INVOLVEMENT OF BIOACTIVE PEPTIDES IN THE CONTROL OF CELL SURVIVAL, PROLIFERATION AND PLASTICITY IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS,

2nd Edition

EDITED BY: David Vaudry and Dora Reglodi

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INVOLVEMENT OF BIOACTIVE PEPTIDES IN THE CONTROL OF CELL SURVIVAL, PROLIFERATION AND PLASTICITY IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS,
2nd Edition

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EDITORIAL
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Editorial: Involvement of Bioactive Peptides in the Control of Cell Survival, Proliferation and Plasticity in Physiological and Pathological Conditions

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Keywords: neuropeptides, cell survival, proliferation, plasticity, bioactive peptides, peptides, physiology, pathology

Editorial on the Research Topic

Involvement of Bioactive Peptides in the Control of Cell Survival, Proliferation and Plasticity in Physiological and Pathological Conditions

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The scope of this Research Topic is to highlight the involvement of bioactive peptides in the control of cell survival, proliferation, and plasticity in physiological and pathological settings. Bioactive peptides are small amino acid chains that act on target effector cells and their sources can be diverse. As illustrated here, bioactive peptides include neuropeptides (PACAP, oxytocin, substance P, ODN), gastrointestinal peptides (secretin, VIP, ghrelin, galanin, gastrin), plant peptides (caryophyllaceae-type cyclopeptides), and fragments of proteins (FAM19A5, cerebrolysin). But wherever they come from and whatever the functions they were discovered for, it appears that a growing number of peptides can promote neuronal survival and plasticity. For example, secretin was initially identified for its gastrointestinal functions but also promotes cell survival in the hippocampus and the cerebellum during development and regulates neuronal plasticity and memory in these two brain regions in adulthood (Wang and Zhang). Other peptides such as oxytocin, known to stimulate milk ejection and uterine contraction, or ODN, known for its involvement in stressful behavior or food intake, are now the subject of studies for their ability to reshape neural circuitry or to promote neuroprotection (Pekarek et al.; Masmoudi-Kouki et al.), paving the way for therapeutic applications for the treatment of neurodevelopmental, neuropsychiatric and neurodegenerative diseases. Some researchers are looking for the potential role of other peptides, such as ghrelin in neurodegenerative disease (Kim et al.), and putative neuropeptides, such as FAM19A5, for the treatment of traumatic brain injury (Shahapal et al.). This suggests that we may still discover further bioactive peptides controlling cell survival, proliferation, and plasticity.

For some peptides such as PACAP, the neuroprotective activity seems to be well established (1) to the point of looking for the most efficient administration routes to reduce the volume of the infarct lesion and to promote functional recovery after stroke (Cherait et al.). However, Maugeri et al.

highlight rightly that this peptide often has contradictory effects on neuronal survival depending on the tissue or the duration of the treatment. For instance, in the early phase of amyotrophic lateral sclerosis (ALS) progression, PACAP promotes motor neuron survival and axonal regeneration, whereas at later stages of the disease it promotes neuro-inflammation responsible for motor neuron degeneration (Maugeri et al.). PACAP is thought to protect cells through autocrine or paracrine mechanisms, but the interpretation of its action can be complicated by expression levels of the peptide and its receptors that vary depending on the cell type and the time after injury. While in ALS patients PAC1R is downregulated (Maugeri et al.), Woodley et al. show that PAC1R, VPAC1R, and VPAC2R are up-regulated for at least 2 weeks in the distal part of the sciatic nerve after transection. PACAP and VIP expression are also upregulated with a peak of expression after 2 and 7 days, respectively. Based on these kinetics of expression and functional studies, the authors conclude that PACAP could promote the expression of early proinflammatory mediators required to attract macrophages to the injured sciatic nerve, while VIP would prevent excessive macrophage recruitment and stop the inflammatory response at later stages. Opposite effects of PACAP and VIP are also reported by Takeuchi et al. on mouse cortical neurons (Takeuchi et al.). Indeed, while PACAP promotes neurite outgrowth, VIP impaires axon outgrowth and decreases dendrite arborization through activation of the VPAC2 receptor. Taken together, these results show to what extent a peptidergic system can be efficient but also extremely complex. On top of that, peptides can act alone but also as a cocktail as shown by Kang et al. with cerebrolysin, which decreases hippocampal neuronal death and increases brain-derived neurotrophic factor (BDNF) expression after one week of administration in a pilocarpine-induced seizure model. The fact that neuropeptides probably work together in a complementary or synergistic manner to fine-tune cell survival, proliferation, or plasticity is an idea taken up in several studies of this Research Topic (Zeng et al.; Wang and Zhang; Takeuchi et al.). As mentioned in those manuscripts, this is supported by recent studies showing co-expression of neuropeptide receptors capable of forming heterodimers leading to a complex pharmacology. Therefore, in future projects, it will not be sufficient to study one particular neuropeptide, and we will have to focus on groups of neuropeptides co-expressed in cells or released sequentially.

Besides acting on neurons, bioactive peptides can also control cell survival, proliferation, and plasticity in peripheral tissues. This is the case of PACAP, which exerts numerous protective effects on peripheral organs such as the airways, liver, heart, kidney, and intestine (Toth et al.). If PACAP could, among others, be a useful tool for the treatment of osteoarthritis formation, this is also the case of substance P, which promotes chondrocyte proliferation and differentiation (Li et al.). All these effects of neuropeptides and some of their similarities of actions in physiological or pathological conditions illustrate the complexity of the regulations involved and the therapeutic potential offered by these molecules when we are able to understand their subtleties.

Endogenous neuropeptides and their receptors are often overexpressed in cancer cells, prompting scientists to consider them as therapeutic targets. This is the case with galanin, the expression of which is found in most brain tumors (Falkenstetter et al.). This could be of interest for the treatment of these cancers since on other cell types, galanin exerts anti-proliferative effects and promotes apoptosis. Exogenous bioactive peptides such as cyclopeptides from plants are also tested for their cytotoxic activities on tumor cell lines (Houshdar Tehrani et al.). This suggests their possible use for cancer treatment, but in view of the numerous effects that certain peptides can have on the organism, it will be necessary to carry out in-depth studies to exclude the existence of undesirable effects at the central or peripheral level. To conclude, the most difficult issue is probably not to find functions of interest for a bioactive peptide, but to succeed to find the relevant clinical application.

AUTHOR CONTRIBUTIONS

DV and DR have co-chaired this Research Topic. DV and DR wrote together this editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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FAM19A5 Expression During Embryogenesis and in the Adult Traumatic Brain of *FAM19A5-LacZ* Knock-in Mice

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FAM19A5 is a secretory protein that is predominantly expressed in the brain. Although the *FAM19A5* gene has been found to be associated with neurological and/or psychiatric diseases, only limited information is available on its function in the brain. Using *FAM19A5-LacZ* knock-in mice, we determined the expression pattern of FAM19A5 in developing and adult brains and identified cell types that express FAM19A5 in naïve and traumatic brain injury (TBI)–induced brains. According to X-gal staining results, FAM19A5 is expressed in the ventricular zone and ganglionic eminence at a very early stage of brain development, suggesting its functions are related to the generation of neural stem cells and oligodendrocyte precursor cells (OPCs). In the later stages of developing embryos and in adult mice, FAM19A5 expression expanded broadly to particular regions of the brain, including layers 2/3 and 5 of the cortex, cornu amonis (CA) region of the hippocampus, and the corpus callosum. X-gal staining combined with immunostaining for a variety of cell-type markers revealed that FAM19A5 is expressed in many different cell types, including neurons, OPCs, astrocytes, and microglia; however, only some populations of these cell types produce FAM19A5. In a subpopulation of neuronal cells, TBI led to increased X-gal staining that extended to the nucleus, marked by slightly condensed content and increased heterochromatin formation along the nuclear border. Similarly, nuclear extension of X-gal staining occurred in a subpopulation of OPCs in the corpus callosum of the TBI-induced brain. Together, these results suggest that FAM19A5 plays a role in nervous system development from an early stage and increases its expression in response to pathological conditions in subsets of neurons and OPCs of the adult brain.

Keywords: FAM19A5, traumatic brain injury, brain development, neuron, oligodendrocyte precursor cells

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INTRODUCTION

FAM19A5, also called TAFA5, is a member of the TAFA family of secreted proteins that are predominantly expressed in the brain (Tom Tang et al., 2004). Due to the presence of conserved CC motifs, this family has been considered as an atypical member of the CC-chemokine family (Tom Tang et al., 2004). In addition, FAM19A5 is also regarded as a putative neuropeptide because

it is co-localized with vasopressin and oxytocin in magnocellular and parvocellular neurons of the hypothalamic paraventricular nucleus, which are involved in fluid homeostasis (Paulsen et al., 2008). FAM19A5 expression in the mouse brain was found to increase at the later stages of embryonic development (Yue et al., 2014), suggesting a role of FAM19A5 in brain development. Genome-wide association studies have demonstrated an association of *FAM19A5* with late-onset Alzheimer disease in humans (Herold et al., 2016; Mez et al., 2017). Mosaic monosomy of chromosome 22—which includes disruption of the *FAM19A5* gene—leads to skeletal abnormalities, low body weight, and neuropsychiatric problems, including attention deficit hyperactivity disorder (ADHD), aggression, or autistic symptoms (Kashevarova et al., 2018). Multiple gene copies of the *FAM19A5* gene seems to be associated with glioma in some patients (Díaz De Ståhl et al., 2005), implying the role of FAM19A5 in tumorigenesis of the central nervous system (CNS). These observations indicate that FAM19A5 has roles in neural development and the pathological conditions of neurological and/or psychiatric diseases.

In addition, FAM19A5 may have functions at the peripheral tissues. For instance, FAM19A5 likely inhibits the RANKL-induced differentiation of osteoclast precursor cells by interacting with formyl peptide receptor 2 (Park et al., 2017). A recent report showed that FAM19A5 is secreted from adipose tissues and inhibits the proliferation and migration of smooth muscle cells. In particular, FAM19A5 was found to suppress neointima formation in injured rat carotid arteries by interacting with sphingosine-1-phosphate receptor 2 (Wang et al., 2018).

Since the first report on FAM19A5 expression in the brain (Tom Tang et al., 2004), only limited information is available on the function of FAM19A5 in the brain and peripheral tissues. In this study, using *FAM19A5-LacZ* knock-in (KI) mice, we determined the expression pattern of FAM19A5 during embryogenesis and in the adult brain, and identified cell types in the brain that express FAM19A5. Traumatic brain injury (TBI) is a sudden insult to the brain from an external force which may result in permanent or temporal brain dysfunctions. Various cellular mechanisms are activated after TBI to regenerate or replace the damaged or dead cells. Some genes important in development reactivate under injury condition (Mierzwa et al., 2014; Chaboub et al., 2016). Therefore, we analyzed changes in FAM19A5 expression in response to TBI.

MATERIALS AND METHODS

Generation of *FAM19A5-LacZ* Knock-in Mice

FAM19A5-LacZ KI mice were generated by the UC Davis Mouse Biology Program. The *FAM19A5*-targeting vector was constructed as shown in **Figure 1A**. The gene-trap method using *LacZ* as a reporter gene was employed to visualize FAM19A5 expression in tissue sections (Mountford et al., 1994). Briefly, the target vector containing IRES-*lacZ* gene was inserted in front of exon 4 of the *FAM19A5* gene. The *LacZ* gene is expressed independently of the target *FAM19A5* gene due to the IRES element. This *FAM19A5*-targeting vector was delivered to embryonic stem cells by electroporation. We confirmed the incorporation of this vector into the target chromosome by genotyping and chromosome counting of transgenic embryonic stem cells. Selected transgenic embryonic stem cells were injected into blastocysts, and the embryos were implanted into the uterus of female recipient mice. We performed a germline transmission test to check for stable germline expression in the chimeric generation. The following primers were used for genotyping: FAM19A5-F1, 5′-TGG TCA GAA CTG TGT GAG TGC-3′; FAM19A5-R1, 5′-CAC CAT GGG CAA GTT TAA CA-3′; and FAM19A5-R2, 5′-CCA ACC CCT TCC TCC TAC AT-3′ (**Supplementary Figures 1A,B**). The generated *FAM19A5-LacZ* KI chimeric mice were backcrossed onto C57BL/6J genetic background. Wild-type C57BL/6J female mice were purchased from Orient Bio, Inc. (Seongnam, South Korea) and mated with heterozygous KI males. To obtain homozygous *FAM19A5-LacZ* KI mice, the heterozygous male mice were mated with the heterozygous female mice. Their wild-type littermates produced by the backcrossing were used for the control groups.

Animals

Mice were housed in temperature-controlled (22–23°C) conditions with a 12-h light/12-h dark light cycle (lights on at 8:00 am). The mice were given *ad libitum* supplies of standard chow and water. All animal experiments were designed to use the fewest mice possible, and anesthesia was administered. All animal procedures were approved by the Institutional Animal Care and Use Committee of Korea University (KOREA-2016-0091-C3).

Traumatic Brain Injury

8-to-9-week-old *FAM19A5-lacZ* KI mice were anesthetized with sodium pentobarbital (50 mg/kg). Cryogenic TBI was performed by placing a prechilled iron rod on the calvarium for 1 min (Moon et al., 2011). Animals were sacrificed at 7 days post-injury.

X-gal Staining for Embryo, Postnatal, and Adult Brains

For embryonic X-gal staining, the pregnant mice were sacrificed by cervical dislocation, and the embryos were isolated. Whole embryos at embryonic day 10.5 (E10.5), E12.5, and E14.5 were fixed in 4% paraformaldehyde (PFA) and 0.2% glutaraldehyde (GTA) in phosphate buffer (PB) at 4°C for 10, 15, and 30 min, respectively. For embryos older than E14.5, the heads were cut and the skins were removed. The heads of the embryos were fixed in the same fixative for 1∼2 h at 4°C. For postnatal mice, the brains were isolated from the skulls and fixed in the same fixative for 1∼2 h at 4°C. The fixed tissues were then washed with phosphate-buffered saline (PBS) twice for 5 min and incubated in X-gal staining solution, 1 mg/ml of X-gal, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.01% sodium deoxycholate, and 0.02% Non-idet-P40 in 0.1 M PB at pH 7.4 for 48 h at

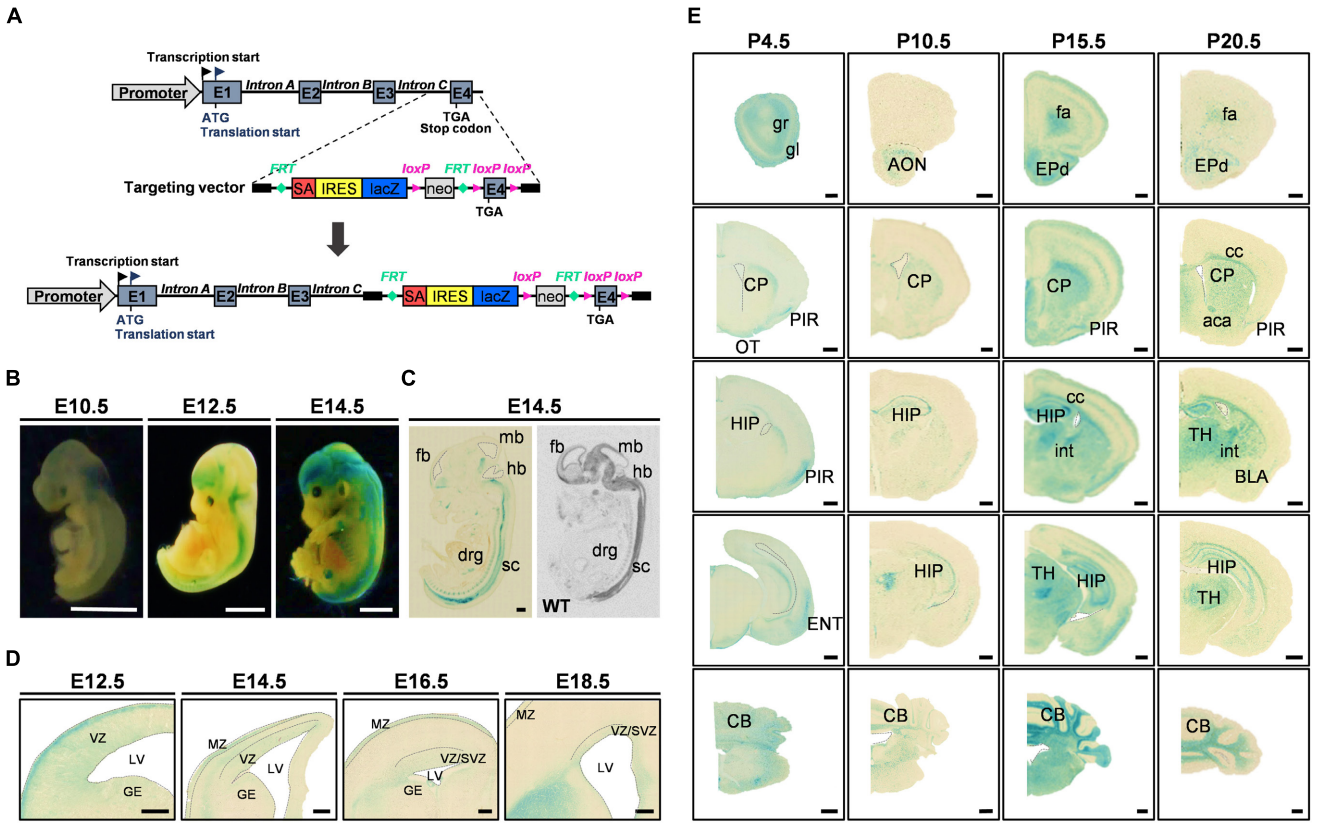

FIGURE 1 | *FAM19A5-LacZ* knock-in (KI) gene construct and FAM19A5 expression in the nervous system at developmental stages. **(A)** Schematic diagram of the *FAM19A5-LacZ* KI mouse gene construct. The target vector containing the *LacZ* gene is inserted in front of exon 4 (E4) of the *FAM19A5* gene by homologous recombination, producing β-galactosidase enzyme under control of the *FAM19A5* gene promoter. **(B)** Whole embryo X-gal staining at embryonic days E10.5, E12.5 and E14.5 of *FAM19A5-LacZ* KI homozygote (+/+) mice. X-gal staining occurred from the early embryonic stage E10.5, with increased and continuous staining in the later embryonic stages. **(C)** Comparison of sagittal views between X-gal staining (left) and *in situ* hybridization pattern (right) at E14.5. Mouse and rat embryos at E14.5 were subjected to X-gal staining and *in situ* hybridization, respectively, revealing a similar pattern of expression. **(D)** FAM19A5 expression in the germinal zones; ventricular zone (VZ), subventricular zone (SVZ) and ganglionic eminence (GE). Coronal view of E12.5, E14.5, E16.5, and E18.5 X-gal–stained sections. **(E)** X-gal staining for coronal brain sections at postnatal stages P4.5, P10.5, P15.5, and P20.5, suggesting broad and continuous FAM19A5 expression throughout the postnatal stages. aca, anterior commissure, anterior part; AON, anterior olfactory nucleus; BLA, basolateral amygdaloid nucleus, anterior part; CB, cerebellum; cc, corpus callosum; CP, caudate putamen; drg, dorsal root ganglion; ENT, entorhinal cortex; EPd, dorsal endopiriform nucleus; E1, exon 1; E2, exon 2; E3, exon 3; E4, exon 4; fa, forceps minor of the corpus callosum; fb, fore brain; GE, ganglionic eminence; gl, glomerular layer of the olfactory bulb; gr, granular layer of the olfactory bulb; hb, hind brain; HIP, hippocampal region; int, internal capsule; IRES, internal ribosome entry site; LV, lateral ventricle; mb, mid brain; MZ, marginal zone; neo, neomycin phosphotransferase gene; OT, olfactory tubercle; PIR, piriform cortex; SA, splice acceptor site; sc, spinal cord; SVZ, subventricular zone; TH, thalamus; VZ, ventricular zone; WT, wild-type. Ventricles (lateral ventricle, 3rd ventricle and 4th ventricle) are demarcated by dashed lines in panel C-E. All animals used were *FAM19A5-LacZ* KI mouse model otherwise labeled. Scale bars represent 2 mm for panel B, 500 μm for panels C and E, and 100 μm for panel **D**.

37°C in the dark. The stained tissues were post-fixed with 4% PFA in PBS overnight at 4°C and washed, then whole brain images were obtained.

For X-gal–stained sections from E12.5 to postnatal day (P4.5), the stained whole brains were cryoprotected with 30% sucrose in PBS and sectioned at 40 μm using a cryostat (Leica, Wetzlar, Germany). For X-gal–stained sections from P10.5, P15.5, P20.5, and 10-week old adult male mice, animals were perfused with 4% PFA and 0.2% GTA in PB. The brains were isolated and post-fixed in 0.2% GTA in PB for 24 h at 4°C. The brains were then cryoprotected in 30% sucrose in PBS and serially cross-sectioned in 40 μm using the cryostat. The sectioned tissues were then incubated in X-gal staining solution for 48 h at 37°C in the dark. The images of the sections were taken using a slide scanner (Axio scan Z1, Zeiss).

Immunofluorescence Analysis of Brain Sections

For X-gal staining with multiple fluorescence labeling, 10-week old adult male mice were perfused with 4% PFA in PBS, and isolated brains were post-fixed in the same solution for 3 h. Brains were then cryoprotected in 30% sucrose in PBS, serially sectioned with the cryostat (20 μm slices), and stored in 50% glycerol in PBS at −20°C. For X-gal staining, sections were brought to room temperature and washed three times in PBS for 5 min each, then transferred to X-gal

staining solution at 37°C overnight. After X-gal staining, sections were blocked with 3% bovine serum albumin and 0.1% Triton X-100 in PBS for 30 min and incubated in primary antibodies overnight at 4°C. The primary antibodies used in this study were mouse anti-NeuN (1:1000, Millipore), rabbit anti-NeuN (1:500, Millipore), rabbit anti-GFAP (1:1000, Wako), rabbit anti-NG2 (1:500, Millipore), rabbit anti-Iba1 (1:500, Dako), sheep anti-Sez6l2 (1:500, R&D Systems), rabbit anti-Tuj1 (1:1000, SIGMA), mouse anti-nestin (1:500, Millipore), rat anti-CD31 (1:500, BD Biosciences), rabbit anti-PDGFRβ (1:500, Abcam), goat anti-CD45 (1:500, R&D Systems), rabbit anti-MBP (1:500, Abcam), rabbit anti-MAP2 (1:500, Millipore), rabbit anti-Olig2 (1:500, Millipore), rabbit anti-Ki67 (1:500, Abcam), rabbit anti-Active caspase-3 (1:500, Cell signaling), and mouse anti-O4 (1:500, Millipore). Following several PBS washes, the appropriate secondary antibody was applied for 30 min. Nuclei were labeled with Hoechst 33342 (Invitrogen, Carlsbad, CA, United States). For the fluorescent TUNEL assay, co-labeled sections were stained using an *in situ* cell death detection kit (Roche) according to the manufacturer's instructions. The sections were washed, mounted, and imaged using a confocal microscope (TCS SP8, Leica) as previously described (Levitsky et al., 2013). Briefly, the X-gal–stained sections were excited at 633 nm, and the fluorescence signals emitted at 650–770 nm were visualized. Differential interference contrast microscopy images were also obtained to confirm real X-gal fluorescence precipitates. Confocal acquisition of the additional fluorescence labels was conducted as follows: Hoechst (excited at 405 nm and detected between 415–450 nm) for the nucleus; Alexa 488 (excited at 488 nm and detected between 489– 550 nm) and Alexa 555 (excited at 561 nm and detected between 563 and 620 nm) for other cell type–specific markers. For the quantification of nuclear and cytoplasmic X-gal fluorescence, Z-stack images of between 10 and 15 μm in depth were acquired at 1 μm intervals using a 20 X objective.

Immunoenzyme (HRP) Analysis of Brain Sections

For co-staining of X-gal and 3, 3′-diaminobenzidine (DAB) staining, the X-gal–stained brain sections were incubated in 0.3% H_2O_2 solution for 10 min and washed in PBS for 5 min at room temperature. Sections were blocked with 3% bovine serum albumin and 0.1% Triton X-100 in PBS for 30 min and incubated in primary antibodies overnight at 4°C. The primary antibodies used in this study were mouse anti-NeuN (1:1000, Millipore), rabbit anti-GFAP (1:1000, Wako), rabbit anti-NG2 (1:500, Millipore), and rabbit anti-Iba1 (1:500, Dako). Following several PBS washes, the appropriate biotinylated secondary antibody was applied for 30 min. The sections were subjected to the avidin-horseradish peroxidase complex method (Vector Laboratories, Burlingame, CA) for 30 min at room temperature and finally treated with DAB (SIGMA) until desired dark brown color was generated. The images of the sections were taken using a slide scanner (Axio scan Z1, Zeiss).

Nissl Staining

For Nissl staining following the X-gal staining, the sections were placed in PBS for 5 min each, then hydrated in 1% Cresyl violet at 50°C for 20 min. The sections were rinsed with distilled water, dehydrated, and mounted with Permount (Thermo Fisher Scientific). The slide was imaged using an optical microscope.

In situ Hybridization

Adult mice and rats were sacrificed, and mouse brains and embryonic rats were removed and quickly frozen in isopentane on dry ice. Tissue sections were cut to 20 μm thickness with a cryostat, thaw-mounted on Superfrost Plus slides (Thermo Fisher Scientific, United States), and stored at −70°C until use. Sections were fixed in 4% PFA, washed with PBS, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% NaCl (pH 8.0). Samples were hybridized overnight with a radiolabeled probe (1.2 × 106 cpm) and washed four times with 2 × standard sodium citrate (SSC). A template for the FAM19A5 probe was prepared by subcloning the RT-PCR products into a pGEM-T vector (Promega). For the preparation of a radiolabeled mouse FAM19A5 cDNA probe, the following primers were used: mFAM19A5-F, 5′-ATG CAG CTC CTG AAG GCG CT-3′; mFAM19A5-R, 5′-TCA GGA GAC CGT GGT GGT CT-3′. For the preparation of a radiolabeled rat FAM19A5 cDNA probe, the following primers were used: rFAM19A5-F, 5′-ATG CAG CTC CTG AAG GCG CTC-3′; rFAM19A5-R, 5′-TCA GGA GAC CGT GGT GGT CT-3′.

Sense and antisense riboprobes were prepared using an *in vitro* transcription system (Promega) in the presence of [α-^{35}S] UTP (Amersham Pharmacia Biotech, United States). After RNase A treatment, slides were rinsed with 2×, 1×, 0.5×, and 0.1× standard sodium citrate containing 1 mM dithiothreitol for 10 min each at room temperature, then washed with 0.1× standard sodium citrate at 60°C. The samples were dehydrated in ethanol and exposed to X-ray film (Biomax MR, Kodak, United States).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

TRI Reagent (Molecular Research Center, United States) was used to isolate total RNA from mouse brain tissue. 1 μg of RNA was reverse−transcribed to complementary DNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, United States). Primer sequences used for qRT-PCR were as follows: mFAM19A5-F, 5′-AGG TGA ATG ACC CCC TTC GT-3′; mFAM19A5-R, 5′-TGA CTC TGC TCC CCA GCT TC-3′; mGAPDH-F, 5′-ATC CTG CAC CAC CAA CTG CT-3′; mGAPDH-R, 5′-GGG CCA TCC ACA GTC TTC TG-3′. Real−time polymerase chain reaction was performed on CFX96 Touch™ Real-Time PCR detection system using the SsoAdvanced Universal SYBR®Green Supermix (Bio-Rad, United States). Gene expression was normalized to GAPDH level, and the relative quantity of mRNAs was calculated based on the comparative Cq method.

Quantification and Statistical Analysis

To quantify the cytoplasmic and nuclear X-gal precipitates of the naïve and TBI-induced brain, the acquired confocal images from the cortex and corpus callosum were converted to 3D images using IMARIS software (IMARIS9.0, Bitplane AG, Zurich, Switzerland). The "Surface tool" of the IMARIS software was used to mark all the signals detected in the X-gal fluorescence channel, and the total X-gal surface was set according to the result. Then, the "Coloc tool" was used to filter and generate the channel indicating the overlap regions of the Hoechst and X-gal signals. The cytoplasmic X-gal surface was calculated as the total X-gal surface minus the "Coloc" surface, and the nuclear X-gal was calculated as the surface areas of the total X-gal surface minus the cytoplasmic X-gal surface. Then the number and the volume of the cytoplasmic and nuclear surfaces were calculated. The number and volume of the X-gal signal were acquired from the central part of the brain tissue section images, which included the injury lesion sites.

The images were taken of three mice from each group (naïve and TBI). For qRT-PCR, total RNA was extracted from 9 naïve and 12 TBI-induced mice brains. All statistical analyses were performed using GraphPad Prism 5 software (GraphPad software, Inc., La Jolla, CA). Data are shown as the means ± standard errors of the mean. For multiple comparisons, one-way ANOVA was performed, followed by Newman-Keuls multiple comparisons test. The criterion for statistical significance was set at a p value less than 0.05.

