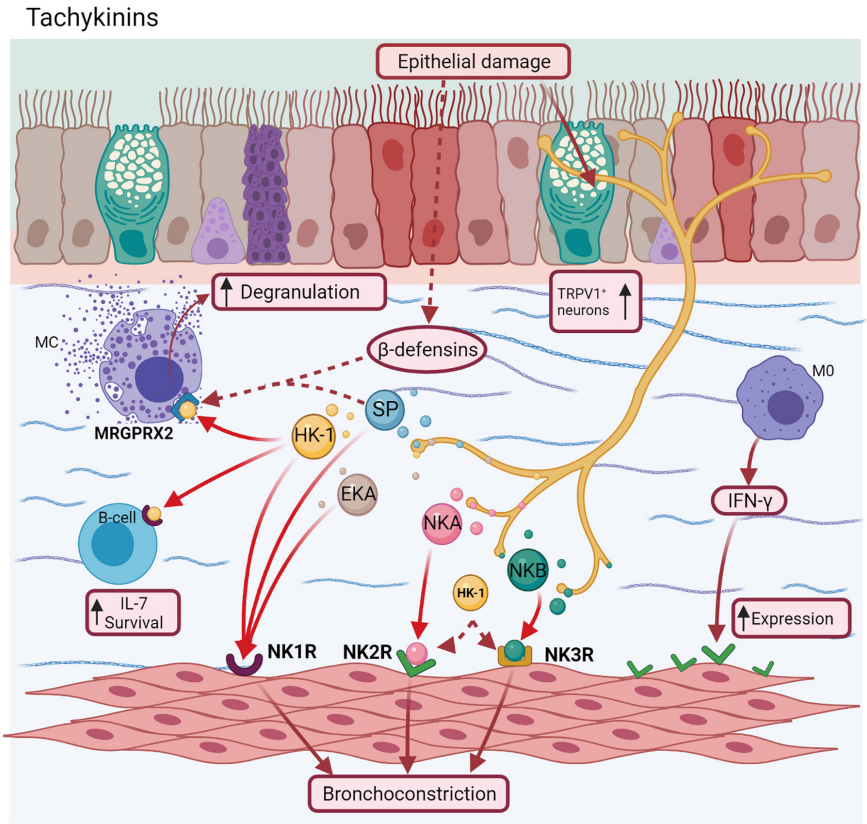

FIGURE 5 | Tachykinins. Neurokinin A (NKA) and Neurokinin B (NKB) bind to NK2R and NK3R receptors, respectively, whereas Hemokinins (HK-1), Endokinins (EKA/B), and SP bind to NK1R. However, SP, NKA, and NKB can couple to all receptors, localized mainly in airway smooth muscle inducing airway bronchoconstriction. Additionally, HK-1 and other ligand as β-defensins, can join a novel receptor implicated in allergic asthma (MRGPRX2) of mast cells, favoring their degranulation.

cells (Li et al., 2014) and immune cells, such as Th9 cells (Mikami et al., 2013). In MMAA, DCs are localized next to vagal sensory neurons, where there is a CGRP neuron proliferation (**Figure 6**; Le et al., 2014).

The association between TRPV1 in lung tissues and an increase of CGRP in the BALF of OVA-sensitized mice has been described (Kim et al., 2020). The induction of the internalization of its receptor in airway epithelium and the subsequent expression of inflammatory interleukins, including IL-6, are among the effects of CGRP. Interestingly, biopsies from asthma patients support this observation, with reduced levels of RAMP1 compared to the controls (Bonner et al., 2010).

Calcitonin-gene related peptide modulates the Th9 response (response related to TIHS), inducing the expression of GATA3 and PU.1 (transcription factor of Th9 cells) and IL-9 production, enhancing airway inflammation (Mikami et al., 2013). Higher concentrations of CGRP could be released by CCL17 more than other inflammatory interleukins (IL-1, TNF-α, and IL-13) by a CCR4-dependent mechanism, which plays a role in the late asthmatic reaction. CCL17 may amplify the vascular component of the inflammatory response by stimulating epithelial cells to release CGRP (Bonner et al., 2013). This mechanism represents a possible therapeutic target for vascular events in patients with asthma and allergic inflammation (Bonner et al., 2013).

On the other hand, ILC2 express RAMP1. When the axis CGRP/RAMP1 interacts, it induces an increase in IL-5 production from these cells in an IL-25 and IL-33 microenvironment, inducing the maturation and activation of ILC2, but does not affect their proliferation. Similarly, CGRP is recruited to eosinophils and promotes the synthesis of leukotriene C4 triggering the Th2 response (**Figure 6**; Sui et al., 2018).

In allergen-induced late reactions, CGRP increases in both BALF and biopsies from allergic asthma patients after the inhalation of allergen-derived T-cell peptide epitopes, in comparison to SP and NKA levels, causing vasodilatation and edema (Kay et al., 2007). There is also evidence that this NP enhances the edema induced by histamine and SP (Brain and Williams, 1985). In addition, CGRP could exert other effects involved in asthma, such as AHR. In a rabbit model of ozone-induced AHR, CGRP stimulates an early inflammatory response that contributes to cleaning up of irritants (Ren et al., 2004).

Depending on the context, this NP has anti-inflammatory effects. For example, CGRP activates adenylate cyclase, which

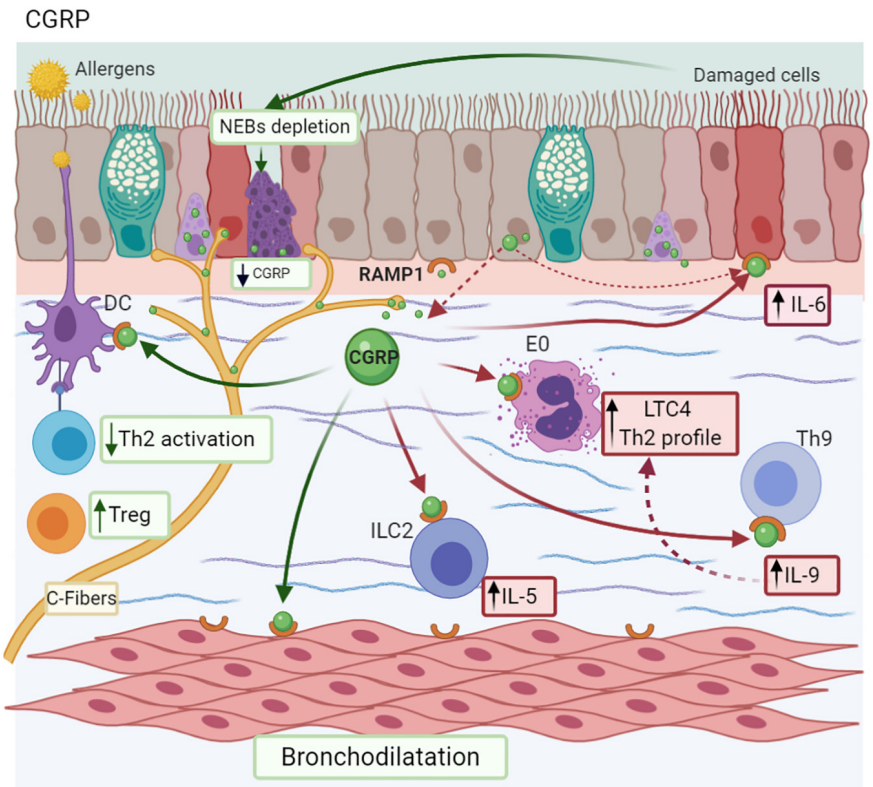

FIGURE 6 | Calcitonin gene-related peptide-CGRP. CGRP is synthesized and stored in C-fibers, neuroepithelial bodies (NEBs), and the epithelium. CGRP promotes a Th9 profile after allergen exposure by RAMP1 activation; IL-6 secretion by the bronchial epithelium; and IL-5 synthesis by ILC2 cells. Additionally, epithelial damage provokes NEBs depletion with the subsequent decrease in CGRP levels. Depending on the context, it also causes bronchodilation and promotes Treg differentiation, reducing Th2 activation.

results in increased cellular levels of cyclic AMP, a pathway usually associated with bronchodilation (Dakhama et al., 2002). On the other hand, AHR induced by allergen exposure results in the depletion of NEB and submucosa plexus, followed by a decrease in CGRP. Interestingly, the exogenous administration of α-CGRP reduced both AHR and inflammation induced by eosinophils, comparable to anti-IL-5 antibody (Dakhama et al., 2002).

Calcitonin-gene related peptide inhibited DC maturation in mice lungs, followed by the decrease in antigen-specific T cell activation (specifically Th2) and the increase in Treg cells (Rochlitzer et al., 2011; Peng et al., 2018). Likewise, reduces the eosinophil counts and increases the levels of IL-10 in BALF (Rochlitzer et al., 2011). These mechanisms suggest that CGRP could also represent a new therapeutic target in asthma therapy, as an anti-inflammatory mediator.

SEROTONIN

Serotonin (5-HT) is an NT and vasoactive amine that participates in numerous physiological processes. Intestinal enterochromaffin cells synthesize ∼90% of this NT (Arreola et al., 2015). However, is stored in dense granules of platelets 5-HT has seven receptor families (5-$HT_{1\text{-}7}$), with their subtypes mainly associated with G proteins, except for 5-HT_3, which is a ligand-controlled cation channel. Owing to the great variety of receptors and their extensive distribution, they are involved in a wide range of functions (Andrade et al., 2019).

Platelets are the main source of 5-HT in the lungs (Dürk et al., 2013). These cells are capable of active extravasation in this organ (Pitchford et al., 2008), where they release this NT (Dürk et al., 2013). Both processes promote platelet recruitment via the expression of *P*-selectin and its respective ligand (integrins), localized in eosinophils and lymphocytes, as observed in MMAA (Pitchford et al., 2005).

However, PNEC (Fu et al., 2002) and MCs are able to synthetize it (Kushnir-Sukhov et al., 2007). This effect increases in the presence of hypoxia and IL-33, respectively (Sjöberg et al., 2015). In mature DCs, 5-HT modulates the production of IL-1β and IL-8 through 5-$HTR_{3/4/7}$ receptors (Idzko et al., 2004). A similar effect has been reported in peripheral mononuclear blood cells (PMBC) (Cloëz-Tayarani et al., 2003). In addition, NT increases the migration of pulmonary DCs to draining lymph nodes and induces the expression of a Th2 profile in these cells (Müller et al., 2009).

Additionally, ASM cells express 5-$HT_{2A/3/4/7}$ (Fernandez-Rodriguez et al., 2010; Segura et al., 2010) in MMAA, which

mediates bronchoconstriction (Arreola-Ramírez et al., 2013), activating its receptors on parasympathetic ACh-containing neurons, resulting in the release of ACh (**Figure 4**; Fernandez-Rodriguez et al., 2010). Interestingly, TNF-α up-regulates the contraction mediated by 5-HT via the 5-HT_{2A} receptor (Adner et al., 2002). On the other hand, some reports have shown that patients with asthma showed increased levels of 5-HT in BALF compared to healthy control subjects (Dürk et al., 2013). Likewise, lung function was negatively correlated with an increase in 5-HT (Lechin et al., 1996). Consequently, the reduction of the plasma concentration of free 5-HT could be useful in the treatment of asthma patients. For example, there is an anecdotic report that evaluated the use of Tianeptine (an antidepressant), a drug that decreases plasma 5-HT by enhancing its reuptake. In a double-blind placebo control developed in patients with a weak response to conventional asthma treatment, this therapeutic approach was found to improve lung function and diminish symptoms in asthma patients (Lechin et al., 1998).

GAMMA-AMINO BUTYRIC ACID

Traditionally, Gamma-aminobutyric acid (GABA) exerts inhibitory neuronal functions (Xu et al., 2017). GABA is stored in vesicles and then released by exocytosis into the synaptic space. Its coupling to GABA receptors-GABARs (α/A, β/B, γ/C) and their subunits induces the opening of K^+ ion channels to allow for the efflux of K^+ and the influx of Cl^-, resulting in hyperpolarization and a decrease in neuronal excitability (Sarasa et al., 2020). By contrast, $GABA_BR$ are GPCRs (Lu and Inman, 2009).

Epithelial cells express all the components for local GABA synthesis, release, and coupling with $GABA_A$ and $GABA_B$ receptors, creating an autocrine and/or paracrine system on airway epithelium and ASM (Mizuta et al., 2008b; Zaidi et al., 2011). In the epithelium, GABA exerts effects associated with bronchial remodeling. Biopsies of MMAA have found the aberrant innervation in airways induced by Neurotrophin 4 (NT4), inducing the hypersecretion of GABA by PNEC, mainly in mice later in life. This GABA effect is reversed when NT4 is blocked (Barrios et al., 2017). Likewise, allergen exposure results in an increase in the expression of $GABA_A$ receptor subunits in airway epithelium cells from patients with asthma, but not in ASM (Xiang et al., 2007). This NT is associated with an increase in MUC5AC secretion by goblet cells (Barrios et al., 2019). Similar effects were observed in airway epithelium exposed to cigarette smoke (Fu et al., 2011), apparently promoted by the IL-13 microenvironment (Barrios et al., 2017).

T cells also have a complete GABAergic intrinsic system that includes GAD and other proteins identified in neurons, and express $GABA_A$Rs. Activated lymphocytes showed a greater uptake of GABA than resting ones. This NT inhibits T cell proliferation *in vitro*, an effect that may contribute to the modulation of T cell activation (Dionisio et al., 2011). Likewise, macrophages express the α1 subunit receptor, and the presence of GABA is associated with a reduction in IL-6 and IL-12 production by these cells (Reyes-García et al., 2007).

ASM express $GABA_A$Rs (α4, α5, β3, γ2, γ3, δ, π, and θ) (Mizuta et al., 2008c). Specifically, the stimulation of α4 and α5 subunits induces a membrane potential change that promotes the relaxation of ASM (Gallos et al., 2012). In a similar context, GABA agonists are capable of reducing AHR induced by SP and histamine in mice (Mizuta et al., 2008c). Muscimol, a $GABA_A$R agonist, blocks the bronchoconstriction induced by ACh and NKA in guinea pigs, and potentiated isoproterenol-mediated relaxation. By another hand, α5β3γ2, other GABA agonist, caused relaxation in ASM *ex vivo* and attenuated AHR in MMAA. In addition to phenolic α4β3γ2, GABA agonists reduced eosinophil counts in BALF, but did not increase mucus production in the bronchial epithelium (Forkuo et al., 2017). Other candidates with similar effects on ASM are MIDD0301, an agonist of the A receptor. However, it has the advantage of being almost undetectable in the CNS, without causing sedation (**Figure 7**; Yocum et al., 2019).

VASOACTIVE INTESTINAL POLYPEPTIDE

Vasoactive intestinal peptide is a neuropeptide of NANC system; which it has been proposed as an anti-inflammatory agent (Misaka et al., 2010) with theoretical therapeutic potential due to its bronchodilator effects (Lindén et al., 2003). In murine lungs, the epithelium and arteriolar smooth muscle are the sites with the highest VIP production (Samarasinghe et al., 2010). However, other immune cells, such as Th2 lymphocytes (Delgado and Ganea, 2001) and eosinophils (Metwali et al., 1994), are also able to synthesize VIP. In allergen challenge, the levels of VIP and NEP (the enzyme responsible for degrading VIP) decrease in the first days. However, in later phases, VIP increases, but not NEP (Delgado and Ganea, 2001).

VIP and PACAP have ∼70% homology and an equal affinity for the same receptors (Rangon et al., 2005), namely VIP receptor 1/2 (VPAC1/VPAC2), members of the GPCR family (Yadav et al., 2011). The expression and affinity of VIP receptors depends on the cell type and the activation stage. For example, resting T CD4 + cells and monocytes in humans express higher VPAC1 levels constitutively, while VPAC2 can be induced after T CD4 + stimulation by downregulating VPAC1 expression (Lara-Marquez et al., 2001). At VIP binding sites, plenty of VPAC1 and VPAC2 localize in the submucosal glands, airway epithelium, ASM, and alveolar walls (Groneberg et al., 2001; Ren et al., 2004). However, immune cells, such as MC, express VPAC receptors (Kulka et al., 2008).

Among the anti-inflammatory effects of VIP are the attenuation of IL-1β-induced neutrophil recruitment (Sergejeva et al., 2004). The increase of mRNA *E*-cadherin expression in airway epithelium is necessary to accelerate the repair of bronchial injuries (Guan et al., 2006) and the inhibition of IL-8 synthesis *in vitro* through NF-κB modulation (Delgado and Ganea, 2003a) with the subsequent decrease in monocyte chemotaxis via VPAC1 (Delgado and Ganea, 2003b). As mentioned above, the effects of CGRP related to inflammation by irritants are also described with VIP (Ren et al., 2004). In MMA, mice treated with VIP showed less bronchial wall thickening,

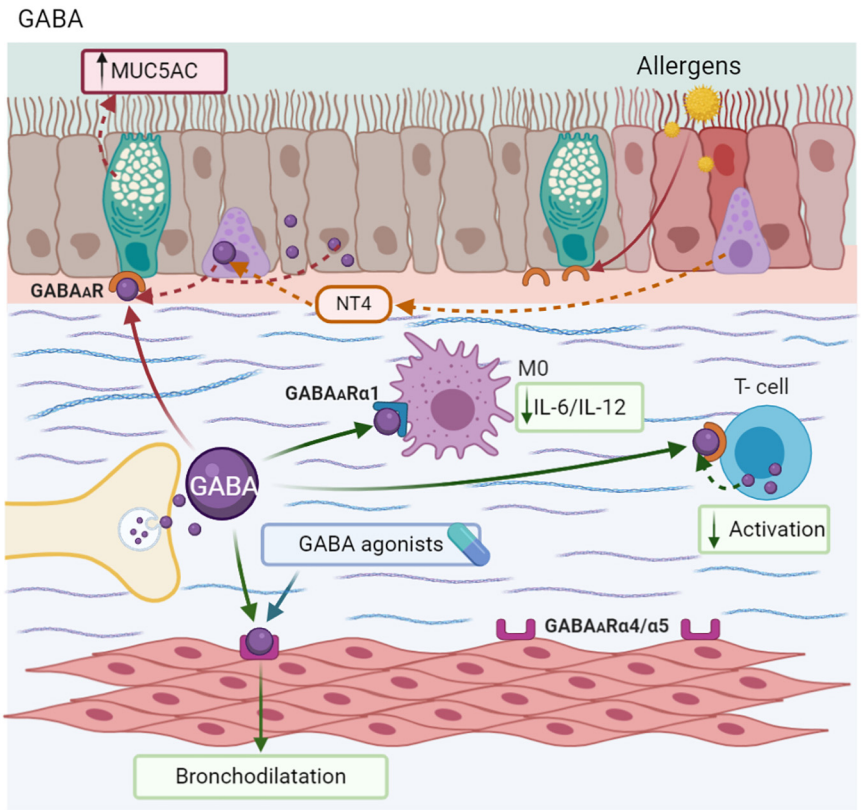

FIGURE 7 | Gamma-Aminobutyric acid-GABA. GABA is synthesized in nerve endings and also by PNEC, epithelium, and ASM. The aberrant production of GABA induced by Neurotrophin 4 (NT4) causes MUC5AC hypersecretion. However, GABA modulates T-cell activation and decreases the synthesis of IL-6 and IL-12 in macrophages (M0). This neurotransmitter and its agonist induce bronchodilatation.

cilia detachment, inflammatory cell infiltration, and a reduction in IL-13-induced ASM proliferation, while the use of a VPAC1 antagonist blocked these effects (Wang et al., 2018). In a similar context, the addition of alpha-alumina nanoparticles to VIP (α-AN/VIP) prevented its enzymatic degradation; α-AN/VIP induced a marked decrease in AHR, BALF-eosinophilia, mucus hypersecretion, goblet cell hyperplasia, IgE, and low levels of the cytokines IL-1, IL-5, IL-6, and IL-13, in comparison with ICS, such as beclomethasone (**Figure 8**; Athari et al., 2016).

Vasoactive intestinal peptide is one of the most potent endogenous bronchodilators and is more potent than adrenergic substances, such as isoproterenol (Palmer et al., 1986). NP and its agonists attenuate the bronchoconstriction induced by histamine through VPAC2 (Schmidt et al., 2001). The VPAC2 agonist (*Ro 25-1553*) induces bronchodilatation in patients with moderate asthma (**Figure 8**) (Lindén et al., 2003). Although, in comparison with formoterol, it is less potent, the combination of these two agents doubles the relaxant action (Källström and Waldeck, 2001). Despite these beneficial actions on ASM, the limitation of VIP as a bronchodilator drug is due to its immediate degradation and its cardiovascular effects, including high blood pressure, tachycardia, prolonged QT segment, or alterations in serum potassium (Lindén et al., 2003).

NOCICEPTIN/ORPHANIN FQ

Nociceptin/orphanin FQ (N/OFQ) is peptide (IUPHAR/BPS, 2020), classified as a "non-classical or non-opioid member (Singh et al., 2016). This NP has ∼60% homology with other opioids and, its receptor, the N/OFQ receptor (NOP), is structurally similar to other opioid receptors (Corboz et al., 2000). The N/OFQ-NOP axis has several biological functions, including nociception, stress, and anxiety, among others (Basso et al., 2005). In the airways, N/OFQ blocks NANC excitatory responses mediated by SP and NKA (Shah et al., 1998).

T and B lymphocytes and monocytes express the NOP receptor (Peluso et al., 1998; Thomas et al., 2014). Patients with severe asthma show an increase in the NOP mRNA in ASM, bronchial epithelium, eosinophils, and MC. In this group of patients, an increase of N/OFQ in the sputum, sub-epithelium, and extracellular matrix have been observed compared to the control group or patients with mild asthma (Singh et al., 2016). The lymphocyte synthesizes N/OFQ (Arjomand et al., 2002). This NP reduces IL-4 + CD4 + T cells and IL-13 in the lungs of MMAA, modulating the physiopathology of asthma (Borges et al., 2016).