RESULTS

FAM19A5 Expression During Embryogenesis and in the Postnatal Mouse Brain

To understand the functional mechanism of FAM19A5 in the brain, we first investigated the expression pattern of FAM19A5 in the developing mouse brain. To assess this pattern, X-gal staining was employed on the brain tissue of *FAM19A5-LacZ* KI mice. *FAM19A5-LacZ* KI homozygote mice exhibited stronger X-gal staining than heterozygote mice, and wild-type littermates did not generate blue precipitations (**Supplementary Figure 1C**). These results were further corroborated by those of the *in situ* hybridization assay (**Supplementary Figure 2**).

The whole mount X-gal staining at embryonic stages E10.5, E12.5, and E14.5 demonstrated predominant expression of FAM19A5 in the brain and spinal cord (**Figure 1B**). X-gal signal was observed from E10.5. The sagittal sections of E14.5 displayed positive X-gal staining in the brain, spinal cord, and dorsal root ganglion, which is consistent with the *in situ* hybridization result showing expression of FAM19A5 at the mRNA level (**Figure 1C**). Both X-gal staining (**Figure 1D**) and the *in situ* hybridization technique (**Supplementary Figure 2A**) showed FAM19A5 expression in the ventricular zone as well as marginal zone during the neurogenesis period, including at E12.5, E14.5, and E16.5. X-gal staining was also observed in the ganglionic eminence at E12.5, but this staining became weaker at E14.5 and E16.5. FAM19A5 expression was maintained during the late neurogenesis stage (E18.5) in the ventricular zone and subventricular zone. Furthermore, the coronal section views at postnatal stages P4.5, P10.5, P15.5, and P20.5 revealed the broad expression pattern of FAM19A5 in the brain, including the olfactory bulb, corpus callosum, piriform cortex, caudate putamen, hippocampus, amygdala, thalamus, entorhinal cortex, and cerebellum (**Figure 1E**). These results are consistent with those of the *in situ* hybridization assay (**Supplementary Figure 2A**), showing continuous whole brain expression of FAM19A5 from the early embryonic stages to the postnatal periods.

Characterization of Cell Types Expressing FAM19A5 in the Adult Brain

In adult mice, FAM19A5 was broadly expressed in many regions of the brain. However, relatively high-intensity X-gal signals were found in several brain areas, such as the olfactory bulb, anterior olfactory nucleus, piriform cortex, hippocampus, thalamus, amygdala, entorhinal cortex, superior colliculus, and inferior colliculus (**Figure 2A**). In the cerebral cortex, X-gal precipitates were broadly distributed in layers 2/3 and 5 where pyramidal neurons are enriched; X-gal signals were also found in some neurons in layer 4. In the hippocampus, pyramidal neurons in the CA regions (CA1, CA2 and CA3) displayed the presence of X-gal signals. However, the granular neurons of the dentate gyrus hardly exhibited X-gal precipitation (**Figure 2A** and **Supplementary Figure 1C**). Besides, brain regions with white matter such as the corpus callosum, anterior commissure, and internal capsule also showed X-gal precipitation (**Figure 2A**).

FAM19A5 appears to be expressed not only in neurons but also in glial cells, as these cells are widely spread across the white matter along with the axon bundles (**Figure 2A** and **Supplementary Figure 1C**). To explore the identity of cells expressing FAM19A5, X-gal–stained sections were further immunostained with various brain cell-type markers: NeuN for neurons, GFAP for astrocytes, NG2 for OPCs, and Iba1 for microglia. X-gal and DAB double staining displayed FAM19A5 expression in the subpopulations of all cell types, including neurons, astrocytes, OPCs, and microglia (**Figure 2B**). In particular, a large portion of $NeuN^+$ cells in the cortical regions and $NG2^+$ cells in the corpus callosum were double stained with X-gal.

To quantify the portion of $X\text{-}gal^+$ cells out of the total population of each cell type, direct confocal fluorescence acquisition technique was applied to the X-gal staining (Levitsky et al., 2013). Fluorescence dots were observed in both *FAM19A5-LacZ* KI (+/−) and *FAM19A5-LacZ* KI (+/+) mice but not in wild-type mice. The fluorescence dots mostly overlapped with the X-gal signals seen by differential interference contrast microscopy, with few exceptions of autofluorescent signals (**Figure 3A**). X-gal staining combined with immunofluorescence staining again showed X-gal fluorescence dots in various types of cells, including neurons, astrocytes, OPCs, and microglia (**Figure 3B**).

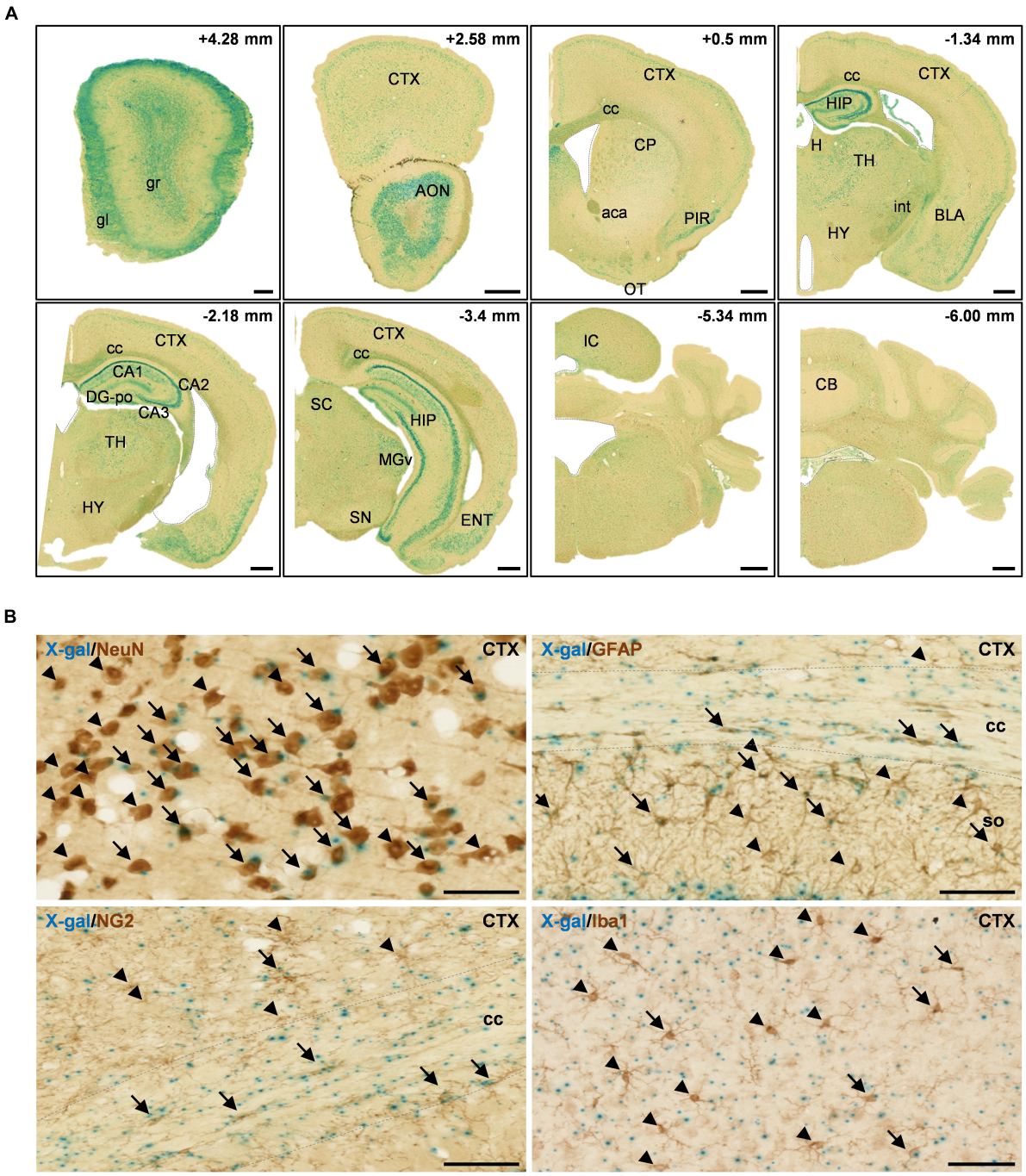

FIGURE 2 | X-gal staining of the adult *FAM19A5-LacZ* knock-in (+/+) mouse brain. **(A)** X-gal staining of coronal sections of 10-week old adult male mice. The section's distance from the Bregma point is indicated at the upper right hand corner of each box. The lateral ventricle, third ventricle, fourth ventricle, and aqueduct of Sylvia are demarcated by dashed lines. **(B)** X-gal staining combined with 3, 3′-diaminobenzidine staining on adult mouse brain sections. Brain sections incubated in X-gal staining solution for 24 h were immune-labeled for various cellular markers: NeuN for neuron, GFAP for astrocyte, NG2 for oligodendrocyte precursor cell, and Iba1 for microglia. X-gal signals (blue) are seen in subpopulations of $NeuN^+$, $GFAP^+$, $NG2^+$, and $Iba1^+$ cells in the adult mouse brain. Arrows and arrow heads depict cell marker positive cells with and without X-gal signals, respectively. The areas for the corpus callosum are indicated by dashed lines. aca, anterior commissure, anterior part; AON, anterior olfactory nucleus; BLA, basolateral amygdaloid nucleus, anterior part; CA1, field CA1 of the hippocampus; CA2, field CA2 of the hippocampus; CA3, field CA3 of the hippocampus; CB, cerebellum; cc, corpus callosum; CP, caudate putamen; CTX, cerebral cortex; DG-po, polymorphic layer of the dentate gyrus; ENT, entorhinal cortex; gl, glomerular layer of olfactory bulb; gr, granular layer of olfactory bulb; H, habenula; HIP, hippocampal region; HY, hypothalamus; int, internal capsule; IC, inferior colliculus; MGv, medial geniculate nucleus, ventral part; OT, olfactory tubercle; PIR, piriform cortex; SC, superior colliculus; SN, substantia nigra; so, stratum oriens of the hippocampus; TH, thalamus. Scale bars represent 500 μm in panel **A** and 50 μm in panel **B**.

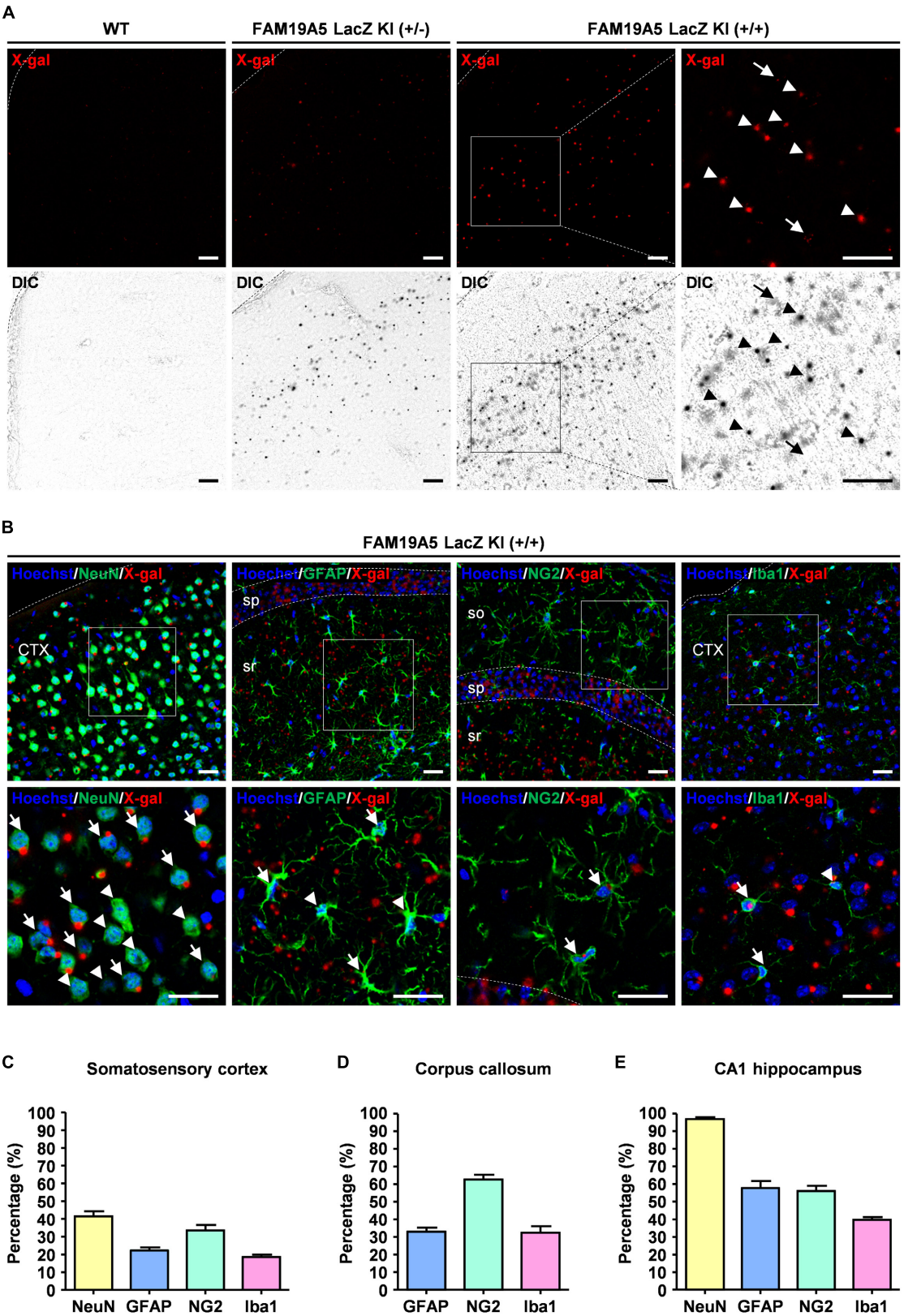

FIGURE 3 | Continued

FIGURE 3 | Quantification of X-gal$^+$ neuronal and glial cells via direct confocal acquisition of X-gal fluorescence. **(A)** Confocal acquisition of fluorescence from X-gal precipitates in the wild-type (WT) and *FAM19A5-LacZ* knock-in (KI) mouse brains. Fluorescence signals were stronger in *FAM19A5-LacZ* KI homozygote (+/+) mice than in heterozygote (+/−) mice. However, these fluorescence signals were not observed in the WT mouse brains. Differential interference contrast (DIC) images were used to determine whether the florescence signal originated from X-gal precipitates. Arrow heads indicate florescence from X-gal precipitates but arrows represent auto-fluorescence signals that are not driven from X-gal precipitates. **(B)** X-gal staining combined with immunofluorescence staining for cell type-specific markers of the adult *FAM19A5-LacZ* KI (+/+) mouse brain. Brain sections incubated for 24 h in X-gal solution were immunostained for cell markers, including NeuN, GFAP, NG2, and Iba1. X-gal fluorescence signals were observed in subpopulations of neurons (NeuN), astrocytes (GFAP), oligodendrocyte precursor cells (NG2), and microglia (Iba1). Arrows and arrow heads represent cell-type marker$^+$ cells with and without X-gal fluorescence signal, respectively. **(C–E)** Quantification of X-gal$^+$ cells in the somatosensory cortex **(C)**, medial corpus callosum **(D)**, and CA1 region of the hippocampus **(E)**. Number of X-gal$^+$ cells out of cell-type marker$^+$ cells were counted based on both fluorescence and DIC images. Data were presented as the means ± standard errors of the mean (n = 5). CTX, cerebral cortex; so, stratum oriens of the hippocampus; sp. pyramidal cell layer of the hippocampus; sr, stratum radiatum of the hippocampus. Scale bar represents 50 μm.

Using this technique, the proportion of FAM19A5 expressing cells out of NeuN$^+$, GFAP$^+$, NG2$^+$, and Iba1$^+$ cells were quantified in three different brain areas: the somatosensory cortex, corpus callosum, and CA1 region of the hippocampus. In the somatosensory cortex, X-gal precipitations were found in 41% of NeuN$^+$, 22% of GFAP$^+$, 33% of NG2$^+$, and 18% of Iba1$^+$ cells (**Figure 3C** and **Supplementary Figure 3A**). In the medial corpus callosum, 62% of NG2$^+$, 32% of GFAP$^+$, and 32% of Iba1$^+$ cells exhibited X-gal staining (**Figure 3D** and **Supplementary Figure 3B**). In the CA1 region of the hippocampus, most (97%) NeuN$^+$ neuronal cells exhibited X-gal$^+$, and 30∼50% of glial cells around the CA1 region were co-stained with X-gal (**Figure 3E** and **Supplementary Figure 3C**).

Increased FAM19A5 Expression in Response to Traumatic Brain Injury

To investigate the transcriptional regulation of the *FAM19A5* gene in a pathological condition, we determined changes in X-gal staining intensity in the TBI-induced brain of *FAM19A5-LacZ* KI mice. Changes in the numbers and morphologies of microglia/macrophages, astrocytes, and OPCs are typical pathohistological characteristics of the injured brain (Shechter and Schwartz, 2013; Burda and Sofroniew, 2014). Therefore, we first examined such pathohistological changes in the TBI-induced brain of *FAM19A5-LacZ* KI mice. TBI significantly induced a large accumulation of Iba1$^+$ microglia (or blood-born macrophages) in the injury core and penumbra of the cortex, as well as the corpus callosum (**Figure 4A**). To further distinguish these changes by cell type, the injured brain was double stained with anti-Iba1 antibody favoring microglia and anti-CD45 antibody preferring blood-born leukocytes. Microglia having strong staining for Iba1 but faint staining for CD45 were primarily distributed in the penumbra but also present in the injury core (**Supplementary Figures 4Aa,a'**). By contrast, blood-born macrophages with weak Iba1 staining but strong CD45 staining were predominantly present in the injury core along the injury border (**Supplementary Figures 4Ab,b'**). Round-shaped non-microphage leukocytes were stained by only anti-CD45 antibody. The majority of these cells were found in the injury core but some were also observed in the injury penumbra (**Supplementary Figures 4Ac,c'**). An increased number of reactive astrocytes labeled with anti-GFAP antibody also gathered along the border between the penumbra and the injury core but primarily were collected in the penumbra (**Figure 4A**). In addition, the intensity of NG2$^+$ signals in OPCs were increased in the penumbra and corpus callosum (**Figure 4A**). By contrast, the TBI-induced brain exhibited lower signals for myelin basic protein representing fully mature oligodendrocytes than the naïve brain (**Supplementary Figure 4B**). Neurons were immunostained with two different marker proteins (Yaguchi et al., 2014). Neurons in the penumbra of the TBI-induced mouse brain exhibited lower intensity of Tuj1 immunostaining than those in the cortex of the naïve mouse brain (**Figure 4B**). In addition, the nuclei of the neurons in the penumbra looked largely shrunken in size. Interestingly, unlike Tuj1, Sez6l2 was relatively well stained in both the TBI-induced and naïve brains with similar intensities (**Figure 4B**).

Because TBI increases senescence-associated β-gal activity at pH 6.0 (Tominaga et al., 2019), we performed X-gal staining at pH 7.4 in the TBI-induced brain of the wild-type mice. No X-gal staining was observed in the TBI-induced brain of the wild-type mouse, indicating no senescence-associated β-gal activity in this assay system. Then we compared the X-gal staining pattern between the naïve and TBI-induced brain of *FAM19A5-LacZ* KI mice, revealing increased X-gal staining in the penumbra and corpus callosum of the TBI group (**Figure 5A**).

To distinguish the cell types with increased X-gal staining in the TBI-induced brain, tissue sections were further stained with Cresyl violet (Nissl) to examine the morphology of X-gal–stained cells (**Figure 5B**). Nuclear sizes and morphologies are the well-known criteria for distinguishing neurons from non-neuronal glial cells in the brain. In general, neurons have a large nucleus with high euchromatin content, while non-neuronal glial cells contain a small condensed nucleus with heterochromatin (García-Cabezas et al., 2016). A variety of cell types were determined based on the nuclear morphology and X-gal–staining pattern. In the cerebral cortex of naïve mice, some glial cells exhibited a small amount of X-gal precipitate in the cytoplasm (**Figure 5B**, a) while others showed none (b). Some neurons also showed X-gal precipitate in the cytoplasm (c) but others did not (d). Likewise, some populations of glial cells in the corpus callosum exhibited a weak X-gal staining in the cytoplasm (g). Thus, under the normal condition, some populations of neuronal and non-neuronal cells of *FAM19A5-LacZ* KI mice may express low level of β-gal, giving rise to a single small punctual shaped X-gal precipitate within the cytoplasm outside of nucleus (a and c). In the TBI-induced brain, however, increased X-gal precipitations were observed, expanding to the nucleus and covering a large area of cytoplasm in both neuron-like cells (e and f) in the penumbra of the cortex and glia-like cells in the corpus callosum (k).

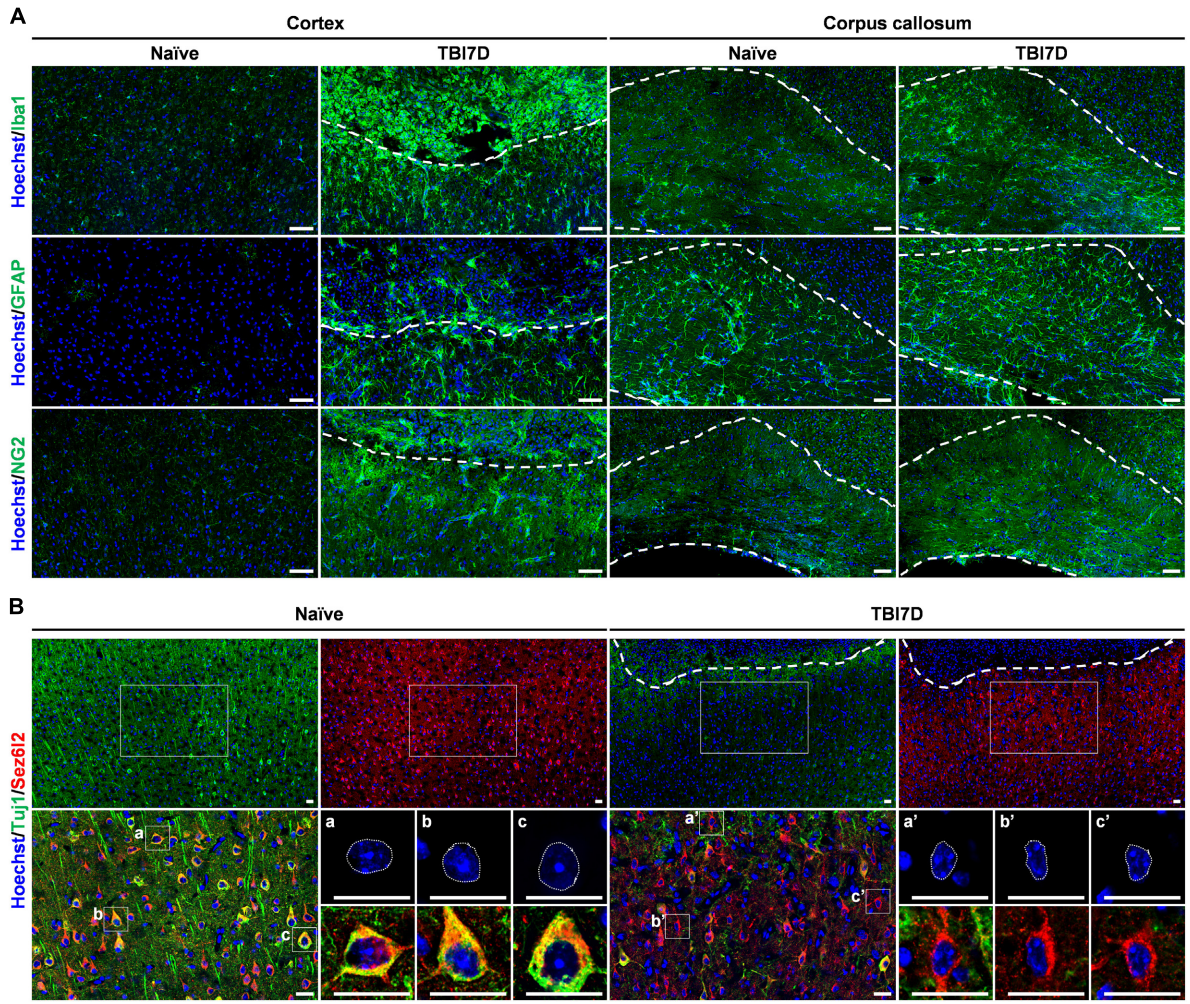

FIGURE 4 | Pathohistological changes of the brain in response to traumatic brain injury. **(A)** Immunostaining for Iba1$^+$ microglia/macrophage, GFAP$^+$ astrocytes and NG2$^+$ oligodendrocyte precursor cells in the cortex and corpus callosum of naïve and TBI-induced brain tissue of *FAM19A5-LacZ* knock-in (+/+) mice at 7 days after TBI (TBI7D). Dashed lines in the cortex of the TBI mouse brain show the border between the injury core (upper part) and penumbra (lower part); dashed lines in the corpus callosum (right panels) indicate their border with the cortex (upper) or with the lateral ventricle (lower). Scale bar, 50 μm. **(B)** Immunostaining for Tuj1$^+$ and Sez6l2$^+$ neurons in the cortex of naïve and TBI-induced brain tissue. Antibodies for Tuj1 (green) and Sez6l2 (red) were double immunostained and nuclei were stained with Hoechst (blue). Dashed lines in the cortex of the TBI mouse brain show the border between the injury core and penumbra. Magnified images (left bottom of each group) show colocalizations of Tuj1 and Sez6l2 in the neuronal cells. Nuclear morphology of individual neurons was further magnified (right bottom of each group). Sez6l2$^+$ neurons in the TBI group were marked by condensed nuclei and decreased Tuj1 signal. Scale bar, 20 μm.

We then determined the number of X-gal-precipitated regions in the cytoplasm and nucleus in the cortex and corpus callosum. TBI significantly increased the number of X-gal precipitated regions that entered the nucleus of cells in both the injury penumbra (**Figure 5C**) and the corpus callosum (**Figure 5D**). The number of the X-gal dots (which occurred only in the cytoplasm) slightly increased in the TBI group. We examined FAM19A5 mRNA levels using qRT-PCR. The result showed a significant increase in FAM19A5 mRNA levels in the corpus callosum of the TBI mice (**Figure 5E**). However, FAM19A5 mRNA levels in the cortical area were slightly decreased in the TBI mice compared to that of the wild type mice. This result is likely that the cortical region of the wild type mice containing the layer 2/3 region exhibits a relatively higher level of FAM19A5, while TBI mice largely lost the layer 2/3 region.

Increased FAM19A5 Expression in the Subpopulation of Neurons and Oligodendrocyte Precursor Cells of the TBI-Induced Brain

To identify cell types with nuclear X-gal staining by TBI, the brain tissue of *FAM19A5-LacZ* KI mice was further immunostained with various cell type–specific markers. It is important to note that insoluble blue precipitates of X-gal hamper immune reactions, therefore immunostaining could not occur in the X-gal–stained region (Levitsky et al.,

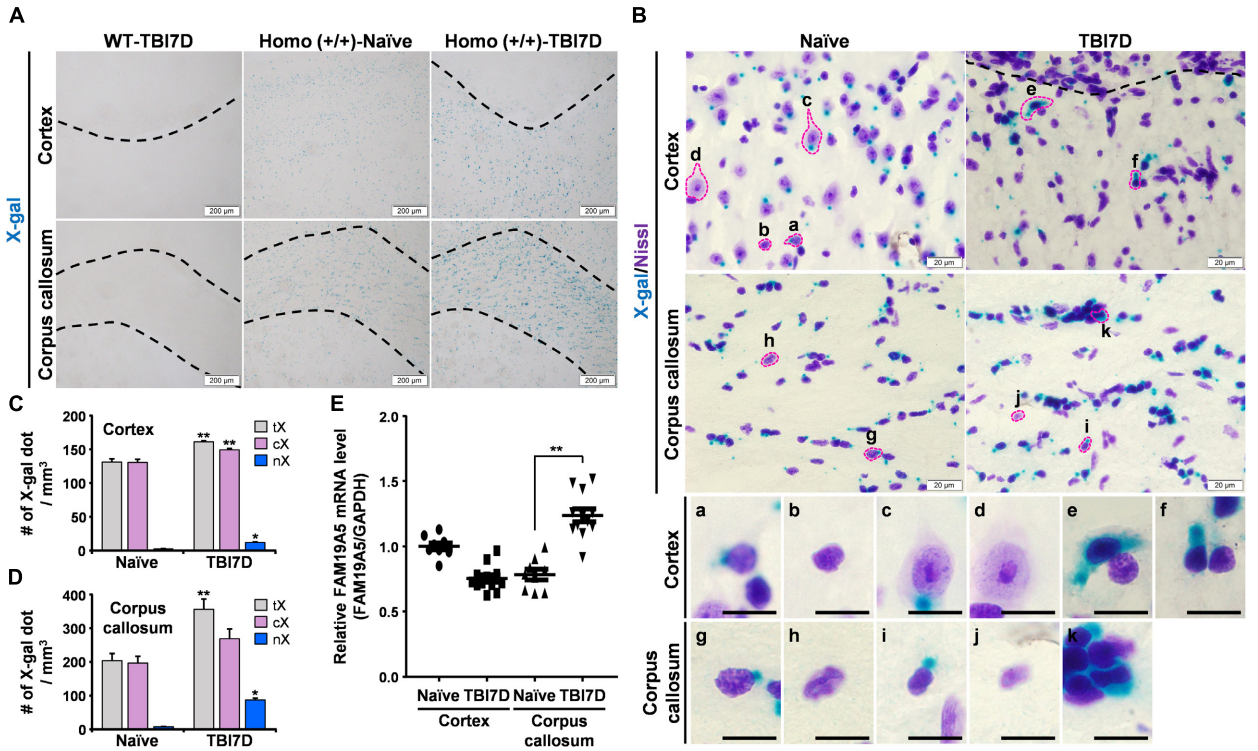

FIGURE 5 | TBI increased X-gal staining in the *FAM19A5-LacZ* knock-in (+/+) mouse brain. **(A)** X-gal staining of the naïve and injured brain at 7 days after TBI (TBI7D). Dashed lines in the cortex of the TBI mouse brain show the border between the injury core and penumbra (left and right top). Areas between dashed lines are the corpus callosum (bottom). **(B)** X-gal and Cresyl violet (Nissl) staining in the cortex and corpus callosum. Images for individual cells were magnified depending on nuclear morphology and X-gal staining pattern (a-k). Scale bar, 10 μm. **(C,D)** Number of X-gal dots in the cortex **(C)** and corpus callosum **(D)**. Number of total X-gal (tX), cytoplasmic X-gal (cX), and nuclear X-gal (nX) was measured from confocal images for fluorescence of X-gal and Hoechst using IMARIS software. Values are the means ± standard errors of the mean. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. naïve ($n = 3$). **(E)** qRT-PCR analysis of FAM19A5 mRNA levels in the cortex and corpus callosum of naïve and TBI-induced mice. Values are the means ± standard errors of the mean. $^{**}P < 0.01$ vs. naïve (naïve, $n = 9$; TBI, $n = 12$).

2013). Out of many cell-type markers, anti-Sez6l2 antibody, a neuronal cell marker, immunostained cells with nuclear X-gal signals in the penumbra of the TBI-induced brain (**Figure 6A**), whereas it stained healthy neuronal cells with cytoplasmic X-gal signals in the naïve brain section. The nuclear X-gal^{+} cells in the penumbra region have nuclei that are slightly larger than those of glial cells but smaller than those of normal neuronal cells. In addition, Hoechst staining showed that heterochromatins were primarily located along the nuclear envelope border in the nuclear X-gal^{+} cells, whereas heterochromatins were in the center near nucleoli in normal healthy neurons (**Figure 6A**). The neuronal markers such as NeuN, Tuj1, and Map2, however, were hardly stained in the cells with nuclear X-gal signals (**Supplementary Figures 5A–C**). The lack of signals is likely due to decreased expression of these marker proteins under an injury condition. Indeed, our study revealed a drastic decrease in signal intensity for Tuj1 in the penumbra region in the absence of X-gal staining (**Figure 4B**). Alternatively, the increased X-gal precipitation may interfere with immunostaining when these marker antibodies are used (Levitsky et al., 2013). In addition, the nuclear X-gal^{+} cells were not co-stained with markers for non-neuronal cells, such as GFAP and nestin for astrocytes (**Figure 6C** and **Supplementary Figures 5D,E**), Iba1 for microglia/macrophage (**Figure 6C** and **Supplementary Figure 5F**), NG2 and Olig2 for oligodendrocytes or their precursor cells (**Figure 6C** and **Supplementary Figures 5G,H**), CD31 for endothelial cells (**Figure 6C** and **Supplementary Figure 5I**), or PDGFRβ for pericytes (**Figure 6C**). Furthermore, nuclear X-gal^{+} cells located in the injury penumbra were not stained with Ki67 for a cell proliferation marker (**Supplementary Figure 5J**) or TUNEL/active caspase-3 for cell death markers (**Supplementary Figure 5K**).

We were able to see a large portion of GFAP^{+} astrocytes in the injury core were cytoplasmic X-gal^{+}, albeit no nuclear X-gal staining (**Supplementary Figure 6A**). In the injury core, some of NG2^{+} and PDGFRβ^{+} pericytes, and CD31^{+} endothelial cells were cytoplasmic X-gal^{+} (**Supplementary Figures 6B–D**). However, we were unable to observe CD45^{+}/Iba1^{-} cells with cytoplasmic X-gal staining (**Supplementary Figure 6E**).

In contrast to the penumbra region, some nuclear X-gal^{+} cells in the corpus callosum of the TBI-induced brain were co-stained with NG2^{+} OPCs (**Figure 6B**) and O4^{+} postmitotic oligodendrocytes (**Supplementary Figure 7A**). However, antibodies for Sez6l2 (**Supplementary Figure 7B**), GFAP (**Figure 6C** and **Supplementary Figure 7C**), and Iba1 (**Figure 6C**

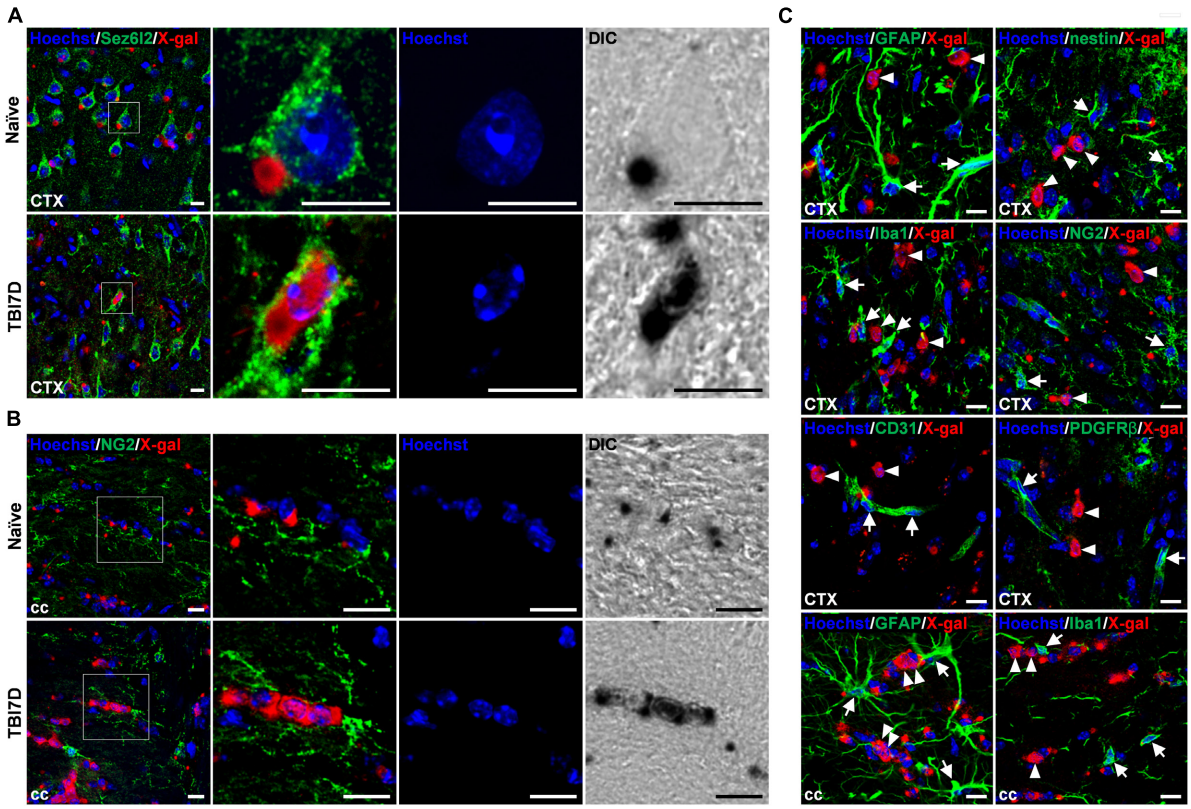

FIGURE 6 | Nuclear X-gal precipitation in Sez6l2$^+$ neurons and NG2$^+$ oligodendrocyte precursor cells of the TBI-induced brain. **(A)** Fluorescence and differential interference contrast (DIC) images of the cortical region in naïve and TBI7D brain sections after X-gal staining combined with Sez6l2 immunostaining and Hoechst staining. Boxed regions are magnified in the right panels showing nuclear morphology and X-gal staining pattern. **(B)** Fluorescence and DIC images of NG2$^+$ OPCs in the corpus callosum. Nuclear X-gal was stained in NG2$^+$ OPCs of the TBI-induced group. Boxed regions are magnified in the right panels showing nuclear morphology and X-gal staining pattern. **(C)** Fluorescence images for X-gal staining combined with GFAP, nestin, Iba1, NG2, CD31, or PDGFRβ immunostaining of the penumbra and corpus callosum of TBI7D brain section. Arrows and arrowheads indicate cell type–specific marker$^+$/nuclear X-gal$^-$ and cell type–specific marker$^-$/nuclear X-gal$^+$ cells, respectively. Scale bar, 10 μm.

and **Supplementary Figure 7D**) failed to double stain the cells with the nuclear X-gal signal.

DISCUSSION

Using *FAM19A5-LacZ* KI mice, we demonstrated that the *FAM19A5* gene is primarily expressed in the CNS from an early stage (E10.5) of embryogenesis. In particular, X-gal staining of embryo brains indicates that FAM19A5 is expressed in germinal zones such as the ventricular zone and ganglionic eminence, suggesting a role of FAM19A5 in proliferation and differentiation of neuroglial progenitor cells. Development of the CNS starts with the formation of the neural tube from the neural plate (Sakai, 1989). The neuroepithelial cell layer adjacent to the ventricle, named the ventricular zone, is the pool for neural stem cells (NSCs) that are capable of self-renewal and differentiate into various types of brain cells including neurons and glial cells to form an organized and functional brain. In rodents, the corticogenesis period ranges from E10.5 to E18.5, followed by the gliogenesis period which completes after birth (Götz and Huttner, 2005; Molyneaux et al., 2007; Agirman et al., 2017). X-gal staining at E12.5 also shows FAM19A5 expression in the ganglionic eminence, the place for the first emergence of OPCs that can differentiate into either oligodendrocytes or astrocytes depending on context (Ffrench-Constant and Raff, 1986). In addition, progenitor cells for GABAergic interneurons in the ganglionic eminence at E12.5 migrate tangentially to the neocortex and occupy layer 4 (Anderson et al., 1997).

In the adult brain, X-gal staining combined with immunostaining for cell-type markers suggests that FAM19A5 is expressed in diverse cell types, including neurons, astrocytes, OPC, and microglia. Expression of FAM19A5 in diverse cell types is consistent with the results of single cell RNA-sequencing obtained from mouse and human brains (Zhang et al., 2014, 2016). It is noteworthy that FAM19A5 is expressed only in the subpopulations of these cell types. For instance, FAM19A5 is broadly expressed in pyramidal neurons in layers 2/3 and 5 of the cortex and in the CA regions of the hippocampus, but not in granular neurons in the dentate gyrus of the hippocampus. In addition, more than 62% of NG2$^+$ OPCs in the corpus callosum are X-gal$^+$ but only 30% of NG2$^+$ OPCs in the cortex are X-gal$^+$.

This restricted expression of FAM19A5 in diverse cell types is likely due to the heterogeneity of NSCs (Alvarez-Buylla et al., 2008; Agirman et al., 2017).

It is well established that the presence of various types of NSCs are morphologically and transcriptionally distinct, including neuroepithelial cells, radial glial cells (RGCs), intermediate progenitor cells, short neural precursor cells, and outer RGCs (oRGCs). They give rise to the distinct subtypes of neurons and glial cells in a time-scaled manner (McCarthy et al., 2018). Furthermore, such differentiation of NSCs is regulated by multiple intrinsic and extrinsic cues including sonic hedgehog (Shh), Wnt/β-catenin, bone morphogenic proteins, and fibroblast growth factors (Li et al., 1998; Morrow et al., 2001; Panchision et al., 2001; Hirabayashi et al., 2004; Shen et al., 2006; Kang et al., 2009; Xu et al., 2010; Draganova et al., 2015). However, the reason for FAM19A5 expression in pyramidal neurons but not in granular neurons of the dentate gyrus is unclear and needs to be further investigated.

In the mouse forebrain, OPCs emerge first from the medial ganglionic eminence at E12.5, second from the lateral ganglionic eminence at E15.5 (Tekki-Kessaris et al., 2001; Chapman et al., 2013), and third from the subventricular zone around birth (Kessaris et al., 2006). In particular, OPCs from the subventricular zone migrate locally to populate the corpus callosum, as well as cortical areas (Kessaris et al., 2006). Therefore, OPCs originate from multilineage competent neuroepithelial precursors in a stereotypic fashion during mouse embryogenesis (Goldman and Kuypers, 2015). High levels of X-gal staining in the medial ganglionic eminence at E12.5 but lower levels of X-gal staining in lateral ganglionic eminence at E16.5 may account for the heterogeneity of FAM19A5-expressing cells among OPC populations. Furthermore, enriched X-gal$^+$ OPCs in the corpus callosum may suggest the large portion of FAM19A5-expressing OPCs in the corpus callosum originate from the subventricular zone around birth.

The present study also showed substantial X-gal staining in the subpopulation of astrocytes, even though the proportion of X-gal$^+$ astrocyte differ depending on brain regions. Astrogenesis has been known to start after completion of the neurogenic period and before generation of oligodendrocytes (Malatesta et al., 2000). However, a recent report provided evidence for the appearance of first astrocyte around E16 (Bayraktar et al., 2015), increasing abruptly after cessation of neurogenesis around E18.5 and continuing postnatally (Qian et al., 2000; Molyneaux et al., 2007; Ge et al., 2012). Thus, X-gal staining in the germinal zone at E18.5 may suggest roles of FAM19A5 in gliogenesis from NSCs. In addition, astrocytes can arise from OPCs (Ffrench-Constant and Raff, 1986; Zhu et al., 2008; Suzuki et al., 2017). OPCs can give rise to both oligodendrocytes and astrocytes, which are modulated by extracellular signals temporospatially (Zhu et al., 2008; Tanner et al., 2011).

Unlike neurons, astrocytes, and oligodendrocytes, microglia arise during the first wave of yolk sac hematopoiesis at E7.5, which occupies the brain around E9.5 (Ginhoux et al., 2010; Gomez Perdiguero et al., 2015; Sheng et al., 2015). However, a study by De et al. (2018) showed the presence of two subpopulations of microglia, non-Hoxb8 and Hoxb8 microglia. The non-Hoxb8 microglia are generated from the first wave while the Hoxb8 microglia are from the second wave of yolk sac hematopoiesis, which can be detected in the brain only from E12.5.

One interesting observation of the present study is that TBI leads to increased FAM19A5 expression in a subset of neuronal populations in the injury penumbra of the cortex and some OPCs in the corpus callosum. TBI induces morphological and functional changes in cells surrounding lesion sites, in addition to recruiting reactive astrocytes, OPCs, microglia, blood-born macrophages, and leukocytes (Werner and Engelhard, 2007). In particular, neurons in the injury penumbra undergo dramatic nuclear condensation with increased heterochromatin formation (**Figure 4B**) accompanied by decreased expression of NeuN and Tuj1, two typical neuronal marker proteins (Kim et al., 2016). Importantly, neurons with a condensed nucleus exhibited increased X-gal staining that invaded the area of the nucleus. Nuclear X-gal staining is quite unusual because the *LacZ gene*-driven β-gal protein is relatively heavy (116 kDa in molecular weight) and cannot cross over the nuclear envelope under normal conditions (Strasser et al., 2012). Therefore, disruption of the nuclear pore complex (NPC), which is specialized in transporting cellular components larger than 40 kDa, may be responsible for the diffusive activity into the nucleus (Kotwaliwale and Dernburg, 2009). Cellular stress or aging is known to cause nuclear leakage by deterioration of the nuclear pore complex, which would allow cytoplasmic proteins larger than 70 kDa to enter the nucleus (D'Angelo et al., 2009; Fichtman and Harel, 2014). Thus, it can be postulated that neurons under severe stress conditions produce more FAM19A5 promoter–driven β-gal proteins that cross over the nuclear envelope due to nuclear leakage. Because the neurons in the injury penumbra exhibited nuclear condensation and presumptive nuclear envelope leakage, we further examined whether these neurons entered the cell death process using TUNEL and active caspase-3 immunostaining (Toné et al., 2007; Strasser et al., 2012). However, the neurons with nuclear X-gal staining were neither TUNEL$^+$ nor active caspase-3$^+$, indicating that these neurons were not destined to die yet.

TBI also induced increased X-gal staining in the OPCs of the corpus callosum, even though this place was distant from the lesion site. The neurons in the cortex project axons to a variety of brain regions, including the thalamus and cortex in the other hemisphere, through the corpus callosum where extensive myelination occurs (Tau and Peterson, 2010). TBI is known to cause demyelination of the axons projecting through the white matter of the brain, resulting in axonal degeneration and death of both neurons and oligodendrocytes (Flygt et al., 2013; Dent et al., 2015). After the loss of mature oligodendrocytes by axon degeneration following brain injury, OPCs are increased in number, are activated in response to the changes of the microenvironment, and differentiate to mature oligodendrocytes (Flygt et al., 2016; Bonfanti et al., 2017). The *FAM19A5* promoter–driven X-gal signals were elevated in the NG2$^+$ OPCs and O4$^+$ postmitotic oligodendrocytes in the corpus callosum. Thus, elevated FAM19A5 expression is likely to be involved in OPC-mediated repair process machinery against injury (Ohtomo et al., 2018).

In summary, the FAM19A5 expression pattern revealed by X-gal staining during mouse embryogenesis and in the adult brain following TBI suggests FAM19A5 is a key regulator in both CNS development and the injury response of the brain. Understanding the function of FAM19A5 in the brain is of particular importance because recent clinical studies have revealed the genetic association of *FAM19A5* with brain development-related symptoms, such as ADHD and autism (Kashevarova et al., 2018), and degenerative disease, such as Alzheimer disease (Herold et al., 2016; Mez et al., 2017). FAM19A5 is expressed in a variety of brain cell types but primarily in pyramidal neurons in the cortex and hippocampus and in OPCs in the corpus callosum, as well as in astrocytes and microglia. Increased expression of FAM19A5 in response to TBI suggests a presumptive role of FAM19A5 in the wound healing process of the brain after injury.

DATA AVAILABILITY

All the data generated for this study are included in the manuscript and/or the **Supplementary Files**. The raw data supporting this manuscript will be available from the authors by reasonable request without undue reservation.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committee of Korea University (KOREA-2016-0091-C3). The protocol was approved by the Institutional Animal Care and Use Committee of Korea University.

AUTHOR CONTRIBUTIONS

AS and EC performed the experiments, analyzed the results, and wrote the manuscript. HY, IJ, and HK performed the experiments and analyzed the results. JL designed the experiments and provided the analysis methods. WL wrote the draft of the manuscript. H-CP, HK, and J-IH provided the study conception and performed the data analysis. BK, WK, and JS provided the study conception, designed the experiments, and wrote the manuscript. All authors read the manuscript, contributed to the revisions, and approved its submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.00917/full#supplementary-material

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ORIGINAL RESEARCH
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Distinct VIP and PACAP Functions in the Distal Nerve Stump During Peripheral Nerve Regeneration

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Vasoactive Intestinal Peptide (VIP) and Pituitary Adenylyl Cyclase Activating Peptide (PACAP) are regeneration-associated neuropeptides, which are up-regulated by neurons following peripheral nerve injury. So far, they have only been studied for their roles as autocrine signals for both neuronal survival and axon outgrowth during peripheral nerve regeneration. In this report, we examined VIP and PACAP's paracrine effects on Schwann cells and macrophages in the distal nerve stump during peripheral nerve regeneration. We show that VPAC1, VPAC2, and PAC1 are all up-regulated in the mouse distal nerve following peripheral nerve injury and are highly expressed in Schwann cells and macrophages within the distal sciatic nerve. We further investigated the effect of VIP and PACAP on cultured rat Schwann cells, and found that VIP and PACAP can not only promote myelin gene expression in Schwann cells but can also inhibit the release of pro-inflammatory cytokines by Schwann cells. Furthermore, we show that VIP and PACAP inhibit the release of pro-inflammatory cytokines and enhance anti-inflammatory cytokine expression in sciatic nerve explants. Our results provide evidence that VIP and PACAP could have important functions in the distal nerve stump following injury to promote remyelination and regulate the inflammatory response. Thus, VIP and PACAP receptors appear as important targets to promote peripheral nerve repair following injury.

Keywords: VIP and PACAP, receptor expression, Schwann cells, macrophages, remyelination, inflammatory cytokines

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INTRODUCTION

The peripheral nervous system has a remarkable ability to regenerate following injury and the up-regulation of regeneration-associated genes in neurons contributes significantly to the success of repair following injury (Navarro et al., 2007; Ma and Willis, 2015). Neuropeptides such as galanin, neuropeptide Y, Vasoactive Intestinal Peptide (VIP), and Pituitary Adenylyl Cyclase Activating Peptide (PACAP) are important regeneration-associated neuropeptides for peripheral nerve regeneration (Navarro et al., 2007; Waschek, 2013). VIP is a 28 amino acid peptide, first isolated from porcine intestine, which has the ability to induce vasodilation in the canine femoral artery (Said and Mutt, 1970, 1972). PACAP is a 38 amino acid peptide and was discovered some years later, sharing 68% homology at the N-terminus with VIP (Miyata et al., 1989).

Subsequent studies have revealed that both VIP and PACAP are widely distributed peptide hormones with a variety of biological activities by acting as neurotransmitters in many different organs and tissues (Harmar et al., 1998). Three receptors, named as VPAC1, VPAC2, and PAC1, have been identified for the VIP and PACAP ligands. Both VIP and PACAP can act through VPAC1 and VPAC2 whereas PAC1 selectively binds PACAP. These receptors are G-protein coupled receptors with seven transmembrane domains and the binding of VIP and PACAP to these receptors typically activates Gs and Gq/11 proteins and increases both intracellular cyclic AMP (cAMP) and inositol1,4,5-trisphosphate/diacylglycerol levels in target cells (Harmar et al., 1998; Castorina et al., 2014).

Many studies have reported that both VIP and PACAP are dramatically up-regulated in motor and sensory neurons after peripheral nerve injury (Zhou et al., 1999; Xiao et al., 2002; Armstrong et al., 2003). VIP mRNA induction in motor neurons is detectable 6 h after peripheral nerve injury and reaches a maximum up-regulation on day 7. Thereafter, the level decreases slightly but VIP expression remains high up to 30 days following injury (Armstrong et al., 2003). cDNA microarray analysis in DRG (dorsal root ganglion) at 2, 7, 14, and 28 days following injury also showed at least a 10 fold VIP up-regulation at all investigated timepoints (Xiao et al., 2002). The up-regulation of PACAP mRNA in motor neurons is also detectable 6 h after injury but PACAP peaks at 48 h with more than a 20-fold up-regulation. PACAP levels then decrease slightly from day 2 following injury, but remain more than 10-fold elevated for as long as 30 days during regeneration (Zhou et al., 1999). Previous studies have confirmed that VIP and PACAP are secreted by regenerating axons (Reimer et al., 1999), and upon release it can promote neuronal survival and axon outgrowth via receptors localized to the growth cone of regenerating axons (Lioudyno et al., 1998; Zhou et al., 1999; Armstrong et al., 2003, 2008). Reports also showed that both VIP and PACAP are up-regulated in sympathetic neurons after peripheral nerve injury, but the up-regulation is not as strong as seen in motor and sensory neurons (Zhou et al., 1999; Armstrong et al., 2003).

So far, all studies have focused on the autocrine effect of VIP and PACAP on neuronal survival and axon outgrowth during peripheral nerve regeneration (Armstrong et al., 2003; Navarro et al., 2007). However, the question remains unanswered as to why neurons dramatically up-regulate VIP and PACAP ligands and yet down-regulate their receptors, if the effects of VIP and PACAP are purely upon on regenerating axons (Zhou et al., 1999; Waschek et al., 2000). It is well known that large numbers of macrophages infiltrate into the distal nerve stump during peripheral nerve regeneration (Martini et al., 2008), and both VIP and PACAP have well characterized immunomodulatory functions on macrophages in other tissues to regulate pro- and anti-inflammatory factor expression (Delgado et al., 1999; Ganea and Delgado, 2002). There is also some evidence to show that VIP and PACAP have a direct effect on cultured Schwann cells and schwannoma cells to promote cell survival, induce laminin synthesis as well as regulating myelin protein expression (Zhang et al., 1996; Castorina et al., 2008, 2014; Lee et al., 2009).

Given the large amount of VIP and PACAP ligand released by the growth cone of regenerating axons and the down-regulation of VIP and PACAP receptors in neurons, we hypothesized that VIP and PACAP could have important paracrine effects upon Schwann cells and macrophages in the distal nerve stump to promote peripheral nerve regeneration. Therefore, in this work, we examined VPAC1, VPAC2, and PAC1 mRNA expression following mouse sciatic nerve transection injury and found that all three receptors are up-regulated in both Schwann cells and macrophages within the distal nerve stump. Next, we took *in vitro* approaches and investigated the effects of VIP and PACAP on cultured primary rat Schwann cells and mouse sciatic nerve explants. Our studies showed that VIP and PACAP could not only promote myelin gene expression in Schwann cells but also inhibited the release of pro-inflammatory cytokines by Schwann cells. Furthermore, we showed that VIP and PACAP inhibited the release of pro-inflammatory cytokines and promoted anti-inflammatory cytokine expression in sciatic nerve explants. Thus, our findings indicate that VIP and PACAP have important paracrine effects in the distal nerve stump to promote remyelination and resolve the peripheral nerve inflammatory response in order to restore nerve tissue homeostasis following repair.

MATERIALS AND METHODS

Animals and Peripheral Nerve Surgery

All work involving animals was carried out according to Home Office regulation under the United Kingdom Animals (Scientific Procedures) Act 1986. Ethical approval for all experiments was granted by the University of Plymouth Animal Welfare and Ethical Review Board. Sprague Dawley rats and C57BL/6 mouse breeding pairs were purchased from Charles River United Kingdom Ltd. PLP-GFP mice were described before (Mallon et al., 2002; Dun et al., 2019). All animals were housed in a controlled laboratory environment (temperature 22 ± 2°C, humidity 50–60%, 12-h light/dark cycle). All animals were fed with standard rodent diet and water *ad libitum*. Two month old male and female mice were randomized. Total animals used for each experiment were stated in the figure legends. For sciatic nerve transection injury, mice were anesthetized with isoflurane, the right sciatic nerve was exposed and transected at approximately 0.5 cm proximal to the nerve trifurcation site and no re-anastamosis of the severed nerve was performed. Overlying muscle was sutured and the skin was closed with an Autoclip applier. All animals undergoing surgery were given appropriate post-operative analgesia, 0.05% bupivacaine solution, topically applied above the muscle suture before applying the surgical clip. Meloxicam (5 mg/kg) injection was given just before recovery from anesthetic. All animals undergoing surgery were given nesting material and enrichment in the cage to prevent autotomy. All animals under surgery were monitored daily. At the indicated time points post-surgery for each experiment described, animals were euthanased humanely by CO2 in accordance with United Kingdom Home Office regulations.

VIP, PACAP and Receptor Selective Agonists, cAMP, PolyI:C and LPS

Vasoactive Intestinal Peptide (Catalog No.: 1911) and VPAC2 selective agonist (Catalog No.: Bay 55-9837) were purchased from TOCRIS. PACAP38 (Catalog No.: H-8430), VPAC1 selective agonist (Catalog No.: H-5802), and PAC1 selective agonist Maxadilan (Catalog No.: H-6734), VPAC2 selective antagonist (Catalog No.: H-7292), and PAC1 selective antagonist M65 (Catalog No.: H-6736), were purchased from Bachem. cAMP (Catalog No.: D0627), PolyI:C (Catalog No.:P0913), and LPS (Catalog No.: L3024) were purchased from Sigma.

Primary and Secondary Antibodies

Primary antibodies against VIP (Abcam, ab78536), PACAP (Abcam, ab216627), VPAC1 (Santa Cruz, sc-30019; Abcam, ab123517), VPAC2 (Santa Cruz, sc-30020), PAC1 (Santa Cruz, sc-30018), myelin protein zero (Mpz) (Sigma, SAB2500665), myelin basic protein (Mbp) (Abcam, ab40390), neurofilament heavy chain (NF) (Abcam, ab4680), F4/80 (Abcam, ab6640), and GAPDH (EMD Millipore, MAB374) were used. Hoechst and species specific secondary antibodies conjugated with Alexa Fluor 488 or 568 dyes were purchased from Invitrogen. Horseradish peroxidase conjugated secondary antibodies for western blotting were purchased from Sigma.