The exogenous administration of N/OFQ in human lung tissue reduced the activation, recruitment, and eosinophil counts,

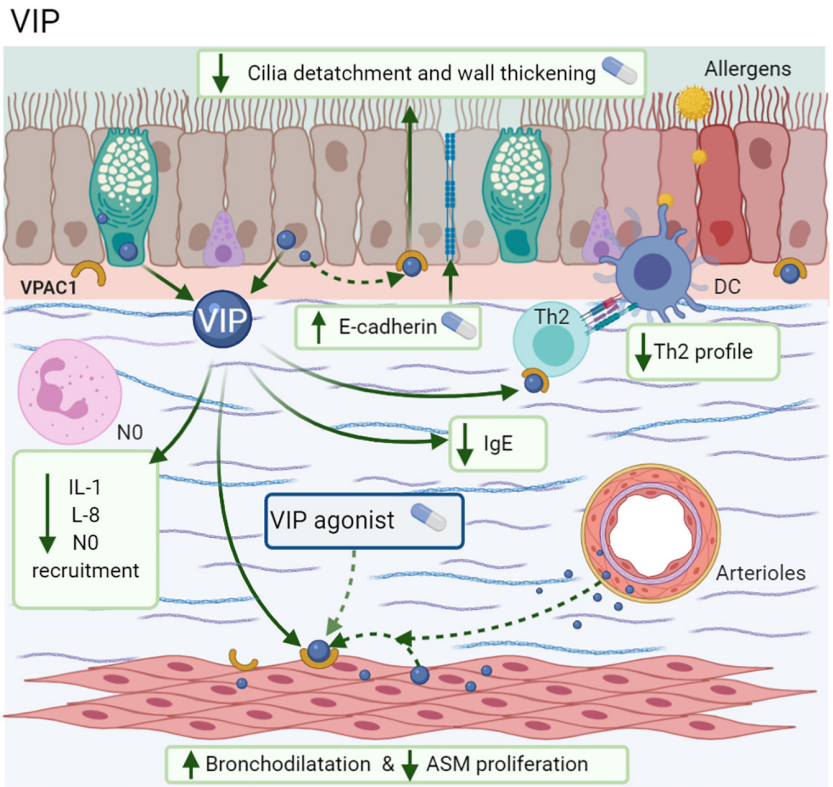

FIGURE 8 | Vasoactive intestinal polypeptide (VIP). VIP is synthesized by the epithelium, glands, ASM, arteriolar muscle, Th2 cells, and eosinophils; exerts its actions by VPAC1. Improves the bronchodilatation and repair of epithelium by *E*-cadherin expression; also decreases ASM proliferation, IgE, IL-1, IL-5, IL-13, diminishes the neutrophil recruitment.

as well as the peribronchial inflammatory infiltrate, with a decrease in IL-8, CCL11, and CCL26 (Singh et al., 2016). In a similar way, in an MMAA, the NOP receptor agonist *UFP-112*, administered in OVA-sensitized mice, reduced eosinophilic infiltration and T cell proliferation, with a decrease in the Th2 profile and increased IFN-γ levels, effects that were blocked by the antagonist *UFP-101* (Sullo et al., 2013). This NP had beneficial structural effects, including a reduction in ASM proliferation and bronchial wall thickness in OVA-sensitized mice (**Figure 9**; Tartaglione et al., 2018).

In relation to bronchoconstriction, N/OFQ and its agonist decrease ACh-induced AHR in human lung tissue (Sullo et al., 2013; Singh et al., 2016). In guinea pig lungs, the administration of N/OFQ inhibited capsaicin-induced bronchoconstriction in a dose-dependent manner, but it has no effect on the NKA-induced AHR. The use of the NOP receptor antagonists *J11397* (Corboz et al., 2000) and *UFP-101* inhibits this phenomenon. However, naloxone, an opioid antagonist, has no effect (Basso et al., 2005).

NEUROPEPTIDE Y

Neuropeptide Y (NPY) is found mainly in the CNS and sympathetic nerves (Chen et al., 2020), where it is co-stored in DVC and co-released with norepinephrine (Ekblad et al., 1984). Its receptors (Y1R-Y6R) (Beck-Sickinger et al., 2019) belong to the CGRP protein family, with the YIR being the most studied, which is expressed on immune cells (leukocytes, lymphocytes, DC, and MC), but is not detectable in airway epithelium and ASM under basal conditions (Wheway et al., 2005; Makinde et al., 2013).

This NP has pleiotropic effects depending on the cells where it exerts its functions. For example, macrophages favor its adhesion and oxidative burst (De la Fuente et al., 2001). In immature DCs, it helps migration in addition to CCL3, inhibits IL-12 and INF-γ production, and promotes the release of the Th2 profile (MacIa et al., 2011; Buttari et al., 2014; Oda et al., 2019). Additionally, in MMAA, NPY increases the eosinophil counts, CD11c +, and cytokines, such as IL-4, IL-5, and IL-13. Its Y1R antagonist (BIBO-3304) suppresses these effects, suggesting that this receptor mediates all these mechanisms (Oda et al., 2019).

However, its effects on asthma are not yet fully understood (Oda et al., 2019). There is evidence that patients with the *NPY*-399C/T polymorphism and obesity have a higher probability of suffering from asthma (Jaakkola et al., 2012). Some reports have shown that the expression of NPY and the NPY/Y1 axis is elevated in allergic asthmatic airways (MacIa et al., 2011; Makinde et al., 2013), an effect that is modulated by NGF

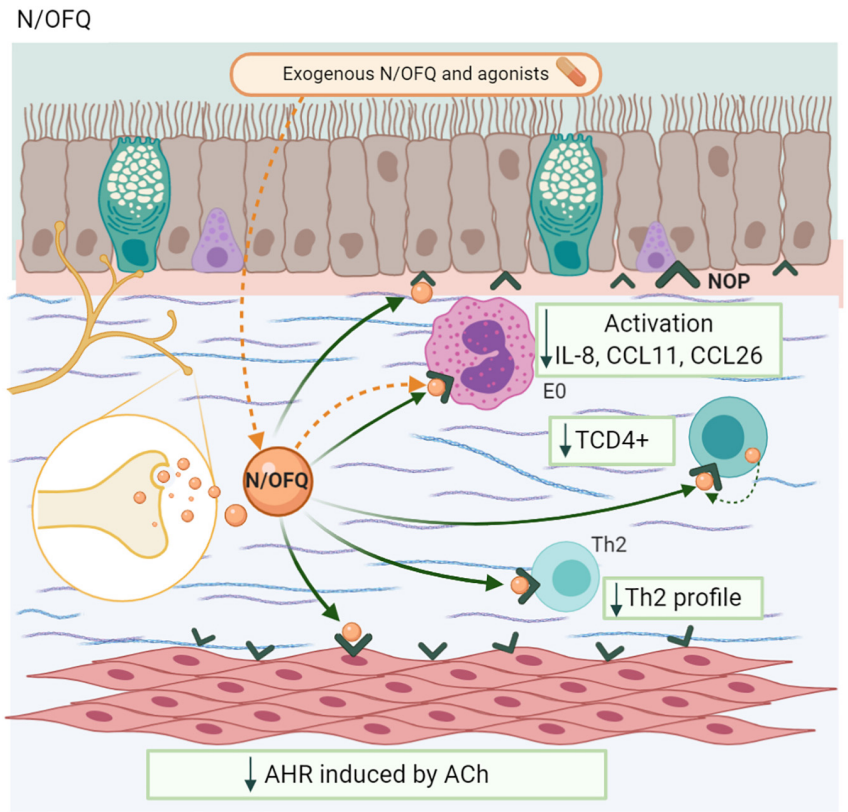

FIGURE 9 | Nociceptin/orphanin FQ-N/OFQ. Neurons and lymphocytes produces this NP. The coupling with its receptor (NOP) decreases TCD4 + population, Th2 profile synthesis, the eosinophil chemotaxis as well as its activation, and the airway hyperresponsiveness (AHR) induced by ACh.

(Wu et al., 2012). Likewise, chronic allergen exposure and stress in MMAA increases NPY, eosinophils, and leukocyte counts in BALF (Lu and Ho, 2016), suggesting a positive correlation between NPY levels during a stress episode in an asthmatic exacerbation and AHR. Interestingly, the loss of *FOXP1* and *FOXP4* in the epithelium of patients with a non-Th2 asthma phenotype induces ectopic NPY production and other proteins associated with airway remodeling, such as MUC5AC. NPY acts in a paracrine manner between the epithelium and ASM. In fact, there is evidence that it enhances the bronchoconstriction induced by methacholine (**Figure 2**; Li et al., 2016).

CONCLUSION

NP and NT are usually associated with mental diseases and mood disorders (Lietzén et al., 2011). However, both molecules contribute to enhancing and/or modulating the inflammatory response to asthma. For example, the association between stress and asthma symptoms is well documented (Rietveld et al., 1999; Sandberg et al., 2000). Negative psychological stress has been found to increase the risk of asthma attacks in children (Liu et al., 2002), characterized by a high number of eosinophils in the sputum, EDN, IL-5 (Ritz and Steptoe, 2000), as well as decreased lung function during period of stress (Von Leupoldt et al., 2006). Although the molecular immunological mechanisms involved in the pathophysiology of asthma are well studied, the role of NT/NP has yet to be fully elucidated.

This general review presents the relevant mechanisms of NT/NP in the pathophysiology of asthma at different levels. NT/NP and their receptors are not synthesized exclusively in the nervous system (Ehrhard et al., 1993; Kobayashi et al., 2002). They can also be expressed in immune cells, the airway epithelium (PNEC) (Branchfield et al., 2016), and ASM (Ricci et al., 2004). Bronchial remodeling is closely linked to neuronal remodeling in the airway, generating longer nerves, branch points (Drake et al., 2018), and higher expression levels of TRPV1 receptors, mainly in C fibers (Nassenstein et al., 2018), which can be activated by several stimuli, causing coughing via the vagus nerve (Narula et al., 2014). These fibers store NT/NP, which, when released, participate in a range of functions at the local level (Voedisch et al., 2012).

Substantial knowledge on NT/NP comes from murine models of both allergic and non-allergic asthma. For example, some TACs as NKA and its receptor increased by stimulus of IFN-γ concomitantly with AHR in murine models of severe asthma (asthma resistant to classical treatment) (Kobayashi et al., 2012), even this bronchonconstrictor effect is similar to the induced by

ACh in guinea pigs (Daoui et al., 2000); this effect is reproducible with other TACs (HK-1, EKA, EKB, and NKB agonist) in both animal and human models (Grassin-Delyle et al., 2010). Probably, this effect is due to the increase in cytoplasmic Ca^{2+} as well as the ASM proliferation reported with SP, contributing to airway remodeling (Li M. et al., 2011). The use of NKR antagonists favors ASM relaxation, relieving this symptom (Nishi et al., 2000). In contrast, VIP has anti-inflammatory effects, such as reducing the AHR and diminish of airway mucus secretion by the inhibition of ERK1/2 signaling pathway in murine models (Wang et al., 2018). Exogenous VIP administered, decreases airway inflammation in an allergic asthma murine model, effect comparable ICS (Athari et al., 2016). Likewise, N/OFQ reduce the bronchial wall thickness in its hyperplastic phase (Tartaglione et al., 2018) and GABA with its agonists block bronchoconstriction induced by ACh/NKA in guinea pigs (Gleason et al., 2009). However, there are NT/NP with dual effects in asthma. For example, in both human and murine with allergic asthma, the axis ACh/M1-M3 receptor is involved in the increasing of ASM mass (Kistemaker et al., 2014), enhances IL-8 synthesis (Oenema et al., 2010), mucin expression (Kistemaker and Gosens, 2015), and collagen synthesis by fibroblasts (Matthiesen et al., 2006). But, the ACh/α7nAChR axis exerts anti-inflammatory effects, suppressing NF-κB in macrophages (Wang et al., 2003) and ILC2 with the subsequent reduction of a similar Th2 profile attenuating bronchial inflammation (Galle-Treger et al., 2016). Other NP with same dual effects in MMAA CGRP induces to Th9, that mimics a type I hypersensitivity response (Mikami et al., 2013) and stimulate ILC2 (Sui et al., 2018). But equally to ACh, the exogenous CGRP reduce the airway inflammation induced by eosinophils (Dakhama et al., 2002). Thus, in a didactic way, the NT/NP could be classified based on their effect on the immunological mechanisms in asthma (**Supplementary Table 1**).

Some NT/NPs, such as 5-HT (Lechin et al., 1998) and NKA (Mostafa et al., 2008), can be used as biomarkers, since they are correlated with low lung function and associated with asthmatic exacerbation. Likewise, the use of exogenous NP or the blockade/activation of NT/NP receptors has shown beneficial effects, attenuating the inflammatory mechanisms and decreasing AHR (Dakhama et al., 2002; Krishnakumar et al., 2002; Lindén et al., 2003; Mahn et al., 2009; Li and Shang, 2019). Although there are some studies evaluated experimental drugs that block NP/NT receptors, their limited efficacy in these clinical trials is possibly due to unanticipated changes in signaling, its metabolism as short half-life (Ho et al., 1997; Cattaruzza et al., 2009) or the presence of adverse reactions inherent to the CNS and other organs where these receptors are expressed (e.g., sedation or arrhythmias) (Lindén et al., 2003; Drake et al., 2018) (**Supplementary Table 1**).

An exception is the group of drugs that block M2 and M3 receptors. The inclusion of anticholinergics drugs, such as tiotropium, in the treatment of asthma has been supported by medical consensus since 2016. This is an example of how NT/NP and their receptors are involved in asthma physiopathology, but they can also serve as therapeutic targets for the benefit of asthma patients.

AUTHOR CONTRIBUTIONS

GP-R, NS-P, and LG-S: review of literature, manuscript redaction, and figure elaboration. FR-J: manuscript redaction. LT: review of literature, manuscript redaction.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.663535/full#supplementary-material

Supplementary Table 1 | Summary of main effects of neurotransmitters/neuropeptides andtheir experimental drugs in asthma.

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frontiers in Cell and Developmental Biology

OPINION
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Multiple Origins of Neurons From Secretory Cells

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WHY ARE NEURONS DIFFERENT? INTRODUCTION

The elusive diversity of neurons puzzled neuroscientists since discovering the first nerve cells in the 1830s. Quantitative information about neuronal diversity began to flow from the middle of the twentieth century. At that time, microelectrode and histochemical tools were applied to vertebrate and invertebrate preparations. Simpler nervous systems of some gastropod mollusks, annelids, and nematodes revealed identified neurons with defined transmitter specificity and functions. Early systematic studies pointed out that most of the neurons composing their nervous systems might be unique (Bullock and Horridge, 1965). That revelation provided tractable experimental preparations to decipher cellular bases of behaviors (Kandel, 1976, 2001; Kuffler and Nicholls, 1976).

Today, with advances in single-cell (epi)genomics and transcriptomics, the astonishing diversity of neuronal cell types exceeds any imagination (Moroz, 2018). *The most straightforward question is, how different are the neurons? But more fundamental questions are: Why are neurons different? Why are there so many neurotransmitters? Why are neurotransmitters different?* These questions have been addressed by many (Kandel, 1979; Van Vallen, 1982; Bloom, 1984), aiming for functional aspects.

In 1968–1974 these questions were asked from an evolutionary standpoint, and Dmitry Sakharov had proposed the hypothesis of neuronal polygeny (=multiple origins of neurons) (Sakharov, 1970a,b, 1972, 1974a,b). Using minimal comparative data available 50 years ago, Sakharov suggested that **neurons evolved from *genetically different* secretory cells.** The evolutionary view of neuronal evolution can be summarized as follows. Each of these populations of secretory cells could use chemically distinct transmitter(s) and different (distant) receptors for communications in early neural systems, where synapses *are not* required. Ancestral diversity of secretory cell types (=secretory phenotypes) has been preserved over 500+ million years of biological evolution, forming lineages of homologous neurons across phyla. Thus, neurons are different because they have different genealogies. Subsequent functional "demands" and specifications could further tune these different ancestral neurosecretory phenotypes. In other words, the traditional *one-root genealogy* of neurons was transformed into *multiple genealogies* or a net of phyletic cell/neuronal lineages, as schematically presented in **Figure 1**.

The polygeny hypothesis stated that the transmitter-based primordial diversity of chemical signaling networks and mechanisms is the cornerstone for any nervous system organization. It was also suggested that neurons could evolve from heterogenous secretory cells in different tissues, or embryonic layers. In other words, neurons could evolve more than once within early precursors of ectoderm, endoderm, or mesoderm and/or other tissues. The presence of many transmitters in extant nervous systems reflects the complex chemical organization or *chemical wiring* of early neural systems. Extant neural systems might preserve at least some ancestral secretory lineages (and gene regulatory networks/modules) over many millions of years.

The polygeny hypothesis also proposed the criteria and predicted neuronal homologs across taxa or within evolutionary different cell type lineages using modern terms.

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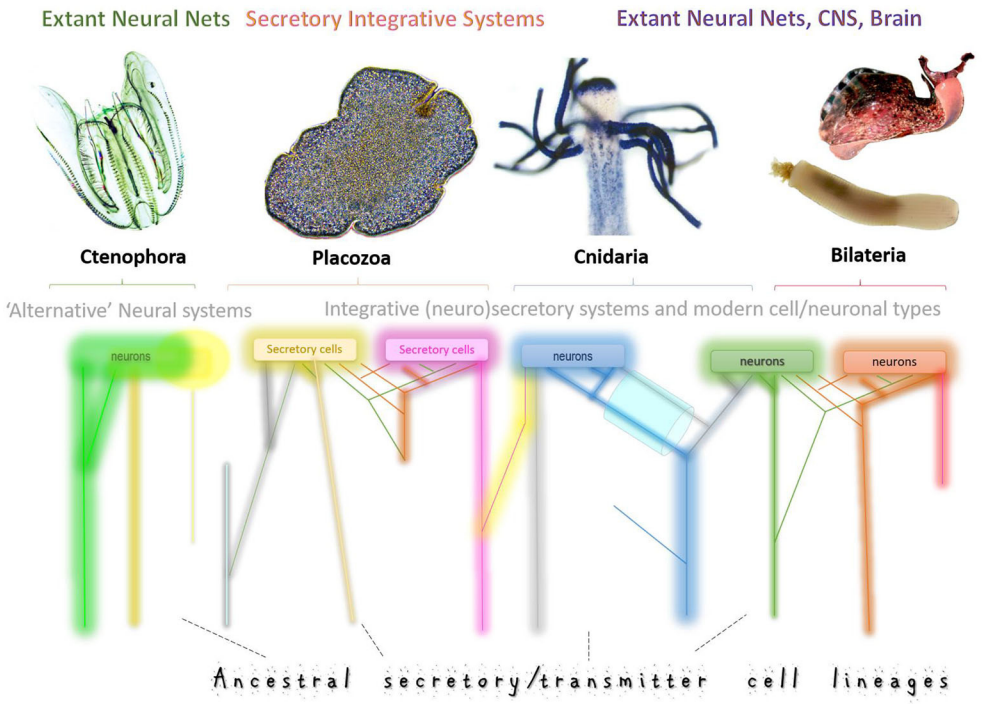

FIGURE 1 | Multiple origins of neurons and secretory cells. Schematic illustration of the ancestral cell lineages (different color trajectories) that led to the exant neural systems in four basal metazoan clades with Placozoa as nerveless animals. Neural systems might consist of genetically diverged cell types with different ancestries, gene regulatory networks, and signal molecules. This diagram integrates both the hypothesis of independent origins of neurons (as in ctenophores, Moroz et al., 2014) and the sister-cell model (Arendt et al., 2016), which suggests that novel neuronal types arise in pairs, through sub-specialization of ancestral cell types. Thus, sister neuronal subtypes can share gene-regulatory networks, perhaps, evolutionary conserved developmental pathways, and are expected to have more similar expression profiles than each of them compared to other neuronal types. The key prediction of this model is that gene expression profiles from sister-cell types will form a hierarchical tree structure in phylogenetic reconstructions. A complementary model predicts that neurons and novel neuronal subtypes arise through "co-options" or "fusions" of regulatory modules and pathways "recruited" from genetically unrelated cell types. As a result, their expression profiles would be substantially different, leading to net-type rather than tree-type cellular genealogies in phylogenetic reconstructions. We expect that both scenarios can coexist in any given nervous system. But the tremendous diversity of neural systems across phyla suggests variable contributions of each historical scenario. Combining tools of (i) statistical geometry, artificial intelligence and (ii) modern phylogenomics with (iii) massive parallel single-neuron transcriptome profiling would allow us to unbiasedly reconstruct the genealogy of neurons by testing the treeness statistics as it was recently used for cancer and placental cells. The top illustrations are photos of *Mnemiopsis*, *Trichoplax*, *Podocoryne*, *Priapulus*, and *Aplysia*. Some cell lineages (different colors) might become eliminated in the course of evolution (loss) or be expanded or evolved in parallel from different secretory cell types.

Unique serotoninergic, catecholaminergic, and peptidergic homologous neurons had been identified in mollusks (Sakharov, 1974b, 1976; Weiss and Kupfermann, 1976). Thus, even in the 1970–1990s, these findings provided clear illustrative examples for **the existence of conservative neuronal cell types separated by million years of divergent evolution**. Again, gastropod mollusks were used as reference species (Sakharov, 1976; Weiss and Kupfermann, 1976; Gillette and Davis, 1977; Moroz et al., 1997; Sudlow et al., 1998). Remarkably, some homologous neuronal cell types (such as a pair of serotonergic MCC interneurons in Euthyneura) preserved their neurotransmitter identities and functions for 380 million years (Moroz, 2018). This hypothesis provided the versatile chemical foundation to reconstruct neuronal evolution where evolutionary innovations in neurosecretory and behavioral phenotypes multiply.

Below, we summarize three conceptual aspects of electrical vs. chemical signaling paradigms to understand neuronal evolution. (1) History of the idea to trace the origins of neurons from secretory cells. (2) Postulates that substantiate different evolutionary scenarios for neuronal evolution. (3) Prospectives of multi-transmitter brain organization and transmitter-dependent behaviors essential to understanding the grammar of neural systems. The elusive chemical syntax of neural ensembles can explain brain operations' emerging properties, eventually leading to basal cognition (Levin et al., 2021; Lyon et al., 2021).

BRIEF HISTORY OF THE IDEA

The diversity of low molecular weight and peptide transmitters in all animals triggered several attempts to incorporate transmitter signaling in neuronal evolution models. Several recent publications provided different historical outlines and perspectives of this endeavor (Moroz, 2009, 2014; Moroz and Kohn, 2015, 2016; Arendt, 2021; Jekely, 2021; Moroz et al., 2021),

which are not yet integrated into a comprehensive and unbiased review of the subject.

Electrical Paradigm

The sensory-effector-contractility scheme of neuronal operation was the dominant model in considering the neuronal evolution, which can be traced to the Parker's elementary neural system (Parker, 1919; Pantin, 1956; Grundfest, 1959, 1965; Passano, 1963; Horridge, 1966, 1968). Mackie (1970) outlined the elegant theory of the neuronal origin from myoepithelial-type cells (like in extant cnidarians, Mackie, 1970). Within this theoretical framework, Mackie and his colleagues further developed the concept of parallel electrical signaling systems (often coupled to contractility), supported by the widespread distribution of the epithelial conductive pathways (Anderson, 1980; Satterlie and Spencer, 1987; Mackie, 2004; Satterlie, 2015). Ancestral neuron-muscle relationships have been emphasized in these models.

Chemical Paradigm

In 1954–1959 Clark, Haldane, and Grundfest were the first students of neuronal evolution who proposed *the origin of neurons from secretory cells* (Haldane, 1954; Clark, 1956a,b; Pantin, 1956; Grundfest, 1959). These hypotheses provided transmitter-centric prospects in deciphering neuronal evolution, in contrast to earlier concepts, which were primarily based on electrical, reflective paradigms (Kleinenberg, 1872; Claus, 1878; Hertwig and Hertwig, 1878, 1879, 1880; Chun, 1880; Hertwig, 1880; Parker, 1919; Wyman, 1925; Pantin, 1956). At that time, in the 1950–1960s, the distribution of signal molecules across phyla was mostly unknown, but neurotransmitter functions of acetylcholine, monoamines, and several neuropeptides have been established (Valenstein, 2005).

Nevertheless, the dominant view was that neurons had a single origin, and later in the evolution, the diversity of transmitters increased following the classical single Tree of Life model. Lenz specifically stressed this point in his influential work and the book (Lentz, 1966, 1968). And the neuronal monophyly model has not been challenged by other authors. For example: "The conceptual model of the ancestral neuron, considered as the phylogenetic derivative of an undifferentiated and pluripotential epithelial cell, is that of a functionally versatile structure, equally endowed for the dispatch of long-distance and localized chemical signals. The neurosecretory neuron has remained closer to the nerve cell precursor than has the conventional neuron with its specialization for synaptic transmission." (Scharrer, 1976).

In contrast, the independent origins of neurons from multiple types of secretory cells is a more realistic reconstruction of neuronal evolution (Sakharov, 1974b; Moroz, 2009, 2014; Moroz and Kohn, 2016). The postulates derived from this hypothesis are summarized below. Electrical vs. chemical-centered hypotheses of neuronal origins are complementary. But it was stressed that **transmitters made neural systems** as integrative ensembles, where the transmitter operated as a "*versatile glue*" recruiting many proto-neurons and their effectors together to form biologically relevant behaviors (Moroz et al., 2021).

POSTULATES OF THE POLYGENY HYPOTHESIS

1) ***In early metazoans, neurons evolved from genetically (genealogically) different secretory cells that used multiple transmitters to communicate and integrate behaviors without synapses.*** From this starting point, the evolutionary innovations multiply. Nerveless Placozoa and Porifera are two animal lineages that preserved such ancestral intercellular communications and, likely, the integration of behavior in a "pure" non-synaptic form (i.e., without any recognized electrical or chemical synapses, Moroz et al., 2021). In other words, both **the diversity of transmitters and their receptors *predated* the origins of neural systems**. The recruitments of classical transmitters and (neuro)peptides in early developmental control (Buznikov, 1990) might reflect this ancestral pre-neuronal integrative functions of these intercellular signal molecules (Koshtoyants et al., 1961; Buznikov et al., 1964, 1968, 1970, 2001, 2005, 2010; Buznikov and Shmukler, 1981; Buznikov, 1991; Shmukier and Buznikov, 1998; Levin et al., 2006).
2) In evolutionary terms, **a neuron is a functional (not genetic) category**. The genetic category is referred to the scenario that all neurons are derived from the *same* ancestral cell lineage. Therefore, all neural systems and neuronal cell types are homologous because of their shared *genetic* ancestry. Alternatively, the functional category signifies examples of convergent evolution when similar chemical and physiological constraints resulted in similar neuronal phenotypes. This view does not prevent establishing evolutionary lineages of homologous cell types within particular taxonomical units such as classes, subclasses, orders, families, and genera. The cell-lineage-specific homologies across phyla are still a challenge (Tarashansky et al., 2020).
3) *What is a neuron?* As their "ancestors," all modern **neurons are polarized secretory cells specialized for directional active conducting and release of more than one transmitter**: usually 2–5 peptides and a low-molecular transmitter(s) (Weiss et al., 1992; Moroz et al., 2005, 2006; Moroz and Kohn, 2010; Cropper et al., 2018; Merighi, 2018; Nassel, 2018; Svensson et al., 2018). These features enable neurons to convey signals, primarily chemical, beyond their immediate neighbors and without affecting all intervening cells *en route*. Evolutionary elaborated memory capabilities of neurons are essential to generate stereotyped and learned behaviors within the same cell ensembles (Kandel, 2001; Walters and Moroz, 2009; Walters and Williams, 2019).
4) **An ancestral mode of intercellular communication mediated by early neurons was a non-synaptic transmission** (= volume or paracrine secretion; Moroz, 2009, 2014). The early directional signaling was achieved due to

the differential cell-specific expression of receptors for secreted signaling molecules and diffusion/microanatomical constraints.

5) As neurons, **synapses evolved independently in animal lineages** and later in evolution (Moroz and Kohn, 2016). Early neural systems were without synapses but with dozens and even hundreds of signal molecules (=small transmitters and secretory peptides) and multiplicity of their receptors (Moroz et al., 2021). These classes of signal molecules formed the chemical and dynamic connectome or a sort of multi-transmitter "*glue*" uniting neurons to generate stereotyped and learned behaviors.

6) **Early neurons were primarily genetically different because of their genealogy**. The first level of evolutionary constraints can be traced back to deep ancestry of complex life cycles of eukaryotes and the nerveless ancestor of all animals (=Urmetazoan). Alexey Zakhvatkin (1906–1950) originally proposed this hypothesis (https://www.si.edu/object/siris_sil_363532), which obtained additional evidence (Mikhailov et al., 2009; Tikhonenkov et al., 2020). The subsequent functional specification within distinct cellular lineages results from parallel evolutionary processes, perhaps similar to the cell-sister type hypothesis (Arendt, 2008; Arendt et al., 2016), and **Figure 1**.

   Of note, even unicellular eukaryotes have many cell types because of their complex life cycles. Cell types in a given unicellular eukaryote are separated in time of development, including the formation of colonial organisms. In evolution of the lineage that led to animals' multicellularity, the preexisting *temporal separation* of cell types was switched to the *spatial co-existence* of similar ancestral cell types (Mikhailov et al., 2009; Tikhonenkov et al., 2020). The Urmetazoan could possess 10–50 distinct cell types (Moroz, 2018; Sebe-Pedros et al., 2018; Musser et al., 2019). Some of these early cell types could be traced back to the complex life cycles of unicellular and colonial eukaryotes.

7) **Every neural system is chemically and genetically chimeric**. This prediction is the most straightforward consequence of the neuronal polygeny hypothesis. Some ancestral neural lineages were lost in evolution, but the core genomic regulatory modules (transcription factors, enhancers, etc.) were preserved in extant nervous systems as decedents of early cell types. Most invertebrate ganglia, neural "circuits" or neural ensembles are composed of different cell lineages with distinct secretory phenotypes and evolutionary histories. I predict the reconstruction of hundreds of genealogies for metazoan secretory cells and neurons in particular. Neurons might evolve from ectodermal, entodermal, and mesodermal-type derivatives. See illustrative examples from the sea urchin (Wei et al., 2011), cnidarians (Nakanishi et al., 2012), including recent scRNA-seq work (Arendt, 2019; Siebert et al., 2019), and ctenophores (Moroz et al., 2014; Moroz, 2015a). Trans-differentiation with transmitter phenotype switching both in development and adult brains is possible (Spitzer, 2017; Bertuzzi et al., 2018; Meng et al., 2018; Ferrarelli, 2020; Li et al., 2020). But it might be a relatively rare event stressing both modularity and substantial evolutionary conservation of secretory specificity within the lineages of homologous neurons.

8) By acting within synaptic clefts and beyond, the transmitters are **multi-level integrators of behaviors and behavioral choice**. *Transmitters could be versatile integrative factors* that non-synaptically unite different effectors (ciliated, secretory, contractile, immune cells, etc.) in early animals. As a result, the tightly coupled integrative *transmitter systems (secretory phenotypes) are evolutionary conservative*. Thus, the transmitter specificity can be instrumental in deciphering the *homologous behaviors* (=transmitter-induced motor outputs and behaviors). For example, serotonin acts as the integrator of behavioral [feeding] arousal in annelids (Lent, 1974, 1984, 1985; Lent et al., 1991) and mollusks (Kabotyanskii and Sakharov, 1991; Moroz, 1991; Gillette et al., 2000) and many other bilaterians (e.g., Sakharov, 1990). Serotonin has one of the most evolutionary conservative systemic functions across bilaterians. Dopamine and other catecholamines also integrate behaviors in various evolutionary lineages (e.g., Livingstone et al., 1980; Kravitz, 1988; Moroz, 1991), but the systemic functions of dopamine are less evolutionary conserved than those for serotonin. These functional differences might be related to the different chemical reactivity and stability of two transmitter molecules. ***Serotonin is an antioxidant*** capable of terminating free radical oxidative reactions (therefore, be more "resistant" to bioenergetic perturbations and more evolutionary stable). In contrast, dopamine is easily oxidized with several potentially toxic products (often leading to neurodegeneration).

9) With **more than 20 small and 100+ peptide transmitters in nearly every nervous system**, their chemical balances provide unprecedented opportunities for evolutionary innovations, behavioral controls, and behavioral choice—all uniquely realized in different animal groups. Thus, "**transmitters made nervous system**" (Moroz et al., 2021). The foundation of brain languages is the *multi-transmitter organization of early neural systems*. Thus, ***both*** low molecular weight transmitters (amino acids such as glutamate, aspartate, glycine, as well as ATP, NO, protons) and short peptides were the first transmitters or co-transmitters. Multi-transmitter chemical wiring and integration were imperative both in Precambrian metazoans and the present-day animals. Some parasitic groups might have a reduced set of their neurotransmitters because of secondary simplification (e.g., orthonectids with about two dozen neurons in the entire CNS; Slyusarev and Starunov, 2016; Slyusarev et al., 2020).

10) **The ancestral non-synaptic transmission has not disappeared in the course of evolution**, contributing

to the neuronal integration in extant neural systems. Paracrine, non-synaptic communication is also known as the volume transmission (Agnati et al., 1995, 2006, 2010; Zoli et al., 1998; Nieuwenhuys, 2000; Ridet and Privat, 2000; Sykova, 2004; Trueta and De-Miguel, 2012; Taber and Hurley, 2014; Noble et al., 2018). Even non-synaptic organization of central pattern generators is theoretically possible; it can be illustrated by mathematical modeling of chemical gradients and generation of rhythmic behaviors without synapses. Changeable chemical gradients and oscillations of extra-synaptic neurotransmitters have also been experimentally detected *in vivo* using physically isolated neurons as a biosensors (Chistopol'skii and Sakharov, 2008; Chistopolsky et al., 2008; Dyakonova et al., 2015).

Ctenophores or comb jellies seem to present the most extreme case of multiple origins of neurons and synapses (Moroz et al., 2014; Moroz and Kohn, 2016), with the remarkably different multi-transmitter set. In this early-branching animal lineage (Whelan et al., 2017), there are two morphologically functional, molecularly and, perhaps, genetically different neural systems: (i) skin nerve net and (ii) even more diffused cells in the mesoglea. The mesogleal neuroid elements share their phenotypes with muscle cells (Norekian and Moroz, 2019a,b, 2020). This situation might be a relict; does it reflect the origins of some populations of neurons and muscles from the same evolutionary predecessors? A similar situation might be in cnidarians as outlined in the hypothesis of G. Mackie (1970). However, in this scenario, the evolutionary predecessors of neurons and muscles were myoepithelial cells. The emerging single-cell sequencing data in *Hydra* (Siebert et al., 2019) showed that selected muscle and neuronal cells in cnidaria might share some transcriptional factors summarized in the recent review (Arendt, 2021). Of note, striated muscle cells also evolved at least two-three times in evolution (Steinmetz et al., 2012).

QUESTIONS AND PROSPECTIVE FOR EXPRERIMENTAL VALIDATION

The polygenesis hypothesis, and many of its predictions related to reconstructions of cellular genetic relationships, can be tested using single-cell "omics" approaches. The initial data from different phyla and observed unprecedented diversity of molecular phenotypes (Sebe-Pedros et al., 2018; Cocanougher et al., 2019; Musser et al., 2019; Siebert et al., 2019) seems to favor the hypothesis of multiple origins of neurons and the existence of numerous cell-type-specific phyletic lineages. However, the challenge is integrating vast comparative data (with expected hundreds of cell-type-specific lineages across thousands of species) with real-time physiology of individual cells and their ensembles in each representative species. It might take decades, but a new evolutionary theory for neural diversity and functions needs interdisciplinary studies. I envision a Periodic System of Cell Types—the natural genealogical classification of cell phenotypes and states integrated with evolutionary cell trees of Life and *predictive power*. It can be a conceptual analog to the Periodic System of Chemical Elements; that is, the position of an element in the Periodic System predicts its properties (e.g., inert gases or metals). Similarly, the ideal classification of cell types can predict their functional features and constraints (Moroz, 2018). The fundamental questions to be addressed can be broadly divided into two overlapping long-term objectives: (i) deciphering neural evolution vs. (ii) decoding chemical networks for intercellular communications, including methodology to reveal numerous chemoconnectomes unbiasedly.

Deciphering Neuronal Evolution

Single-cell comparative data and novel informatics theory are needed for multiple cross phyla genealogies. But, no criteria for cell-specific homologies across phyla have been established and experimentally validated. Some approaches are suggested (Tarashansky et al., 2020), but true homology can only be found with multiple cross-validated criteria, including identifying potential continuity of homologs tracing intermedial species. Here, the lack of required comparative data from "minor" phyla and classes is a significant bottleneck. There are ∼35 metazoan phyla and 100+ classes with dozens of eukaryotic lineages related to metazoans. The most critical basal metazoan groups to be investigated are Placozoa, Porifera, and Ctenophores. For representatives of these groups and other reference species (Striedter et al., 2014), the following questions need to be addressed.

Are there yet unknown transmitters? Prediction: it can be dozens of novel small (neuro)transmitters and many thousands of novel neuropeptides. The secretory organelles are highly conserved across eukaryotes. The recent comparative study on the choanoflagellates (the sister group to Metazoa) clearly illustrated a polarized localization of putative but quite diverse secretory vesicles in two model species *Salpingoeca rosetta* and *Monosiga brevicollis* (Gohde et al., 2021). However, it is unclear how many signal molecules can be co-released? What are the functions of such paracrine secretion in choanoflagellates?

What is the contribution of synaptic vs. non-synaptic release across different animal lineages? For most transmitters, we anticipate a broad spectrum of variations. A synapse can be at one part of the spectrum, with the highly localized transmission within the synaptic cleft constraints, to "true" hormonal distance signaling. Volume transmission is not only restricted by diffusion rates of signal molecules. Tissue micromechanics, cilia-induced vortexes, and dynamic extracellular space can also clearly increase passive diffusion rates in unicellular and colonial organisms and multicellular, primarily nerveless, animals such as sponges and placozoans.

How many types of synapses, and what is their natural/evolutionary classification? Synapses in ctenophores and cnidaria are poorly analyzed. Nothing is known about volume transmitters in these organisms as in the majority of bilaterians. Volume transmission in nerveless animals such as placozoans and sponges has not been quantitatively measured.

Do transmitters evolve? Are there any constraints and trends in the evolutionary selection of (neuro)transmitters and synapses? Both functional (e.g., chemical stability vs. reactivity [antioxidant/prooxidant properties], synthesis, inactivation, etc.)

and evolutionary constraints (pre-adaptations, ecology, and lifestyles) have to be considered. The chemical/secretory organization and relationships among the digestive, immune and neural cell types are unknown for basal metazoans.

Deciphering Chemoconnectomes and Chemical Syntaxis of Neural Systems

A *chemoconnectome* is defined as an entire set of neurotransmitters, neuromodulators, neuropeptides, and receptors supporting chemical transmission in an animal (as illustrated for *Drosophila* by Deng et al., 2019). However, visualization of dynamic chemoconnectomes (which can change in time: from milliseconds to hours and days), is a much greater challenge than reconstructing traditional connectomes, static descriptions of synaptic wiring. This challenge demands conceptually new and innovative methods and theories to simultaneously image dozens of specific molecules over broad ranges of transmitter concentrations (nanomoles to micromoles) in real-time. Unfortunately, most current bioanalytical approaches measure one or a few neurotransmitters at a time, and only for narrow concentration ranges. The 4D dynamic (3D space+time) of complex (extracellular) *milieus inferiors* with hundreds of signal molecules (spread from nanoliter to milliliter volumes) is the Frontier in cell, developmental, and evolutionary biology as well as biomedicine.

Multiplexed nanotools (Farsi et al., 2016; Jing et al., 2018; Wu et al., 2018; Dinarvand et al., 2019; Zeng et al., 2020) are instrumental in visualizing cell-specific secretion of co-transmitters and the actual balance of neurotransmitters as stereotyped and learned behaviors are generated.

The combinatorial power of chemical interactions is enormous, but constraints of neurotransmitters signaling also exist, observed in well-defined phenomena of transmitter-dependent behaviors (Dyakonova and Sakharov, 2019). Methodological and theoretical efforts would decipher still elusive "neuronal syntax" (Buzsaki, 2010) of the electrochemical brain grammar (as "words," "sentences," or other hierarchically organized "quanta" of bio-information), which, I think, is the primary chemical, and transmitter-based, in its nature.

I would conclude that the current biodiversity of species with the astonishing diversity of secretory and signaling mechanisms, neurons and synapses, neural and alternative integrative systems are true *Gifts of Nature* to neuroscientists and humankind. We are only getting the first surprises from these gifts. We are only starting to taste novel fundamental insights and paradigm shifts in this endeavor. It might be controversial, but the shortcut to better understanding our brains and neurological disorders and regenerative medicine of the future is studying small creatures in the world ocean. Admittedly, not all marine creatures can be brought to the lab and cultured. But we now have the capacity to bring labs to the sea (Moroz, 2015b) and expand frontiers of the living world and ourselves.

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The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Stress Hormones: Emerging Targets in Gynecological Cancers

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In the past decade, several discoveries have documented the existence of innervation in ovarian cancer and cervical cancer. Notably, various neurotransmitters released by the activation of the sympathetic nervous system can promote the proliferation and metastasis of tumor cells and regulate immune cells in the tumor microenvironment. Therefore, a better understanding of the mechanisms involving neurotransmitters in the occurrence and development of gynecological cancers will be beneficial for exploring the feasibility of using inexpensive β-blockers and dopamine agonists in the clinical treatment of gynecological cancers. Additionally, this article provides some new insights into targeting tumor innervation and neurotransmitters in the tumor microenvironment.

Keywords: perineural invasion, noradrenaline, epinephrine, dopamine, neurotrophic factors, glucocorticoids, tumor immune microenvironment

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INTRODUCTION

Cervical cancer and ovarian cancer are two major gynecological malignancies. Preliminary and secondary strategies for the prevention of cervical cancer have reduced its rates of incidence and mortality. However, in 2018, there were 106,000 cases of cervical cancer in China and 48,000 deaths (Arbyn et al., 2020). Therefore, cervical cancer remains the second leading cause of cancer-related death among young and middle-aged women (Bray et al., 2018; Siegel et al., 2020). Ovarian cancer is the seventh most common cause of cancer and the eighth leading cause of death in women. As ovarian cancer is difficult to diagnose early and is associated with high malignancy and drug resistance, it has the worst prognosis and highest mortality rate among all gynecological cancers (Coburn et al., 2017; Webb and Jordan, 2017; Torre et al., 2018). Therefore, a better understanding of the biological behaviors of cervical cancer and ovarian cancer is urgently needed, and novel therapeutic targets need to be identified.

Perineural invasion (PNI) has emerged as a novel research hotspot and is a harbinger of a poor prognosis in multiple cancers, including cervical cancer and ovarian cancer.

Abbreviations: SNS, sympathetic nervous system; PNI, perineural invasion; NE, noradrenaline; E, epinephrine; DR, dopamine receptor; PGE2, prostaglandin E2; MMPs, metalloproteinases; MMP-2, metalloproteinase 2; MMP-9, metalloproteinase 9; IL-6, interleukin 6; IL-8, interleukin 8; VEGF, vascular endothelial growth factor; ADAM17, A Disintegrin and Metalloproteinase 17; DEX, dexamethasone; MDSCs, myeloid-derived suppressor cells; TAMs, tumor -associated macrophages.

Cervical cancer and ovarian cancer promote their own PNI via the release of neurotrophins (Allen et al., 2018; Long et al., 2018), axonal guidance molecules (Madeo et al., 2018), and exosomes (Madeo et al., 2018; Lucido et al., 2019; Vermeer, 2019; Kovacs et al., 2020). In addition, Schwann cells and cervical cancer cells can work in concert to promote tumor innervation (Huang et al., 2020). Evaluations of clinical specimens have also confirmed the presence of innervation in cervical cancer and ovarian cancer (Lucido et al., 2019; Kovacs et al., 2020; Reavis et al., 2020). In these evaluations, PNI in cervical cancer has a detection rate of 7.0% to 35.1% (Zhu et al., 2018; Zhu et al., 2019). Furthermore, existing studies suggest that there is a positive correlation between chronic stress and cancer progression. Long-term stress stimulation activates the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal axis (HPA), leading to the release of stress hormones, especially catecholamines and glucocorticoids. Catecholamine hormones can be further divided into norepinephrine (NE), epinephrine (E), and dopamine. These hormones act on β-adrenergic receptors, dopamine receptors (DRs), and glucocorticoid receptors. The interactions between stress hormones and receptors can produce a series of physiological effects on tumor cells and stromal cells.

The β-adrenergic receptors (β1, β2, β3) are a group of G protein-coupled receptors that mediate SNS signal transduction and activate downstream signaling pathways to prepare the body for "fight or flight." β2-Adrenergic receptor (ADRB2) is overexpressed in ovarian cancer and cervical cancer and is positively correlated with a poor prognosis in patients (Lutgendorf et al., 2009; Huang et al., 2016; Chen et al., 2017). Ovarian cancer patients with high glucocorticoid receptor expression also have shorter progression-free survival and overall survival (Veneris et al., 2017; Veneris et al., 2019). The DRs include DR1 and DR2, both of which are highly expressed in ovarian cancer (Peters et al., 2020). Currently, no evidence has directly demonstrated that intratumoural infiltrating nerves are involved in the effect of stress on tumor cells. However, we hypothesize that under chronic stress, tumor innervation and receptors on the tumor cell surface may function via stress hormones to establish cross-talk and promote tumor progression together.

EPIDEMIOLOGICAL STUDIES

Epidemiological studies have reported that depression, social isolation, and posttraumatic stress disorder, which cause long-term activation of the SNS, are closely related to the incidence of ovarian cancer. In patients with high depressive symptoms and low social support, the levels of NE in ovarian cancer tissues are significantly increased, and the risk of ovarian cancer or cancer progression is increased (Lutgendorf et al., 2009, 2011; Huang et al., 2015; Roberts et al., 2019). In contrast, eudaimonic well-being is negatively correlated with the NE levels in ovarian cancer tissues. Improving the eudaimonic well-being of patients with ovarian cancer has certain physiological protective effects (Davis et al., 2015). Although the specific mechanism has yet to be clearly elucidated, the possible explanation is that in ovarian cancer, the levels of circulating NE or intratumoural NE gradually increase due to the presence of chronic stress, which causes tumor vascularization, metastasis, invasion, and other effects.