Schwann Cell Culture and Sciatic Nerve Explants

Schwann cells were prepared from sciatic nerve and brachial plexus of nine postnatal day 3 Sprague Dawley rats as previously described (Brockes et al., 1979; Dun et al., 2019). Schwann cells were cultured in low glucose (1 g/ml) DMEM containing 3% fetal bovine serum (FBS), 10 ng/ml NRG-1 (R&D, Cat No. 396-HB-050), and 2 μM forskolin (Sigma, Cat No. 344270). Intact sciatic nerve or distal sciatic nerve 10 day after transection injury were dissected out in L15 medium, the epineurium was removed under the dissection microscope and the nerves were slightly teased in L15 medium, subsequently, teased nerve segments were incubated with VIP, PACAP or receptor selective agonists in low glucose (1 g/ml) DMEM containing 5% FBS.

Immunohistochemistry and Immunocytochemistry

Sciatic nerve and DRG samples were dissected out and fixed overnight in 4% paraformaldehyde (in PBS, PH7.2) at 4°C. Samples were then washed in PBS (3 × 10 min) and dehydrated in 30% sucrose (in PBS) overnight at 4°C. Subsequently, samples were embedded in OCT medium and sectioned on a cryostat at a thickness of 12 μm. Sections were permeabilized with 0.25% Triton X-100 plus 1% bovine serum albumin (BSA) in PBS for 45 min and then blocked with blocking buffer (3% BSA plus 0.05% Triton X-100 in PBS) for 1 h at room temperature. Sections were incubated with primary antibodies (1:100 diluted in blocking buffer) overnight at 4°C. The next day, sections were washed with PBS (3 × 10 min) and then incubated with species-specific secondary antibodies plus Hoechst dye (1:500 diluted in blocking buffer) for 1 h at room temperature. Finally, sections were washed with PBS (3 × 10 min) and mounted with Citifluor (Agar Scientific, R1320) for imaging with a Leica SP8 confocal microscope.

Western Blot

Nerve samples were directly sonicated into 1 X SDS loading buffer. Cells were lysed in an appropriate volume of radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl, pH 7.4, 0.1% SDS, 1% NP-40, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium deoxycholate) and phosphatase inhibitor cocktails used at 1:100 (Santa Cruz Biotechnology, sc-45045 and sc-45065) on ice, then spun down at 16,000 × *g* for 15 min at 4°C. Supernatant was transferred to new 1.5 ml microcentrifuge tubes and the protein concentration was determined using the Pierce™ BCA Protein Assay Kit. An appropriate volume of sample containing 20 μg of protein was added to 4X sample buffer. Proteins were separated on 10% or 12% SDS polyacrylamide running gels and transferred onto a polyvinylidene fluoride (PVDF, 0.45 μm) transfer membrane using the wet transfer method. Membranes were blocked in 5% fat free milk in TBST (Tris buffered saline plus 0.1% Tween-20) for 1 h at room temperature. Primary antibodies were diluted (1:500) in 5% milk (in TBST) and the membranes was incubated in primary antibodies overnight at 4°C. Next day, membranes were washed in TBST (3 × 10 min) and then incubated with HRP conjugated secondary antibody (1:5000 in 5% milk, TBST) for 1 h at room temperature. After three TBST washes (10 min each), Pierce ECL western blotting substrate was added onto the membrane and incubated for 5 min to develop the chemiluminescent signal. Amersham Hyperfilm™ ECL films were used to capture the intensity of the chemiluminescent signal. Exposed films were then developed in a Compact X4 automatic processor. The intensity of protein bands was quantified using the free ImageJ software available from https://imagej.nih.gov/ij/.

mRNA Purification, cDNA Synthesis, RT-PCR and qRT-PCR

Total mRNA was extracted using a miRNeasy Mini Kit (Qiagen, 217004) and first stand cDNA was synthesized with M-MLV reverse transcriptase (Promega, M368) using random hexamer primers (Promega, C1181). RT-PCR was performed in the G-Storm GS4M, qRT-PCR was performed in the PCR LightCycler480 Real-Time PCR Instrument (Roche Applied Science) using SYBR Green I Master with primers showing in **Table 1**. Cross point (Cp) values were calculated by using the software of the LightCycler480 Real-Time PCR Instrument. Relative mRNA levels were calculated by the 2[-Delta Delta C(T)] method (Livak and Schmittgen, 2001) using GAPDH as a reference gene for normalization. All reactions were carried out in triplicate for statistical analysis.

Statistical Analysis

Samples for Western blotting and qRT-PCR in **Figures 1**, **8** were prepared by grouping three nerves from three different mice together for each time point to create a pooled sample as $n = 1$,

TABLE 1 | Primer sequences.

Primers	Forward: 5′—3′	Reverse: 5′—3′	Accession numbers	Size (bp)
Mouse VPAC1	GCCTCCACACAAGGCAAATG	GTGTTTCCAGGTAGGGCACA	NM_011703	160
Mouse VPAC2	ATAGGCGCGAGACTGAGGAA	CAACCAGCAGTAGCAGGTCA	NM_009511	135
Mouse PAC1	CCGGACCAAGTCTGGATGAC	AGCCATCCTCAGTGCAGTTC	NM_001025372	112
Mouse MCP-1	AGGTCCCTGTCATGCTTCTG	TCTGGACCCATTCCTTCTTG	NM_011333	249
Mouse TNFα	CGTCAGCCGATTTGCTATCT	CGGACTCCGCAAAGTCTAAG	NM_013693	206
Mouse ILα	GCAACGGGAAGATTCTGAAG	TGACAAACTTCTGCCTGACG	NM_010554	177
Mouse ILβ	GCCCATCCTCTGTGACTCAT	AGGCCACAGGTATTTTGTCG	NM_008361	230
Mouse TNFγ	ACTGGCAAAAGGATGGTGAC	TGAGCTCATTGAATGCTTGG	NM_008337	237
Mouse IL4	CCATATCCACGGATGCGACA	AAGCACCTTGGAAGCCCTAC	NM_021283	166
Mouse IL6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC	NM_031168	159
Mouse IL10	GCTCTTGCACTACCAAAGCC	CTGCTGATCCTCATGCCAGT	NM_010548	112
Mouse IL13	GGCAGCATGGTATGGAGTGT	CTTGCGGTTACAGAGGCCAT	NM_008355	132
Mouse GAPDH	AAGGTCATCCCAGAGCTGAA	CTGCTTCACCACCTTCTTGA	XM_017321385	222
Rat VPAC1	GCTCCTTAAAACTGGCCCCT	TCAAACACCTCAGTGCCGTT	NM_012685	149
Rat VPAC2	GAAGGCAGAGAGGGCGATAG	CAACCAGCAGTAGCAGGTCA	NM_017238	152
Rat PAC1	AGCATTCACCCCCTTTCCTCA	GGAGAGAAGGCGAATACTGTGT	NM_001270579	175
Rat MCP-1	CAGGTCTCTGTCACGCTTCT	GGCATTAACTGCATCTGGCTG	NM_031530	87
Rat TNFα	CATCCGTTCTCTACCCAGCC	AATTCTGAGCCCGGAGTTGG	XM_008772775	151
Rat ILα	CCTCGTCCTAAGTCACTCGC	GGCTGGTTCCACTAGGCTTT	NM_017019	105
Rat ILβ	GACTTCACCATGGAACCCGT	GGAGACTGCCCATTCTCGAC	NM_031512	85
Rat IL6	AGCGATGATGCACTGTCAGA	GGAACTCCAGAAGACCAGAGC	NM_012589	106
Rat Krox20	AGGAGCAAATGATGACCGCC	CATGCCATCTCCAGCCACTC	NM_053633	185
Rat Mbp	TGTGGGGGTAAGAGAAACGC	AAGGTCGGTCGTTCAGTCAC	NM_001025291	126
Rat Mpz	ATGACCGAGGACCAATGACG	CTGTGCTCCAGAGTGGTCAG	NM_017027	102
Rat GAPDH	AGTGCCAGCCTCGTCTCATA	GGTAACCAGGCGTCCGATAC	XM_017593963	77

and then repeated the process using another six animals to reach $n = 3$. Therefore, we have used pooled biological replicates for the repetition of these experiments. Statistical significance was analyzed using the Student's *t*-test and ANOVA by comparing the test groups with the control groups. All data are represented in the figures as mean value ± SEM. *P* values are indicated with single asterisk ($^{*}<0.05$), double asterisks ($^{**}<0.01$) and triple asterisks ($^{***}<0.001$) on graphs. Where graphs are not labeled with an asterisk, any differences between the test groups and the control groups were non-significant. The *n* number for each experiment has been stated in each figure legend.

RESULTS

Up-Regulation of VPAC1, VPAC2 and PAC1 in the Mouse Distal Nerve Stump Following Transection Injury

We first used an RT-PCR method to detect expression of VPAC1, VPAC2, and PAC1 mRNAs in the intact mouse sciatic nerve and in the distal sciatic nerve stump 7 days following transection injury. RT-PCR result showed that VPAC1, VPAC2, and PAC1 mRNAs are all present in the intact mouse sciatic nerve and are all seemingly increased in the distal nerve stump following injury (**Figure 1A**). After confirming the presence of their mRNAs in the intact sciatic nerve and in the distal sciatic nerve stump, we used qRT-PCR to examine the time course of their mRNA changes at 4, 7, 10, and 14 days following sciatic nerve transection, using the contralateral intact sciatic nerve as control. VPAC1, VPAC2, and PAC1 mRNA levels are all significantly up-regulated in the distal nerve stump following injury compared to their expression in control nerves (**Figure 1B**). VPAC1 and VPAC2 show similar expression profiles and their expression becomes significantly elevated compared to control nerve at day 7 and peaks at day 10. PAC1 expression is significantly up-regulated at day 4 and peaks at day 10. Expression of VPAC1, VPAC2, and PAC1 begins to decrease at day 14 but remains significantly increased compared to control nerves (**Figure 1B**).

Next, we investigated changes in protein expression by western blot in the proximal nerve stump and in the distal nerve stump at 7, 10, and 14 days following transection injury. Consistent with their mRNA up-regulation in the distal nerve stump at day 7, 10, and 14, western blot results confirmed the up-regulation of VPAC1, VPAC2, and PAC1 proteins in the distal nerve stump at day 7, 10, and 14 post-injury (**Figures 1C–F**). In contrast, there were no significant changes of their protein levels in the proximal nerve stump following injury (**Figures 1C–F**). Thus, our RT-PCR, qRT-PCR, and western blot results not only confirmed the expression of VPAC1, VPAC2, and PAC1 in the mouse distal nerve stump but also revealed their up-regulation in the distal nerve stump following injury.

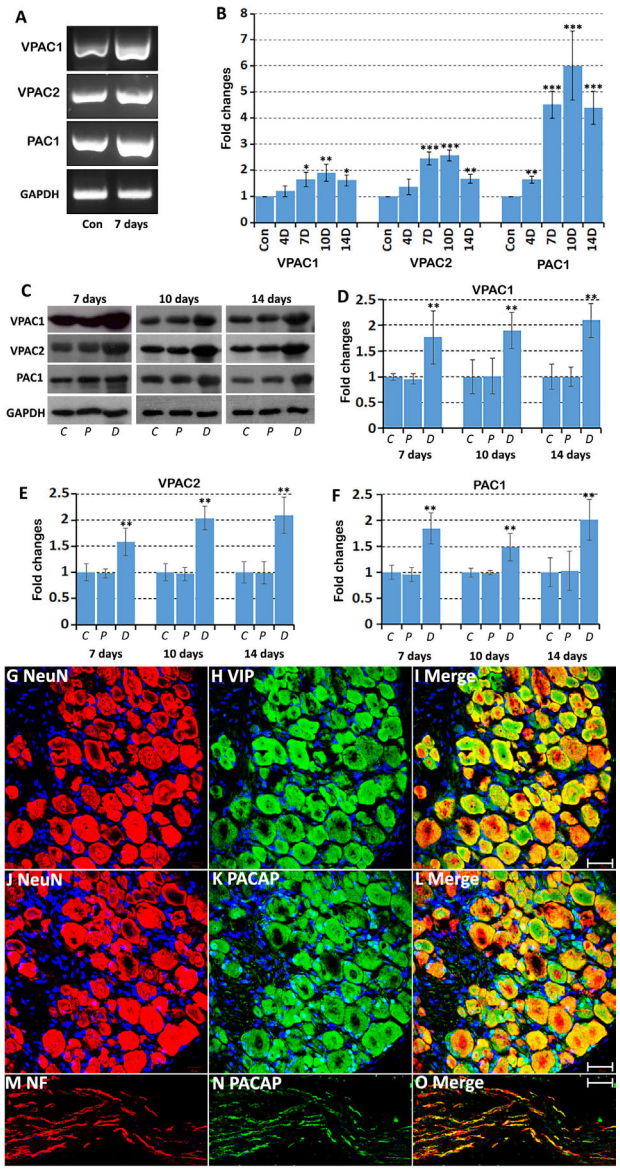

FIGURE 1 | Up-regulation of VPAC1, VPAC2, and PAC1 following injury in the mouse distal sciatic nerve. **(A)** RT-PCR showing the presence of VPAC1, VPAC2, and PAC1 mRNAs in the intact (Con) mouse sciatic nerve and in the distal sciatic nerve 7 days after transection injury. **(B)** qRT-PCR showing VPAC1, VPAC2, and PAC1 mRNA up-regulation in the mouse distal sciatic nerve at 4, 7, 10 and 14 days following transection injury, $n = 3$. **(C)** Western blot showing VPAC1, VPAC2, and PAC1 protein expression in control uninjured (*C*), proximal (*P*), and distal (*D*) mouse sciatic nerve at 7, 10, and 14 days following transection injury. **(D–F)** Quantification of VPAC1 **(D)**, VPAC2 **(E)**, and PAC1 **(F)** protein levels from three independent western blot results showing VPAC1, VPAC2, and PAC1 protein up-regulation in the distal nerve stump. All samples were normalized to GAPDH and control samples were normalized to 1. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. **(G–I)** Double staining of Vasoactive Intestinal Peptide (VIP) with the neuronal marker NeuN showing that sensory neurons in the DRG express VIP at 7 days after sciatic nerve transection injury. **(J–L)** Double staining of Pituitary Adenylyl Cyclase Activating Peptide (PACAP) with the neuronal marker NeuN showing that sensory neurons in the DRG express PACAP 7 days after sciatic nerve transection injury. **(M–O)** Double staining of PACAP with neurofilament (NF)

(Continued)

FIGURE 1 | Continued
showing that PACAP is present in leading regenerating axons in the nerve bridge 7 days after sciatic nerve transection injury. Scale bars in I and L 20 μm. Scale bar in O 40 μm. Thirty six mice were used in **A** and **B** for RT-PCR and qPCR experiments. Twenty seven mice were used in **C-F** for western blot experiments. Three mice were used in **(G–O)** for immunostaining and three sections from each mouse were used for each staining.

Vasoactive Intestinal Peptide and PACAP are almost undetectable in motor and sensory neurons of the adult peripheral nervous system, but both are significantly up-regulated after peripheral nerve injury (Zhou et al., 1999; Xiao et al., 2002; Armstrong et al., 2003). The up-regulation of VIP and PACAP in DRG tissue after peripheral nerve injury has been very well documented using immunostaining, *in situ* hybridization and microarray methodologies (Zhou et al., 1999; Xiao et al., 2002; Armstrong et al., 2003). To confirm VIP and PACAP up-regulation in sensory neurons of our mouse sciatic nerve transection injury model, we stained VIP and PACAP in sensory neurons of DRG tissue at 7 days after sciatic nerve transection injury. Our staining has confirmed that VIP and PACAP are expressed in sensory neurons of DRG (**Figures 1G–L**). We also stained for PACAP in sciatic nerve following injury and confirmed that PACAP is present in regenerating axons (**Figures 1M–O**). Thus, VIP and PACAP released from regenerating axons could interact with their receptors expressed in the distal nerve stump during peripheral nerve regeneration.

Expression Pattern of VPAC1, VPAC2 and PAC1 in the Intact Mouse Sciatic Nerve

Our PCR and Western blot results have revealed VPAC1, VPAC2, and PAC1 expression in intact mouse sciatic nerve. To understand more clearly the cell-specific expression of VPAC1, VPAC2, and PAC1 proteins in intact adult mouse sciatic nerve, we performed VPAC1, VPAC2, and PAC1 double staining with an axonal marker, NF, on adult sciatic nerve transverse sections. Double staining of VPAC1 with NF showed that VPAC1 strongly co-localized with NF (**Figures 2A–C**), indicating that VPAC1 is strongly expressed in axons of the peripheral nerve. VPAC1 also showed staining surrounding axons indicating that it is also expressed in Schwann cells (**Figures 2A–C**). Similarly, double staining of VPAC2 with NF showed that VPAC2 is expressed in axons and Schwann cells (**Figures 2D–F**). In contrast, double staining of PAC1 with NF showed that PAC1 is only expressed in Schwann cells (**Figures 2G–I**). To confirm the expression of VPAC1, VPAC2, and PAC1 in Schwann cells, we stained VPAC1, VPAC2, and PAC1 on sciatic nerve transverse sections from PLP-GFP mice, which label Schwann cells GFP-positive (Mallon et al., 2002; Carr et al., 2017; Dun et al., 2019). Once again, the staining showed that VPAC1 is high expressed in axons and weakly expressed in Schwann cells (**Figures 3A–I**). VPAC2 staining shows stronger expression in Schwann cells than in axons (**Figures 3D–M**). In contrast, PAC1 is only expressed in Schwann cells with clear cell membrane localization comparing to the cytoplasmic GFP signal (**Figures 3G–I**).

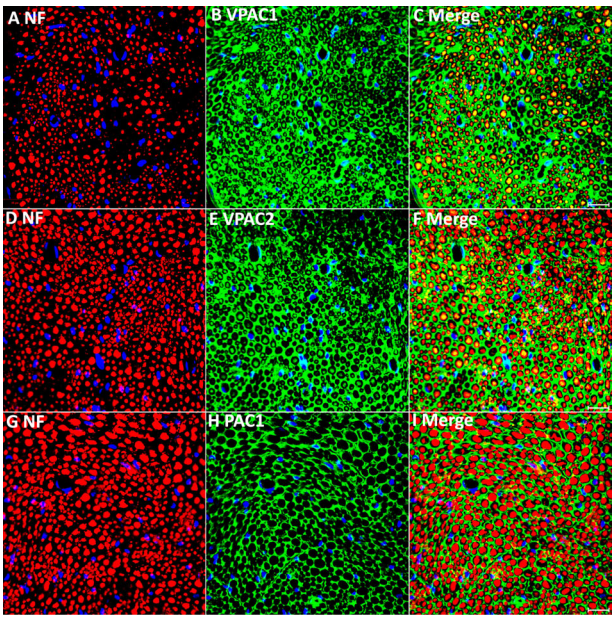

FIGURE 2 | Double staining of VPAC1, VPAC2, and PAC1 with neurofilament heavy chain (NF) on transverse sections from intact mouse sciatic nerve. VPAC1 **(A–C)** and VPAC2 **(D–F)** staining both show co-localization with neurofilament (NF) but PAC1 **(G–I)** does not. Yellow color in **(C,F)** shows VPAC1 and VPAC2 co-localize with NF, respectively. VPAC1, VPAC2, and PAC1 also show positive staining in areas surrounding axons. Scale bars in **(C,F,I)** 20 μm. Three mice were used and three sections from each mouse were stained for each receptor.

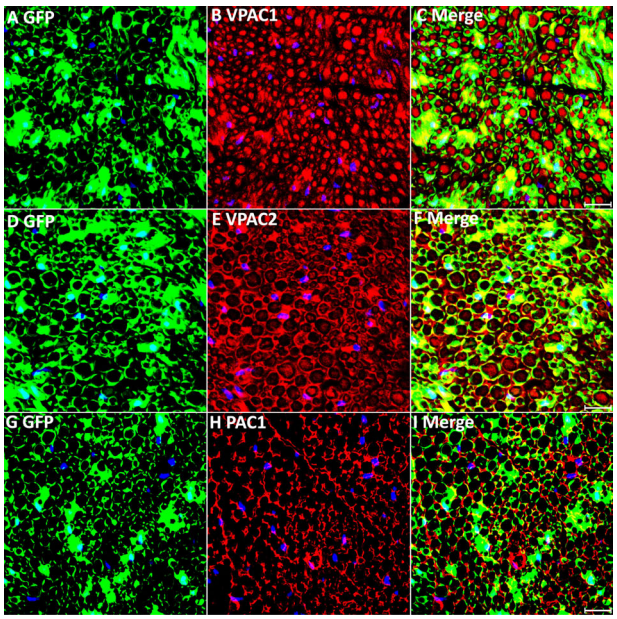

FIGURE 3 | VPAC1, VPAC2, and PAC1 staining on transverse sections from uninjured sciatic nerve of PLP-GFP mice. VPAC1 **(A–C)**, VPAC2 **(D–F)**, and PAC1 **(G–I)** staining all co-localize with GFP-positive Schwann cells. VPAC1 and VPAC2 also show positive staining in axons but PAC1 **(G–I)** does not. Scale bars in **(C,F,I)** 20 μm. Three mice were used and three sections from each mouse were stained for each receptor.

Expression Pattern of VPAC1, VPAC2, and PAC1 in Mouse Distal Sciatic Nerve Following Injury

Next, we transected the sciatic nerve in PLP-GFP mice to study VPAC1, VPAC2, and PAC1 expression in Schwann cells of the distal nerve stump. Immunostaining of VPAC1, VPAC2, and PAC1 on transverse sections of distal nerve at 7 days post-injury showed that all GFP positive cells express VPAC1, VPAC2, and PAC1 receptors, confirming that VPAC1, VPAC2, and PAC1 are all still expressed in Schwann cells of the distal sciatic nerve after transection injury (**Figures 4A–I**). Thus, VIP and PACAP released from regenerating axons could interact with their receptors that are expressed in Schwann cells of the distal nerve stump to potentially regulate Schwann cell function during peripheral nerve regeneration.

In addition to Schwann cells, macrophages are another major cell type in the distal nerve stump following injury, acting to promote peripheral nerve regeneration (Martini et al., 2008; Stierli et al., 2018). VIP and PACAP have well characterized functions in macrophages to promote anti-inflammatory cytokine expression (Ganea and Delgado, 2002), and studies in the immune system showed that macrophages express both VIP and PACAP receptors (Delgado et al., 2004; Vaudry et al., 2009). Therefore, we studied the expression of VPAC1, VPAC2, and PAC1 in macrophages of the distal nerve stump by immunohistochemistry on day 7 following mouse sciatic nerve transection injury. Double staining of VPAC1, VPAC2, or PAC1 with two well characterized macrophage markers, F4/80 and CD68, on transverse sections revealed that VPAC1 (**Figures 5A–C,J–L**) and PAC1 (**Figures 5G–I,P–R**) are expressed by most macrophages of the distal nerve stump and that some macrophages express also VPAC2 (**Figures 5D–F,M–O**, indicated by arrows). Thus, VIP and PACAP released from regenerating axons could also interact with receptors that are expressed in macrophages in the distal nerve stump to regulate the immune response within the nerve during regeneration.

VIP and PACAP Increase Myelin Protein Expression in Cultured Schwann Cells

It has been previously been described that VIP and PACAP treatment increases myelin protein expression in the rat RT4 schwannoma cell line (Castorina et al., 2014), but this has not been tested in primary Schwann cells. Therefore, we studied the effect of VIP and PACAP on myelin gene expression in primary rat Schwann cells. First, the relative expression levels *in vitro* of the three receptors was compared in primary rat Schwann cells by qRT-PCR. This showed that VPAC1 has the lowest expression, with VPAC2 expression 3.0 fold and PAC1 expression 10.4 fold higher compared to VPAC1 (**Figure 6A**). Next, we investigated if VIP or PACAP treatment could induce mRNA expression of three key markers of myelinating Schwann cells, the transcription factor Krox20, Mpz, and Mbp in primary

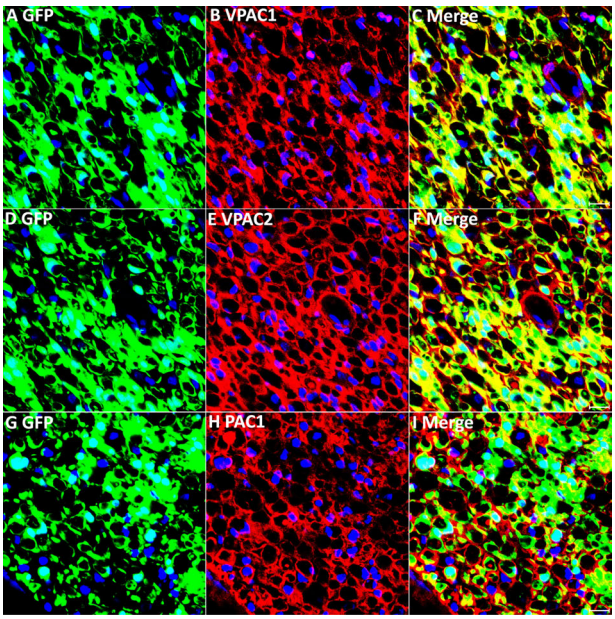

FIGURE 4 | VPAC1, VPAC2, and PAC1 expression in Schwann cells of the distal nerve stump following injury. VPAC1, VPAC2, and PAC1 staining on transverse sections from distal sciatic nerve of PLP-GFP mice 7 days after transection injury. VPAC1 **(A–C)**, VPAC2 **(D–F)**, and PAC1 **(G–I)** all co-localize with GFP in Schwann cells, confirming that VPAC1, VPAC2, and PAC1 are all expressed in Schwann cells of the distal nerve stump. Scale bars in **(C,F,I)** 20 μm. Three mice were used and three sections from each mouse were stained for each receptor.

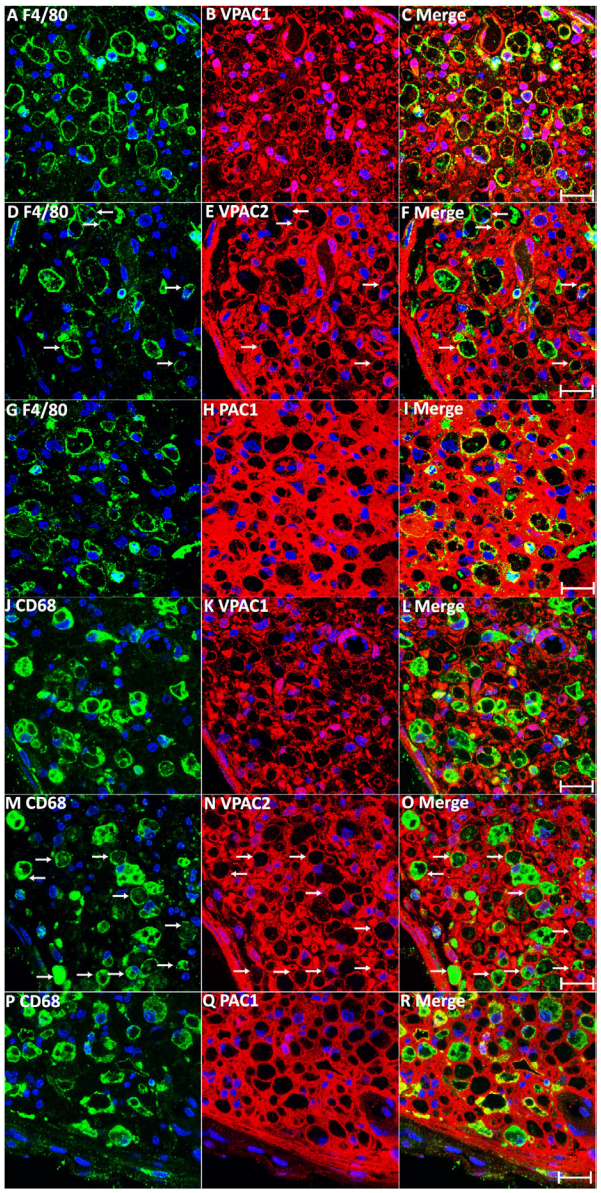

FIGURE 5 | Expression of VPAC1, VPAC2, and PAC1 in macrophages of the mouse distal sciatic nerve at 7 days post-injury. **(A–I)** Double staining of VPAC1, VPAC2, and PAC1 with macrophage marker F4/80 on transverse sections from mouse distal sciatic nerve at 7 days post-transection injury. **(J–R)** Double staining of VPAC1, VPAC2, and PAC1 with macrophage marker CD68 on transverse sections from mouse distal sciatic nerve 7 days post-transection injury. VPAC1 **(A–C,J–L)** and PAC1 **(G–I,P–R)** are expressed in most macrophages of the distal nerve stump. Macrophages expressing VPAC2 are indicated by arrows in **(D–F,M–O)**. Scale bars 20 μm. Three mice were used and three sections from each mouse were stained for each receptor.

rat Schwann cells. Schwann cells were treated with VIP or PACAP (100 nM) every 24 h for 3 days with cAMP treatment used as a positive control (Morgan et al., 1991; Monje et al., 2006). At the mRNA level, both VIP and PACAP significantly increased the expression of Krox20, Mpz, and Mbp (**Figure 6F**). This indicates that both VIP and PACAP may function to induce remyelination during peripheral nerve regeneration, however, PACAP appears to have a much stronger ability than VIP in driving Krox20, Mbp, and Mpz expression (**Figure 6F**). To investigate which receptor may be responsible for promoting Schwann cell myelination, Schwann cells were treated with VPAC1, VPAC2, and PAC1 specific agonists every 24 h for 3 days. All three receptor-specific agonists significantly increased Krox20, Mbp, and Mpz mRNA levels, indicating that this VIP and PACAP function in Schwann cells may not be receptor specific (**Figure 6G**). However, selective activation of PAC1 induced the biggest increase of Krox-20 and Mbp expression in Schwann cells (**Figure 6G**). We further used western blotting and measured Mpz and Mbp protein levels in Schwann cells after VIP, PACAP or VPAC1, VPAC2, and PAC1 specific agonist treatment. This showed that VIP, PACAP, VPAC1, VPAC2, and PAC1 specific agonists are all able to increase Mpz and Mbp protein expression (**Figures 6B–E**). The treatment showed that signaling through VPAC2 and PAC1 receptors has a much stronger ability to increase Mpz and Mbp protein expression in Schwann cells (**Figures 6B–E**). Taken together, both VIP and PACAP are able to induce Krox20, Mbp, and Mpz expression in Schwann cells, while PACAP appears to have a much stronger ability in promoting Krox20, Mbp, and Mpz expression, and VPAC2 and PAC1 receptor are the major receptors mediating their function in Schwann cells to regulate myelination.