Continuous human papillomavirus (HPV) infection is the main reason for the occurrence and development of cervical cancer. Severe types of stress, such as bereavement (loss of a parent, spouse, or child), may increase the risk of cancers related to HPV infection, such as cervical cancer. Continuous exposure to these severely stressful life events can increase the susceptibility of the host to cancer-causing HPV infection or accelerate the occurrence of established infectious cancers and ultimately lead to cervical cancer (Coker et al., 2003; Fang et al., 2011; Lu et al., 2016, 2019). Although behavioral changes after stressful life events may also play a role in cervical cancer, chronic stress-induced neuroendocrine disorders leading to changes in the biological behavior of tumor cells have been increasingly considered to be one of the biological mechanisms linking psychological stress with the occurrence and development of cervical cancer (Kennedy et al., 2014). Hence, regardless of the cause of cervical cancer, psychotherapy may be an important part of its prevention or treatment.

NORADRENALINE AND EPINEPHRINE

In response to stress, the levels of circulating catecholamines will increase. However, the local sympathetic nerve appears to provide most of the catecholamine content in tumor tissue, as we did not find any significant difference in circulating NE levels among tumor patients, nor did we find a significant correlation between plasma NE levels and intratumoural NE levels. However, these studies also had some limitations. Blood sampling was performed 2∼3 h before surgery, so parallel analyses of NE levels in the tumor and plasma could not be performed (Lutgendorf et al., 2009, 2011; Cole et al., 2015). In another study, mice were treated with hexamethonium bromide, a compound that can block ganglionic transmission in the peripheral nervous system. As expected, hexamethonium bromide completely eliminated the effect of stress on tumor growth. Tumor samples from animals that routinely faced restraint stress had significantly more innervation than tumor samples from control animals, and this increase could also be completely blocked by hexamethonium bromide. Adrenalectomy also failed to significantly inhibit stress-induced tumor growth, intratumoural nerve counts, and blood NE levels (Allen et al., 2018). All these results confirm the role of nerve endings in catecholamine-mediated tumor growth. Therefore, we concluded that under chronic stress, nerves in the tumor parenchyma can release neurotransmitters, such as NE and E, into the tumor microenvironment. Then, these neurotransmitters bind to receptors on the tumor cell surface and produce a series of effects on tumor cells. The effects are described below.

Activation of Oncogenes

The increases in the levels of NE and E induced by chronic stress can act on ADRB2 to promote tumor cell growth, metastasis, and angiogenesis (Sood et al., 2006; Thaker et al., 2006;

Hassan et al., 2013; Cole et al., 2015; Jiang et al., 2020). These effects involve the activation of multiple tumor genes, including Src and signal transducer and activator of transcription-3 (STAT3). The Src protein plays important roles in the regulation of cell growth and differentiation, but abnormal activation of the Src protein is closely related to the occurrence of several tumors. Elevated NE levels lead to the abnormal phosphorylation of Src through ADRB2, followed by regulation of downstream pathways to enhance the proliferation, migration, and angiogenesis of ovarian cancer cells (Nilsson et al., 2007; Sood et al., 2010; Armaiz-Pena et al., 2013; Choi et al., 2015; Cole et al., 2015). It has also been confirmed that there is a positive correlation between high levels of NE in tumors and high Src phosphorylation levels in ovarian cancer tissues (Armaiz-Pena et al., 2013). STAT3 is another important oncogene. Abnormal activation of STAT3 triggers a variety of pathological events, including tumorigenesis (Calo et al., 2003). Norepinephrine and E induce STAT3 phosphorylation through ADRB2; STAT3 then translocates into the nucleus to activate target genes, leading to the proliferation, infiltration, and metastasis of ovarian cancer cells (Landen et al., 2007). Mitogen-activated protein kinase phosphatase-1 (MKP-1), also known as DUSP1, participates in the inactivation of MAPK and leads to the inhibition of apoptosis. High expression of MKP-1 is related to resistance to chemotherapy in ovarian cancer (Denkert et al., 2002). NE activates the cAMP-PKC-CREB signaling pathway through ADRB2 to induce the expression of the MKP-1 gene, which inhibits the responsiveness of ovarian cancer cells to paclitaxel chemotherapy (Wu et al., 2005; Kang et al., 2016). NE and E can also upregulate the expression of silent information regulator-1 (Sirt1) by activating ADRB2. Sirt1 can block the acetylation of p53, thereby conferring chemotherapy resistance to cervical cancer cells (Reed and Quelle, 2014; Chen et al., 2017) (**Figure 1**).

Metastasis, Invasion, and Epithelial-Mesenchymal Transition

Metalloproteinase (MMP)-2 and MMP-9 play key roles in the invasion of malignant tumors (Davidson et al., 1999; Bergers et al., 2000; Huang et al., 2002; Sood et al., 2004). Norepinephrine and E can directly increase the invasive ability of ovarian cancer cells through upregulation of MMP-2 and MMP-9 via ADRB2. Propranolol (a non-selective β-blocker) can block this process (Sood et al., 2006; Thaker et al., 2006). In addition to being an inflammatory mediator, prostaglandin E2 (PGE2) is related to tumor cell proliferation, metastasis, and angiogenesis. Norepinephrine and E induce Nf-kb phosphorylation through ADRB2, and then p-Nf-kb enters the nucleus and binds with the PTGS2/PTGES gene to increase the synthesis of PGE2, which ultimately drives the proliferation and metastasis of ovarian cancer (Nagaraja et al., 2016). Epithelial-mesenchymal transition (EMT) plays an important role in embryonic development, damage repair, and cancer metastasis. Upregulation of the expression of Slug is an EMT hallmark (Hajra et al., 2002; Onder et al., 2008; Casas et al., 2011; Villarejo et al., 2014). Human telomerase reverse transcriptase (hTERT), apart from stabilizing the length of telomeres, is believed to promote malignant transformation independent of telomere lengthening. Norepinephrine upregulates hTERT-mediated Slug expression through Src and ultimately promotes the occurrence of EMT in ovarian cancer (Choi et al., 2015) (**Figure 1A**).

Angiogenesis

Angiogenesis refers to the formation of new blood vessels by original endothelial cells and is an important physiological process in the repair of tissue damage. In cancer, angiogenesis is a key process for the growth and metastasis of most solid tumors, as it ensures a supply of oxygen and nutrients to the tumor tissue and transports metabolic waste from the tumor microenvironment (Lim et al., 2020). Although tumor angiogenesis is mainly driven by vascular endothelial growth factor (VEGF), it is also affected by MMPs, interleukin (IL)-6, IL-8, and so on. Norepinephrine can increase the expression of VEGF in ovarian cancer cells (Lutgendorf et al., 2003; Thaker et al., 2006; Chakroborty et al., 2009; Szubert et al., 2016) and promote the migration of endothelial cells by inducing the expression of MMP-2 and MMP-9 (Bergers et al., 2000; Huang et al., 2002; Thaker et al., 2006; Landen et al., 2007; Gonzalez-Villasana et al., 2015), thereby inducing the formation of new blood vessels in tumors. The cytokines IL-6 and IL-8 are vital in inflammation and can increase tumor angiogenesis (Browning et al., 2018; Taher et al., 2018; Kim, 2020; Fousek et al., 2021). Norepinephrine can induce ovarian cancer cells to produce IL-6 and IL-8 through effects on the Src protein and FosB protein, respectively, and thus promote angiogenesis in ovarian cancer (Nilsson et al., 2007; Shahzad et al., 2010) (**Figure 1A**).

Cell Survival

Anoikis refers to the process of programmed cell death that occurs after the separation of normal cells from the extracellular matrix and neighboring cells. Evasion of anoikis improves the chances of survival of metastatic cancer cells, allowing the cancer cells to proliferate at new sites of attachment (Liotta and Kohn, 2004). Focal adhesion kinase (FAK) is a widely expressed protein tyrosine kinase that participates in the malignant invasion of tumors. Norepinephrine and E initiate Src-related FAK phosphorylation through ADRB2 and thus protect ovarian cancer cells from anoikis (Sood et al., 2010). Norepinephrine can also induce YAP1 dephosphorylation and nuclear translocation via ADRB2, thus protecting cervical cancer cells from anoikis (Li et al., 2020). Propranolol can also inhibit this NE-mediated process (Gong et al., 2019) (**Figure 1**). In addition to NE, neurotrophic factors and their ligands, such as BDNF/TrkB, can induce escape from anoikis in ovarian cancer, cervical cancer, and endometrial cancer cells (Yu et al., 2008; Bao et al., 2013; Yuan et al., 2018a).

DOPAMINE

Dopamine is another catecholamine neurotransmitter and regulates various physiological functions of the central nervous system. Disorders related to the regulation of the dopamine system include Parkinson's disease and schizophrenia. In a

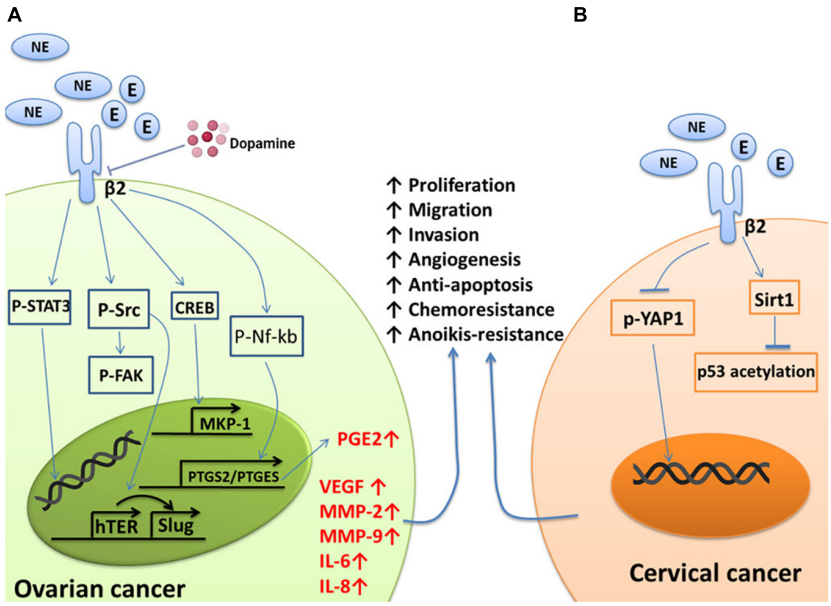

FIGURE 1 | Summary of the effects of NE/E on pathways involved in cancer cell survival, metastasis, and chemoresistant signaling. **(A)** NE binds to ADRB2 to activate Src, which then induces the phosphorylation of FAK and the expression of VEGF, IL-6, and IL-8, conferring anoikis resistance, metastasis, and angiogenesis in ovarian cancer cells. STAT3 is phosphorylated and translocates to the nucleus to transactivate the target genes MMP-2 and MMP-9. NE can also activate ADRB2 to transcriptionally activate PTGS2 and PTGES via Nf-kb to produce PGE2. Finally, NE can induce ovarian cancer cells to become resistant to chemotherapy by acting on a target gene to induce MKP-1 expression through CREB. Additionally, NE-mediated tumor growth and angiogenesiscan be blocked by dopamine. **(B)** YAP1 is dephosphorylated and translocates from the cytoplasm to the nucleus in response to NE signaling, which results in anoikis resistance, a process initiated by the activation of ADRB2. Norepinephrine can also activate ADRB2 to induce chemoresistance by suppressing the acetylation of p53 through the upregulation of Sirt1 in cervical cancer cells.

restraint stress model, intratumoural NE levels were found to remain elevated, whereas dopamine levels were dramatically decreased in the stress group compared with the control group (Moreno-Smith et al., 2011). The possible reason for the drop in the dopamine levels is that dopamine is a precursor for the synthesis of NE and E.

Norepinephrine-mediated tumor growth and angiogenesis were completely blocked with daily dopamine administration (Moreno-Smith et al., 2011) (**Figure 1A**). The signaling pathway that involves dopamine is the dopamine-mediated reversal of NE-induced Src phosphorylation. In addition, dopamine reduces the stress-mediated growth and microvessel density of ovarian cancer through tumor cell DR2 and inhibits the mobilization of endothelial progenitor cells from the bone marrow cavity into the peripheral circulation through DR2 on endothelial progenitor cells (Basu et al., 2001; Chakroborty et al., 2008; Moreno-Smith et al., 2011). In addition, dopamine can promote the maturation and normalization of the ovarian cancer vascular system through the DR1, allowing greater intake of chemotherapeutic drugs (Moreno-Smith et al., 2013). Based on these findings, dopamine replacement therapy may represent a novel treatment strategy to block the detrimental effects of chronic stress. Interestingly, the incidence of cancer in patients with schizophrenia may be lower than that in the general population (Mortensen, 1989; Barak et al., 2005; Asada et al., 2008; Chou et al., 2011). Patients with schizophrenia have high levels of the dopaminergic system, and preclinical studies have confirmed that dopamine can inhibit tumor angiogenesis. However, this view is still controversial, and it remains to be confirmed whether the lower incidence of cancer in schizophrenia patients is related to the hyperactivity of their dopaminergic system.

NEUROTROPHIC FACTORS

Neurotrophic factors are protein molecules that are necessary for the growth and survival of nerve cells. Neurotrophic factors belong to the small polypeptide growth factor family composed of five members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophic factor-3 (NT-3), neurotrophic factor-4/5 (NT-4/5), and neurotrophic factor-6 (NT-6). Neurotrophic factors interact with two types of receptors: p75 and Trk receptors. The Trk receptors are necessary for neurite growth and cell survival. Different Trk receptors bind to specific neurotrophic factors with high affinity: NGF binds to TrkA, BDNF, and NT4/5 bind to TrkB, and NT-3 binds to TrkC (Chao and Hempstead, 1995; Retamales-Ortega et al., 2017).

The expression levels of NGF and its receptor TrkA in ovarian cancer and cervical squamous cell carcinoma are significantly increased and related to the proliferation and metastasis of ovarian cancer as well as the clinical grade and nerve infiltration of cervical cancer (Tapia et al., 2011; Streiter et al., 2016; Retamales-Ortega et al., 2017; Long et al., 2018; Faulkner et al., 2020). Ovarian cancer cells express and secrete NGF, which

directly stimulates endothelial cell proliferation by activating TrkA receptors to induce angiogenesis. Nerve growth factor also acts on the receptor TrkA on the surface of cancer cells in an autocrine manner to increase the protein expression levels of VEGF, COX-2, and A Disintegrin and Metalloproteinase 17 (ADAM17). These three proteins are related to angiogenesis, migration, and cell proliferation in epithelial ovarian cancer (Vera et al., 2014; Retamales-Ortega et al., 2017) (**Figure 2**). The activation of the receptor TrkB by BDNF also plays an important role in tumor progression. BDNF and TrkB are overexpressed in epithelial ovarian cancer tissues. Activation of the BDNF/TrkB pathway induces ovarian cancer cell migration, invasion, angiogenesis, and anoikis resistance (Qiu et al., 2006; Au et al., 2009; Siu et al., 2009). In addition to ovarian cancer, cervical cancer, endometrial cancer, and uterine leiomyosarcoma also exhibit high expression of BDNF and TrkB, which are closely related to adverse clinical phenomena, such as lymph node metastasis (Yu et al., 2008; Moon et al., 2011; Makino et al., 2012; Bao et al., 2013; Yuan et al., 2018a,b).

Moreover, neurotrophins released by tumor cells can stimulate adjacent nerve cells to develop nerve endings in the tumor. For example, NE can bind to ADRB3 expressed by ovarian cancer cells to produce BDNF, and then BDNF acts on TrkB receptors on host neurons to increase the innervation of the tumor (Entschladen et al., 2006; Allen et al., 2018) (**Figure 2**). These nerve endings may release catecholamines, which initiate the migratory and angiogenic activity of tumor cells, prerequisites for invasion and metastasis.

GLUCOCORTICOIDS

Glucocorticoids are another type of hormone that increase during a stress response. They are widely used clinically as anti-inflammatory and immunosuppressive agents. Glucocorticoids can also be used as adjuvant drugs with chemotherapy to reduce the side effects of chemotherapy. However, *in vitro* studies have demonstrated that glucocorticoids can promote tumor cell survival, metastasis, and drug resistance. The expression of receptor tyrosine kinase-like orphan receptor 1 (ROR1) is closely related to the phenotype of ovarian cancer stem cells, peritoneal metastasis, and the development of resistance to chemotherapy (Zhang et al., 2012; Zhang H. et al., 2014; Zhang S. et al., 2014; Henry et al., 2017; Karvonen et al., 2019). Dexamethasone (DEX), a synthetic glucocorticoid, can promote the expression of ROR1, fibronectin, and MUC1 by activating glucocorticoid receptors, thereby mediating stemness, adhesion, and drug resistance in cancer cells, respectively (Yin et al., 2016; Karvonen et al., 2020). The activation of glucocorticoid receptors can also upregulate the expression of serum and glucocorticoid-regulated kinase 1 (SGK1) and MKP-1, both of which can promote the survival of ovarian cancer cells (Melhem et al., 2009;

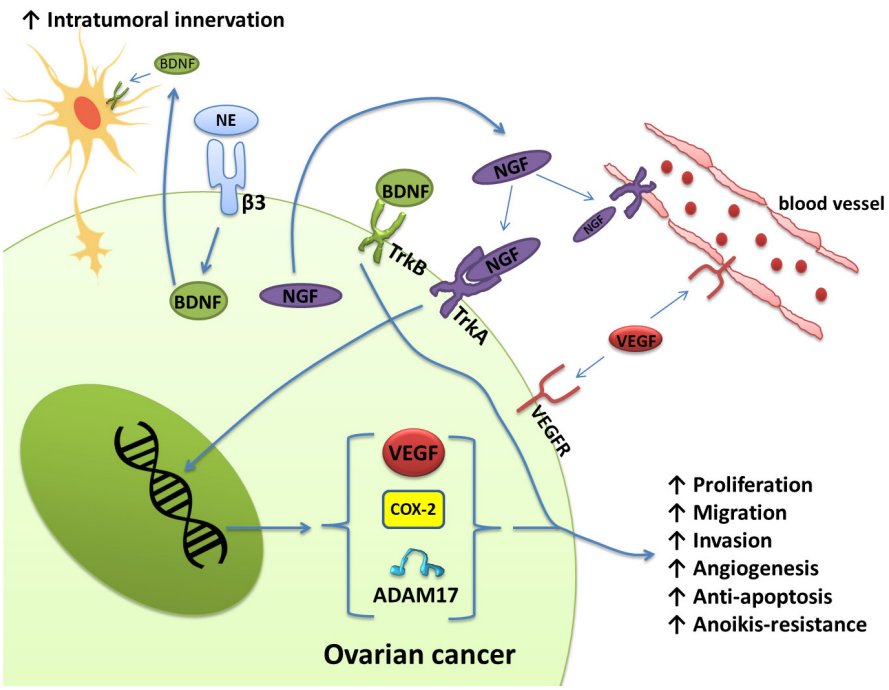

FIGURE 2 | Schematic representation of the effects of NGF/TrkA and BDNF/TrkB, which are involved in several signaling pathways in ovarian cancer. Ovarian cancer cells express and secrete NGF. Through the activation of TrKA, NGF induces angiogenesis by directly stimulating the proliferation of endothelial cells. Nerve growth factor also regulates angiogenesis indirectly through the production of VEGF by ovarian cancer cells. In addition, NGF increases COX-2 levels, which induces the production of PGE-2. PGE-2 has been associated with invasion in cancer cells. ADAM17 also appears to be regulated by the activation of NGF/TrkA. Activation of the BDNF/TrkB pathway also confers migration, invasion, angiogenesis, and anoikis resistance to ovarian cancer cells. Norepinephrine can also bind to ADRB3 expressed by ovarian cancer cells to induce the production of BDNF, which then acts on TrkB receptors on nerve cells to increase the innervation of tumor tissues.

Stringer-Reasor et al., 2015) (**Figure 3A**). Glucocorticoids can also affect the life cycle of HPV, interfere with the function of p53, and reduce the expression of miR-145, thus playing direct roles in the persistence of HPV infection and resistance to chemotherapy in cervical cancer patients (Feng et al., 2012; Shi et al., 2012) (**Figure 3B**).

As a common drug used for abortion in clinical practice, mifepristone has anti-glucocorticoid activity separate from its anti-progesterone effect. The addition of mifepristone to a combination cisplatin and paclitaxel regimen can prevent the development of drug resistance in ovarian cancer cells and cervical cancer cells (Jurado et al., 2009; Gamarra-Luques et al., 2012; Ponandai-Srinivasan et al., 2019). This also suggests that the activation of the glucocorticoid signaling pathway negatively impacts gynecological cancers.

Consistency between preclinical and clinical studies on ovarian cancer supports the hypothesis that glucocorticoid signaling has a promotive effect on solid tumors. However, cervical cancer patients with higher expression of glucocorticoid receptors have longer progression-free survival and overall survival (Block et al., 2017; Kost et al., 2019). The reason for the contradiction between clinical and experimental studies on cervical cancer is unclear, and whether other signaling pathways are involved remains to be studied.

TUMOR IMMUNE MICROENVIRONMENT

It is clear that the tumor microenvironment, which is composed of a series of stromal cells [including macrophages, T cells, myeloid-derived suppressor cells (MDSCs), and fibroblasts] and their secreted products, has a significant impact on cancer progression. In this section, we will briefly discuss the effects of sustained stress on the immune microenvironment of gynecological cancers. A previous section explains that NE can induce the production of IL-6 and IL-8 in ovarian cancer cells and promote angiogenesis and metastasis. Additional effects of IL-6 include attenuation of Th1 responses in the tumor microenvironment (Johnson et al., 2018; Tsukamoto et al., 2018), activation of cancer-associated fibroblasts (Karakasheva et al., 2018), reductions in CD8 + cytotoxic T lymphocyte populations, increases in immunosuppressive FOXP3 + regulatory T cell populations (Kato et al., 2018), and enhanced generation of MDSCs (Hanazawa et al., 2018). In combination with chemotherapy, propranolol potentially results in improvements in circulating CD8 + T cells (Ramondetta et al., 2019). IL-8 also has a strong ability to recruit macrophages or MDSCs to the tumor microenvironment (Fousek et al., 2021). Macrophages have two different phenotypes: a tumor-suppressive phenotype (M1) and a tumor-supportive phenotype (M2). Tumor-associated macrophages (TAMs) mainly exhibit M2 characteristics. IL-8 can polarize macrophages toward the CD163 + M2 phenotype, which may contribute to poor survival in ovarian cancer (Ning et al., 2018). At the same time, stress hormones can also directly bind to β2-adrenergic receptors on the surface of macrophages (Sloan et al., 2010; Allen et al., 2018; Colon-Echevarria et al., 2020). Ultimately, this will exacerbate the infiltration of TAMs (**Figure 4**). In a study, treatment of mice with hexamethonium bromide resulted in a marked reduction in macrophage infiltration. In contrast, cytisine, a neuronal nicotinic acetylcholine (nACh) receptor agonist, could mimic the effects of restraint stress on macrophage infiltration (Allen et al., 2018). Therefore, macrophage infiltration mediates stress-enhanced progression.

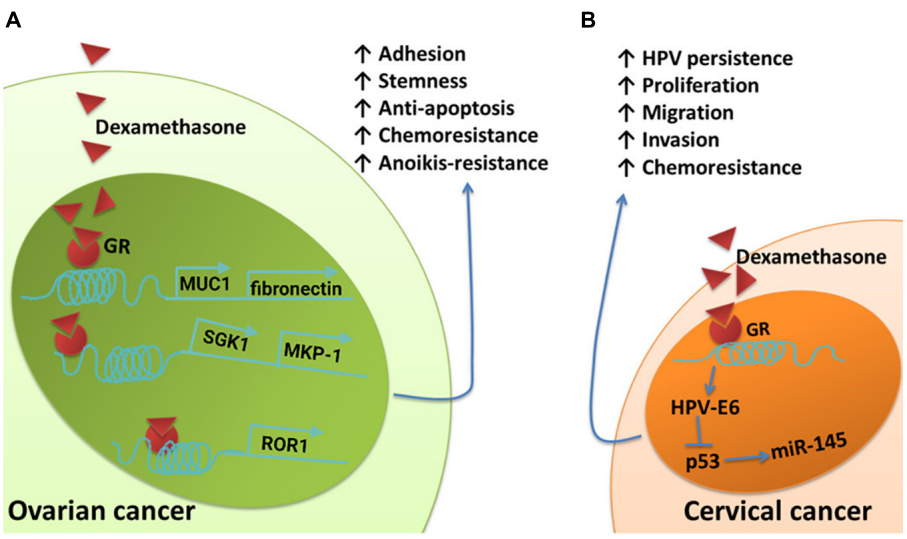

FIGURE 3 | Schematic of the effects of glucocorticoids on pathways involved in cancer cell survival, metastasis, and chemoresistant signaling. **(A)** The upregulation of fibronectin and MUC1 induced by DEX contributes to DEX-induced pro-adhesion effects and protects ovarian cancer cells from chemotherapy. Dexamethasone induces increased expression of SGK1 and MKP-1, both of which promote cell survival. Dexamethasone induces anti-apoptotic features and drug resistance in ovarian cancer by promoting ROR1-mediated stemness. **(B)** Glucocorticoid-induced HPV–E6 expression effectively suppresses the upregulation of p53-dependent miR-145 and cellular apoptosis.

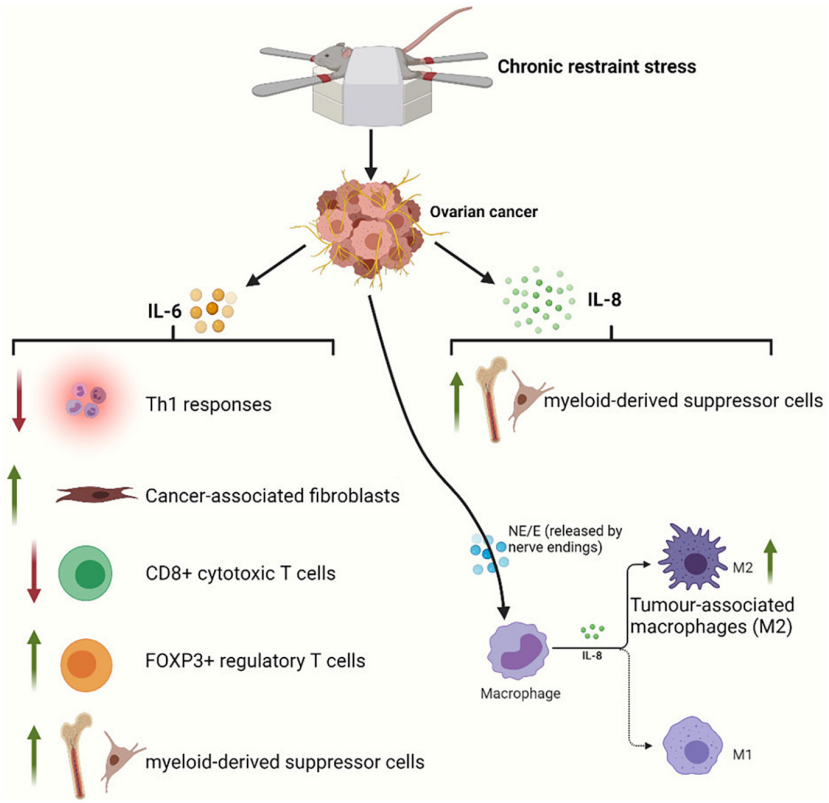

FIGURE 4 | Restraint stress can act through a variety of immune mechanisms to promote tumor progression. IL-6 released by ovarian cancer cells can inhibit adaptive antitumor immunity by suppressing Th1 responses and CD8 + T cell activation and by driving and recruiting regulatory T cells. IL-6 also initiates cancer-associated fibroblast and MDSC infiltration of the tumor microenvironment. IL-8 released by ovarian cancer cells can inhibit innate immunity by polarizing macrophages toward a type 2 tumor-associated phenotype and by supporting MDSCs into the tumor microenvironment. NE and E also exacerbate the infiltration of M2 macrophages via β2-adrenergic receptors on macrophages.

CLINICAL TRIALS

As mentioned above, several experiments have confirmed that the activation of β-adrenergic receptors can promote the malignant progression of ovarian cancer. However, the existing clinical research results are still conflicting. Some studies have reported that patients with epithelial ovarian cancer who used β-blockers have a lower chance of death and longer overall survival than patients who did not use β-blockers (Diaz et al., 2012; Al-Niaimi et al., 2016; Ramondetta et al., 2019). In contrast, other clinical studies have observed no association between the use of β-blockers and a reduction in ovarian cancer mortality (Heitz et al., 2013; Johannesdottir et al., 2013; Cho et al., 2020). One study even reported that patients who used β-blockers during the perioperative period had an increased risk of death (Gonzalez et al., 2020). Notably, almost all patients in the above studies were using selective β1-receptor blockers, but it is more likely that non-selective β-blockers can benefit patients with ovarian cancer. However, the use of non-selective β-blockers has been limited due to well-known side effects. Hence, these contradictory research results highlight the importance of stratification studies based on the type of β-blocker. Otherwise, the results are unreliable (Hefner and Csef, 2016). After categorizing the selectivity of β-blockers, we observed that ovarian cancer patients who used non-selective β-blockers showed reduced cancer-specific mortality. Selective β-blocker intake did not affect prognosis and even produced reduced overall survival (Watkins et al., 2015; Heitz et al., 2017; Harding et al., 2019). The reason underlying this finding is still unclear. However, the patients taking selective β-blockers tended to be older and have various chronic underlying diseases, which might make them more intolerant of cancer therapy.

Glucocorticoids have been included in standard treatment plans because they can reduce the side effects of chemotherapy. However, an increasing number of experiments have proven that glucocorticoids can promote the survival of tumor cells. These results have caused concerns among clinicians to some extent, resulting in the question: Is the adjuvant application of glucocorticoids safe during chemotherapy? However, when DEX is used during the perioperative period or chemotherapy administration, there is currently no evidence to indicate that the application of this glucocorticoid will negatively impact the prognosis of patients (Munstedt et al., 2004; De Oliveira et al., 2014; Djedovic et al., 2018). We have yet to determine whether the small sample size affected the results of the study or whether the benefits of glucocorticoids, such as an increased white blood cell count and increased patient compliance, concealed its protective effect on tumor cells. In general, before further

research is performed to address this question, we should at least allay fears related to the use of glucocorticoids; after all, their benefits are obvious.

DISCUSSION

Several preclinical experiments have demonstrated overexpression of stress hormone receptors in ovarian cancer cells and cervical cancer cells. Various stress hormones produced under chronic stress exert protective effects on cancer cells through these receptors, which eventually leads to adverse clinical results. Simultaneously, cancer cells can also initiate their own innervation by releasing neurotrophic factors. Under chronic stress, these nerve endings release stress hormones (mainly NE and E), which in turn bind to the overexpressed receptors on tumor cells and induce various effects (Faulkner et al., 2019). Therefore, it is theoretically feasible to try to eliminate tumor innervation or block stress hormone receptors on the surface of tumor cells. Drugs that block these receptors are common in clinical treatment and therefore have the greatest potential. However, the relatively small cohort of studies evaluating non-selective β-blockers have led us to question the effectiveness of these drugs in treating cancers. Hence, whether to use non-selective β-blockers in gynecological cancer patients has not yet been determined. Likewise, there is no sufficient evidence indicating that using glucocorticoids will shorten the lifespan of chemotherapy-treated patients. Therefore, we do not support the aversion to using DEX for gynecological cancer treatment; after all, several preliminary studies have demonstrated that DEX is effective in preventing postoperative nausea, vomiting, and the side effects of chemotherapy. Dopamine and DR agonists are widely used in the treatment of Parkinson's disease, hyperprolactinemia, and other non-neoplastic diseases; they are inexpensive and have few side effects. Therefore, the prospect of dopamine being used to treat cancer patients in the future is also very encouraging.

In summary, we should view a tumor as a complete organism. This "organism" contains tumor cells, stromal cells, and vascular and neural connections to its host. This provides not only mechanisms for disease progression but also opportunities for therapeutic intervention. Further studies are needed to clarify the exact relationships between PNI and stress hormones in gynecological cancers. Only through this work can the process of using these inexpensive drugs to treat gynecological cancers be accelerated.

AUTHOR CONTRIBUTIONS

GC and LQ contributed equally to this manuscript. GC contributed to conception and design of the study and wrote the first draft of the manuscript. LQ screened the relevant literature and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Shedding Light on the Role of Neurotransmitters in the Microenvironment of Pancreatic Cancer

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Pancreatic cancer (PC) is a highly lethal malignancy with a 5-year survival rate of less than 8%. The fate of PC is determined not only by the malignant behavior of the cancer cells, but also by the surrounding tumor microenvironment (TME), consisting of various cellular (cancer cells, immune cells, stromal cells, endothelial cells, and neurons) and non-cellular (cytokines, neurotransmitters, and extracellular matrix) components. The pancreatic TME has the unique characteristic of exhibiting increased neural density and altered microenvironmental concentration of neurotransmitters. The neurotransmitters, produced by both neuron and non-neuronal cells, can directly regulate the biological behavior of PC cells via binding to their corresponding receptors on tumor cells and activating the intracellular downstream signals. On the other hand, the neurotransmitters can also communicate with other cellular components such as the immune cells in the TME to promote cancer growth. In this review, we will summarize the pleiotropic effects of neurotransmitters on the initiation and progression of PC, and particularly discuss the emerging mechanisms of how neurotransmitters influence the innate and adaptive immune responses in the TME in an autocrine or paracrine manner. A better understanding of the interplay between neurotransmitters and the immune cells in the TME might facilitate the development of new effective therapies for PC.

Keywords: pancreatic cancer, tumor microenvironment (TME), neurotransmitter, immunotherapy, immune cells

INTRODUCTION

Although the accumulation of genetic and epigenetic defects is believed to drive carcinogenesis, the progression of cancer is indeed highly dependent on the interactions between cancerous and non-cancerous cells in the tumor microenvironment (TME). Immune cells make a large contribution to the non-malignant cellular components in the TME (Ho et al., 2020). Tumor-infiltrated immune cells bring out an important and complicated regulatory function in cancer progression. The TME also includes multiple secreted non-cellular components, such as cytokines, neurotransmitters and extracellular matrix (Dey et al., 2020; Winkler et al., 2020). Among them, neurotransmitters are recently emerging as a novel non-cellular portion of the TME that have been appreciated in cancer progression, especially in pancreatic cancer (PC) (Tan et al., 2021).

Pancreatic cancer is a devastating malignant disease with a very dismal prognosis (Ligorio et al., 2019). PC has a unique TME characterized by a markedly increased neural density. Neural remodeling and perineural invasion (PNI), the term describing the neoplastic invasion of tumor cells into nerves, are two common adverse histological characteristics of PC. As a group of chemical substances released by neurons, neurotransmitters have been documented to play a vital role in PC (Renz et al., 2018b). Altered concentration of several neurotransmitters is usually observed in the TME of PC and is associated with increased cancer aggressiveness and worsened overall prognosis (Biffi et al., 2019; Jurcak and Zheng, 2019; Hosein et al., 2020). PC cells showed chemotaxis toward neurotransmitters. Neurotransmitters can directly regulate the biological behavior of PC cells via binding to their corresponding receptors on tumor cells and activating the intracellular downstream signals (Entschladen et al., 2008). Moreover, recent studies have revealed that neurotransmitters do not only act on cancer cells, but also communicate with the immune cells in the TME (Binnewies et al., 2018), which suggests an indirect role of neurotransmitters in regulating the fate of PC by the crosstalk between neurotransmitters and the immune microenvironment in PC.

In this review, we will summarize the present progresses on the functions of neurotransmitters in the TME of pancreatic cancer. We will not only present the literatures that support direct effects of neurotransmitters on PC cells, but also discuss the interplay between neurotransmitters and the tumor immune microenvironment. Lastly, we will provide our perspectives on the potential therapeutic strategies the targeting neurotransmitter-immune cell crosstalk in PC.

NON-NEUROLOGICAL ROLES OF NEUROTRANSMITTERS IN CANCERS

Classification, Origin and Operation of Neurotransmitters

Neurotransmitters are biochemical molecules that carry information between neurons or between neurons and effector cells (Orrego, 1979). Most neurotransmitters are typically water-soluble molecules with dissociating groups. Based on their chemical structure, the critical classification of neurotransmitters can be summarized as follows (Spitzer, 2015): biogenic amines, amino acids, peptides, and other categories. Biogenic amine neurotransmitters are composed of dopamine (DA), norepinephrine (NE), epinephrine (E), and serotonin (5-HT). Amino acid neurotransmitters contain gamma-aminobutyric acid (GABA), glycine, glutamate, histamine, and acetylcholine (Ach). Peptide neurotransmitters include substance P (SP), neuropeptide Y (NPY), calcitonin gene related peptide (CGRP) and many others. Also, neurotransmitters can be classified by their function (excitatory or inhibitory) or by their action (direct or neuromodulator). Excitatory neurotransmitters (such as NE) activate the postsynaptic neuron and facilitate interneuronal information transduction, while inhibitory neurotransmitters (such as GABA) inhibit the postsynaptic neuron and hinder information transduction. Some neurotransmitters can be both excitatory and inhibitory. Neuromodulators (such as 5-HT and DA) do not directly participate in interneuronal information transduction, but work together with excitatory or inhibitory neurotransmitters to modify the postsynaptic cell's response (Boto and Tomchik, 2019). Differ from neuromodulators in the extent of actions, neurohormones (such as oxytocin and vasopressin) are secreted by neurosecretory cells into the blood steam and exert their effect on distant peripheral targets (Iovino et al., 2019).

Emerging evidence demonstrates that neurotransmitters can be released not only by the neurons from the central or peripheral nervous system, but also by non-neuronal cells (**Table 1**). For instance, Ach, the first established neurotransmitter, has been found to be synthesized in a variety of non-neuronal cells such as epithelial cells (from airway, digestive tract, urogenital tract, or epidermis), mesothelial cells (from pleura or pericardium), endothelial cells, fat cells, and fibroblasts (Reijmen et al., 2018; Elyada et al., 2019). The primary and secondary lymphatic organs of the immune system can be innervated by nerves. The local neurotransmitter thus can act as an immunomodulatory messenger to regulate the interaction between peripheral nerves and the lymphocytes (Zhang et al., 2014; Blanchart et al., 2017; Sung et al., 2017; Hujber et al., 2018). This peripheral innervation has been reported to participate in the development of immune cells (Allen et al., 2020). Meanwhile, the immune cells can also produce specific neurotransmitters such as 5-HT (Chen et al., 2015) to regulate the immune cells function and remodel the surrounding microenvironment through autocrine and paracrine approaches (Briggs et al., 2016).

In the nervous system, neurotransmitters mediate interneuronal communication in synaptic transmission. Although whether nerve-to-non-neuronal cell synapses or synapse-like structures exist outside of the nervous system is not yet known, the nervous system can influence non-neuronal cells through changing circulating neurotransmitter levels (Monje et al., 2020). In the tumor microenvironment, neurotransmitters may be also secreted from non-neuronal cells and confer both paracrine and autocrine effects on cancer cells, as well as immune cells.

The Role of Neurotransmitters in Cancer

Similar to the process of neovascularization and lymphangiogenesis, the formation of new nerve endings in the tumor is called neurogenesis. Neurogenesis is one of the determinants in tumorigenesis and cancer development (Boilly et al., 2017). Neurotransmitters serve as a link between intratumoral nerves and tumor cells in the TME. Tumor cells express various neurotransmitter receptors. Neurotransmitters released from nerve fibers in the TME can directly act on tumor cells by binding to their specific neurotransmitter receptors (Hanoun et al., 2015; Renz et al., 2018a,b). Meanwhile, tumor cells can also produce endogenous neurotransmitters in response to diverse stimuli from the microenvironment. For example, various types of tumor cells have been revealed to produce GABA. The elevated intratumoral level of GABA has been

TABLE 1 | The origin of neurotransmitters.

Neurotransmitters	Non-neuronal secretion	References
Norepinephrine	Thymic cells	Roggero et al., 2011; McBurney-Lin et al., 2019
Serotonin	Enterochromaffin (EC) cells of the gastrointestinal tract, thymic epithelial cells, cancer cells, immune cells, platelet	Lifantseva et al., 2017; Spohn and Mawe, 2017
Dopamine	Gastrointestinal tract, thymic epithelial cells, immune cells	Lifantseva et al., 2016; Liu C. et al., 2021
GABA	Cancer cells, the endocrine cells of pancreatic islets	Soltani et al., 2011; Kim et al., 2015
Substance P	Enterochromaffin (EC) cells of the gastrointestinal tract, thymic epithelial cells	Heitz et al., 1976; Zaidi and Matthews, 2013
NPY	Cancer cells	Gotzsche and Woldbye, 2016
CGRP	Immune cells	Bracci-Laudiero et al., 2002; Messlinger, 2018
Acetylcholine	Epithelial cells, immune cells, cancer cells	Brenner and Sakmann, 1978; Kawashima and Fujii, 2004
Histamine	Mast cells	Haas et al., 2008; Misto et al., 2019
Glutamate	Endocrine cells of pancreatic islets	Cabrera et al., 2008

observed in PC as well as ovarian cancer and breast cancer (Zhang et al., 2014; Jiang et al., 2019).

Neurotransmitters can affect almost all aspects related to tumor development including cell proliferation, angiogenesis, and metastasis (Boilly et al., 2017). Peripheral 5-HT generates a mitotic effect on a variety of tumor and non-tumor cells such as fibroblasts, smooth muscle cells, osteoblasts, mesangial cells, and endothelial cells (Alpini et al., 2008; Moon et al., 2020). Many studies have shown a potential stimulatory effect of 5-HT on cancer cell proliferation, invasion, dissemination, and tumor angiogenesis (Herr et al., 2017). Abnormal glutamate signaling showed carcinogenic potential in glioma, melanoma, breast cancer, and prostate cancer (Ribeiro et al., 2017; Sung et al., 2017; Yu et al., 2017; Anastas and Shi, 2019). Substance P and SP/NK-1 system have also been involved in the development and progression of many cancers such as glioma, colon cancer, and lung cancer (Munoz et al., 2011; Covenas and Munoz, 2014). Elucidating their specific roles in tumor biology especially in the TME may open up new windows for the diagnosis and treatment of cancers.

NEUROTRANSMITTERS IN THE TME OF PANCREATIC CANCER

Nerves and Neurotransmitters Are Key Components of Pancreatic TME

The TME of pancreatic cancer is characterized by nutrient deficiency, connective tissue hyperplasia and high nerve distribution. Paracrine signals derived by cancer cell promote nerve axonogenesis or neurogenesis in the TME. The infiltrated nerve fibers can control cancer initiation, growth and metastasis. Nerve fibers in the pancreatic TME include axons originating from the sympathetic, parasympathetic, enteropancreatic or hepatic plexus, afferent nerve fibers and newly developed nerve fibers. Neuron as presynaptic cell can secrete neurotransmitters such as E/NE, which act on specific receptors to regulate tumor proliferation and metastasis. In PC cells, sensory (Saloman et al., 2016; Sinha et al., 2017) and sympathetic nerves activate the growth of PC cells through the liberation of substance P and E/NE. On the contrary, parasympathetic nerves inhibit PC cell growth via ACh, leading to the inhibition of PI3K/AKT and EGFR/ERK in cancer cell (Renz et al., 2018b). This opposite impact of sympathetic and parasympathetic nerves suggests that the development of PC is regulated through a balance of neural innervation.

Neural remodeling and PNI are important pathological characteristics of PC (Guo et al., 2013). Neural remodeling is manifested as the increased size and density of infiltrated nerves in the pancreas. PNI is considered as one of the main routes for PC recurrence and metastasis after surgical resection since it presents a structural conduit for cell migration. Recent studies have illustrated that multiple types of cells in the TME of PC contribute to neural remodeling and PNI (Li et al., 2014). New nerve fibers in the TME act as a rich source of neurotransmitters and neurotrophic factors (Zahalka and Frenette, 2020), which substantially affects the malignant potential of tumor cells and the disease progression. For example, the classical neurotransmitters NE and 5-HT have been found to be significantly increased in PC tissues (Zhao et al., 2014; Jiang et al., 2017), and the altered levels of different neurotransmitters have been demonstrated to be associated with PC recurrence, metastasis, and survival (Guo et al., 2013), suggesting that microenvironmental neurotransmitters function as an essential non-cellular component that contributes to PC progression (Schuller et al., 2012).

Direct Effects of Neurotransmitters on Pancreatic Cancer

Neurotransmitters in the TME can be released by tumor-infiltrating fibers, cancer cells and non-cancerous cells such as immune cells and epithelial cells (Entschladen et al., 2008; Boilly et al., 2017). Neurotransmitters have numerous regulatory functions on PC cells, which we summarized in **Table 2**.

Epinephrine and Norepinephrine

The classical neurotransmitters E and NE have been found to give promotion to PC progression through multiple mechanisms. E and NE are stress molecules produced by the sympathetic nervous system and linked to PC growth via β-adrenergic signaling in both *in vitro* and *in vivo* studies (Renz et al., 2018b). Specifically, E indirectly enhanced β-AR-dependent neurotrophin secretion, which in turn increased NE levels and promoted PC growth (Zhao et al., 2014). The activation of β-AR can promote tumor growth and angiogenesis via VEGF and metalloproteinase MMP2/MMP9 signaling pathways (Thaker et al., 2006). NE also tended to promote

TABLE 2 | Summary of the studies on the role of neurotransmitters in pancreatic cancer.