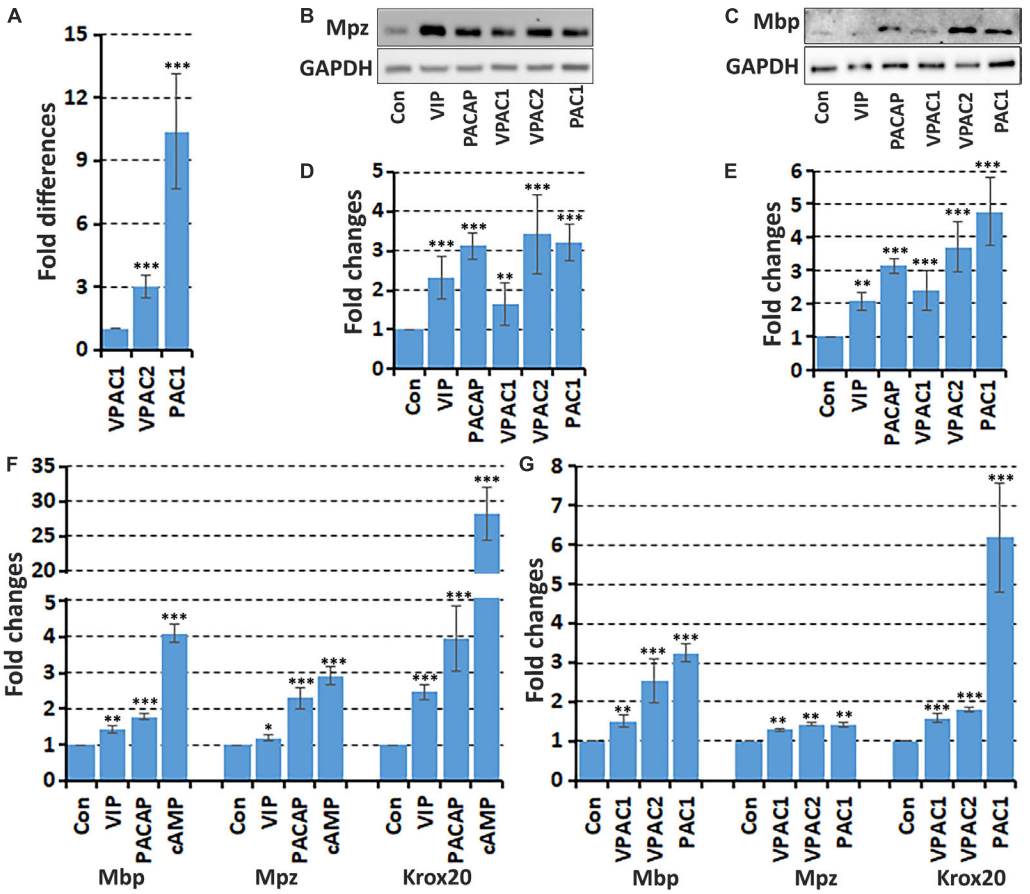

FIGURE 6 | VIP and PACAP increase myelin gene expression in cultured Schwann cells. **(A)** Relative expression level of VPAC1, VPAC2, and PAC1 in cultured primary rat Schwann cells, $n = 3$. **(B,C)** Western blot results show VIP, PACAP and receptor specific agonist up-regulation of myelin protein zero (Mpz) and myelin basic protein (Mbp) protein expression in Schwann cells. **(D,E)** Quantification of Mpz and Mbp levels from three independent western blot results. **(F)** qRT-PCR data showing VIP and PACAP increase the mRNA expression of myelinating Schwann cell markers Mbp, Mpz, and Krox20 after 24 h treatment, $n = 3$. **(G)** VPAC1, VPAC2, and PAC1 receptor specific agonist treatment increases Mbp, Mpz, and Krox20 mRNA expression in Schwann cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

VIP and PACAP Inhibit the Release of Pro-inflammatory Cytokines in Schwann Cells Induced by PolyI:C and LPS

After peripheral nerve injury, Schwann cells in the distal nerve stump release pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), Interleukin 6 (IL6), Interleukin 1 alpha (IL1α), Interleukin 1 beta (IL1β), and monocyte chemoattractant protein-1 (MCP-1) to recruit macrophages for clearance of both myelin and axonal debris (Martini et al., 2008; Zigmond and Echevarria, 2019). Pro-inflammatory cytokine production has to be carefully controlled in order to prevent excessive macrophage recruitment. VIP and PACAP have well characterized functions on macrophages to inhibit pro-inflammatory cytokine expression (Ganea and Delgado, 2002). To study if VIP and PACAP could inhibit pro-inflammatory cytokine expression in Schwann cells, we used both polyinosinic-polycytidylic acid (PolyI:C) and lipopolysaccharide (LPS) to induce pro-inflammatory cytokine expression in primary rat Schwann cells. Schwann cells express high levels of the toll-like receptors 3 and 4 (TLR3, TLR4), and PolyI:C activates TLR3 while LPS activates TLR4 in Schwann cells to induce pro-inflammatory cytokine expression (Goethals et al., 2010).

As expected, PolyI:C (10 μg/ml) and LPS (100 ng/ml) treatment significantly induced TNFα, IL6, IL1α, IL1β, and MCP-1 expression in primary rat Schwann cells compared to untreated Schwann cells (**Figure 7A**). VIP and PACAP treatment on un-stimulated Schwann cells had no effect on TNFα, IL6, ILα, ILβ, and MCP-1 expression (**Figure 7A**). Adding VIP or PACAP together with PolyI:C and LPS significantly reduced the induction of TNFα, IL6, ILα, ILβ, and MCP-1 by PolyI:C, and LPS in Schwann cells (**Figure 7A**). In contrast, adding VPAC2 and PAC1 receptor antagonists 1 h before VIP and PACAP treatment inhibited this VIP and PACAP function (**Figures 7B,C**). To investigate which receptors may be responsible for VIP and PACAP inhibiting pro-inflammatory cytokine expression in Schwann cells, Schwann cells were treated with receptor specific agonists together with PolyI:C and LPS. qRT-PCR results showed that VPAC1 and VPAC2 specific agonists significantly inhibited

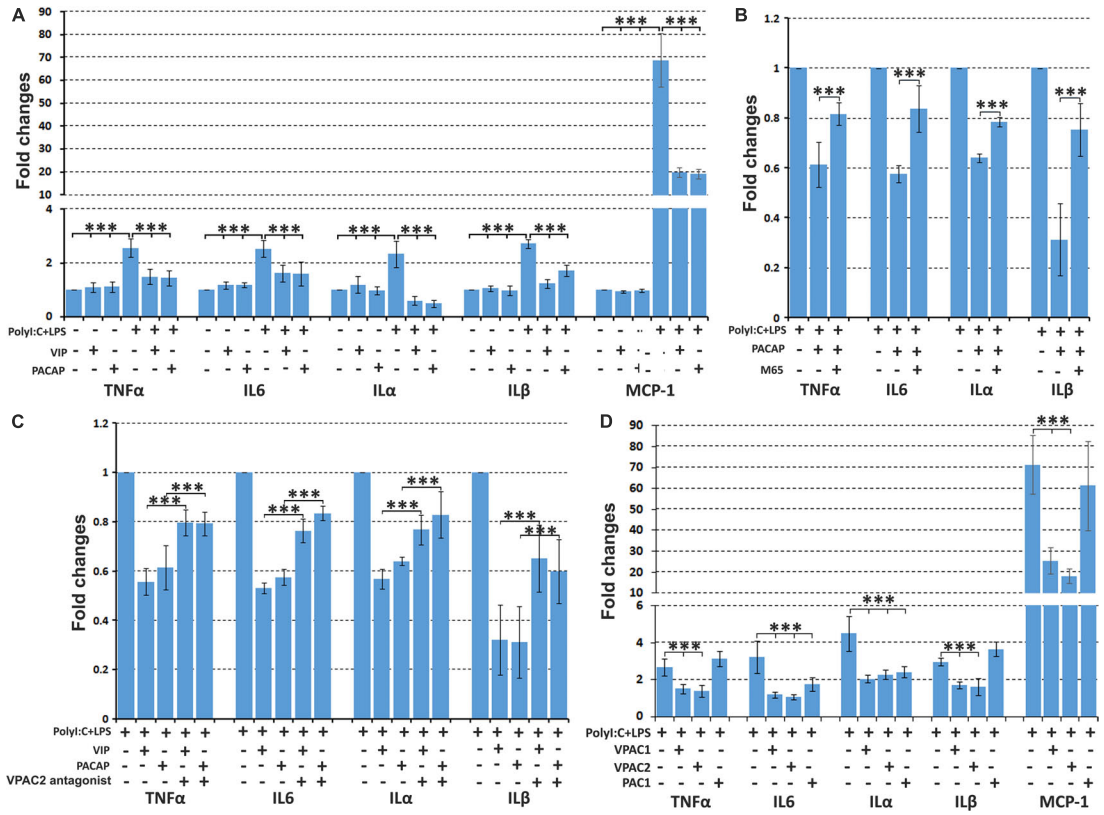

FIGURE 7 | VIP and PACAP inhibit pro-inflammatory cytokine expression in cultured Schwann cells. **(A)** VIP and PACAP inhibit Poly:IC and lipopolysaccharide (LPS) induction of TNFα, IL6, ILα, ILβ, and MCP-1 expression in Schwann cells. **(B)** PAC1 receptor antagonist (M65) pre-treatment (1 h before adding PACAP) inhibits PACAP function. **(C)** VPAC2 receptor antagonist pre-treatment (1 h before adding VIP and PACAP) inhibits VIP and PACAP function. **(D)** VPAC1 and VPAC2 receptor specific agonist inhibits TNFα, IL6, ILα, ILβ, and MCP-1 expression in Schwann cells induced by PolyI:C and LPS stimulation. Note that PAC1 receptor specific agonist inhibits IL6 and ILα but not TNFα, ILβ, and MCP-1 expression. All samples were normalized to GAPDH and control samples were normalized to 1. $n = 4$. $^{***}P < 0.001$.

PolyI:C and LPS-induced TNFα, IL6, ILα, ILβ, and MCP-1 expression (**Figure 7D**), but a PAC1 specific agonist had no effect (**Figure 7D**). Thus, VIP and PACAP appear to inhibit pro-inflammatory cytokine expression through VPAC1 and VPAC2 receptors on Schwann cells.

VIP and PACAP Inhibit the Release of Pro-inflammatory Cytokines in Sciatic Nerve Explants

Peripheral nerve injury rapidly triggers secretion of pro-inflammatory cytokines such as TNFα, IL6, ILα, ILβ, and MCP-1 by Schwann cells in the distal nerve stump to recruit macrophages for myelin and axonal debris clearance (Martini et al., 2008; Zigmond and Echevarria, 2019). The pro-inflammatory cytokine expression in the distal nerve peaks at 24 h following peripheral nerve injury (Rotshenker, 2011). To test whether VIP and PACAP could inhibit pro-inflammatory cytokine secretion in the injured peripheral nerve, we used mouse adult sciatic nerve explants and incubated with VIP and PACAP peptides. The nerve dissection and explant culture triggers the injury response and induces TNFα, IL6, ILα, ILβ, and MCP-1 secretion from Schwann cells of the nerve explants (**Figure 8A**). After 24 h of VIP and PACAP incubation, mRNA was extracted for subsequent qRT-PCR analysis to measure TNFα, IL6, ILα, ILβ, and MCP-1 expression. qRT-PCR results showed that both VIP and PACAP significant inhibited TNFα, IL6, ILα, ILβ, and MCP-1 expression in sciatic nerve explants (**Figure 8A**). To determine which receptor VIP and PACAP may be acting through to decrease pro-inflammatory cytokine expression in the nerve explants, the same experiments were performed but nerve explants were incubated with receptor-specific agonists. Treatment with each receptor-specific agonist showed that stimulation with VPAC1 and VPAC2 specific agonists significantly down-regulated the expression of pro-inflammatory cytokines investigated (**Figure 8B**). Treatment with a PAC1-specific agonist had no effect upon TNFα and ILα expression (**Figure 8B**), but, to our surprise, the PAC1-specific agonist significantly up-regulated IL6, ILβ, and MCP-1 expression in sciatic nerve explants (**Figure 8B**). As PACAP up-regulation reaches its peak on day 2 following injury, which is much earlier than the peak of VIP expression on day 7, the regulation of IL6, ILβ, and MCP-1 by PACAP in nerve explants indicates that PACAP may promote early pro-inflammatory cytokine production in the sciatic nerve following injury. In line

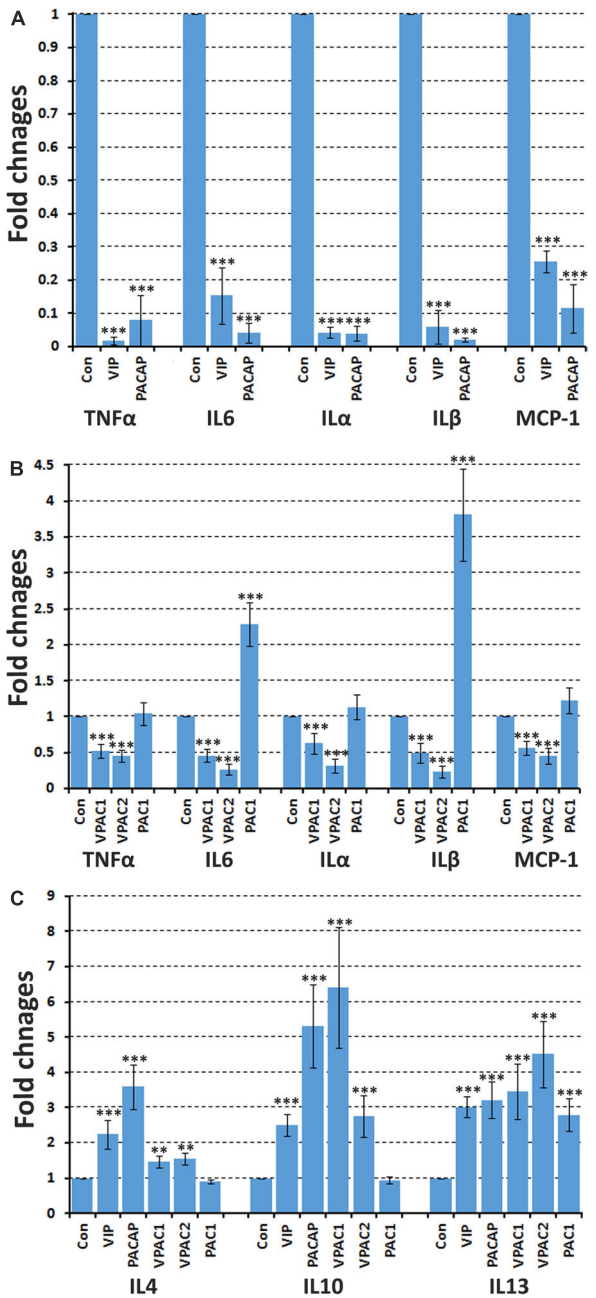

FIGURE 8 | VIP and PACAP regulate pro-inflammatory and anti-inflammatory cytokine expression in nerve explants. **(A)** 24 h treatment with VIP or PACAP inhibit TNFα, IL6, ILα, ILβ, and MCP-1 expression in cultured sciatic nerve explants. Sciatic nerve explants were cultured from uninjured mouse sciatic nerve. **(B)** 24 h treatment with VPAC1 or VPAC2 receptor-specific agonist treatment inhibits TNFα, IL6, ILα, ILβ, and MCP-1 expression in cultured sciatic nerve explants. In contrast, PAC1 receptor specific agonist treatment induces IL6 and ILβ expression. Sciatic nerve explants were cultured from uninjured mouse sciatic nerve. **(C)** 24 h treatment with VIP, PACAP or receptor specific agonists induce anti-inflammatory cytokines IL4, IL10, and IL13 expression in nerve explants. Nerve explants were cultured from the distal nerve stump at 10 days post-transection injury. All samples were normalized to GAPDH and control samples were made relative to 1. $n = 3$. $^{**}P < 0.01$, $^{***}P < 0.001$. 27 mice were used in **(A,B)** for nerve explant culture, and 54 mice were used in **(C)** for nerve explant culture.

with above results for IL6, ILβ, and MCP-1 production in cultured Schwann cells, VIP and PACAP appear to act through VPAC1 and VPAC2 receptors in Schwann cells in the nerve explants to inhibit pro-inflammatory cytokine expression.

VIP and PACAP Enhance Anti-inflammatory Cytokine Production in Mouse Nerve Explants

Macrophages in the distal nerve stump secrete anti-inflammatory cytokines such as IL4, IL10, and IL13 to promote peripheral nerve regeneration (Martini et al., 2008; Ydens et al., 2012; Zigmond and Echevarria, 2019). Macrophages infiltrate the injured nerves from day 3 and the total macrophage number within the injured nerve peaks between day 10 and day 14 post-injury and decreases thereafter (Martini et al., 2008; Zigmond and Echevarria, 2019). A previous study showed that 20.3% cells are macrophages in the mouse distal sciatic nerve at 10 days following nerve transection (Stierli et al., 2018). To investigate if VIP and PACAP could act on mouse distal nerve and promote anti-inflammatory cytokine expression, we treated nerve explants taken from the distal nerve stump 10 days post-injury with VIP and PACAP for 24 h and then investigated the expression of anti-inflammatory cytokines IL4, IL10, and IL13. qRT-PCR results showed that both VIP and PACAP are able to significantly increase the expression of anti-inflammatory cytokines IL4, IL10, and IL13 in distal nerve explants (**Figure 8C**). To determine which receptor VIP and PACAP may be acting through to increase IL4, IL10, and IL13 expression in the nerve explants, we incubated the nerve explants with receptor specific agonists and then measured the levels of IL4, IL10, and IL13 expression. We found that VPAC1 and VPAC2 activation significantly up-regulated the expression of IL4, IL10, and IL13. However, PAC1 stimulation only up-regulated IL13, it had no effect on IL10 and IL4 expression (**Figure 8C**), indicating that VPAC1 and VPAC2 are the major receptors increasing anti-inflammatory expression in the distal nerve explants.

DISCUSSION

Vasoactive Intestinal Peptide and PACAP are regeneration-associated neuropeptides that are up-regulated in motor, sensory, and sympathetic neurons following peripheral nerve injury (Xiao et al., 2002; Armstrong et al., 2003; Navarro et al., 2007). Their up-regulation in motor neurons is detectable 6 h after peripheral nerve injury and both reach more than a 20-fold peak increase (Zhou et al., 1999; Xiao et al., 2002; Armstrong et al., 2003). Although previous studies have been focused upon studying the autocrine effect of VIP and PACAP on neuronal survival and axon outgrowth during peripheral nerve regeneration, these studies have also revealed that the increase of VIP and PACAP expression in neurons was accompanied by a decrease in expression of their receptors (Zhou et al., 1999). For instance, PAC1 mRNA expression was decreased to 50% in motor neurons by 6 h after facial nerve axotomy comparing to uninjured site, and by 1 week it had decreased to about 25%. Thereafter, the level of PAC1 mRNA remained low at about 20–25% for up to

30 days to the levels of control animal motor neurons (Zhou et al., 1999; Waschek et al., 2000). The down-regulation of PAC1 has been explained as G protein-coupled receptor desensitization in response to ligand binding (Zhou et al., 1999; Waschek et al., 2000). Our new data reveals a plausible reason for the receptor down-regulation in neurons, namely to allow VIP and PACAP to execute their important function on Schwann cells and macrophages in the distal nerve stump during regeneration. Using qRT-PCR and western blot methods, we confirmed that VPAC1, VAPC2, and PAC1 are all up-regulated in the distal nerve stump after peripheral nerve injury. By using cell-specific markers, we further showed that the receptor proteins are highly expressed in Schwann cells and infiltrating macrophages of the distal nerve stump.

Previous reports have shown that VPAC1, VPAC2, and PAC1 are expressed in cultured Schwann cells and schwannoma cells (Zhang et al., 1996; Castorina et al., 2008, 2014; Lee et al., 2009). Lee et al. (2009) compared VPAC1 and VPAC2 expression levels in cultured Schwann cells and showed that Schwann cells expressed higher levels of VPAC2 mRNA than VPAC1 mRNA. In line with these findings, our results also showed that the VPAC2 mRNA level is 3 fold higher than VPAC1 mRNA expression in Schwann cells. We also showed that PAC1 has the highest level of expression in Schwann cells (**Figure 8A**). Zhang et al. (1996) were the first to report a VIP function on Schwann cells. They showed that VIP treatment on cultured primary Schwann cells induced laminin synthesis (Zhang et al., 1996). More than 10 years later, Lee et al. (2009) published the second report showing that VIP has a direct effect upon Schwann cells. Lee et al. (2009) reported that VIP pre-treatment inhibited LPS-induced nitric oxide (NO) synthase gene expression and NO production in Schwann cells. In addition to these two papers studying the direct effect of VIP on primary Schwann cells, two other reports have shown that the schwannoma cell lines CRL-2768 and RT4-P6D2T both express VPAC2 and PAC1 receptors (Castorina et al., 2008, 2014). VIP and PACAP treatment on rat RT4 schwannoma cells not only prevented cell apoptosis but also induced myelin protein expression (Castorina et al., 2008, 2014). *In vivo* studies also showed that injection of VIP into the mouse sciatic nerve gap following nerve transection and delivery of VIP-expressing mesenchymal stem cells into a nerve guidance conduit for rat sciatic nerve gap repair accelerated Schwann cell re-myelination (Zhang et al., 2002; Hernandez-Cortes et al., 2014). Binding of VIP and PACAP to their receptors increases intracellular cAMP (Harmar et al., 1998; Castorina et al., 2014), and cAMP is a key signaling molecule to induce Schwann cell myelination during peripheral nerve development (Jessen and Mirsky, 2005; Monk et al., 2009). Thus, VIP and PACAP could be important signals to promote peripheral nerve re-myelination during regeneration. Their effects on Schwann cell re-myelination could be mediated by increase intracellular cAMP levels. Previous studies also showed that binding of PACAP to PAC1 had a much stronger ability to induce cAMP production than binding to VPAC1 and VPAC2 receptors (Vaudry et al., 2009). In light of this, then it is perhaps not surprising that we observed PAC1 as the most effective at inducing Krox20, Mbp, and Mpz expression in Schwann cells. Thus, it appears that PACAP has more important role in Schwann cell re-myelination although VIP does still apparently have the ability to promote re-myelination during peripheral nerve regeneration.

After peripheral nerve injury, large numbers of macrophages infiltrate into the distal nerve stump to clear axonal and myelin debris; infiltrated macrophages also release pro-inflammatory cytokines to recruit more macrophages to the distal nerve (Ip et al., 2006; Zigmond, 2012; Zigmond and Echevarria, 2019). The rapid inflammatory response after peripheral nerve injury must be carefully controlled in order to prevent excessive macrophage recruitment and unnecessary inflammation and tissue damage. Currently, signals that are required to balance the pro-inflammatory cytokines and anti-inflammatory cytokines production in the distal nerve stump have not been well studied (Martini et al., 2008). In the later stages of regeneration, macrophages in the distal nerve stump undergo a stage transition to completely down-regulate pro-inflammatory cytokines production and further up-regulate anti-inflammatory cytokines expression (Fry et al., 2007). Again, signals that regulate macrophage stage transition in the peripheral nerves during regeneration have not been characterized. Staining on mouse distal nerve, at 7 days post-injury, showed that macrophages within the distal nerve stump express VPAC1, VPAC2, and PAC1. Incubation of distal nerve explants, taken at 10 days post-injury, with VIP or PACAP for 24 h up-regulated anti-inflammatory cytokine IL4, IL10, and IL13 expression. Thus, our studies have identified VIP and PACAP as key signaling molecules to balance pro-inflammatory and anti-inflammatory cytokine production and potentially macrophage stage transition in the distal nerve stump. These are key steps for the resolution of the inflammatory response of the injured peripheral nerve and re-establishment of tissue homeostasis. Indeed, a study in the PACAP knockout animals not only showed that axon regeneration following injury is reduced, but also found that pro-inflammatory cytokine down-regulation is delayed and anti-inflammatory cytokine production is impaired in the distal nerve stump following injury (Armstrong et al., 2008).

Interestingly, the up-regulation of VIP and PACAP in neurons was found to be regulated by cytokines present in the distal nerve stump (Habecker et al., 2009; Zigmond, 2012). Given the well characterized role of VIP and PACAP in macrophage stage transition in other tissue and organs together with a large amount of VIP and PACAP secretion by the regenerating axons to the distal nerve stump, this implies a possible feedback mechanism that, upon the up-regulation of pro-inflammatory cytokines in the distal nerve stump, they not only recruit macrophages to the distal nerve stump but also stimulate VIP and PACAP secretion from neurons. Subsequently, VIP and PACAP act as immunomodulators on infiltrating macrophages to balance pro-inflammatory and anti-inflammatory cytokines production in the distal nerve stump. In support of this idea, the time and the peak of VIP expression not only coincides with macrophage accumulation in the distal nerve stump during regeneration, but also matches with the kinetics of IL-10 production (a classic anti-inflammatory factor) in the distal nerve stump. IL-10 expression starts to increase considerably in the distal nerve stump 4 days post-injury, peaks at day 7 and remains elevated during the course of regeneration (Be'eri et al., 1998).

Peripheral nerve injury triggers TNFα, MCP-1, ILα, ILβ, and IL6 expression in Schwann cells and they peak at 24 h following injury (Rotshenker, 2011). Increasing evidence shows that there are remarkable similarities between inherited peripheral neuropathies and peripheral nerve trauma in term of inflammatory response although the former is chronic and the latter is acute (Martini et al., 2013; Klein and Martini, 2016). In pathological situations such as Charcot-Marie-Tooth (CMT) disease, acute inflammatory demyelinating polyneuropathy (AIDP), and chronic inflammatory demyelinating polyneuropathy (CIDP), Schwann cells also release pro-inflammatory cytokines and recruit macrophages to the peripheral nerves. Macrophages are the major immune cells to be found in the peripheral nerves of animal models for CMT diseases (Fledrich et al., 2012; Martini et al., 2013). In response to chemokines released by Schwann cells in CMT animal models, macrophages enter into the peripheral nerves and phagocytose myelin to leave the demyelinated axons intact and, thus, they strongly contribute to the pathogenesis of CMT disorders (Ip et al., 2006).

Investigations in human nerve biopsies have revealed that abnormal macrophage recruitment plays a key role in the demyelination process in neuropathies such as CMT, AIDP, and CIDP diseases (Martini et al., 2013; Klein and Martini, 2016). Inherited peripheral neuropathies in humans are still incurable and lead to muscle wasting, sensory dysfunction and progressive disability (Fledrich et al., 2012). As such, the development of novel therapeutic approaches becomes important. In this study, we show that VIP and PACAP treatment not only inhibits TNFα, MCP-1, ILα, ILβ, and IL6 expression in Schwann cells induced by Poly:IC and LPS stimulation but also significantly reduced TNFα, MCP-1, ILα, ILβ, and IL6 expression in nerve explants. Our findings from this study have indicated the potential for using exogenous VIP and PACAP through VPAC1 and VPAC2 receptors to inhibit the release of pro-inflammatory cytokines by Schwann cells in such pathological situations. Thus, further investigation of VIP and PACAP immunomodulatory function on CMT mouse models could potentially develop VIP and PACAP as novel molecules for the treatment of peripheral neuropathies.