Neurotransmitters	Receptor	Effect on pancreatic cancer cells	PC cells or animal models	References
Norepinephrine	α, β adrenergic receptors	Norepinephrine promotes proliferation, migration, invasion and inhibits apoptosis of pancreatic cancer cell	Panc-1, MIAPaCa-2, BxPC-3, AsPc-1, HPAC, SW1990 cells	Huang et al., 2012; Guo et al., 2013; Qian et al., 2018
Serotonin	5-HT receptors	5-HT increased proliferation and prevented apoptosis of PDAC cell lines	Kras/Trp53/Pdx1-Cre (KPC) mice, BxPC-3, HPAC, Panc-1, and SW1990 cells	Jiang et al., 2017
Dopamine	D1-like receptors D2-like receptors	The antagonists of DRD2, pimozide and haloperidol, inhibited the proliferation and migration of pancreatic cancer cells	BxPC-3, Panc-1, MIAPaCa-2, Capan-1, CFPAC-1 cells	Jandaghi et al., 2016
GABA	$GABA_A$, $GABA_B$	GABA stimulates pancreatic cancer growth through overexpressing GABAA receptor pi subunit	KLM-1, SUIT-2, KP-1N, PK-1, PK-45P, PK59, MIA PaCa-2, Panc-1 cells	Takehara et al., 2007
Substance P	NK-1 receptor	SP induces pancreatic cancer cell proliferation and invasion via NK-1 receptor and the high expression of NK-1receptor was associated with poor prognosis in patients	CAPAN-1, ASPC-1, PA-TU 8902, BxPC-3, MIAPaCa-2 cells	Munoz and Covenas, 2014, 2015
NPY	Y1-4 receptors	Y2 is strongly overexpressed in pancreatic cancer and may modulate angiogenesis	LsL-$Kras^{G12D}$, LsL-$Trp53^{R172H}$, Pdx1-Cre (KPC) mice	Zhou, 1993; Waldmann et al., 2018
CGRP	Calcitonin-like receptor (CLR). Receptor activity-modifying protein 1 (RAMP1). Receptor component protein (RCP)	CGRP stimulates the growth of PU-PAN-1 tumor cells, and CGRP receptor antagonist CGRP8-37 inhibited this effect	PU-PAN-1 cells	
Acetylcholine	nAchR, mAchR	Administration of a muscarinic agonist suppresses pancreatic cancer tumorigenesis	LSL-$Kras^{+/G12D}$; Pdx1-Cre (KC) and LSL-$Kras^{+/G12D}$; LSL-$Trp53^{+/R172H}$; Pdx1-Cre (KPC) mice, Panc-1 cells	Renz et al., 2018b
Histamine	Histamine1-4 receptors	When bound to H1HR, histamine induces proliferation and metastasis of PANC-1 cells. Activation of H2HR in PANC-1 cells tends to have the opposite effect of H1HR activation.	Panc-1 cell	Francis et al., 2013
Glutamate	AMPA receptors. Kainite receptors. NMDA receptors.	Glutamate increases pancreatic cancer cell invasion and migration	Su86.86 cells, BxPC-3 cells	Herner et al., 2011

PC progression through β-AR/PKA/STAT3 signaling pathway (Coelho et al., 2017).

Serotonin

The neurotransmitter 5-HT, as well as its receptors, was found to be elevated in PC tissues (Jiang et al., 2017). Knockdown of 5-HT receptors inhibited the proliferation and invasion of human PC cells *in vitro* (Gurbuz et al., 2014). In contrast, the activation of 5-HT receptors enhanced glycolysis under metabolic stress, and thus promoted the growth of PC. Regarding to its molecular mechanism, 5-HT stimulation increased the Warburg effect through PI3K-Akt-mTOR signaling (Jiang et al., 2017). In addition, the increased levels of type 1 tryptophan hydroxylase (TPH1), which was a key enzyme for peripheral 5-HT synthesis, and the decreased level of MAOA, which is responsible for 5-HT degradation, in PC tissues were correlated with the poor survival of patients (Jiang et al., 2017). Of importance, the metaplasia of acinar-to-ductal metaplasia (ADM) is a key determinant in PC development (Liou et al., 2017). Serotonin uptake by acinar cells could promote the activation of the small GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1), which is required for the transdifferentiation of acinar cells into ADM (Saponara et al., 2018).

Dopamine

The effect of DA on cancer cells is tumor type-specific. DA mainly reduced the proliferation and migration of endothelial cells in TME (Hoeppner et al., 2015). In PC, the dopamine receptor D2R is abnormally highly expressed and the antagonists of D2R (pimozide and haloperidol) were able to prevent the proliferation of PC cells, suggesting a PC-promoting effect of DA (Jandaghi et al., 2016).

Gamma-Aminobutyric Acid

Gamma-aminobutyric acid is a major inhibitory neurotransmitter in the central nervous system (CNS). Different GABA receptors play different roles in tumor growth. GABA was found to enhance prostate cancer cell proliferation through the GABA-A receptor pathway (Blanchart et al., 2017) and to inhibit cancer cell growth through the GABA-B receptor pathway in

liver cancer (Wang et al., 2008; Hujber et al., 2018). GABRP, a subunit of the GABA-A receptor, was abnormally highly expressed in PC cells (Jiang et al., 2019). GABA treatment in GABRP-positive PC cells increased intracellular Ca^{2+} levels and activated the MAPK/Erk cascade, which led to a pro-tumor effect on PC (Takehara et al., 2007).

Neuropeptides

Neuropeptides, such as substance P, CGRP, and NPY, were also found to have a direct effect on PC cells. Substance P was a powerful regulator to PNI in PC during the early stage of primary tumor formation via the MMP1/PAR1/SP/NK-1R paracrine loop (Huang et al., 2018). Besides, substance P induced cancer cell proliferation and invasion as well as the expression of MMP-2 in PC cells, and sensory nerves in TME may help PC progression in part through up-regulation of its receptor (Sinha et al., 2017). Human PC cells possess distinct CGRP receptors. CGRP can stimulate the proliferation of human PC cells, suggesting a role of CGRP in the growth of PC cells (Zhou, 1993). What's more, CGRP and substance P derived from pancreatic stellate cells mediated the PC pain via activation of sHH signaling pathway (Han et al., 2016), which provided a novel therapeutic option for PC pain. NPY could be detected in both human and murine pancreatic samples. Its receptor Y2 was significantly increased in PanIN lesions and PC samples both in murine and human. The enhanced Y2 receptor-mediated NPY signaling may modulate the angiogenesis of PC (Waldmann et al., 2018).

Effects of Neurotransmitters on Non-malignant Cells in Pancreatic TME

In addition to affecting tumor cells, neurotransmitters can also improve angiogenesis, lymphangiogenesis, and inflammatory responses via exerting influence on endothelial cells and stromal cells in the TME. For instance, the activation of β-AR (β2 and β3) expressed on stromal cells promotes the survival of prostate cancer cells via TGF-β signaling (Magnon et al., 2013). Neovascularization is a vital process involved in tumor growth and metastasis. There is substantial evidence indicating that vascular endothelium infiltrated in the TME expresses various neurotransmitter receptors such as E and NE (Sarkar et al., 2013). NE could also stimulate endothelial cell metabolism and drive angiogenesis in tumors (Zahalka et al., 2017; Hondermarck and Jobling, 2018). DA was shown to mobilize endothelial progenitor cells from the bone marrow and participate in angiogenesis in the TME (Chakroborty et al., 2008). Neuropeptide Y released by tumor cells interacts with receptors on endothelial cells or immune cells, modulating tumor-related angiogenesis and local inflammatory responses (Medeiros and Jackson, 2013). Meanwhile, neurotransmitters can also regulate stromal cells in the microenvironment. Using a high-throughput drug screening system that focuses on the pancreatic stellate cells, Sagara et al. found that dopamine antagonist could inhibit the activation of pancreatic stellate cells and suppressed the invasion of pancreatic cancer cells by disrupting tumor-stromal interaction (Sagara et al., 2021). In addition, 5-HT was demonstrated to be essential for the survival and activation of hepatic stellate cells. Serotonin-activated stellate cells could promote carcinogenesis and contribute to sex disparity in hepatic cell carcinoma (Yang et al., 2017).

INTERPLAYS BETWEEN NEUROTRANSMITTERS AND THE IMMUNE CELLS IN PANCREATIC TME

Immune Cells in the TME of Pancreatic Cancer

Intratumoral immune heterogeneity is considered as a hallmark feature of the TME (Riggan et al., 2021). The tumor immune microenvironment has an immense influence on tumor initiation, progression and therapeutic response. Immune effector cells such as $CD8^+$ cytotoxic T cells (CTL) and natural killer (NK) cells infiltrated in the TME keep the malignant cells under surveillance and form "barriers" to restrain cancer cell metastasis (Kurtulus et al., 2019). The secretion of multiple cytokines in pancreatic TME effect T helper (TH) cells, especially switching the balance of TH1/TH2, and contribute to the immunosuppressive microenvironment (Ho et al., 2020). Dendritic cells (DCs) are also considered as a significant component in adaptive anti-tumor immunity. DCs get involved in the proliferation of CTL in the TME (Puleo et al., 2018; Binnewies et al., 2019; Wculek et al., 2020) due to their role in tumor antigen recognition and presentation that stimulates T cell activation.

On the other hand, the TME can provide an immunosuppressive niche to negatively regulate immune effector cells and facilitate the malignant progression of cancer (Ho et al., 2020), and causes the resistance of PC against various treatments such as chemotherapy, targeted therapy, and immunotherapy (Balachandran et al., 2019). The major immunosuppressive cell types in the TME of pancreatic cancer are tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg), TH17, and tolerogenic DCs (Bronte and Tortora, 2016; Zhu et al., 2017; Ligorio et al., 2019; Zhang et al., 2020). These immunosuppressive cells promote tumor progression through a variety of mechanisms, including the direct mediation, inhibition of tumor-killing immune cells, induction of angiogenesis and lymphoangiogenesis (Ho et al., 2020), and promotion of metastasis. TAMs and MDSCs predominate in the TME and continuously communicate with PC cells to propagate disease progression (Lin et al., 2019). TAMs foster immune escape in the TME by suppressing TH1 cell and the antitumor responses of CTL. These contribute to matrix remodeling and facilitate tumor cell migration and invasion and promote tumor angiogenesis and growth (Qian and Pollard, 2010; Binnewies et al., 2018; Halbrook et al., 2019). MDSCs are known to exert immunosuppressive effects on T cells via secreting arginase, nitric oxide synthase, TGF-β, IL-10, and COX2 (Zhang et al., 2017). Tregs suppress tumor immunity in PC through a variety of pathways including the secretion of IL-10, TGF-β, and granzyme B, the activation of the TRAIL pathway and enhancement of T cells' dysfunction (Zhang et al., 2020). TH17 cells are able to promote tumor cell

growth by secreting IL-17, IL-23, and CCL20 (Wu H.H. et al., 2015), and inhibit the $CD8^+$ T-mediated immune response by IL-17 and IL-22 (Yan and Richmond, 2020). Tumor-infiltrating lymphocytic B cells (TIL-B) resident in tertiary lymphoid structures were associated with better survival in PC patients, while TIL-B got involved in the initiation and progression of PC (Roghanian et al., 2016; Wouters and Nelson, 2018; Mirlekar et al., 2020).

As more TAMs were found in PC with PNI compared to that without PNI (Li et al., 2014; Alrawashdeh et al., 2019), the infiltration of immunosuppressive cells has been considered to relate to the PNI, which is the prominent characteristic of PC. Being the key molecular mediators of neuroimmune interactions, neurotransmitters might mediate the PNI-induced infiltration of immunosuppressive cells in PC. The PNI of pancreatic cancer could mediate β-AR signaling, and the released Ach enhanced tumor growth by establishing an immune-suppressive TME characterized by impaired CTL infiltration and a reduced TH1/TH2 ratio (Yang et al., 2020).

Immune Cells as Non-neuronal Sources of Neurotransmitters

In recent years, promising studies have drawn the attention that immune cells in different activated states can synthesize or store neurotransmitters participate in neuroimmune regulatory circuits (Marino and Cosentino, 2013). Immune cells such as activated TH cells, Treg cells, and mature DC are able to synthesize or release serious classical neurotransmitters and their metabolites, including Ach, DA, and 5-HT. The synthesis of Ach was firstly observed in T cells (Ogawa et al., 2003). Compared with $CD8^+$ T cells or B cells, $CD4^+$ T cells contain more Ach (Kawashima and Fujii, 2003). TH cells could express tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of DA, and store DA in the intracellular vesicles (Cosentino et al., 2000, 2007; Nakano et al., 2009). The inhibitory neurotransmitter such as 5-HT could be secreted by T cells, DCs, and macrophages (O'Connell et al., 2006; Wu et al., 2017).

Of interest, immune cells such as activated T cells have the capacity to synthesize 5-HT, and this potential is enhanced during their activation (O'Connell et al., 2006; Chen et al., 2015). $CD8^+$ T cells were found to selectively express the highest level of type 1 tryptophan hydroxylase (TPH1), an enzyme that catalyzes the conversion of L-tryptophan (a direct precursor of 5-HT), indicating that they are capable of producing 5-HT. DCs and B cells can accumulate 5-HT through a regulated uptake mechanism from the microenvironment or activated T cells via serotonin transporters (SERTs) (Chen et al., 2015). Specifically, the expression of SERT on the surface of DC cells would increase as DCs matured or activated (Katoh et al., 2006), and also dynamically adjusted with the change level of 5-HT in the microenvironment (Arreola et al., 2015). Once DCs contact with T cells, their SERT expressions would significantly up-regulate (Chen et al., 2015). The stored 5-HT in DCs within $LAMP\text{-}1^+$ vesicles were subsequently released via Ca^{2+}-dependent exocytosis. Thus, DCs could sequester 5-HT, which are released from the microenvironment or directly from activated T cells, and transmit this 5-HT to naive T cells. This process suppressed cAMP production and thereby facilitated the activation and differentiation of naive T cells (O'Connell et al., 2006; Sacramento et al., 2018).

The Regulatory Effect of Neurotransmitters on Immune Cells Infiltrated in the Pancreatic TME

Neuro-immune interactions rely on soluble signaling molecules between cells, which including cytokines, chemokines, neurotransmitters, and neurotrophins. Neurotransmitters can regulate both the local and systemic immune responses against cancers. There is a comprehensive neuro-immune regulatory network existing in the TME, and the communication between neurotransmitters and immune cells influences the fate of cancer with either promoting or inhibiting the cancer growth and metastasis. The well-studied immune mediations caused by neurotransmitters are summarized in **Table 3** and **Figure 1**. Understanding the regulatory effects of various neurotransmitters on cancer immunity can help to design new strategies for cancer therapy.

Epinephrine and Norepinephrine

E and NE are mainly secreted by the adrenal medulla and sympathetic nerves, respectively. Local sympathetic innervation provides the bulk of the catecholamine content within the tumor (Zahalka and Frenette, 2020). Excessive activation of sympathetic nerves could damage the anti-tumor immune response, increase the invasion ability of tumor cells, and accelerate the occurrence and development of tumors (Nissen et al., 2018).

E and NE mainly exert immunosuppressive effects. The receptor β-ARs is present in most immune cells such as T cells (including Treg), B cells, macrophages, NK and DC cells (Sarkar et al., 2013). E and NE can inhibit the activity of $CD8^+$ T and NK cells via inducing the apoptosis of these lymphocytes (Zhao et al., 2014). They are known to directly suppress the production of cytokines and T cell proliferation, and potently inhibit TCR-mediated integrin activation on human antigen-specific $CD8^+$ T cells (Leone et al., 2015; Dimitrov et al., 2019). β-AR signal suppressed the production of IL-2 and IFN-γ and proliferation of $CD4^+$ T cells depending on their stage of differentiation (Muthu et al., 2007). The combined application of endogenous E and prostaglandin could reduce the anti-tumor activity of NK cells, thereby promoting the progression of leukemia (Inbar et al., 2011; Meron et al., 2013). Besides, several studies have demonstrated that chronic adrenergic signaling suppresses NK cell activity in solid tumors (Melamed et al., 2005; Tarr et al., 2012; Graff et al., 2018). In addition, β-AR-mediated hormonal signaling could reduce the deformability of macrophages, resulting in the acceleration of tumor metastasis (Kim et al., 2019). NE and E contribute to the macrophage polarization, recruitment, and cytokine production like IL-6 and TNF-α (Izeboud et al., 1999; Chiarella et al., 2014). Activation of β-AR signal could increase the infiltration of macrophages in the primary tumor parenchyma and induce the M2 polarization of macrophages (Dimitrov et al., 2019), which subsequently promoted tumor metastasis and

TABLE 3 | Summary of the functions of neurotransmitters on immune cells.

Neurotransmitters	Immune cells	Effect	References
Epinephrine, norepinephrine	T-lymphocytes	Inhibit the activity of $CD8^+$ T cells	Inbar et al., 2011; Dimitrov et al., 2019; Kim et al., 2019
	NK cells	Inhibit the activity of NK cells	
	Macrophages	Activation of β-AR signal could increase the infiltration of macrophages in the primary tumor parenchyma and induce the M2 polarization of macrophage, which subsequently promoted tumor metastasis	
Serotonin	T-lymphocytes	Releases IL-2, 16 and IFN-γ, T-cell proliferation	Uyttenhove et al., 2003; Yin et al., 2006; Mikulski et al., 2010; Inoue et al., 2011
	Dendritic cells	Increasing the Ca^{2+} concentration in immature cells	
		Activating 5-HT4 and 5-HT7 promotes the differentiation and maturation of DCs, up-regulates intracellular cAMP levels, and promotes the secretion of cytokines, such as IL-1β, IL-6, IL-8, and IL-10	
	Macrophages	Down-regulating chemokine CCR5 expression and up-regulating chemokine CCL2 and MIP1α expressions. Activating 5-HT2 increases the production and release of M2 cytokines. Inhibits release of TNF-α, inhibits NK cell suppression.	
	Neutrophils	Inhibits tumor cell phagocytosis and oxidative burst	
Dopamine	T-lymphocytes	T cells express functional dopamine receptors (DR) D1R-D5R, but their level and function are dynamic and context-sensitive; DA affects Th1/Th2/Th17 differentiation; D2 activation induces T cells to secrete IL-10;	Sarkar et al., 2006, 2013; Levite, 2016; Talhada et al., 2018
		D3 activation promotes T cells to secrete TNF-α, IFN-γ	
		D5 activation promotes T cells to secrete TNF-α and IL-10	
		Activation of D1 and D4 up-regulate the activity of cAMP and transcription factors STAT5 and GATA3, and promote the differentiation of T cells to TH2	
		DA inhibits the suppressive activity of Treg	
		DA activates resting effector T cells (Teffs) resulting in their proliferation and cytokine production. T cells produce DA (Tregs >>> Teffs), release DA, mainly after mitogen/antigen/CD3 ± CD28/PKC activation, uptake extracellular dopamine, and need DA. DA is important for antigen-specific interactions between T cells and dendritic cells.	
	Dendritic cells	Activating the D1 receptor on DC promotes ERK/JNK/NF-κB signaling, and inducing cytokine production	
	B-lymphocytes	Activating the D1 receptor on B cells increases the expression of its inducible ICOSL and CD40L	
GABA	T-lymphocytes Macrophages	Suppressing the immune effect by inhibiting the activation of NF-κB. Inhibiting the activation of macrophages by blocking calcium signals. Up-regulating the expression of the chemokines CXCL5 and CCL20.	Bergeret et al., 1998; Prud'homme et al., 2015
Substance P	T-lymphocytes	Affecting the activity and migration ability of $CD8^+$ T cells	Mashaghi et al., 2016; Shahzad et al., 2018
	Dendritic cells	Regulating the movement of dendritic cells toward lymph nodes via modulating the expression of chemokine receptors and adhesion molecules.	
	Macrophages	Activating NF-κB signaling in macrophages increases the production of pro-inflammatory cytokines, and therefore amplified the inflammatory response mediated by Th1 or Th17	
	Peripheral blood monocytes	Producing pro-inflammatory cytokines, such as IL-1, IL-6, IL-12 and TNF-α	
	Neutrophils	Participating in migration of neutrophils	
NPY	T-lymphocytes	Promoting the polarization of Th2 polarizing by upregulating the production of IL-6 and IL-10	Wheway et al., 2007a,b
	Dendritic cells	Inducing migration of immature DCs through the engagement of NPY Y1 receptor and the activation of ERK and p38, and exerting proinflammatory effects through the recruitment of immature DCs	
CGRP	Dendritic cells	Inhibiting the capacity of dendritic cells to produce inflammatory cytokines and to present antigens to T cells	Fox et al., 1997; Holzmann, 2013
	Macrophages	Inhibition macrophages and upregulation of IL-10, IL-10-independent induction of the inducible cAMP early repressor (ICER) and inhibition of NF-κB activity	

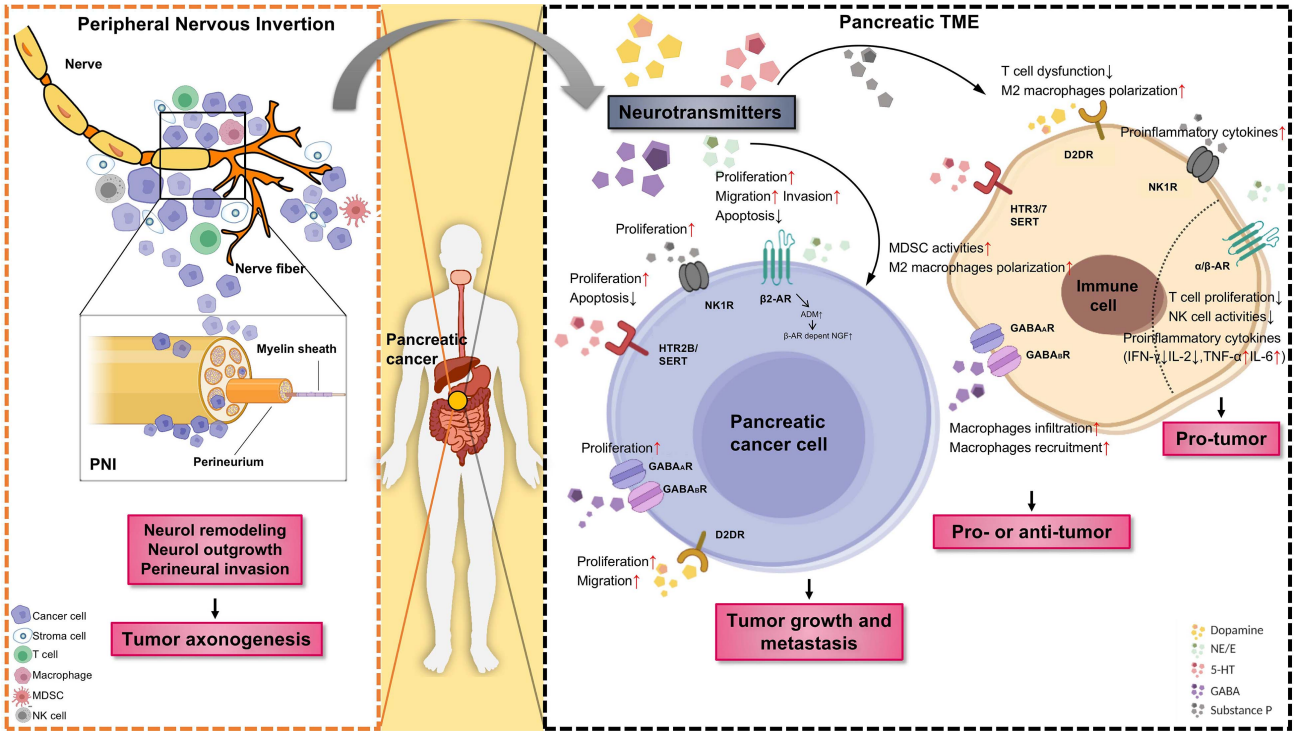

FIGURE 1 | The role of neurotransmitters and the emergence of nerves in the pancreatic tumor microenvironment (TME). Tumor innervation is present as shown by the extension of nerve fibers into the solid tumor, while perineural invasion (PNI) is present as shown by the invasion of cancer cells into the perineurium of the nerve **(Left panel)**. The outgrowth of nerves in TME (axonogenesis) is partly driven by the secretion of neurotransmitters released by sympathetic nerves and other tissues or cells. In return, nerve fibers as branches of neuron infiltrate the TME, could regulate tumor growth and metastasis. Schematic representation of different neurotransmitters, their respective receptors, target cells as well as pro/antitumor activity of specific neurotransmitter/receptor axis **(Right panel)**. Neurotransmitters are an active component of the TME. Neurotransmitters released by neuro-endocrine-immune system and other tissues or cells can promote cancer cell proliferation, migration, and invasion through the stimulation of specific membrane receptors. Moreover, immune cells infiltrated in the TME likewise express diverse neurotransmitter receptors and react with neurotransmitter, are known to have a strong impact on tumor angiogenesis and inflammation. PNI, perineural invasion; TME, tumor microenvironment; MDSC, myeloid-derived suppressor cells. The figure was created with BioRender.com.

stimulated tumor cells to produce chemokines like M-CSF (Van Overmeire et al., 2016). The use of β-blocker propranolol reduced the immunosuppression function of MDSC in breast cancer and enhances the effect of other cancer therapies like anti-PD-1 treatment and irradiation (Mohammadpour et al., 2019).