We show here that up-regulated VIP and PACAP could act on both Schwann cells and macrophages in the distal nerve stump and potentially regulate peripheral nerve regeneration. Although both VIP and PACAP were up-regulated in neurons following peripheral nerve injury, PACAP reaches its peak expression at day 2 while VIP reaches its peak expression at day 7 (Armstrong et al., 2003; Navarro et al., 2007). Their peak difference indicates that VIP and PACAP may execute distinct function in the distal nerve stump at different time points during regeneration. Our treatment with PAC1 specific agonist significantly up-regulated IL6, ILβ and MCP-1 expression in sciatic nerve explants (**Figure 8B**). This result indicated that PACAP may have the ability to promote early pro-inflammatory cytokine production in the sciatic nerve following injury. However, activation of PAC1 has no effect on the release of pro-inflammatory cytokine in cultured Schwann cells (**Figure 7B**), indicating that PACAP may act on a different cell type in the sciatic nerve explants and promote pro-inflammatory cytokine release. About 8% cells in the peripheral nerves are known to be resident macrophages and their activation contributes significantly to the early release of pro-inflammatory cytokine following peripheral nerve injury (Stierli et al., 2018; Zigmond and Echevarria, 2019). We showed that macrophages in the mouse sciatic nerve express PAC1 the receptor (**Figure 5**), thus, PACAP peaking at day 2 following injury may have an important function by acting on resident macrophages to promote early pro-inflammatory cytokine production. In contrast, the peak expression of VIP on day 7 following peripheral nerve injury well matches with the time point of pro-inflammatory cytokine down-regulation. Therefore, VIP could have more a important function than PACAP in terms of the resolution of the distal nerve inflammatory response.

Taken together, in this study, we showed that VPAC1, VPAC2, and PAC1 are up-regulated in Schwann cells and macrophages of the distal nerve stump after peripheral nerve injury. Neuron secreted VIP and PACAP could bind to these receptors on Schwann cells and macrophages in the distal stump to execute distinct functions at different stages of regeneration. In the early stage of peripheral nerve injury, VIP and PACAP could balance pro-inflammatory cytokine production and prevent unnecessary macrophage recruitment. In the later stage of regeneration, VIP and PACAP may down-regulate the expression of pro-inflammatory cytokines and up-regulate the production of anti-inflammatory cytokines in macrophages to trigger the macrophage stage transition and eventually terminate the inflammatory response in the distal nerve stump. In the later stages of peripheral nerve regeneration, VIP and PACAP may also have important function in promoting Schwann cell re-myelination.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Plymouth Animal Welfare and Ethical Review Board.

AUTHOR CONTRIBUTIONS

XD designed the research. PW, QM, YL, and NM performed the experiments and analyzed the data. XD and DP wrote the manuscript.

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REVIEW
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Oxytocin and Sensory Network Plasticity

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An essential characteristic of nervous systems is their capacity to reshape functional connectivity in response to physiological and environmental cues. Endogenous signals, including neuropeptides, governs nervous system plasticity. Particularly, oxytocin has been recognized for its role in mediating activity-dependent circuit changes. These oxytocin-dependent changes occur at the synaptic level and consequently shape the cellular composition of circuits. Here we discuss recent advances that illustrate how oxytocin functions to reshape neural circuitry in response to environmental changes. Excitingly, recent findings pave the way for promising therapeutic applications of oxytocin to treat neurodevelopmental and neuropsychiatric diseases.

Keywords: oxytocin, plasticity, sensory, synapse, disease

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INTRODUCTION

Our brains continually organize a multitude of sensory inputs into meaningful outputs. As humans, we subconsciously handle an ever-changing array of sensory information. Sensory inputs can range from the tantalizing smell of freshly prepared food, to the complexity of making an involved decision in a social hierarchy. Moreover, these inputs not only change rapidly over time but are often presented simultaneously. Human brains have evolved to accommodate rapid influxes of information through multiple forms of plasticity. Synaptic plasticity has long been accepted as the basis of learning and memory (Makino et al., 2016). However, within the past 30 years we have begun to appreciate that adult-born neurons have the capacity to continually integrate into existing circuitry in the adult mammalian brain to provide an additional means of plasticity (Alvarez-Buylla et al., 1990; Lois and Alvarez-Buylla, 1994). Both of these plasticity mechanisms work on two largely different timescales but serve the same purpose of adapting existing brain circuitry to benefit the organism.

Synaptic neuroplasticity is broadly defined as the property of synapses to strengthen or weaken in response to changes in presynaptic activity. This plasticity often manifests as a change to the number of synapses on a particular neuron, thereby altering brain-wide neural connectivity on a long-term scale. This process is guided by both environmental cues and gene expression within cells. Effectively, plasticity at both the synaptic and the cellular level allows for remodeling or rewiring of existing circuitry. Circuit remodeling can be guided by natural development or something more malevolent such as disease or injury. Interestingly, a number of genes associated with neuroplastic control are mutated in patients with psychiatric and autism spectrum disorders (ASDs). This connection suggests that brains that either cannot adapt appropriately to their environment or brains that adapt too quickly both manifest disease (Bourgeron, 2015). Therefore it is vital to understand neuroplastic mechanisms to treat these types of illnesses.

Almost exclusively, scientists have directed their attention toward small, fast-acting neurotransmitters when studying neuroplasticity and neurogenesis. This is largely due to their ability to transmit synaptic information quickly and efficiently in combination with their global presence in plastic brain regions. Decades of research dedicated to how fast neurotransmitters regulate synaptic plasticity has yielded great results, but somewhat at the expense of ignoring the contribution from neuropeptides. Recent research recognizes neuropeptides as key orchestrators of both initiating and maintaining plastic states within a diverse array of sensory systems. Neuropeptides are particularly interesting candidates because their release often correlates physiological stimuli, and brain state to changing circuit dynamics. Notably, work in the hippocampus links local neuropeptide release to environmental and social learning stressors or enrichment (Ögren et al., 2010; Grégoire et al., 2014; Li et al., 2017). Furthermore, neuropeptides have a long-lasting nature and broad effective areas, which make neuropeptides ideal brain state sensors and circuit regulators (Neumann, 2008; van den Pol, 2012).

Recently, the neuropeptide oxytocin has garnered interest as a potent modulator of both adult-born neurogenesis and synaptic plasticity (Zheng et al., 2014; Marlin and Froemke, 2017). Here, we describe a wide array of neuromodulatory functions that oxytocin serves in the context of synaptic plasticity and adult-born neuron integration. Though the behavioral effects of oxytocin are both species dependent and brain region specific, we focus this review on oxytocin-driven plasticity from experimental data in mice or rats. Additionally, we illustrate the consequences of oxytocin-dependent functions on the macroscopic scale of animal behavior. Finally, we reflect on this information and its therapeutic potential to intelligently rewire or replace diseased brain circuitry.

OXYTOCIN AT THE SYNAPSE

Oxytocin is a neurohypophysial ring nonapeptide produced centrally within the hypothalamus (Sofroniew, 1983). The processed form of the peptide is loaded into dense core vesicles and dispersed via axonal projections to distant brain regions. Oxytocin release may be synaptic or extrasynaptic, with broad-acting effects due to its long half-life and extensive release sites (Landgraf and Neumann, 2004; Ludwig and Leng, 2006). Supporting a predominant role for volume transmission of oxytocin, many studies have struggled to identify traditional peptide-expressing presynaptic and receptor-expressing postsynaptic partners. This conundrum has led the field to view oxytocin receptor expression as a key mode of regulation within the oxytocin system.

Oxytocin Receptor (OXTR) is a 7-transmembrane domain G-protein-coupled-receptor that is broadly expressed throughout the mammalian brain (**Figure 1**; Gimpl and Fahrenholz, 2001; Busnelli and Chini, 2018). In neurons, OXTR can couple to both G_q and G_o/G_i protein subunits, leading to variable phosphorylation of PLCβ and PKC, activation of EGFR and MAPK cascade, or activation of inward rectifying potassium

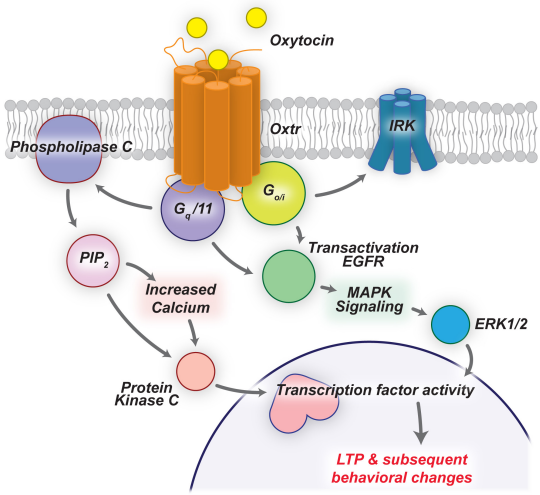

FIGURE 1 | Oxytocin signaling cascades lead to LTP and behavioral changes. OXTRs are G-protein-coupled-receptors that can activate a number of downstream pathways upon ligand binding. Here we highlight two such pathways that drive LTP in the mammalian brain.

channels (Busnelli and Chini, 2018). Interestingly, Busnelli and Chini (2018) show that coupling of different G proteins, and ultimately the intracellular cascade of events caused by OXTR activation, depends on the expression levels of OXTR, the concentration of oxytocin present, and the expression of specific G protein subunits. Therefore, due to the diverse repertoire of *OXTR* associated signaling cascades, OXTR activation has a wide variety of downstream effects, which can readily be seen by comparing differential *Oxtr* and G protein subunit expression.

Expression of *Oxtr* in distinct brain regions facilitates species-specific behaviors (Gimpl and Fahrenholz, 2001; Grinevich et al., 2016). One notable illustration of this is the monogamous prairie vole, in which well-defined expression of *Oxtr* in only a few distinct brain regions drives the development of monogamous behavior in prairie voles; however, lack of *Oxtr* expression in meadow voles yields a more solitary animal (Young and Wang, 2004). Indeed, we can see that *Oxtr* expression is enriched in sensory systems important for social behavior on a species-by-species basis (**Figure 2**). For example, animals that primarily communicate via visual cues have an enrichment of OXTR in visual-responsive brain regions (**Figure 2**). The correlation between regional expression of *Oxtr* and species-specific behavior suggests a common evolutionary function of oxytocin. Specifically, oxytocin has the capability to induce or re-open a critical period within existing brain circuitry in sensory systems (Nardou et al., 2019). The consequence of this can be seen both molecularly at the synapse, and also as modified behavioral outputs.

Oxytocin's most famous role in popular science culture is as "the love hormone" due to the trust and positive feelings Oxytocin engenders toward others (Magon and Kalra, 2011). For example, Oxytocin effects rodent maternal behavior by inducing potent and long-lasting synaptic changes to drive and

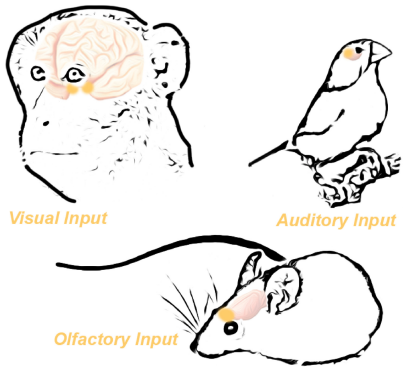

FIGURE 2 | Oxytocin receptor expression in the brain is functionally specific across species. High *Oxtr* expression in the visual pathways of primates correlates with the heavy reliance of primates on the visual system. Similarly, birds demonstrate high *Oxtr* expression in the auditory system, mirroring their heavy reliance on auditory signaling. Finally, mice potently express *Oxtr* in the olfactory bulb, reflecting their reliance on olfactory cues. Adapted from Grinevich et al. (2016).

maintain this nurturing behavior. Recently Marlin et al. (2015) demonstrated that *Oxtr* enrichment in a small domain of the mouse auditory cortex (A1) sensitizes mothers to newborn pup ultrasonic vocalizations; endogenous oxytocin release and subsequent OXTR activation in A1 induces both temporal and spatial gating of A1 activity in the maternal mice in response to their newborn pup calls. Additionally they showed that oxytocin, when paired with pup calls, triggers a disinhibitory effect (Marlin et al., 2015). Importantly, this group later demonstrated that this particular oxytocin signaling mechanism is mediated largely by NMDA-dependent long-term potentiation (LTP) (Mitre et al., 2016), whereby oxytocin promotes a disinhibitory effect that facilitates spiking of normally sub-threshold responses, which is then further strengthened and consolidated by NMDA-dependent LTP. Taken as a whole, oxytocin sensitizes maternal mice to the sounds of their pup calls by increasing the signal-to-noise ratio via disinhibition in the auditory cortex. This drives a precise, unambiguous and meaningful response by A1 neurons of the mother to her own pups, establishing a basis for maternal behavior.

In other sensory cortices, oxytocin increases meaningful excitation, thus driving canonical synaptic plasticity. Sensory-deprived animals demonstrate reduced oxytocin synthesis in the hypothalamus and decreased transmission of oxytocin to the corresponding sensory cortex (ex. S1 in whisker-deprived mice). This reduction in synthesis and overall transmission results in lower excitatory activity within the sensory cortex. However, upon excitation of oxytocin-carrying fibers, or *in vivo* oxytocin application, increased excitatory activity rescues the synaptic effects of sensory deprivation. This effect correlates strongly with cortical synaptic plasticity that manifests from environmental enrichment, which also drives increased oxytocin levels. Thus, oxytocin mediates cortical synaptic plasticity that accompanies sensory enrichment (Zheng et al., 2014). These findings further corroborate the view that oxytocin drives plasticity by enhancing the signal-to-noise ratio throughout various brain regions.

In a similar fashion, but via a different mechanism, oxytocin increases the signal-to-noise ratio of rat socio-sexual olfactory cues. The main olfactory bulb (MOB) inhibitory interneurons, or granule cells, laterally inhibit the principle MOB excitatory neurons to filter olfactory signaling prior to downstream cortical processing. Oettl et al. (2016) demonstrated that oxytocin recruits top-down cortical inputs to drive activation of the MOB granule cells. This sensory modulation drives a greater representation of rat social odorants, facilitating better social memory and recognition (Oettl et al., 2016). In this respect, olfaction implements oxytocin-mediated circuit level plasticity to drive enhanced social salience.

Recent research indicates that Oxytocin directly drives the formation of stronger synapses, thereby improving the signal-to-noise ratios of regions that mediate memory-related behaviors. Hippocampal synaptic malformation from region- and cell-specific deletion of *Oxtr* results in impaired long-term social recognition memory development (Raam et al., 2017). Conditional loss of oxytocin signaling has no effect on sociability, preference for social novelty, or anxiety-related behaviors. Importantly, neurons with *Oxtr* deletions exhibit defects in LTP induction; however, there are no defects in long-term depression (LTD). *Ex vivo*, OXTR blockers are sufficient to potentiate excitatory synaptic responses that rely on NMDA receptor activation (Lin et al., 2018). Additionally, oxytocin application drives dendritic and synaptic refinement in cultured glutamatergic hippocampal neurons (Ripamonti et al., 2017), which supports a model in which oxytocin drives fewer but stronger synapses. Together, these findings support that oxytocin plays an important role in enhancing excitatory connections within the hippocampus to mediate LTP and social memory formation while also mediating aspects of adult-born neurogenesis in the hippocampus. Thus, in conjunction with mediating mechanisms of synaptic plasticity in developing neurons, oxytocin potently regulates neurogenic plasticity as well.

OXYTOCIN AND ADULT NEUROGENESIS

There are two well-established niches for adult neurogenesis in the mammalian brain: the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ), which supplies new neurons into the olfactory system. Adult neurogenesis in the hippocampus begins in the DG with new-neurons which undergo stereotyped developmental programs to become mature dentate granule cells (Toda et al., 2019). Development and integration of adult-born neurons is largely dependent on the physiological state of the animal, with immature neurons displaying a bona fide activity-dependent critical period at roughly 3 weeks after new neurons are born (**Figure 3**; Ge et al., 2007). Continuous adult neurogenesis in the hippocampus may provide both the structural and functional plasticity necessary for complex behaviors such as learning and memory formation (Kempermann et al., 2004). Although the phenomenon of adult neurogenesis itself is well studied in the hippocampus, the field is only beginning to unravel the presynaptic cues that regulate this process. The discovery of signaling molecules linking an animal's

physiological state with the activity or circuit integration status of newly born neurons is critically important.

Understanding oxytocin-mediated hippocampal plasticity is relatively nascent; however, recent seminal discoveries implicate neuropeptide hormones as potential candidates to bridge the gap between physiological state and newborn neuron integration. Namely, oxytocin encompasses dual roles in both regulating adult-neurogenesis and driving adult-born neuron circuit integration. *Oxtr* is highly expressed in developing neurons in both the CA2 and CA3 regions of the hippocampus (Lin et al., 2018). Additionally, long range oxytocin-containing varicosities extend from the hypothalamus to the hippocampal neurogenic niche, thereby providing a source of oxytocin to newborn neurons (Landgraf and Neumann, 2004). The importance of oxytocin signaling in newborn neurons is highlighted by experiments that genetically delete *Oxtr* from the hippocampus to effectively and dramatically reduce newly generated neurons (Lin et al., 2017). Conversely, exogenous application of oxytocin to the hippocampus increases neuronal proliferation in rats (Sánchez-Vidaña et al., 2016). However, these effects are thought to be non-cell autonomous, as oxytocin increases the excitability of CA3 neurons, whose activity directly regulates hippocampal adult-neurogenesis. Interestingly, when *Oxtr* was removed from newborn hippocampal granule cells during the developmental critical period, marked reduction in dendritic complexity and delays in the excitatory-to-inhibitory GABA switch were observed (Lin et al., 2017). Therefore, oxytocin alters the neuronal activity of surrounding newborn neurons, thereby shaping their dendritic morphologies and firing properties. This concept is further illustrated by experiments in which administration of exogenous oxytocin increases the differentiation and complexity of newborn neuron dendrites (Sánchez-Vidaña et al., 2016).

Oxytocin-mediated cellular changes ultimately alter hippocampal activity, thereby driving behavioral phenotypes. This behavioral change is best illustrated by the attenuation of novel social interactions in mice that have hippocampal deleted *Oxtr*. Such attenuation implies a social memory formation defect (Raam et al., 2017; Lin et al., 2018). Thus, oxytocin-mediated development in newborn neurons may ultimately govern how animals respond to and integrate critical social stimuli.

OXYTOCIN AND ADULT NEUROGENESIS IN THE OLFACTORY SYSTEM

Throughout the rodent brain, regions densely innervated by oxytocin-releasing fibers also strongly express *Oxtr* (Knobloch et al., 2012). This robust overlap indicates functional connectivity and potential oxytocin signaling programs in these brain areas (Grinevich et al., 2016). However, one of the few spots that exhibit ligand-receptor mismatch is the MOB. Notably, the mammalian olfactory system constitutes one of the most potent neurogenic niches, receiving tens of thousands of new adult-born granule cells (abGCs) every day (Alvarez-Buylla and García-Verdugo, 2002; Petreanu and Alvarez-Buylla, 2002). These cells begin as progenitors in the SVZ, migrate through the rostral migratory stream exposed to a milieu of extrasynaptic neurotrophic cues, and arrive in the MOB where they undergo precise developmental programs and integrate into existing olfactory circuitry (Carleton et al., 2003). Similar to embryonic neuronal development, abGCs rely on presynaptic activity for survival

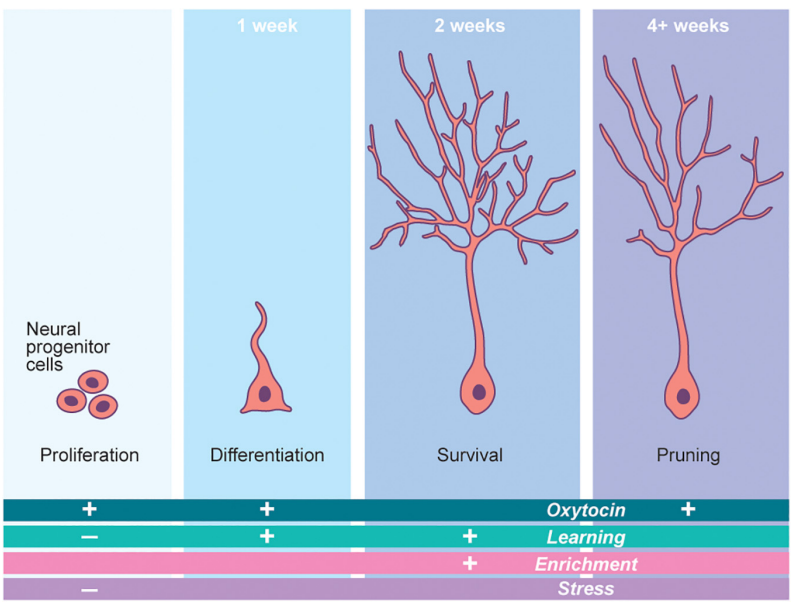

FIGURE 3 | Neurogenesis is a potent driver of neural plasticity in the brain. Adult born neurogenesis follows a stereotyped developmental pathway. Each step of this pathway is modified by a number of outside factors. Oxytocin potently regulates this process in a number of ways, thus governing cellular plasticity in the brain. The time periods marking the top of each developmental step refer to the time at which these steps occur in mice. The "+" and "–" signs denote the increase or decrease, respectively, of each step (Proliferation, etc.) in the presence of the factor (Oxytocin, etc.) associated with each sign. Adapted from Gage (2019).

during their developmental critical period (Yamaguchi and Mori, 2005; Kelsch et al., 2009). Neuropeptides are attractive candidates to convey physiological and/or olfactory activity to incoming abGCs. In fact, abGCs express a number of neuropeptide receptors (Gould and Zingg, 2003; Lein et al., 2007; Garcia et al., 2014). We have found that local neuropeptidergic activity from corticotropin releasing hormone (CRH) producing cells drive synaptic development and survival throughout this critical period. Genetic ablation of CRH signaling in abGCs causes cell death and malformed synapses, while over activation of the CRH receptor (CRHR) in abGCs mediates increased survival and synaptic integration (Garcia et al., 2014). MOB abGCs express moderate to high levels of *Oxtr*, but do not receive direct input from any identifiable oxytocin-releasing projections (Grinevich et al., 2016). The etiology and consequences of this mismatch are not well understood. However, the strong expression of *Oxtr* in developing adult-born neurons offers the potential for functional relevance within the scope of adult-born neuron development, synapse formation, and circuit integration.

Intriguingly, granule cells throughout the entire MOB are highly plastic and exhibit a high turnover of dendritic spines throughout their development (Sailor et al., 2016). This structural plasticity at the synaptic level is thought to underlie the overall flexibility and plasticity of mouse olfactory circuitry. Oxytocin's well-known role in mediating plasticity in other areas of the brain, combined with the high expression of *Oxtr* in the MOB, make oxytocin an intriguing candidate of study. Indeed, early work indicates that local application of oxytocin directly to the MOB enhances the activity of local inhibitory granule cells (Oettl et al., 2016), supporting the notion that OXTR is functional in these neurons; however, it is unclear if oxytocin plays an important role in mediating adult-born neuron plasticity in the MOB.

BEHAVIORAL AND DISEASE STATES AFFECTED BY OXYTOCIN

Oxytocin is known for its role in mediating neural circuits that govern complex mammalian behaviors. Many of these behaviors involve social interactions and rely on social-based learning. Similarly, defective oxytocin-related circuitry can result in human diseases that include social deficits such as Autism Spectrum Disorders (ASDs), which are characterized by deficits in communication and social interactions (Feldman et al., 2016; Guastella and Hickie, 2016; Neumann and Slattery, 2016). The link between oxytocin and ASDs is substantiated by findings that both polymorphisms in *Oxtr* and methylation changes in the *Oxtr* promoter are associated with ASD diagnoses (LoParo and Waldman, 2015; Elagoz Yuksel et al., 2016). Moreover, treating ASD patients with oxytocin has led to symptom improvement (Hollander et al., 2007; Andari et al., 2010; Striepens et al., 2011).

Importantly, mouse models in which oxytocin signaling has been disrupted recapitulate many of the symptoms seen in ASD patients (Lee et al., 2008; Sala et al., 2011). These findings imply that oxytocin signaling potently affects mammalian behavior and offers the field a powerful rodent model to develop therapies for these behavioral disorders. Mouse models of oxytocin signaling may also lead toward a better understanding of other syndromic behavioral disorders. For example, models of fragile-X syndrome show impairments in developmental inhibitory-to-excitatory switch of young neurons regulated by oxytocin signaling, implicating disruptions in the oxytocin pathway (Tyzio et al., 2006). Other models include aberrant dendritic phenotypes seen in *Fmrp* knockout models of fragile-X syndrome, along with other rodent models of ASDs (Galvez et al., 2003; Kulkarni and Firestein, 2012). These data not only supports previous experiments regarding the effects of oxytocin on neurons throughout several brain regions, but it also supports data in which ASD patients manifest aberrant dendritic morphology during development (Bennett and Lagopoulos, 2015; Lin et al., 2017).

Such morphological changes, combined with alterations in LTP downstream of oxytocin signaling, identify oxytocin as a potential mediator of excitatory-inhibitory balance in human neural circuits (Ripamonti et al., 2017). Imbalances between excitation and inhibition have long been associated with neuropsychiatric disorders, including ASD (Selten et al., 2018). Interestingly, a growing body of literature highlights that many of the genes implicated in ASDs act within or around the synapse, further supporting that excitation-inhibition balance is critical in ASD etiology. Furthermore, these findings also suggest oxytocin signaling as a potential therapeutic target for these diseases (Kleijer et al., 2014).

Finally, though much attention has been paid to the link between oxytocin signaling and ASDs, a number of other neuropsychiatric illnesses also show provocative links to oxytocin signaling (Kawamura et al., 2010; Rubin et al., 2014, 2018; Kirsch, 2015). Thus, discovering the neural and circuit-relevant mechanisms of oxytocin signaling will yield important information regarding the etiology of these diseases. Importantly, such efforts will also pave a path toward developing therapies for neuropsychiatric illnesses, and perhaps reshape the way we think about both disease states and patients who suffer from them.

CONCLUSION

We are only beginning to appreciate neuropeptide modulation of plastic brain circuits. From social reward signaling to modulating olfactory representations of kin scents, neuropeptides are essential to relay environmental stimuli that guide changes in brain circuitry. Neuropeptides are one of many factors that contribute to activity-dependent changes in the brain, and oxytocin in particular is a potent regulator of both synaptic and cellular plasticity.

Oxytocin operates through both synaptic and cellular plasticity mechanisms to rewire brain circuitry to increase neuronal representation of sensory stimuli. This increased sensory salience facilitates both the formation and maintenance of complex behaviors. Disruptions in this system, particularly during sensitive critical developmental periods, may cause behavioral malformations due to underlying circuit imbalances.

The information we gain from rodent-based studies of oxytocin and neuropeptide signaling will allow us to continue to dissect the complexities of neural plasticity, which sit at the heart

of many neuropsychiatric and behavioral disorders. Advances in our understanding of neuropeptide signaling provide insight into the mechanisms of neuroplasticity while also offering hope for future treatments of neuropsychiatric illnesses. Excitingly, by understanding oxytocin's influence on plasticity, investigators have begun to co-opt the oxytocin system to treat developmental behavioral disorders (Guastella et al., 2010; Yatawara et al., 2016; Parker et al., 2017). The advent of new methods to better diagnose and understand behavioral disorders will allow us to appreciate neural plasticity as a common underlying mechanism, and provide needed insight toward treating these disorders by leveraging neuropeptide biology.

AUTHOR CONTRIBUTIONS

BP and PH wrote the first draft of the manuscript. BA supplied their technical knowledge to support the manuscript throughout the revision process. PH created the figures for the manuscript. All authors contributed to the manuscript revision and approved the submitted version.

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REVIEW
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The Role of Substance P in the Regulation of Bone and Cartilage Metabolic Activity

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Substance P (SP) is a neuropeptide that is released from sensory nerve endings and is widely present in nerve fibers. It acts on bones and related tissues by binding to receptors, thereby regulating bone metabolism, cartilage metabolism, and fracture healing. SP has attracted widespread attention as a signaling substance that can be recognized by both the immune system and the nervous system. Previous studies have shown that bone and chondrocytes can synthesize and secrete sensory neuropeptides and express their receptors, and can promote proliferation, differentiation, apoptosis, matrix synthesis, and the degradation of target cells through autocrine/paracrine modes. In this paper, we review the research progress made in this field in recent years in order to provide a reference for further understanding the regulatory mechanism of bone and cartilage physiology and pathological metabolism.

Keywords: substance P, Nk-R1 (neurokinin-receptor 1), osteoblasts, osteoclasts, osteoporosis, fracture healing, osteoarthritis

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INTRODUCTION

Bone is a complex and dynamic tissue with a mineralized extracellular matrix and the ability to adapt to its functional demands and repair itself. Bone is abundantly innervated by small diameter sensory nerves in the periosteum, bone marrow, and vascular canals (1, 2). There is increasing evidence that the sensory nervous system is one of the key factors in bone cell differentiation, bone metabolism, and bone remodeling. Neuropeptide plays an important role in the balance between bone formation and bone resorption, and its role in bone repair and reconstruction has gradually become a hot topic. It has been reported that SP is closely related to bone metabolism. SP was accidentally isolated from the brain and intestine extracts of horses by Euler and Gaddum (3). SP is an 11-amino acid peptide that is widely distributed in the peripheral and central nervous system (1, 4). The amino acid sequence is as follows: H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Ple-Gly-Leu-Met-NH2 (5). SP belongs to the tachykinin neuropeptide (TK) family and is the major neuropeptide synthesized from the Tac1 (pre-protachykinin-A) gene. TK mediates its biological effects through three different NK (neurokinin 1, 2, 3) receptors. Among these, NK1 (also known as the tachykinin 1 receptor, TACR1) (6) has the highest affinity for SP and is the main receptor of SP (7–9). SP is widely distributed in the central and peripheral nervous systems as well as in various tissues and organs. SP-like nerve fibers are distributed in various bone tissues of the human body, including long bones, joints and teeth,

and most of the metabolically active parts such as the periosteum and epiphyseal growth plate are distributed, and the cortex and bone marrow are relatively small at the backbone (4, 10). SP is also found in chondrocytes, subchondral bone, and cartilage membranes (11). SP is known to be involved in many physiological and pathophysiological processes including vasodilation, extravasation, smooth muscle contraction (12), pain transmission (13), neurogenic inflammation (14), angiogenesis and bone turnover (15, 16). Cartilage, which contains no blood vessels, nerves, or lymphatics, has long been considered inert tissue in the body. However, an increasing number of studies have shown that cartilage is also regulated by sensory nerves, and sensory neuropeptides can be involved in the regulation of cartilage physiological and pathological metabolism by affecting the proliferation, differentiation, and secretion of chondrocytes (17–20). Although SP and its receptors are widely distributed in the locomotor system, its effects on bone and cartilage metabolism are not well-understood. In the present review, we focus on the effects of SP on bone and cartilage metabolism in some physiological and pathological states. The objective of this article is to review the modulatory effects of SP on the skeletal system and to afford a comprehensive understanding of SP and bone/cartilage metabolism.