In pancreatic TME, it was shown that NE and E could decrease the expressions of MHC-I molecules and the costimulatory ligand B7-1 in PC cells, and increase the expressions of immunosuppressive IDO, PD-1, and PD-L1 (Arreola et al., 2015). Although these changes only last for a short time, these phenotypic changes of cancer cells do not only prevent the antigen recognition by T cells, but also damage T cell function by depleting essential nutrients, such as tryptophan, and inducing the exhaustion of activated lymphocytes (Zhao et al., 2014). β-adrenergic activation directly inhibits the generation of CTL and blocks the recruitment of protective T cells in the tumor microenvironment (Nakai et al., 2014). In PC, stress-induced neural activation is related to increased primary tumor growth and tumor cell dissemination to the normal adjacent pancreas. These effects were associated with increased expression of invasion genes by tumor cells and pancreatic stromal cells in the microenvironment (Kim-Fuchs et al., 2014). Enriching the housing environment for mice could enhance the cytotoxic activity of NK cells and promote tumor-infiltrating NK cells via sympathetic nerve-dependent mechanisms. Application of the β-blocker largely abolished the effects of the enriched environment on NK cells and attenuated its anti-tumor function (Song et al., 2017).

Taken together, adrenergic signaling mainly exhibits pro-tumorigenic properties. This effect is generated in part through the enhanced immune evasion induced by E and NE. The inhibition of adrenergic signaling increases the antitumor immune response via its impact on multiple immune cells, supporting the potential value of adrenergic antagonists in cancer prevention and treatment.

Serotonin

In the brain, 5-HT is synthesized by neurons located in the raphe nucleus of the brainstem (Migliarini et al., 2013). Externally, less than 1% of free 5-HT exists in the blood, and the rest is stored in platelets, presynaptic neurons, and intestinal enterochromaffin cells (Kim et al., 2019). 5-HT is a multifunctional molecule that regulates immune function (Arreola et al., 2015). It is now known

that there are seven different 5-HT receptor subtypes: 5-HT1 to 5-HT7. Except 5-HT3, which is a ligand-gated ion channel, all the other 5-HT receptors belong to the G protein coupled receptor family. Most immune cells express 5-HT receptors. 5-HT1 is mainly expressed in innate immune cells such as the mast cells (Kushnir-Sukhov et al., 2006), macrophages (Nakamura et al., 2008), DCs (Durk et al., 2005), and monocytes (Soga et al., 2007); 5HT-2 is expressed in eosinophils and macrophages (Mikulski et al., 2010). In the adaptive immune system, proliferated T cells mainly express 5-HT1B, 5-HT2A, and 5-HT7, and B cells mainly express 5-HT1A and 5-HT3 (Yin et al., 2006; Inoue et al., 2011).

5-HT is a neuromodulator with neurotransmitter and neuroendocrine functions in cancer (Balakrishna et al., 2021). Meanwhile, it also regulates a variety of immune processes, such as immune cell chemotaxis, activation, proliferation, and cytokine secretion (Herr et al., 2017). SERT blockers including the serotonin selective reuptake inhibitors (SSRI) which increase extracellular 5-HT concentration have immunoinhibitory effects (Gobin et al., 2014). In T cells, 5-HT induces T cell differentiation into Treg cells and promotes the shift of Th17 cells to Tregs. 5-HT acts on Th17 to induce the secretion of IFN-γ and IL-17 and elevates the release of IL-10 from Tregs, which indirectly promote the development of tumors (Sacramento et al., 2018). In DC cells, 5-HT elevates the Ca^{2+} concentration in immature cells, and contributes to the differentiation and maturation of DCs by activating 5-HT4 and 5-HT7. This activation could up-regulate cAMP levels in DCs (Carhart-Harris and Nutt, 2017) and the secretion of related cytokines such as IL-1β, IL-6, IL-8, and IL-10 (Idzko et al., 2004; Katoh et al., 2006). In macrophages, 5-HT regulates the polarization of macrophages through both activated and inhibitory signals. 5-HT2A activation increases the production of M2-type cytokines and migration while 5-HT1A activation enhances the capability of phagocytosis in macrophages (Mikulski et al., 2010; de las Casas-Engel et al., 2013). Activation of 5-HT1A on lung cancer cells could induce immune evasion via autophagy. After the activation, the ratio of TH1/TH2 cells decreased and the number of Tregs increased in TME, which suggesting a resistance to CTL attack (Liu et al., 2019).

In the pancreas, 5-HT is profoundly implicated in acute pancreatitis, pancreas regeneration after pancreatitis and PC. In the pancreatic TME, the SSRI fluoxetine reduced the stromal reaction that surrounds pancreatic lesions, evidenced by decreased fibrosis, inflammation and angiogenesis (Saponara et al., 2018). The intratumoral MAOA expression was also associated with T cell dysfunction and decreased patient survival in a broad range of cancer patients including PC (Wang et al., 2021).

In summary, in most cases, serotonin signaling influences immune cells and facilitates tumor development via suppression of anti-tumor immunity. Therefore, the use of anti-anxiety or anti-depressant drugs targeting serotoninergic system may have potential implications in cancer therapy.

Dopamine

Dopamine contributes to neuroimmune communication and acts on immune cells in an autocrine/paracrine manner through its receptors (Roy et al., 2000). Dopamine receptors are functionally classified into the D1-like subtypes consisting of D1R and D5R and D2-like subtypes (D2R, D3R, and D4R receptors), based on their ability to stimulate the formation or inhibition of cAMP (Kebabian, 1978).

Dopamine mainly acts on T cells and trigger DA receptor-dependent activation of ERK, LCK, FYN, and NF-κB pathways (Ghosh et al., 2003). In T cells, the activation of D2R and D5R can induce IL-10 (Besser et al., 2005; Cosentino et al., 2007), while D3R activation induces TNF-α and IFN-γ secretion (Ilani et al., 2004). Besides, the stimulation of D3R in naive $CD8^+$ T cells also contributed to the regulation of chemotaxis and related cellular function like extravasation and adhesion (Huang et al., 2010; Figueroa et al., 2017). DA affects Th1/Th2/Th17 differentiation. Specifically, the activation of D1R and D4R enhanced the TH2 differentiation by up-regulating the activity of cAMP, STAT5, and GATA3. Besides, DA mediated the chemotactic migration of naive $CD8^+$ T cells by inducing chemokines like CCL19, CCL21, and CXCL12 (Watanabe et al., 2006). It was reported that DA inhibits the proliferation of T cell and the secretion of IL-2, IL-6, and IFN-γ when exposed to a high level of DA (Bergquist et al., 1997; Saha et al., 2001). DA could activate D1-like receptors in Treg, which in turn indirectly activates effect T cell (Cosentino et al., 2007). Except for T cells, inhibition of the DR3-mediated signal in DC cells increased the cross-presentation of antigen to $CD8^+$ T cells (Figueroa et al., 2017).

Dopamine functions as a regulatory component on immune cells in the TME (Yan et al., 2015; Petrilli, 2017). Increased level of plasma DA (40–80 pg/ml) was reported to evidently impair physiological proliferation and cytotoxicity of T cells in cancer patients (Saha et al., 2001). In PC, DA enhanced the chemotherapeutic efficacy of gemcitabine both *in vitro* and in immunocompetent murine models, and changed TME by suppressing the M2 characters of TAMs. Specifically, the activation of D4R in macrophages by DA reduced the production of cAMP, and then inhibited the activation of PKA/p38 signal pathway, which suppressed the transcription of tumor-promoting cytokines of TAMs such as IL-1β and TNF-α (Liu Q. et al., 2021). What's more, DA was found to hinder the function of tumor-induced monocytic MDSCs on the proliferation and IFN-γ production of T cells in lung and melanoma cancer (Hoeppner et al., 2015; Wu J. et al., 2015). DA attenuated NO production by MDSCs directly, mediated by decreased iNOS expression and the downregulation of ERK and JNK signaling pathways. DA-induced activation of resting Teffs and suppression of Tregs seem beneficial for the immunotherapy of cancer. As for the receptors, D2R was identified as an upregulated protein in PC, and D2R antagonists (pimozide and haloperidol) reduced PC growth and particularly metastasis (Jandaghi et al., 2016). The anti-tumor efficacy of ONC201 and ONC212, two small molecule antagonists of D2R, were observed *in vivo* either administrated as a single agent or in combination with 5-fluorouracil, irinotecan, and oxaliplatin. When treated with ONC201, a broad induction of immune cytokines and effector molecules was observed among PC patients with longer progression-free survival (Lev et al., 2017; Stein et al., 2019). Besides, ONC201 has been reported to induce the proliferation of

NK cells and activation via TRAIL and granzyme B in preclinical studies (Stein et al., 2019).

Together, the current findings suggest an important immunomodulatory effect of DA in cancer microenvironment. DA may confer immunopromoting or immunosuppressive effect dependent on the types of immune cells and the specific DA receptor they express.

Gamma-Aminobutyric Acid

Gamma-aminobutyric acid is the main inhibitory neurotransmitter in the central nervous system. In the periphery, GABA is produced by pancreatic β cells, T cells, and macrophages. These cells also express other components of the GABAergic system, including receptors, transporters, and metabolic enzymes (Wu et al., 2017). The GABAergic signaling system affects various functional characteristics of immune cells, such as antigen-induced T cell proliferation (Bjurstom et al., 2008) and LPS-induced cytokine release and effector T cell activity (Lang et al., 2003).

Both *in vivo* and *in vitro* studies have proved that GABA suppresses the immune effect by inhibiting the activation of NF-κB and reducing the production of inflammatory cytokines (Bhat et al., 2010). As a negative regulator in the TME, GABA inhibits the activation of macrophages and T cells by blocking calcium signals and inhibiting NF-κB pathway (Prud'homme et al., 2015). In addition, GABA was shown to regulate the expression of GABA-A receptor subunits on immune cells (Bergeret et al., 1998; Feske et al., 2012). In the pancreatic TME, GABRP expression was remarkably increased in PC tissues among other neurotransmitters' receptors. GABRP expression correlated with macrophages density closely, and the deletion of macrophages largely abrogated the oncogenic functions of GABRP in PC. The GABRP on cancer cells promoted macrophages recruitment by inducing CXCL5 and CCL20 expression. Specifically, GABRP might act as a chaperone protein and regulate the activity of KCNN4 channel to induce Ca^{2+} signaling in PC cells. The GABRP-KCNN4 complex led to the activation of NF-κB, which further facilitated CXCL5 and CCL20 transcription to induce macrophage infiltration in PC (Jiang et al., 2019).

In general, GABA exerts immunosuppressive effects on diverse immune cells and blockade of GABA signaling in the microenvironment may improve the anti-tumor immune response against cancer cells.

Neuropeptides

Most studies related to the immunomodulatory effect of neuropeptides focused on the substance P. In the central system, substance P is released from the brain regions and regulates emotions and specific sensory nerve endings (Cunin et al., 2011; Munoz and Covenas, 2015). In the periphery, substance P is mainly secreted by immune cells such as macrophages and DC cells (Janelsins et al., 2013). Substance P exerts its biological activity through G protein-coupled neurokinin receptors (NKRs), namely NK-1R, NK-2R, and NK-3R (Suvas, 2017). NK-1R mainly exists in the immune system and mediates the effect of substance P on immune cells (Shahzad et al., 2018). Substance P acts on NK-1R to induce a local inflammatory environment in a concentration-dependent manner. Specifically, substance P mediates the migration, proliferation, and activation of immune cells. Substance P activated NF-κB signaling in macrophages, increased the production of pro-inflammatory cytokines such as CCL2, CXCL2, and IL-8 (Levite, 2008). Therefore substance P amplified the inflammatory response mediated by TH1 or TH17 (Cunin et al., 2011; Mashaghi et al., 2016). Recent studies illustrated that administration of substance P during the primary immune response amplifies the secondary immune response by activating $CD8^+$ T cells (Ikeda et al., 2007). Substance P increased the migration of immune cells, including T cells and neutrophils, through a β-arrestin-dependent mechanism (Nichols et al., 2012). Substance P also stimulated human PBMC to produce pro-inflammatory cytokines including IL-1, IL-6, IL-12, and TNF-α (Cunin et al., 2011). As for innate immune cells, substance P activated NK cells and neutrophils by up-regulating their production of cytotoxic-associated molecules, such as perforin and granzyme (Fu et al., 2011; Mashaghi et al., 2016). However, the up-regulation of the NK-1R can be seen in both chronic pancreatitis and PC and enhanced NK-1R expressions were related to advanced tumor stage and a poorer prognosis (Li et al., 2013). In addition, $CD10^+$ fibroblasts can inhibit squamous cancer cells invasion ability by diminishing substance P (Xie et al., 2010).

The peripheral sensory nerves, which mediate pain reflexes, may influence immune responses through the release of neuropeptides CGRP (Holzmann, 2013). CGRP directly acts on macrophages and dendritic cells and inhibits the capacity of these cells to produce inflammatory cytokines and to present antigens to T cells. The molecular mechanisms, by which CGRP acts on innate immune cells, include the upregulation of IL-10, IL-10-independent induction of the inducible cAMP early repressor (ICER) and inhibition of NF-κB activity (Fox et al., 1997). In addition, Liu et al. demonstrated that increased CGRP and neuronal p75 immune-reactivities in tumor-bearing mice, promoting chronic pain in bone metastasis (Liu et al., 2020).

Neuropeptide Y was found to induce a dose-dependent migration of immature DCs through the engagement of NPY Y1 receptor and the activation of ERK and p38. Meanwhile, NPY promoted the polarization of TH2 polarizing by upregulating the production of IL-6 and IL-10 (Wheway et al., 2007a,b). Thus, NPY may exert proinflammatory effects through the recruitment of immature DCs, but it may exert anti-inflammatory effects by promoting a TH2 polarization. In prostate cancer, depression-induced NPY secretion might promote the tumor infiltration of myeloid cells and therefore contribute to cancer progression (Cheng et al., 2019).

Taken together, the neuropeptides CGRP and NPY were suggested to impair the anti-tumor immunity and their inhibition may be a potential strategy for cancer treatment. On the contrary, substance P may facilitate the anti-tumor immune response. However, enhancing the substance P signaling in cancer cells could promote the progression of pancreatic cancer. The distinction between signaling mechanisms of

substance P in immune cells and cancer cells warrants further studies.

NEUROTRANSMITTER-TARGETED DRUGS AS COMBINATORIAL STRATEGIES FOR CANCER IMMUNOTHERAPY

The important role of neurotransmitters in cancer progression suggests that the drugs targeting neurotransmitter signaling may act as promising candidates for cancer treatment (Cole et al., 2015). In fact, the clinical trials of various neurotransmitter receptor antagonists or agonists are already ongoing (**Table 4**). Among them, β-blockers, which are the antagonists targeting adrenergic β receptors, are mostly studied (Botteri et al., 2013; Grytli et al., 2013a,b). Data from prostate cancer and breast cancer studies showed that patients using β-blockers, even former users, had significantly better survival outcomes than the non-users (Grytli et al., 2013b; De Giorgi et al., 2018). In melanoma, pan β-blockers provided more survival benefits than β1- or β2-selective blockers (Livingstone et al., 2013). In PC, long-term use of beta-blockers especially selective β1-blockers may be associated with decreased cancer risk (Saad et al., 2020). Besides, β-blocker drugs may lead to a significantly improved overall prognosis in PC patients, particularly among those with localized disease (Saad et al., 2020). These findings raise the possibility that neurotransmitters-related drugs protect from cancer initiation.

Recently, immunotherapy is emerging as the most promising treatment option for cancers. However, immunosuppressive mechanisms within the TME largely limit its therapeutic efficacy. As aforementioned, neurotransmitters not only have direct effects on tumor cells, but also contribute to the immunosuppressive microenvironment by acting on immune cells in the TME. Therefore, neurotransmitter-targeted drugs have attracted increasing attentions as a combinatorial approach for cancer immunotherapy. In murine tumor models, reducing β-AR signaling was shown to facilitate the conversion of TME to an immunologically active microenvironment, and β-blockers application significantly increased the efficacy of anti-PD-1 checkpoint blockade (De Giorgi et al., 2018). In addition, blocking β-AR signaling also improved the potency

TABLE 4 | Clinical trials related to neurotransmitters in cancer.

Related neurotransmitter	Cancer type and stage	Purpose	Strategy or treatment	Enrolled patients	Therapeutic effects	References
Catecholamine (E/NE)	Head and neck. Esophagus. Stomach. Colon. Prostate cancer.	Investigate the association between propranolol and cancer	Patients with a usage of propranolol >6 months in NHIRD database	24,238	HR:0.58 (95%CI: 0.35–0.95) HR:0.35 (95%CI: 0.13–0.96) HR:0.54 (95%CI: 0.30–0.98) HR:0.52 (95%CI: 0.33–0.83) Reduce cancer risk	Chang et al., 2015
Catecholamine (E/NE)	Locally advanced and metastatic melanoma	Investigate the efficacy of combination with propranolol and pembrolizumab	Propranolol twice a day with pembrolizumab 200 mg every 3 weeks	9	In progress (ORR = 78%) Responders show increased IFN-γ and decreased IL6 level	Gandhi et al., 2021
Serotonin	Breast cancer with obesity or/and overweight	Exam the effects of Mediterranean Diet and Naltrexone/Bupropion Treatment in Obese Breast Cancer Patients	Naltrexone/bupropion combination $(NB)^{+}$ release (ER) combination tablets, Mediterranean Diet	72	The combination of the Mediterranean diet with naltrexone/bupropion treatment Without superior changes	NCT03581630 (Cho et al., 2020)
Dopamine	Pituitary adenoma; Non-functioning pituitary adenoma	Investigate the efficacy of cabergoline in NFPA individuals with remaining tumor after primary neurosurgery	Cabergoline (a DRD2 antagonist and antiparkinson drug)	140	Residual tumor shrinkage: 10.5% vs. 28.8%, stabilization = 66.1% vs. 73.7%, enlargement = 5.1% vs. 15.8% (the control group) PFS: 23.2M vs. 20.8M (the control group)	NCT03271918 (Batista et al., 2019)
Dopamine	Recurrent and stage IV breast cancer	Investigate the effectiveness of cabergoline in treating metastatic breast cancer disease in those who test positive for the prolactin receptor	Cabergoline (a DRD2 antagonist and antiparkinson drug)	20	CBR = 33% (6/18), mPFS = 1.8M, mOS = 10.4M	NCT01730729 (Costa et al., 2017)
Dopamine	Advanced solid cancer refractory to the standard treatment	Evaluate the safety and pharmacokinetics of weekly ONC201	ONC201 (a DRD2 antagonist)	20	Enhance immunostimulatory activity	NCT02250781 (Stein et al., 2019)

of TCR-γ T-cell therapeutics in hematologic malignancies (Baker et al., 2019) and enhanced the antitumor efficacy of STxBE7-based cancer vaccine in a breast cancer model (Daher et al., 2019). Small-molecule MAO inhibitors (MAOIs), used for depression and other neurological disorders, was found to significantly suppress tumor growth and generated synergistic tumor suppression effects when combined with anti-PD-1 treatment. Specifically, MAO-A restrains antitumor T cell immunity through controlling intratumoral T cell autocrine serotonin signaling. Apart from β-blockers and MAOIs (Wang et al., 2021), the results from preclinical studies provide a rationale for testing this combinatorial strategy in cancer patients.

The first prospective study on the combination of immunotherapy and a neurotransmitter receptor antagonist drug was conducted in melanoma patients (De Giorgi et al., 2018; Gandhi et al., 2021). It was shown that the patients taking propranolol, an approved non-selective β-blocker, not only had an 80% risk reduction for recurrence but also tended to be more sensitive to anti-PD-1 treatment (De Giorgi et al., 2018). The enhanced therapeutic efficacy of immune checkpoint inhibitors by β-blocker was also observed in lung cancer patients. The use of β-blockers was significantly associated with improved progression-free survival among non-small-cell lung cancer patients treated with SP-142, a PD-L1 inhibitor (Oh et al., 2021).

Pancreatic cancer is among the most immune-resistant tumor types. Given the potential role of neurotransmitters in the immunosuppressive microenvironment of PC, drugs targeting neurotransmitter signaling may help to limit immune suppression and overcome immunotherapy resistance in PC. The combination of neurotransmitter signaling-targeted drugs and immunotherapy is believed to provide new hope for PC patients (Balachandran et al., 2019).

CONCLUSION

Pancreatic cancer has the unique characteristic of increased neural density. As the messenger molecules, neurotransmitters have emerged as components with significant importance in the TME of pancreatic cancer. Neurotransmitters can directly bind to the cancer cells, generating either promoting or inhibition effects on cancer growth. Meanwhile, the expressions of neurotransmitters and their corresponding receptors in the tumor-infiltrating immune cells imply a complicated relationship between the neurotransmitters and the immune cells in the TME. Further investigation on how neurotransmitters crosstalk with immune cells at the TME level will be of great interest, for it will facilitate our understanding of the mechanism behind the suppressive immune microenvironment of cancer. The tumor immune microenvironment contributes a lot to the initiation and development of cancers, as well as the response to cancer treatments, notably the immunotherapy. Nowadays, immunotherapy is increasingly involved in cancer therapeutic regimes. Breaking immune tolerance using specific receptor antagonists of neurotransmitters is possibly a promising strategy for combinatorial therapy with immune checkpoint inhibitors in PC. It is also plausible that modulating the expression of neurotransmitter receptors on the surface of CAR-T cells may promote the efficacy of CAR-T therapy. Further research into the precise mechanism of how immune cells regulated by different types of neurotransmitters in the TME might open up new avenues toward adjunct therapy against PC.