SP AND ITS RECEPTOR IN BONE DISEASES

Bone Remodeling

The balance between osteoclastic bone resorption and osteogenic bone formation processes in bone metabolism is the key to maintaining normal bone mass (21). Osteoclasts and osteoblasts are derived from bone marrow macrophages and bone marrow stromal cells (BMSCs), respectively (22). Neuropeptides regulate the functions of osteoclasts and osteoblasts by binding to receptors, and participate in bone growth, repair and reconstruction. Nk-R1 is expressed by osteoblasts and osteoclast precursors (22) (**Figure 1**).

SP and Osteoblasts

Osteoblastic progenitors are derived from mesenchymal stem cells (MSCs) (23) and require Runx2 (also known as Cbfa1, runt-related transcription factor2) transcription factors to develop into a mature osteoblastic lineage. They are characterized by an osteoblastic morphology, accompanied by an increase in alkaline phosphatase (ALP) activity and the production of type I collagen and osteocalcin (24). Osteoblasts not only participate in bone formation, but also regulate osteoclast differentiation by secreting macrophage colony stimulating factor (m-csf), receptor activator of nuclear factor-κB ligand (RANKL), and osteopectin (OPG) receptor activators (25). However, the effect of SP on bone formation remains unclear. Interestingly, Goto et al. (26) added SP to rat calvarial osteoblasts to enhance mineralized nodular formation, and two different NK1 antagonists—spantide and FK888—inhibited the expression of Runx2, OCN and type I collagen, which suggested that blocking the SP receptor attenuates osteogenic differentiation. Wang et al. (22) showed that a low SP concentration can promote the proliferation of BMSCs, while a high SP concentration can promote the mineralization of bone marrow stromal cell. SP can improve the bone formation activity of late osteoblasts by acting on Nk-R1 (26). Shih et al. (27) found that *in vitro* culture of bone marrow cells, the number, and size of bone marrow cell colonies increased with SP concentration, indicating that SP could stimulate bone formation. Paradoxically, SP slightly stimulated thymidine incorporation and strongly inhibited calcium accumulation in bone noduli, which was associated with a small decrease in ALP activity (28). SP promoted osteoblast proliferation and inhibited differentiation and mineralization in rats with spinal cord injury through the RANKL/OPG system (29). Although the results of such studies are inconsistent, it is certain that the formation of osteoblasts is influenced by SP. Further research is needed to clarify how SP regulates bone differentiation, what mechanisms are involved in bone formation, and which neurokinin receptors SP activates in osteoblasts and osteocytes.

SP and Osteoclasts

Mature osteoclasts are terminally differentiated, multinucleated cells, formed by the fusion of hematopoietic stem cells through the myelomonocytic precursor cells/macrophage lineage (30). Early stages of osteoclast differentiation are initiated by the binding of m-csf to its receptor c-fos, which, in turn, induces the expression of receptor activator of nuclear factor-κB (RANK), a membrane protein expressed by preosteoclasts. The interaction between RANK and RANKL, which is expressed by cells of the osteoblast lineage, is the major trigger of osteoclast differentiation and activation (31). Tanja et al. (32) found that a lack of SP reduced the bone resorption rate and reduced the numbers of bone marrow precursor cells (BMMs) and multinucleated osteoclasts and reduced cathepsin K activity in tachykinin (Tac)$1^{-/-}$ BMMs/osteoclast cultures. Takaaki et al. (33) proposed a new mechanism for inducing osteoclast formation by stimulating synovial cells with SP. They showed that SP released from peripheral sensory nerve endings is one of the risk factors for the development of arthritis. It induces synovial hyperplasia and hypertrophy, up-regulates RANKL expression and down-regulates the expression of OPG in synovial fibroblastic cells, which results in osteoclastogenesis. SP-like nerve fibers are closely related to the reconstruction of orthodontic periodontal tissue. In patients with severe root resorption of orthodontic teeth, SP can increase the production of pro-inflammatory cytokines and osteoclast formation in pulp fibroblasts (34). Bone loss in capsaicin-treated animals was associated with a decrease in the rate of bone formation and an increase in the number of osteoclasts and the function of osteoclasts (15). Given the above evidence, it is paradoxical that excessive release of SP leads to increased bone absorption, and that extensive reduction of SP in bones also leads to osteoporosis.

SP may maintain a balance between bone resorption and bone formation and mediate bone resorption depending on whether its level is greater or less than a specific range, suggesting that different amounts of SP affect bone metabolism through different mechanisms. Caye-Thomasen et al. found that in the early stages of acute otitis media, it was SP release that caused increased bone tissue absorption in the middle ear (35). NF-κB is an

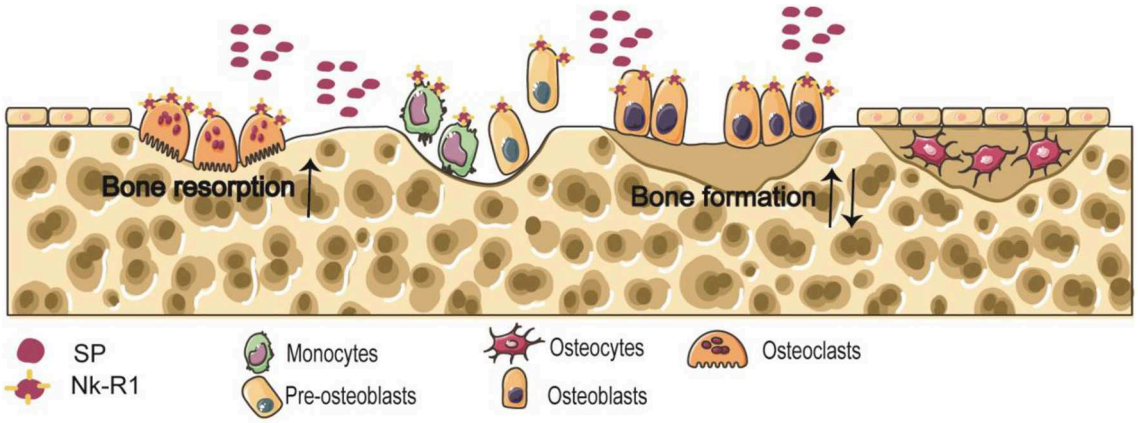

FIGURE 1 | Schema of the effects of released SP on the osteoclasts and osteoblasts.

essential transcription factor for osteoclastogenesis (36). At the same time SP upregulates osteoclastogenesis by activating the NF-κB in osteoclast precursors (36), similar to the effects of RANKL. Wang et al. (22) also observed that SP up-regulated osteoclastogenesis in BMMs and RAW 264.7 cells and increased bone resorption in BMMs. SP appears to enhance RANKL-induced osteoclastogenesis and bone resorption in the same manner as tumor necrosis factor-a (TNF-a) (37). The addition of RANKL to osteoclast cultures induces the release of Ca^{2+} from intracellular storage, leading to an instantaneous increase in intracellular free Ca^{2+}, which accelerates nuclear translocation of NF-κB (38, 39). Similarly, treatment with SP increased cytoplasmic Ca^{2+} levels in rabbit osteoclasts due to an influx of extracellular Ca^{2+} (40). Mori et al. (40) demonstrated that the addition of SP to cultured rabbit osteoclasts resulted in an acute rise in intracellular calcium concentration, which was eliminated by SP receptor antagonists. Intracellular Ca^{2+} mobilization may be a common signaling pathway for RANKL and SP activation of NF-κB in macrophages and osteoclasts. Some scholars have observed that cutting off the sympathetic nerve can promote an increase in peripheral SP and bone absorption (41, 42). The reason may be that after the sympathetic nerve is cut off, the intake of nerve growth factor in the bone tissues it innervates will be reduced, so the sensory nerve will increase the intake of nerve growth factor, which will further promote the synthesis and peripheral release of SP in the sensory neurons. These findings clearly indicate the effects of SP in accelerating osteoclastic bone resorption.

Osteocytes are the main cell in mature bone tissue, equivalent to human adulthood. Osteoclasts/bone-resorbing cells are multinucleated cells formed from differentiated monocytes/macrophages. Osteoblasts/bone-forming cells, derived from pre-osteoblast cells, are the main functional cells of bone formation and responsible for the synthesis, secretion, and mineralization of bone matrix. Nk-R1 has been reported to exist on monocytes, pre-osteoblast cells, osteoblasts, and osteoclasts. Some findings clearly indicate the accelerated effect of SP on osteoclastic bone resorption. And the effect of SP on bone formation is still unclear.

Osteoporosis

Osteoporosis is a critical risk factor for fragility fractures, causing substantial morbidity, and mortality, especially in postmenopausal women and the elderly (43). A large number of neuropeptides regulating bone metabolism may represent a regulatory pathway for the pathogenesis of osteoporosis. Liu et al. (44) reported that after constructing a model of osteoporosis in adult female rats, ovariectomy (OVX) reduced SP in the bone. Liu et al. (45) indicated that epimedium treatment reduced the effects of osteoporosis through a brain/spinal cord/bone axis by increasing bone SP. Interestingly, spinal cord injury (SCI) in experimental rats resulted in an osteoporotic phenotype in the proximal tibia, due to enhanced osteoclast uptake, which was associated with a substantial increase in SP immunoreactive nerve fibers, consistent with *in vitro* observations of SP enhancing osteoclast activity (46). BMD and bone microstructure were significantly reduced at 3 weeks after mechanical stimulation of SCI. The mechanism of OP following SCI is still unclear. Some possible explanations for the pathogenesis of OP after SCI. Firstly, metabolic function changes, such as impaired renal function, hyperlipidemia, and insulin resistance, which may be related to the pathogenesis of OP after SCI. The second is the reduced mechanical loading, the third explanation responsible for this process in the nerve injury itself (46). Chen et al. (47) reported that gelatin microspheres containing different concentrations of SP promoted osteogenesis after 3 months in a rabbit osteoporotic bone defective model. SP increased the amount of trabecular bone and reduced trabecular bone separation. Histological analysis showed that the gelatin microspheres containing SP effectively promoted osteogenesis, regardless of the concentration. Zheng et al. (48) revealed that SP expression decreased in the bone of OVX mice following application of L-703606 (Nk-R1-specific antagonist), and bone loss and the degeneration of bone microstructure in OVX mice was accelerated. Biomechanical analysis showed that blockade

of SP signaling can reduce the maximum stress and maximum load of L3 vertebrae and tibiae. In mice treated with L-703606, there was an increase in the number of osteoclasts, a decrease in the number of osteoblasts and an increase in the osteoid volume in the secondary spongiosa, thereby inhibiting the recruitment of BMSCs to the bone reconstruction site. The OPG/RANKL ratio in the bone of mice treated with L-703606 was also significantly decreased. Kingery et al. (49) suggested that a significant reduction in SP could lead to osteoporosis after sciatic neurectomy. Based on the evidence above, it is possible that the pathogenesis of osteoporosis is associated with the regulation of SP. SP signaling certainly plays an important role in the maintenance of bone mass.

Fractures

SP is considered to be a regulator of angiogenesis that is important for bone repair and remodeling. Ding et al. (50) built a model in which femoral shaft fracture was created 3 weeks after OVX exposure. The fracture healing ability of young mice with OVX-induced bone loss was significantly worse than that in control mice, and SP in the fracture site was significantly decreased at all time points. It was also found that angiogenesis was impaired in OVX mice. The results suggest that neural regulation may play a role in osteoporotic fracture healing and that SP plays an important role during fracture healing, particularly in the early stages. These data contribute to the evidence that SP may play an important role in osteoporotic fracture healing. Tanja et al. (51) used wild type mice and SP-deficient mice ($Tac1^{-/-}$) to establish a fracture healing model to study the effect of SP loss on the process of fracture healing. At day 13 post fracture, they observed a decrease in the area covered by hypertrophic chondrocytes in $Tac1^{-/-}$ mice, indicating that SP deficiency can delay the terminal differentiation of hypertrophic chondrocytes. This research suggested that SP is essential in the process of cartilage ossification during fracture healing. Furthermore, absence of SP reduces pain sensitivity and the mechanical stability of the bone after fracture in general. Guo et al. (52) showed that the NK-R1 antagonist LY303870 partially reversed the vascular and traumatic sequelae of tibial fractures in rats, demonstrating the important role of SP in fractures. Although the effects of SP on osteoblasts and osteoclasts remain controversial, SP plays a critical role in maintaining the balance between bone resorption and bone formation by regulating osteoblasts and osteoclasts during fracture healing (53, 54). Based on the correlation between neuropeptides and fracture healing, the authors hypothesized that SP is secreted into the surrounding bone tissue in a certain way. The amount of this neuropeptide changes during fracture healing, and SP binds to receptors on the cell membrane, activating intracellular signaling pathways, which in turn affect fracture healing and later bone remodeling.

Chronic Inflammation

SP is a neuroinflammatory mediator produced by sensory nerve fibers and local inflammatory cells, such as macrophages, lymphocytes, and dendritic cells (53, 55). SP plays an important role in the skeletal degeneration and damage induced by chronic inflammation (4). Abnormal expression of SP and Nk-R1 in inflammatory diseases provides evidence for SP's involvement in the inflammatory response. Knocking out the Nk-R1 gene and applying an Nk-R1 antagonist in animal models of inflammatory diseases have significant anti-inflammatory effects. SP plays an important role in the development of arthritis as evidenced by a positive correlation between the size and severity of joint destructive changes (56). Rheumatoid arthritis (RA) is a systemic autoimmune disease associated with chronic inflammation of connective tissue. Recent evidence suggests that SP and its receptors are involved in joint inflammation and are involved in the pathophysiology of RA (57). SP levels and Nk-R1 expression were increased in the synovial fluid obtained from RA patients (58). SP stimulated RA synovial cells to release prostaglandin E2 and collagenase, which in turn increased the proliferation of RA synovial cells (59). This suggests that SP plays a role in the development of cartilage destruction and bone damage in arthritis. Experiments with chronic arthritis in rats have shown that sustained inflammatory stimulation can increase SP release in the spinal cord horn (60). Chronic inflammation is a common symptom in OA (osteoarthritis) and RA. A study by Barbara et al. (61) found that serum SP concentration in patients with OA and RA was positively correlated with the intensity of chronic pain.

PHYSIOLOGICAL AND PATHOLOGICAL EFFECTS OF SP ON CARTILAGE METABOLISM

Physiological and Pathological Effects

SP seems to be extremely important for cartilage health because it participates in mechanical transduction through Nk-R1 (62–64). Millward-Sadler et al. (65) demonstrated that adult human articular chondrocytes expressed endogenous pre-protachykinin (PPT, an SP precursor) mRNA, SP, and the corresponding Nk-R1 *in vivo* and *in vitro*. The addition of 1 μmol/L SP to cultured chondrocytes or 0.33 Hz of mechanical stimulation caused hyperpolarization of the cell membrane, suggesting that SP is involved in the mechanical transmission process in chondrocytes. Blockade of SP signaling by a chemical antagonist of Nk-R1 inhibited chondrocyte responses to mechanical stimulation. To sum up, SP secreted by human articular chondrocytes can mediate chondrocyte mechanotransduction via Nk-R1 in an autocrine and/or paracrine manner. Karaha et al. (66) revealed that there was moderate SP expression in articular chondrocytes in a low-exercise group, but SP expression in the cartilage matrix was low or absent. However, SP expression in articular chondrocytes, cartilage matrix, and synovial membrane cells was significantly higher in a high-exercise group, suggesting that SP plays a role in regulating the physiological microenvironment of the cartilage, metabolism, and joint function.

Pathological Effects—Fracture Healing

Opolka et al. (67) conducted studies on costal chondrocytes of 3-week-old mice *in vitro* and found that SP significantly promoted the gene expression of type I, IX, X collagen, and mmp-13, which are closely associated with the terminal differentiation of chondrocytes. In addition, SP also promoted chondrocyte proliferation in a dose-dependent manner. It was also found that the NK1 antagonist L733060 could exert dose-dependent inhibition on the proliferation of chondrocytes, indicating that

endogenous synthesis and secretion of chondrocyte SP could also regulate proliferation through autocrine and paracrine effects. Tanja et al. (51) found at the stage of cartilage nodules formation in fracture healing mice, and a large number of chondrocytes were observed to accumulate. Notably, the fracture chondrocytes expressed high levels of SP and its receptor NK-1 (68), and their expression level was regulated by nerve growth factor and inflammatory factor (11, 51, 69, 70). Future therapeutic targets may involve blocking this particular receptor.

Pathological Effects—Osteoarthritis

Osteoarthritis, also known as degenerative joint disease, is characterized by synovial inflammation, cartilage destruction, and subchondral bone sclerosis associated with aging. Joint replacement is still the only treatment for patients with advanced osteoarthrosis. Recent studies have shown that SP plays an important therapeutic role in the process of OA cartilage degeneration. Higher concentrations of SP were found in the synovial fluid of OA patients, indicating the catabolic effect of SP on articular cartilage (71, 72). Increased substance P levels have been reported in synovial fluid and cerebrospinal fluid obtained from OA patients (73) and immunohistochemistry has demonstrated an increase in SP-immunoreactive nerve fibers in patients with OA (74), also indicating the catabolic effect of SP on articular cartilage (75). Although cartilage is not innervated, increased release of SP in sensory nerve endings during synovial inflammation may affect chondrocyte function. Alternatively, the release of SP by chondrocytes through mechanical stimulation or by other means may affect the activity of various cell types in joints and periarticular tissues (including macrophages, bone cells, and pain fibers), as well as the structural changes associated with OA. Suri et al. (76) found that during the development of OA, new blood vessels could break through the junction of osteochondral cartilage, and sensory nerve fibers could also grow into the diseased cartilage tissue along with blood vessels. At this time, neurogenic SP could act on NK1 in the cartilage cell membrane in a paracrine manner, thus accelerating cartilage degeneration. In animal models and human studies of OA, SP, and NK receptors have been linked to joint pain, inflammation, and injury (77, 78).

Although studies have shown that SP receptor antagonists can help reduce arthritis pain and swelling (79), blocking SP can reduce pain but increase the rate of changes in OA arthritis (80). Therefore, in the treatment of OA, SP may have a dual opposite effect (OA treatment through its anti-inflammatory effect and endogenous stem cell recruitment and pain relief by lowering the pain threshold). A pre-clinical study with a hydrogel implant in a rat knee model revealed that this contradictory potential of SP for therapeutic application in OA can be resolved by the adjustment of the SP dose, the continuity of SP release, and the use of an adequate conjugate to modify the properties of SP. In the same study, SAP conjugates (SP with self-assembled peptide) were used to treat OA. The treatment of OA with SAP-SP significantly improved cartilage regeneration by recruiting MSCs. SAP-SP can prevent apoptosis by secreting anti-inflammatory cytokines, increasing the amount of extracellular matrix involved in chondrogenesis, promoting chondrogenesis and differentiation, and reducing inflammation in OA (78). These studies suggest that SP exhibits anti-inflammatory and regenerative properties through the recruitment of MSCs.

CONCLUSION

Previous studies have confirmed that SP and Nk-R1 are widely present in bone and cartilage tissue and actively participate in bone and cartilage metabolism. Sensory nerve endings can release SP, and SP-positive nerve fibers are distributed in bone and cartilage tissue. SP binds to Nk-R1 to initiate a signal transduction pathway and regulates pathophysiological processes in bone and cartilage tissue.

Due to the current lack of detailed knowledge about the effects of SP on bone, we are still unable to explain the pathophysiology of the most common bone diseases. Although previous studies have shown that SP is involved in bone metabolism, especially in bone resorption, the effect of SP on osteoblast formation is not fully understood. It will be a challenge to elucidate the relationship between bone metabolism and neural regulation. As a substance that can be synthesized by the human body, SP can produce a rapid and efficient response at very low levels. It remains to be determined whether it could be a potential therapeutic agent in osteoporosis and fracture repair. The exact mechanism by which SP is involved in the pathophysiology of bone and related tissues, the interaction of SP with other neuropeptides, cytokines and hormones, and the potential role of SP or Nk-R1 antagonists as effective preventive and therapeutic agents need to be fully determined.

SP plays an important regulatory role in the mechanical response of cartilage, fracture healing, and the pathological degeneration of cartilage by promoting chondrocyte proliferation, adhesion, and secretion and accelerating chondrocyte terminal differentiation. Although cartilage metabolism involves multiple pathways, and SP is not the main pathway that plays a regulatory role, it has been proposed that through SP, NK-R1 antagonists may be promising for the treatment of OA in the future.

Future research should explore areas related to the changes in SP and its receptors in cell proliferation and differentiation, cell signal transduction pathways, and protein and gene expression levels after SP treatment of animals/cells *in vivo* or *in vitro*. Research on SP, the NK1 receptor and the Nk-R1 antagonist will be useful for exploring the mechanism of action of drugs, developing new drugs, and finding new treatments for bone diseases.

AUTHOR CONTRIBUTIONS

L-QY conceived and designed the manuscript. F-X-ZL, FX, XL, FW, J-YZ, YW, BG, M-HZ, and S-KS analyzed the data. L-QY and F-X-ZL wrote the paper.

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REVIEW
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Gastrin, Cholecystokinin, Signaling, and Biological Activities in Cellular Processes

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The structurally-related peptides, gastrin and cholecystokinin (CCK), were originally discovered as humoral stimulants of gastric acid secretion and pancreatic enzyme release, respectively. With the aid of methodological advances in biochemistry, immunochemistry, and molecular biology in the past several decades, our concept of gastrin and CCK as simple gastrointestinal hormones has changed considerably. Extensive *in vitro* and *in vivo* studies have shown that gastrin and CCK play important roles in several cellular processes including maintenance of gastric mucosa and pancreatic islet integrity, neurogenesis, and neoplastic transformation. Indeed, gastrin and CCK, as well as their receptors, are expressed in a variety of tumor cell lines, animal models, and human samples, and might contribute to certain carcinogenesis. In this review, we will briefly introduce the gastrin and CCK system and highlight the effects of gastrin and CCK in the regulation of cell proliferation and apoptosis in both normal and abnormal conditions. The potential imaging and therapeutic use of these peptides and their derivatives are also summarized.

Keywords: gastrin, CCK, G protein-coupled receptor, cancer, imaging, therapeutics

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INTRODUCTION

Multicellular organisms have developed a delicate and efficient cellular regulatory system to maintain a balanced cell proliferation, differentiation, and apoptosis. Aberrant cellular regulation usually results in pathophysiology, for instance, carcinogenesis resulting from infinite cell proliferation, escape from apoptosis, and metastatic invasion. The gut is the largest endocrine organ in the body that expresses over 30 gut hormone genes and a wealth of bioactive peptides (1). As two of the first gastrointestinal peptides discovered, gastrin and CCK are known to play important roles in digestive processes including gastric acid secretion, pancreatic enzyme release, gallbladder emptying, gut motility, and energy homeostasis (2–4).

However, accumulating *in vitro* and *in vivo* studies have demonstrated that gastrin and CCK integrate and coordinate a rich network of information exchange pathways in cellular processes of proliferation and apoptosis, and under certain circumstances, they contribute to the pathogenesis and progression of some types of tumors (5). Indeed, gastrin, CCK and their cognate receptors (CCK2R and CCK1R, discussed in detail in section The Receptors for Gastrin and CCK) have been reported to be expressed and involved in several adenocarcinomas originated in stomach, colon, pancreas, esophagus, and gallbladder, as well as some tumors in the brain (6, 7). However, the gastrin and CCK regulatory systems are complex and intricate due to wide distribution of the hormone genes, cell-specific alternative splicing and post-translational modifications, and

activation of multiple signal transduction pathways. In this review, we aim to briefly introduce the gastrin and CCK systems regarding peptide biosynthesis, cellular expression, receptor activation, downstream signaling pathways, and involvement in proliferative and apoptotic responses in normal and malignant conditions. The underlying mechanisms attributed to the peptide-induced cellular effects and potential imaging and therapeutic applications will be elaborated.

THE GASTRIN AND CCK SYSTEM

Gastrin and CCK

As two of the first gastrointestinal hormones identified, the gastrin and CCK were originally discovered as hormonal regulators in stomach and small intestine in 1906 and 1928, respectively (8, 9). Subsequent isolation and structure determination of gastrin and CCK in the 1960s (10, 11) attracted researchers to investigate the biology, physiology, and pharmacology of these two peptides. In humans, the genes encoding gastrin and CCK precursors are located on chromosome 17q21 and 3p22-p21.3, respectively (12, 13). Both gastrin and CCK exist in multiple molecular forms following cell-specific post-translational processing. Among these peptides, the biologically active gastrin includes progastrin, Gly-extended gastrin-17 or −34 (Ggly), and the amidated gastrin-17 or −34 (Gamide), whereas the biologically active CCKs include Gly-extended and amidated CCK-33, -58, -22, and -8 (4, 14). In humans, amidated G-17 and CCK-33 predominate in plasma, and amidated CCK-8 predominates in the brain (15, 16). It should be noted that all bioactive gastrin and CCK peptides share the same amidated COOH-terminal pentapeptide (Gly-Trp-Met-Asp-Phe-NH2) motif, which is exceedingly well-conserved during evolution and comprises the minimal sequence (pharmacophore) required for biological activity and receptor activation (17).

In response to food intake, gastrin is synthesized and released by mucosal G cells to stimulate enterochromaffin-like (ECL) cells to secrete histamine, which further induces acid release from parietal cells through activation of H_2 histamine receptors, whereas CCK is predominantly produced and secreted by upper small intestinal I cells to stimulate gallbladder contraction and pancreatic enzyme secretion. As classical gut hormones and potent neurotransmitters, gastrin and CCK are widely distributed in gastrointestinal tract, CNS, and peripheral neurons (4, 18). However, CCK has also been suggested to stimulate spermatozoan fertilization, exert anti-inflammatory effects, promote sodium excretion into the urine, and predict the risk of mortality in heart failure, consistent with its expression in male germ cells (19), cells of immune system (20–23), renal cortex and medulla (24), and cardiomyocytes (25), respectively.

In addition to the acute digestive effects, both gastrin and CCK have been suggested to exert potent proliferative and anti-apoptotic effects by contributing to pathogenesis and progression of cancer (26, 27). Indeed, hypergastrinemia induced by proton pump inhibitor (PPI) together with *Helicobacter pylori* (*H. pylori*) infection correlates with higher risk of gastric and colorectal cancer in experimental animal models (28). Increased expression of gastrin and/or CCK were observed in human gastric adenocarcinoma (29, 30), colorectal carcinoma (31, 32), and pancreatic cancer (33), over that of the corresponding normal tissues, indicating potential roles of these peptides in promoting carcinogenesis (6).

The Receptors for Gastrin and CCK

The broad range of physiological functions of gastrin and CCK are mediated by two cognate receptors, which belong to rhodopsin-like G protein-coupled receptors characterized with the hallmark structure of seven transmembrane domains and the alternating NH_2 terminus and COOH tail. Gastrin receptor is also referred as CCK2 receptor (CCK2R, previously also called CCK-B receptor) based on the facts that (1) gastrin and CCK share the core sequence required for biological activities of both peptides and activation of the receptor; (2) gastrin and CCK have comparable affinity and potency for CCK2R; (3) CCK2R mediates almost all the classical physiological activities of gastrin (34, 35). However, CCK1 receptor (CCK1R, previously also called CCK-A receptor) binds and responds to CCK with a 500- to 1,000-fold higher affinity and potency than gastrin (36). Therefore, CCK1R is considered as the cognate receptor for CCK (35).

Using primarily cultured mucosal cells, Sachs and colleagues showed that both gastrin and CCK exert essentially equal potency in stimulating acid secretion from gastric parietal cells and histamine release from mucosal cells (37, 38), which are in line with their comparable affinity and potency for CCK2R. However, non-sulfated CCK-8 is less potent and less effective than gastrin in inducing DNA synthesis of isolated ECL cells, as assessed by 5-bromo-2′-deoxyuridine incorporation and ELISA (38). In addition, infusion of gastrin or CCK-8 in rats stimulates histamine synthesis and ECL hyperplasia except that CCK-8 fails to induce ECL hyperplasia even the circulating CCK-8 levels are 10-fold above normal (39, 40). The differential growth responses between gastrin and non-sulfated CCK-8 might be due to lower stability of non-sulfated CCK-8 caused by endopeptidases from the cell culture and/or different binding states of CCK2R are existing for gastrin and non-sulfated CCK-8 (38). Additional studies employing molecular cloning and radioligand binding might provide further explanations.