AUTHOR CONTRIBUTIONS

YL wrote the manuscript. HL searched the literatures, made the tables, and polished the language. HT and YG conceived the idea and revised the manuscript. All authors read and approved the final manuscript.

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OPINION
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Selective Advantages of Synapses in Evolution

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The common ancestor of all Metazoa (Urmetazoan) was a nerveless animal (Mackie, 1970, 1990; Moroz, 2009). This hypothetical Urmetazoan likely used electrical and chemical communications for behavioral control as in the present-day placozoans or sponges. In placozoans, non-synaptic signaling is mediated by small secretory peptides and low molecular weight transmitters, including NO, ATP, glutamate, glycine, GABA (Nikitin, 2015; Varoqueaux et al., 2018; Moroz et al., 2020a,b, 2021b; Romanova et al., 2020a,b).

Emerging evidence suggests that neurons evolved more than once from secretory cells (reviewed by Moroz, 2014, 2021). Such events might independently occur about 550–540 million years ago in ancestors of three basal metazoan lineages: ctenophores, cnidarians, and bilaterians (Moroz et al., 2021b). The separations of each of five basal metazoan lineages likely happened within a relatively short geological interval, perhaps, even <10 million years. Thus, the outcome of the highly debated topic—the identification of the sister lineage for all Metazoa (i.e., ctenophore-first or sponge-first hypotheses, see details in Whelan et al., 2015, 2017; Halanych et al., 2016; Telford et al., 2016; Kapli and Telford, 2020; Redmond and McLysaght, 2021)—does not challenge the fact of extensive parallel evolution of neural organization within the majority of animal phyla. It also does not challenge the hypothesis of the independent origins of neurons considering remarkably different molecular toolkits for neural cell types across basal metazoans (Moroz et al., 2014) and the broadening definitions of neural systems (Miguel-Tomé and Llinás, 2021).

The surprising corollary of the neural polygeny hypothesis is independent origins of synapses (Moroz and Kohn, 2016; Moroz et al., 2021b). But how had the synaptic organization from secretory cells happened in early animal evolution? What were the selective advantages of synaptic vs. paracrine secretory communications? Some particular "benefits" of synapses are apparent, and some are not. We think that the extension of the endoplasmatic reticulum and growing lipids' diversity in early secretory cells paved the way to versatile and divergent neuronal and synaptic evolution.

ADVANTAGES OF SYNAPSES IN NEURAL EVOLUTION

First, both ***speed*** and more localized, faster delivery of intercellular signals are probably among the most prominent selective advantages of synapses in evolution compared to volume transmission. Consequentially, selective *"benefits"* of shorter and anatomically restricted transmission enabled more precise control [and homeostasis] of transmitter concentrations. There is always a trade-off between the chemical stability vs. the rate of transmitter's chemical inactivation in given microenvironments [e.g., oxidation for monoamines (Burbulla et al., 2017; Riessland et al., 2017), hydrolysis of acetylcholine, or proteolysis for peptides].

Second, both the ***exocytosis*** and regulation of chemical transmission within small contained volumes would require fewer resources and might be energetically more favorable than

producing and releasing a larger pool of transmitters into extracellular spaces to compensate for their diffusion.

Third, many **transmitters are common cellular metabolites** or directly derived from cellular metabolites. In this capacity, signal molecules can also be food/energy sources (Moroz et al., 2021a), (e.g., amino acids such as glutamate and aspartate or small peptides) for specialized cells and symbionts or endoparasites. Thus, more compartmentalized

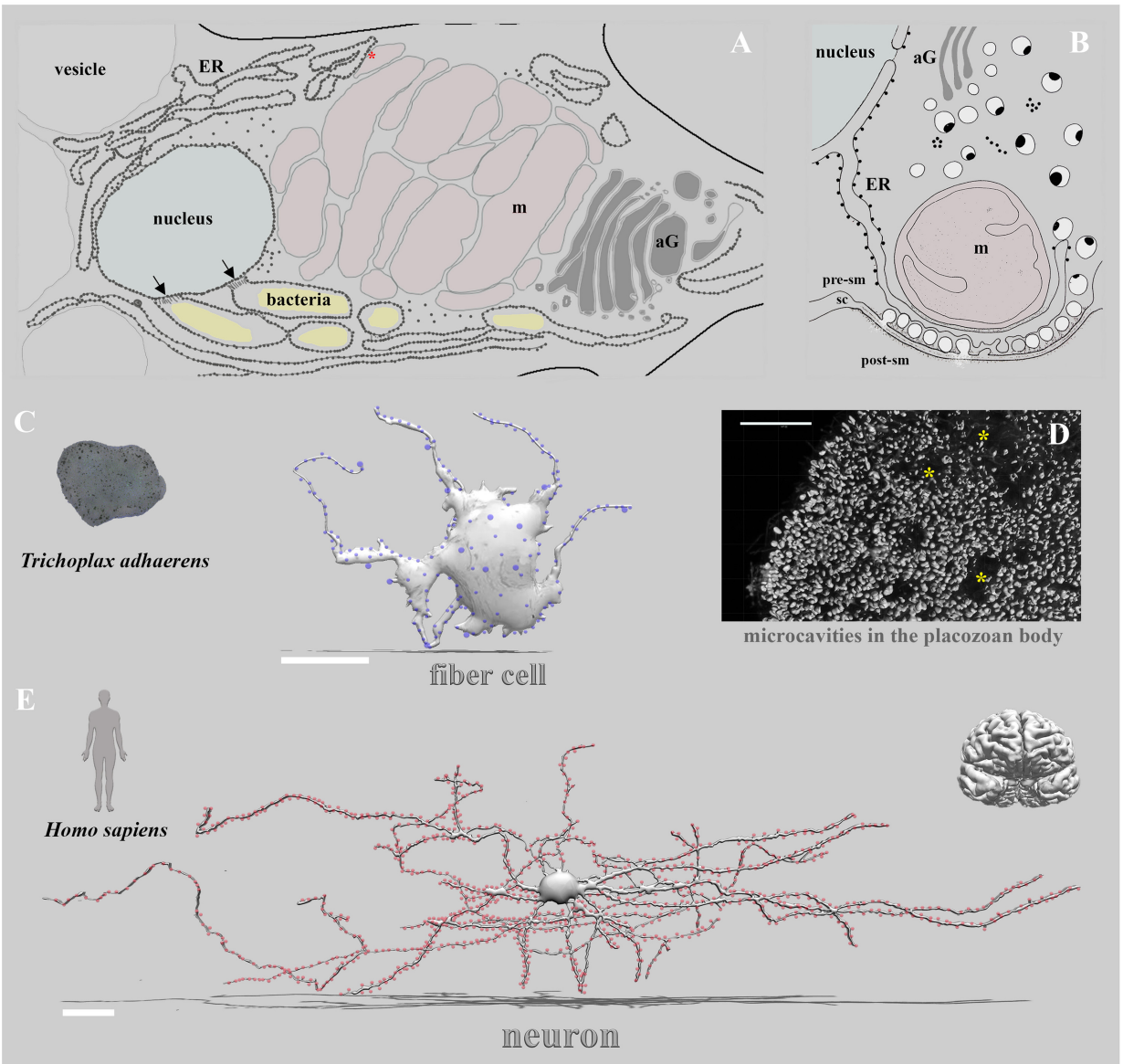

FIGURE 1 | Non-synaptic vs. synaptic transmission: extreme cases of alternative integrative systems. Three remarkable examples of cells involved in the behavioral integrations are illustrated: the placozoan fiber cell **(A,C)**, unique ctenophore tripartite chemical synapse (**B**, modified from Hernandez-Nicaise, 1991, see text), and the pyramidal human neuron **(E)**. **(A)** Details of ultrastructural organization of the fiber cell in *Hoilungia hongkongensis* (modified from Romanova et al., 2021, Figure 5). Inside fiber cells, an extended endoplasmic reticulum entwined all cell compartments, especially mitochondria complex (formed presumed mitochondrial contact site—red asterisk), nucleus, and bacteria. Black arrows indicate specialized contact sites (Dumoux and Hayward, 2016) to the nucleus with encapsulated bacteria within ER-type structures with ribosomes (dotted areas). **(C)** The left image shows the organization disk-shaped nerveless placozoan, *Trichoplax*, which contains no recognized neurons, muscles, or sensory organs but displays coordinated behaviors and action potentials (Smith et al., 2015, 2019; Senatore et al., 2017; Armon et al., 2018; Varoqueaux et al., 2018; Fortunato and Aktipis, 2019; Romanova et al., 2020b). The middle image shows the schematic reconstruction of a placozoan fiber cell with prominent sites (blue dots) of putative secretory/paracrine (non-synaptic) regions (modified from scanning and transmission electrom microscopy datasets (Romanova et al., 2021). The right image **(D)** shows the microcavities (asterisks) (Romanova, 2019) as suggested regions of non-synaptic communications and integration in placozoans (Moroz et al., 2021b). **(E)** The reconstruction of a pyramidal neuron (modified from https://ai.googleblog.com/2021/06/a-browsable-petascale-reconstruction-of.html and Riessland et al., 2017; Shapson-Coe et al., 2021). Red dots are exemplar synapses on the pyramidal neuron. Blue dots are recognized vesicles and exosomes around the fiber cell **(C)**. Scale bars: Fiber cell—20 μm; Neuron—50 μm.

[synapse-type] communication provided potential "protections" of signaling molecules from their consumption by other cells or symbionts/parasites. In the nerveless *Trichoplax* (**Figure 1**), the fiber cells seem to contribute to neuroid integrative, phagocytotic, immune, regenerative, and contractile functions (Grell and Ruthmann, 1991; Romanova et al., 2021). They also contain intracellular symbionts (Gruber-Vodicka et al., 2019; Kamm et al., 2019). But, in placozoans, highly localized signaling might occur without classical synapses.

There are also "*disadvantages*" of the highly localized synaptic transmission related to spatial limitations of integrative functions. Slow diffusion of signal molecules to other (more distant) targets could be "compensated" by the growth of neuroid processes, energetically very costly mechanisms. In complex, relatively large, and mobile animals, there was a parallel development of different systems for long-distance signalings, such as circulatory and immune systems. The predation and larger body sizes were essential factors (Monk and Paulin, 2014), triggering the origins and rapid evolution of the neural and synaptic organizations.

As a result, we might envision multiple trade-offs between speed, efficiency, and associated energy cost of non-synaptic volume transmission vs. highly localized synapses in the early evolution of animal communication systems. These two communication systems always co-exist in most extant animals. But how had chemical synapses evolved? There are three aspects to this question. (i) combinatorial selection of molecular components [modules], including recruitments of various adhesive molecules to bring presynaptic and postsynaptic complexes together, and/or cooption of gap junctions proteins for the same purposes (Ovsepian and Vesselkin, 2014; Ovsepian, 2017; Ovsepian et al., 2020); (ii) reorganization of intracellular and extracellular membrane domains in secretory cells and their targets to enhance signaling and communication efficiency; and (iii) preferential selection of chemically different transmitter classes for paracrine vs. synaptic communications. Below, we will briefly discuss these interconnected components of synaptic evolution.

The Versatility of Secretory and Receptive Modules Is the Core of the Synaptic Origins

Natural selection might take advantage of several "preadaptations" to "build" synapses using a broad array of modular exocytosis machinery and adhesive molecules (cadherins, neurexins, neuroligins, immunoglobulins, complex receptive scaffolds from unicellular eukaryotes, etc.)—all these components were previously selected for other functions rather than to make synapses. It perfectly fits the definition of the ***exaptation*** as "characters evolved for other uses…, and later co-opted for their current role." (Gould and Vrba, 1982). However, we must view a knot of such exaptations within contexts and constraints of each phyletic lineage of animals, their bodyplans, and their development. Equally important would be modeling the energetic cost of synapse formation, growth, and the maintenance of long neural processes (which consume a lot of energy to sustain their homeostasis, propagate electrical signals and secretory events). These factors, plus ecologies and behaviors of particular species, provide additional constraints to synaptic recruitments, synaptic architecture, and even preferential selection of "available" transmitters in one or another type of neural system.

Limited comparative, cell-specific molecular and physiological data from early-branching metazoan lineages prevent making final conclusions about the combinatorial logic and the scope of modularity within different synaptic architectures. However, we can state that most core molecular machinery, sufficient for synapse formation (secretory presynaptic and postsynaptic receptive modules), predated the origin of animals and their neural systems (Ryan and Grant, 2009; Moroz and Kohn, 2015, 2016; Ovsepian, 2017; Ovsepian et al., 2020). In unicellular and colonial eukaryotes, paracrine secretion is widely used for other functions, different from (neuro)transmitter signaling, such as digestion, phagocytosis, defense, immunity, and injury-regenerative responses, control of cell divisions and differentiation, etc.

ENDOPLASMIC RETICULUM IN PROTONEURONAL SECRETORY CELLS MIGHT PROMOTE SYNAPTOGENESIS IN EVOLUTION

Over 60 years, scientists put forward the idea that neurons evolved from secretory cells (reviewed Moroz, 2014, 2021). But what kind of preadaptations in secretory cells (apart from exocytosis itself) might facilitate synaptogenesis? We hypothesize that the endoplasmic reticulum (ER) specialization toward enhanced secretion capabilities and associated dramatic increase of intercellular membranous structures could be essential factors that triggered and shaped early neuronal and synaptic evolution. Three outcomes of enhanced secretory functions relevant to synaptic evolutionary selection are summarized below.

Increased Intracellular Membrane Space in Secretory Cells Drives Organelles' Interactions and Lipid Complexity

ER comprises more than half of the total cell membranes and occupies about 35% of cytoplasmic volume (Valm et al., 2017). However, the intracellular membranous space is more extensive in secretory cells and cells with extended neural processes such as axon-type terminals, which have elaborated ER even in distant neurites (Ozturk et al., 2020). Increased synthesis of peptides and other secretory molecules, their accumulation in ER and vesicles, and Ca^{2+}- dependent vesicular release—all require a dramatic expansion of ER and its tightly coupled interactions with mitochondria, Golgi apparatus, and other organelles (Sassano and Agostinis, 2019). The expanded and highly heterogeneous lipid space (Harayama and Riezman, 2018; Santos and Preta, 2018) facilitates complex phase transitions among biomolecules, organelles (Bag et al., 2020; King et al., 2020), and synaptic vesicles (Rohrbough and Broadie, 2005). Lipid synthesis occurs in ER, and the current estimates suggest that more than 38,000 different identified lipids composed cellular membranes

(Liebisch et al., 2020). The theory predicts ∼180,000 lipid species distributed among eight major lipid categories (Brugger, 2014), supporting astonishing diversity of functions. Considering both enrichment of neural systems in lipids and the fact the ∼75% of lipid diversity is found in the brain, we can say: ***neuronal evolution is the lipid revolution***. The molecular diversity of synapses (Rohrbough and Broadie, 2005) and neurons based on lipidomics can be even greater than described using scRNA-seq, and the plasma lipid composition is a neuron-/cell-type-specific feature (Neumann et al., 2019; Fitzner et al., 2020). The most imperative are the data illustrating that the differences in lipid composition between cell-type plasma membranes are smaller than differences between organellar membranes in a given cell (Symons et al., 2021). In other words, each organelle might have its unique lipidome (Symons et al., 2021), and cell-to-cell communications by extracellular vesicles is a little-explored route of lipid signaling (Barber and Raben, 2019; Skotland et al., 2020).

Different Fates of Lipophilic vs. Lipophobic Transmitters

Increased lipid diversity of plasma and intracellular membranes shaped evolutionary recruitments of different transmitters in synaptic architectures. Small transmitters directly interact with membranes and can be broadly divided into two groups. Lipophilic transmitters (i.e., melatonin, serotonin, histamine, dopamine, noradrenaline, adrenaline, and adenosine) had high lipid partition coefficients (Wang et al., 2011; Postila et al., 2016; Engberg et al., 2020; Josey et al., 2020; Parkkila and Viitala, 2020) and can operate in receptor-independent mechanisms by changing lipid dynamics (Dey et al., 2021). They accumulated within surface membrane layers (e.g., postsynaptic membranes) with enhanced planar 2D diffusions. In other words, they act according to the 3D → 2D diffusion scheme (Postila and Rog, 2020). In contrast, glutamate, aspartate, glycine, and GABA are primarily lipophobic molecules. Accordingly, they work as classical transmitters in 3D diffusion space, but this situation can be changed in the presence of Ca^{2+} (Perez-Isidoro and Ruiz-Suarez, 2016). Acetylcholine has an intermediate position in its interactions with lipids (Postila et al., 2016; Postila and Rog, 2020).

Consequently, there are selection constraints in the synaptic receptor architecture designs (Postila et al., 2016; Postila and Rog, 2020). In many G-protein coupled receptors for lipophilic transmitters, the ligand-binding sites are predominantly (but not exclusively) hidden in the membrane (Postila and Rog, 2020). In contrast, the binding sites are primarily in the extracellular space for lipophobic transmitter receptors (e.g., iGluRs, mGluRs or nicotinic receptors). These physical properties of signal molecules might contribute to the fact that the first synapses utilized lipophobic transmitters (Glu, Asp, GABA), in addition to neuropeptides, as in extant ctenophores and cnidarians (Moroz et al., 2021a,b). The recruitments of lipophilic transmitters in neural systems and synapses seemingly occurred later in evolution, only in bilaterians (Moroz et al., 2021b). Furthermore, glutamate and acetylcholine were recruited primarily as fast excitatory neurotransmitters in the neocortex and neuromuscular junctions of vertebrates, respectively. The reverse situation occurred in insects, where glutamate was recruited as a fast neuromuscular transmitter (Jan and Jan, 1976). More likely, it is a reflection of evolutionary recruitment games under similar physical and chemical constraints for fast transmission in different evolutionary lineages. Together with their chemical stability and fast uptake/inactivation and coupling to bioenergetic, the lipophobic properties of transmitters might provide selective advantages for rapid synaptic communication dynamics.

Integrative Functions of ER as a Hub of Neuronal/Synaptic Innovations

ER physically and chemically interacts with cellular organelles using vesicular and non-vesicular lipid transport (Holthuis and Menon, 2014) at specialized membrane contact sites (MCS, Levine, 2004; Phillips and Voeltz, 2016; Ruiz-Lopez et al., 2021). MCS support complex interactions with mitochondria (Schlattner et al., 2014; Kannan et al., 2017; Wong et al., 2019) via tethered regions of ER known as **mitochondria-associated membranes** or MAM (Allen et al., 1989; Helle et al., 2013).

Specialized lipid chaperons can control Ca-dependent regulations and dynamic composition of lipid rafts at MCS [e.g., Sigma 1 receptor (Zhemkov et al., 2021a)], also acting as hubs of inter-organelle communications and signaling (Zhemkov et al., 2021c). Not surprisingly that such regulations at MCS and mitochondria do control neuropeptide asymmetric distribution and secretion (Valadas et al., 2018; Zhao et al., 2018), cell death (Prudent et al., 2015) and contribute to mechanisms underlying neurological disorders (Schon and Area-Gomez, 2013; Zhemkov et al., 2021b) and synaptopathies (Di Miceli et al., 2020).

ER is major calcium storage and the system for Ca^{2+} homeostasis (Berridge, 1998). A continuous, highly extended ER network is viewed as an "intracellular highway" or a much faster route for Ca^{2+} tunneling over long distances due to little Ca^{2+} buffering in the ER lumen in secretory cells (Petersen et al., 2017) and, perhaps, in neurons too.

In other words, ER has been termed a "***neuron within a neuron***" (Berridge, 1998, 2002). Boosted ER-Ca^{2+}/MCS/MAM systems in early secretory cells [or protoneurons] were ideally suited for developing a polarized release of all classes of transmitters with versatile preadaptations for divergent synaptic evolution in later Precambrian animals.

MAMs could also be viewed as an ancestral prototype of intracellular communication at the synapse. **Cellular stress and immunity responses** form dynamic tethering of ***signaling synapses between ER and MAM*** (Horner et al., 2011). MAM could be further co-evolved to support cell-cell synaptic interactions with high bioenergetic "demands" by coupling the same components [mitochondria (energy) and ER (secretion)] in presynaptic (and even postsynaptic) membranes.

The tripartite synapses in ctenophores (**Figure 1B**), with layered arrangements of secretory vesicles, ER, and mitochondrion (Hernandez-Nicaise, 1991), are the perfect examples of the membranous structural organization in one of the earliest synapse designs (Moroz, 2015). These asymmetrical

synapses contained three distinct layers of organelles, forming a so-called "presynaptic triad" (Hernandez-Nicaise, 1973, 1974, 1991): (i) a single layer of synaptic vesicles lining the presynaptic membrane, (ii) a cistern of agranular endoplasmic reticulum just above the row of vesicles, followed by (iii) one or several mitochondria with presumed MAM type contacts. The postsynaptic density and active zones, however, are less prominent in ctenophore synapses. ER-mitochondria relationships can also be noted in some cnidarian synapses (Anderson, 1985; Anderson and Grunert, 1988; Anderson and Spencer, 1989).

Mitochondria complexes and elaborated ER structures are characteristics of the fiber cells of placozoans (**Figures 1A,C**). These tetraploid cells can coordinate several interrelated functions such as systemic feeding with bacterial phagocytosis and immunity responses. As a result, we view a meshwork of fiber cells [and associated small star-like cells (Romanova et al., 2021)] as an organism-scale integrative or homeostatic system, potentially involved in systemic injury and regeneration responses, perhaps even in morphogenesis. This type of system can be close to the hypothetical protoneuronal organization, which initially evolved to control morphogenesis in first nerveless metazoans (Fields et al., 2020).

QUESTIONS AND PERSPECTIVES

We only scratched the surface of the problem of synaptic selection. Early interdependence and ancestral relationships of innate immune and neural systems is another poorly investigated layer in the evolution of intercellular communications. The landscape for developing immune functions is similar to neural control due to many shared secretory products, lipid and ER rearrangements and conceptually shared features of neuronal and immune synapses with similar adhesive molecules. In due course, both systems (co-)evolved as responses to stress/injury factors and recognition of self vs. foreign RNA, DNA, protein, and cell invasions. Remarkably, ER-MAMs signaling also plays an essential role in innate immunity against RNA virus infection: as a platform for inducing an immune response and regulating viral replication. MAM tethering ER to mitochondria and peroxisome(s) form immune synapses during RNA virus invasion (Horner et al., 2011, 2015). Thus, it would be intriguing to think that some architectures of neural systems evolved as a branch of immune communications. Injury-induced regeneration signaling can be a universal exaptation for immune and neural systems, both adopted for faster responses. The growing diversity and compartmentalization of lipids and ER further promoted cell plasticity, forming more localized immune and neural synapses, often recruiting the same transmitters (e.g., histamine and serotonin, glutamate, and GABA), as well as multiplicity of small signaling peptides. Ultimately, as all things in nature, membrane-membrane and cell-cell communications should be physically closer to be efficient.

AUTHOR CONTRIBUTIONS

DR: visualization. All authors contributed to the article and approved the submitted version.

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