Table 1 summarizes the general distribution of CCK2R and CCK1R in healthy adult mammals. CCK2R is predominantly expressed in the brain and selected regions in the gastrointestinal tract, including gastric epithelial parietal cells, ECL cells, and D cells, pancreatic acinar cells, myenteric neurons, monocytes and T lymphocytes, and human peripheral blood mononuclear cells

Abbreviations: CCK, cholecystokinin; CCK1R, CCK1 receptor; CCK2R, CCK2 receptor; COX-2, cyclooxygenase-2; epidermal growth factor; EGFR, epidermal growth factor receptor; FAK, focal adhesion tyrosine kinase; Gamide, amidated gastrin; Ggly, Gly-extended gastrin; GRS, gastrin receptor scintigraphy; *H. pylori*, *Helicobacter pylori*; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MEN-1, multiple endocrine neoplasia type I; MTC, medullary thyroid carcinoma; NETs, neuroendocrine tumors; NO, nitric oxide; ODC, ornithine decarboxylase; PAI-2, plasminogen activator inhibitor type 2; PKB, protein kinase B; PKC, protein kinase C; PPI, proton pump inhibitor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end labeling; WT, wild-type.

TABLE 1 | Expression of CCK2R and CCK1R in normal tissues.

Receptor	Expression sites	Techniques	Species	References
CCK2R	Present in gastric mucosal parietal cells, enterochromaffin-like, and D cells.	Northern blotting, IHC, and RT-PCR	Canine, guinea pig, and/or human	(41–44)
	Present in pancreatic cells.	Autoradiography	Human	(45)
	Present in cortex, olfactory regions, hippocampal formation, septum, and interpeduncular nucleus, and amygdaloid nuclei.	*In situ* hybridization	Rat	(46)
	Present in duodenum myenteric neurons.	Autoradiography	Canine	(42)
	Present in cells of the immune system including leukemia cell lines derived from myeloid, T- and B- lymphoid, and peripheral blood mononuclear cells.	RT-PCR	Human	(47, 48)
	Present in rat brain and the fundus mucosa but absent in the rest of the digestive tract, pancreas, pancreatic islets, or kidney.	Northern blotting	Rat	(49)
	Present in rat brain and in the mucosa from the fundus and antrum but totally absent in the intestines, pancreas, pancreatic islets, and kidney Present in human brain, stomach, and pancreas but absent in the kidney.	RT-PCR and Southern blotting	Rat and human	(49)
CCK1R	Present in pancreatic acinar cells.	Autoradiography	Guinea pig	(50)
	Present in gallbladder smooth muscles.	Autoradiography	Bovine and human	(45, 51)
	Present in chief and D cells of gastric mucosa and absent in fundic mucosal histamine-containing cells.	Radioligand binding assay	Canine and guinea pig	(52, 53)
	Present in cortex, olfactory regions, hippocampal formation, septum, and interpeduncular nucleus, as well as hypothalamic nuclei including paraventricular nucleus, arcuate nucleus, and medial preoptic area.	*In situ* hybridization	Rat	(46)
	Present in fundus mucosa and pancreas but absent in the remaining GI tract or brain.	Northern blotting	Rat	(49)
	Present in rat brain and the mucosa of the fundus, antrum, duodenum, and colon, kidney, pancreas and pancreatic islets but absent in the lieum; Present in human brain, stomach, pancreas, and kidney.	RT-PCR and Southern blotting	Rat and human	(49)
	Present in gallbladder, intestine, brain, ovary, spleen, thymus, and ductal cells.	RT-PCR and *In situ* hybridization	Human	(54)
	Present in brain capillary endothelial cells.	IHC and Western blotting	Rat	(55)

IHC, immunohistochemistry; RT-PCR, reverse transcriptase polymerase chain reaction.

(18, 41, 42, 47, 48, 56, 57), whereas CCK1R is mainly found in pancreatic acinar cells, gallbladder smooth muscles, gastric mucosal chief and D cells, as well as cerebral and peripheral neurons (6, 46, 50–52).

Table 2 summarizes the localization and actions of gastrin, CCK, and their receptors on different types of human cancers (indicated as percentage of positive tissues expressing the corresponding ligand or receptor). A variety of human adenocarcinomas in the stomach, pancreas, colon, rectum, esophagus, lung, liver, medullary thyroid, overexpress CCK2R and/or CCK1R than the matched normal tissues (61, 62, 71, 79, 81, 82), serving as the basis for CCK2R- or CCK1R-targeted tumor imaging and therapy (84). Indeed, intensive chemical, physiological, and pharmacological studies provide novel diagnostic and therapeutic agents for the tumors overexpressing CCK2R and/or CCK1R (85).

Another line of evidence, the identification and characterization of the CCK2R splice variants and mutations, further confirmed the involvement of the gastrin and CCK system in cell proliferation and cancer pathogenesis. Indeed, CCK2Ri4sv, an CCK2R splice variant containing intron 4 and therefore additional 69 amino acids in the third intracellular loop, was identified in patients with colorectal cancer (86). Further *in vitro* functional studies showed that CCK2Ri4sv exhibits constitutive activation of signaling pathways resulting in enhanced Ca^{2+} levels and cell proliferation in both primary and Balb3T3 human colorectal tumor cells (86). In contrast, *in vitro* expression of CCK2Ri4sv in human epithelial HEK293 cells does not affect cell growth (87). Interestingly, compared to CCK2R-HEK293-xenografted mice, the CCK2Ri4sv-HEK293-xenografted mice have significantly increased tumor growth, which is associated with a constitutive, Src-dependent increase in the transcription factor hypoxia-inducible factor-1α and secretion of vascular endothelial growth factor (87). Other naturally occurring mutations in the *CCK2R* gene, such as V287F and R396C, were also shown to promote cell proliferation or angiogenesis through increase in Src-dependent secretion of cytokines (88).

Several cellular models have been utilized to investigate the cellular effects of gastrin and CCK, including (1) cells endogenously expressing CCK2R and CCK1R including human pancreatic tumor PANC-1 and Capan-1 cells (65–67), rat brain E18 neuroblasts (89); (2) cells endogenously expressing only CCK2R, such as rat pancreatic tumor AR42J cells (90), human pancreatic tumor BxPC-3, MIAPaCa-2, and AsPC-1 cells (67, 68, 91), rat pituitary adenoma GH3 cells (92), and human colon cancer HT-29 cells (93); (3) cells ready to be transiently or stably transfected with CCK2R or CCK1R, such as human gastric

TABLE 2 | Expression and function profiles of gastrin, CCK, and their receptors in human cancer cell lines and tissues.

Tumor types	Percentages of positive expression (positive/total)				Techniques	Effects on tumor cells	References
	Gastrins[a]	CCK	CCK1R	CCK2R			
Gastric cancer							
Tumor cell lines	Present in MKN45G and SGC-7901	NA[b]	NA[b]	Present in ECC10, SGC-7901, TMK-1, and HSC-39; Absent in AGS, ECC12, MKN-1, HGC27, HSK-TC, GCIY, KATOIII, OKAJIMA	FC, IHC, and/or Northern blotting	The gastrin-CCK2R system plays an important role in the elevated morphology of gastric tumors.	(29, 58–60)
Tumor tissues	36% (8/22)	NA[b]	NA[b]	NA[b]	FC and IHC	Treatment of anti-gastrin-17 antiserum significantly reduces proliferation of gastric tumor cells.	(58)
	0 (0/14)	4/14 (29%)	5/14 (36%)	1/14 (7%)	RT-PCR	CCK and CCK1R might play a more important role than for gastrin and CCK2R in gastric cancers.	(61)
	NA[b]	NA[b]	63% (5/8)	88% (7/8)	RT-PCR	Local or systemic originated-CCK might influence the growth of esophageal tumors.	(62)
	100% (15/15)	NA[b]	NA[b]	100% (15/15)	IHC	The expression levels of progastrin, Ggly, Gamide, and CCK2R positively correlates with the degree of gastric lesions.	(30)
	73% (22/30)	NA[b]	NA[b]	100% (30/30)	RT-PCR	Co-expression of gastrin and CCK2R might contribute to progression of gastric cancer.	(29)
	48% (133/279)	NA[b]	NA[b]	57% (158/279)	IHC	Gastric carcinoma tissues expressing both gastrin and CCK2R have a poorer prognosis than those negative for both.	(63)
	NA[b]	NA[b]	NA[b]	65% (31/48)	IHC	The gastrin system plays an important role in the elevated morphology of gastric tumors.	(60)
	NA[b]	NA[b]	NA[b]	0 (0/10)	Northern blotting	CCK2R might not be involved in gastric tumor.	(59)
	NA[b]	NA[b]	0 (0/27)	7% (2/27)	Autoradiography	CCK2R and CCK1R might not be involved in gastric tumor.	(64)
Pancreatic cancer							
Tumor cell lines	Present in PANC-1, BxPC-3, AsPC-1, Capan-1, and MIA PaCa-2	Present in PANC-1, BxPC-3, and AsPC-1 Absent in MIA PaCa-2	Present in PANC-1, Capan-1; Absent in BxPC-3, AsPC-1, and MIA PaCa-2	Present in PANC-1, BxPC-3, AsPC-1, Capan-1, and MIA PaCa-2	Radioligand binding and real time-PCR	The autocrine production of gastrin and CCK are important for stimulating pancreatic tumor cell growth.	(65–69)
Tumor tissues	NA[b]	NA[b]	100% (22/22)	100% (22/22)	RT-PCR and *in situ* hybridization	CCK1R might serve as selective bio-marker for pancreatic adenocarcinoma.	(54)
	NA[b]	NA[b]	90% (27/30)	NA[b]	RT-PCR and *in situ* hybridization	Increased expression of CCK1R might promote pancreatic malignancies.	(70)
	Up to 91%	NA[b]	NA[b]	95% (21/22)	IHC	CCK2R, progastrin, Ggly, and Gamide might promote pancreatic malignancy in an autocrine manner.	(33)
	Up to 74% (14/19)	0 (0/18)	67% (12/18)	100% (18/18)	RIA and RT-PCR	A local regulatory mechanism through gastrin and CCK2R, but no CCK mechanism, might be involved in pancreatic carcinoma.	(71)
	NA[b]	NA[b]	0 (0/32)	9% (3/32)	Autoradiography	Ductal pancreatic tumor cells very rarely express CCK1R and CCK2R.	(72)

(Continued)

TABLE 2 | Continued

Tumor types	Percentages of positive expression (positive/total)				Techniques	Effects on tumor cells	References
	Gastrins[a]	CCK	CCK1R	CCK2R			
Colorectal cancer							
Tumor cell lines	Present in LoVo, HCT-15, HT-29, Caco2, SkCo15 Absent in COLO−201, DLD1, SW403	NA[b]	NA[b]	Present in Caco2, Sk-Co15, HT-29.18 glu, and HT-29.18 gal.	Northern blotting and RT-PCR	Incomplete processing and low level of expression of gastrin were observed in five human colon carcinoma cells.	(32, 59, 73)
Tumor tissues	NA[b]	NA[b]	NA[b]	67% (45/67)	Radioligand binding assay	CCK2R content of colon cancers may have prognostic and therapeutic significances.	(74)
	21% (6/28)	NA[b]	NA[b]	NA[b]	FC and IHC	Gastrin but not CCK promotes growth of human gastric adenocarcinoma cells.	(58)
	100% (15/15)	NA[b]	NA[b]	NA[b]	RIA	Gastrin precursors are more abundant than amidated-G in neoplastic colon.	(75)
	Up to 97% (22/23)	NA[b]	NA[b]	NA[b]	Ribonuclease protection, IHC, Southern blotting, and RT-PCR	About 97 and 87% of colorectal adenocarcinomas express Gamide and progastrin, respectively, which might promote proliferation of colorectal tumor.	(76)
	Up to 100% (44/44)	NA[b]	NA[b]	NA[b]	RIA	Expression of progastrin and Ggly is increased in tumor tissues than controls.	(31)
	Up to 100% (12/12)	NA[b]	NA[b]	NA[b]	Northern blotting and RT-PCR	Solid colonic tumors contain higher levels of progastrin than normal colonic tissues.	(32)
	NA[b]	NA[b]	NA[b]	20% (2/10)	Northern blotting	Indicates a role of CCK2R in growth and differentiation of colorectal carcinomas.	(59)
	86% (96/112)	NA[b]	NA[b]	11% (13/112)	RNase protection assay, radioligand binding	The gastrin system exists in an autocrine proliferative loop in colorectal tumor.	(77)
	87% (26/30)	NA[b]	NA[b]	77% (23/30)	RT-nested PCR, Southern blotting	Gastrin might stimulate the growth of human tumor cells likely through a receptor other than CCK1R and CCK2R.	(73)
	NA[b]	NA[b]	42% (5/12)	17% (2/12)	RT-PCR	Local or systemic originated-CCK might influence the growth of colorectal tumor.	(62)
	NA[b]	NA[b]	0 (0/25)	4% (1/25)	Autoradiography	CCK2R and CCK1R might not be involved in colorectal tumor.	(64)
	44% (35/79)	NA[b]	NA[b]	38% (30/79)	IHC, RT-PCR	Co-expression of gastrin and CCK2R message is significantly increased in colorectal tumor.	(78)
Esophageal cancer							
Tumor tissues	NA[b]	NA[b]	63% (5/8)	0/8	RT-PCR	Local or systemic originated-CCK might influence growth of esophageal tumor.	(62)
	NA[b]	NA[b]	NA[b]	58.3% (7/12)	RT-CPR, Northern blotting	Gastrin-induced signaling through CCK2R promotes tumor cell proliferation.	(79)
	100% (4/4)	NA[b]	NA[b]	75% (3/4)	RT-PCR	Almost all esophageal tumors express gastrin and CCK2R.	(80)

(Continued)

TABLE 2 | Continued

Tumor types	Percentages of positive expression (positive/total)				Techniques	Effects on tumor cells	References
	Gastrins[a]	CCK	CCK1R	CCK2R			
Other types of cancer							
Small cell lung cancer cell lines	NA[b]	NA[b]	NA[b]	Present in H60, Lu134A, and Lu139; absent in PC6, Lu134B, Lu135, and PC14	Northern blotting	The majority of human small cell lung cancer cells express CCK2R.	(59)
Small cell lung cancer tissues	NA[b]	NA[b]	NA[b]	100% (10/10)	RT-PCR	The CCK2R might be a good prognostic and therapeutic target for small cell lung cancer.	(81)
Thyroid cancer tissues	NA[b]	NA[b]	8%(2/23)	92% (21/23)	Autoradiography	CCK2R might be utilized as diagnostic and therapeutic target for thyroid cancer.	(82)
Hepatic metastasis	71% (5/7)	NA[b]	NA[b]	100% (7/7)	RT-nested PCR, Southern blotting	A novel receptor different from CCK1R and CCK2R might be involved in gastrin-induced proliferative effects on hepatic tumor.	(73)
Gallbladder tumor tissues	NA[b]	NA[b]	77% (72/94)	NA[b]	IHC and IMB	CCK1R expression is significantly increased in gallbladder cancer and associated with the degree of tumor differentiation.	(83)

[a] *Includes three forms of gastrins: progastrin, Ggly, and Gamide.*

[b] *Not assessed.*

FC, flow cytometry; RIA, radioimmunoassay; RT-PCR, reverse transcriptase polymerase chain reaction; IHB, immunoblotting; IHC, immunohistochemistry; Gamide, amidated gastrin; Ggly, Gly-extended gastrin.

adenocarcinoma AGS and MKN-45 cells (94, 95), rat intestinal epithelial RIE-1 cells (96), Rat-1 and mouse Swiss 3T3 (also named NIH3T3) fibroblasts (97–99), Chinese hamster ovary CHO cells (99).

Intracellular Signaling Pathways

Once activated by the ligands, the CCK2R and CCK1R located on the cell surface undergo conformational changes and trigger a complex intracellular network of signaling pathways. As shown in **Figure 1**, both CCK2R and CCK1R can signal through Gq protein to activate phospholipase Cβ (PLCβ), resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate into inositol trisphosphate and diacylglycerol. Furthermore, second messenger diacylglycerol, together with inositol trisphosphate-induced Ca^{2+} efflux from endoplasmic reticulum, stimulate the phosphorylation of protein kinase C (PKC) isoforms to activate downstream effector proteins such as mitogen-activated protein kinases (MAPKs, important regulators in cell proliferation, differentiation, survival, and apoptosis) and inflammatory regulator NF-κB (35, 100).

In addition, both receptors are able to induce PKC-independent activation of MAPK and PI3K/AKT/mTOR signaling pathways (35, 101). A variety of non-receptor tyrosine kinases, including protooncogene Src kinase, focal adhesion tyrosine kinase (FAK, involved in cell morphology, cell motility, and invasion), and Janus kinase (JAK, involved in cell proliferation, differentiation, apoptosis, and oncogenesis) have also been reported to be activated by CCK2R and/or CCK1R (97, 102, 103). Furthermore, likely through activation of heparin-binding epidermal growth factor (EGF)-like growth factor, CCK2R activation was also reported to lead to transactivation of the EGF receptor (EGFR), a transmembrane tyrosine kinase receptor that plays important roles in cell growth, apoptosis, and migration (96, 104).

However, only CCK1R can couple to Gs protein to activate adenylyl cyclase, a cell membrane enzyme that catalyzes the cytoplasmic ATP into cAMP. The intracellular cAMP acts as second messenger to activate protein kinase A, which further stimulates the phosphorylation of cAMP response element-binding protein, a well-known transcription factor that affects a wealth of downstream genes (105, 106). In addition, the activation of nitric oxide (NO)/cGMP signaling cascade have been reported to be mediated by CCK1R in CHO cells and rodent pancreatic acini cells expressing CCK1R (107, 108).

GASTRIN AND CCK IN CELL PROLIFERATION

Gastrin and CCK Effects *in vitro*

A number of *in vitro* studies showed that gastrin and/or CCK induce a significant but modest increase in DNA synthesis in CCK2R-expressing cells, about 1.5-fold in AR42J cells, 1.6-fold in GH3 cells, up to 1.6-fold in HT-29 cells, 1.8-fold in

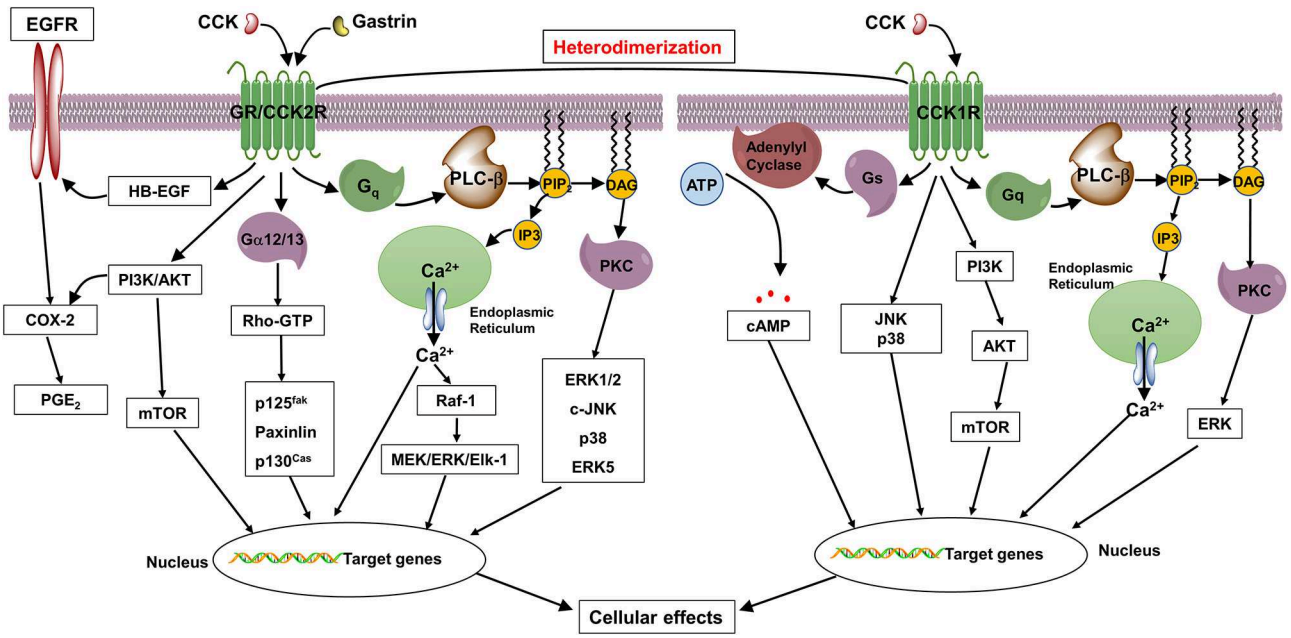

FIGURE 1 | Proposed diagrams of gastrin- and CCK-induced signaling pathways through CCK2R and CCK1R in normal and tumor cells. In response to gastrin and CCK, CCK2R couples to Gq and $G\alpha_{12/13}$ proteins to promote cell proliferation and inhibit apoptosis through activation of PLC/Ca^{2+}/PKC, MAPK, p125fak, Src, and PI3K/AKT cascades, as well as transactivation of EGFR, whereas CCK1R couples to Gq and Gs to exhibit trophic effects through activation of PLC/Ca^{2+}/PKC, AC/cAMP/PKA, MAPK, and PI3K/AKT pathways.

CHO cells, 3-fold in Rat-1 cells, and 4-fold in Swiss 3T3 cells over the corresponding unstimulated control cells (98, 99, 109–111). Although gastrin and CCK exert similar growth-promoting effect in different cell models, the mechanisms attributed for the ligand-stimulated trophic effects seem to be cell specific. In AR42J cells, gastrin was shown to stimulate cell proliferation through MEK/ERK2/Elk-1-induced upregulation of *c-fos* gene, an early response gene associated with cell growth (94, 109). This activation is PKC-dependent and requires the small GTP-binding RhoA, the CA rich G sequence of the SRE promoter, and transcription factors Elk-1 and Sap-1a which bind to the E26 transformation specific motif (112). Interestingly, in GH3 cells with a similar CCK2R expression and gastrin/CCK2R binding kinetics compared to AR42J cells, the same group further showed that gastrin promotes cell growth in Ca^{2+}-dependent manner, without activation of ERK1/2 (110).

In addition, gastrin and CCK stimulate cell proliferation in ERK1/2- and p74raf-1 kinase-dependent but Gi-independent manner in CCK2R-transfected Rat-1 cells (98), whereas it involves PKC/Ca^{2+}- and Src-dependent activation of p38 pathway in CHO-CCK2R cells (103). In CCK2R-expressing Swiss 3T3 cells, gastrin induces G_1/S cell cycle transition and cell proliferation through upregulation of cyclin D1, D3, and E, activation of cyclin-dependent kinases, and hyperphosphorylation of retinoblastoma protein (113). Gastrin-induced cyclin D1 transcription activity is mediated through activation of β-catenin and CREB pathways in AGS-CCK2R cells (114).

Cyclooxygenase-2 (COX-2), an inflammatory regulator critical for prostaglandin synthesis, have been suggested to be a downstream player of CCK2R. Indeed, gastrin increases transcriptional level of COX-2 in several cell lines, although the underlying mechanisms are different. In RIE-1 cells, the mRNA level of COX-2 is increased through activation of ERK5 and transactivation of the EGFR (96), whereas in Swiss 3T3 cells, it does not require PKC activity, activation of ERK1/2, or transactivation of EGFR (115). Gastrin-dependent COX-2 expression is inhibited by pretreatment with CCK2R antagonist L365,260, but not by pretreatment with CCK1R antagonist L364,714, indicating CCK2R but not CCK1R mediates gastrin-induced upregulation of COX-2 (115). In HT-29 cells, gastrin stimulates COX-2 expression via ERK1/2 and PI3K/AKT pathways (93). Together with the evidence that the COX-2 inhibitor L-745,337 reverses gastrin-induced DNA synthesis and cell growth (93), it is reasonable to conclude that COX-2 is responsible for the trophic effects of gastrin.

As the most abundant peptide neurotransmitter in the brain, the effects of gastrin and CCK on neuronal proliferation have been also investigated. In rat brain E18 neuroblasts, the group of Bragado showed that CCK promotes neuroblast proliferation by inducing tyrosine phosphorylation of adaptor proteins $p130^{Cas}$ and paxillin (89), two key components of focal adhesion complexes (116, 117), and phosphorylation of PKB/AKT and ERK1/2, followed by stimulation of DNA-binding activity of AP-1 (89). Furthermore, pharmacological blockade of CCK2R signaling with a potent and selective non-peptide CCK2R antagonist CR2945 inhibits CCK-stimulated ERK1/2

phosphorylation by over 50%, whereas totally antagonizes gastrin-stimulated ERK1/2 phosphorylation, indicating the proliferation-promoting effects of CCK on rat brain neuroblasts are mediated by both CCK1R and CCK2R (89).

Gastrin and CCK Effects *in vivo*

The trophic actions of gastrin on gastric mucosa were demonstrated by a sequence of *in vivo* studies. Two early clinical studies reported that inhibition of gastrin synthesis by gastrectomy results in atrophy of the residual mucosa, suggesting the involvement of gastrin in the regulation of mucosal cell growth (118, 119). Indeed, continuous administration of pentagastrin to male Wistar rats results in an increase in both density and population of gastric mucosal parietal cells (120, 121). Inspired by the above discoveries, Willems and colleagues, using histamine immunocytochemistry and autoradiography after labeling of mucosal specimens with ^{3}H-thymidine, first showed sustained administration of porcine gastrin causes a marked increase in DNA synthesis and in the mitotic index in canine fundic mucosa (122). Similarly, endogenous and exogenous hypergastrinemia, induced by antrocolic transposition, antral exclusion, and subcutaneous infusion of G17 were reported to lead to elevated cell proliferation rates of mucosal cells and oxyntic mucosal thickness in rats (123–125). Using BxPC-3 cell-xenografted mice, Smith et al. first demonstrated that gastrin, but not CCK, stimulates pancreatic tumor growth in a tonic and autocrine fashion (67, 68).

Extensive rodent studies have demonstrated that hypergastrinemia induced by continuous treatment of acid-suppressing drugs, including PPIs and H_2 histamine receptor antagonists, results in mucosal hyperplasia and ECL cell hyperplasia. In rare cases, ECL cell neuroendocrine tumors (NETs) might develop although these generally remain benign (126, 127). However, *Mastomys*, a hypergastrinemic rodent model that is genetically susceptible to spontaneous formation of gastric NETs, can develop gastric carcinoid tumor in the presence of normal serum gastrin levels, likely through the constitutive activation of CCK2R (128, 129). In humans, gastrin is known to promote proliferation of ECL cells and pathogenesis of gastric NETs. Indeed, hyperplasia-dysplasia-neoplasia processes in ECL cell populations were reported in hypergastrinemic conditions (130–132). However, it is still of debate whether hypergastrinemia alone is sufficient to result in ECL cell NETs. Patients with sporadic Zollinger-Ellison syndrome (ZES) rarely develop gastric NETs even though their circulating gastrin levels are over 10-fold above normal for a long period of time (133, 134). In contrast, gastric NETs were reported in 13–30% of patients combined with ZES and familial multiple endocrine neoplasia type I (MEN-1, an autosomal dominantly inherited disorder caused by inactivation of MEN-1 gene), indicating that gastrin and genetic factors are both important for the formation of gastric NETs (135, 136).

In addition, the ability of CCK to induce pancreatic hyperplasia and hypertrophy was also reported by several *in vivo* studies. Sustained subcutaneous injection of cerulein, a structural and functional homolog of CCK, in rats initiates a significant dose- and time-dependent increase in pancreatic weight and DNA, RNA, and protein contents (137, 138). Similar effects of CCK-8 were observed in male Wistar rats by Rosewicz et al., who further reported the trophic actions of CCK analog might be mediated by induction of pancreatic ornithine decarboxylase (ODC) activity and subsequent accumulation of polyamines, which are closely involved in cellular growth and proliferation due to their ability to facilitate almost all aspects of DNA, RNA, and protein syntheses (139, 140). Indeed, the cerulein-induced pancreatic acinar cell growth is inhibited by an irreversible ODC inhibitor α-difluoromethylornithine and this inhibition is further reversed by administration of the small polyamine putrescine (141, 142).

Very recently, an elegant study by Stanic and his colleagues revealed that CCK, signaling through CCK1R, is involved in regulating neurogenesis in the female adult mouse brain (143). In this study, transgenic mice lacking *Cck1r* (*Cck1r*$^{-/-}$) have decreased proliferating cells in the subgranular zone of the dentate gyrus and rostral migratory stream, with 42% and 29% lower number of cells immune-stained with Ki67 (a nuclear protein associated with cell proliferation) and Doublecortin (a microtubule-associated protein expressed in migrating and differentiating neurons), respectively (143–145). The decreased proliferating precursor cells in female *Cck1r* $^{-/-}$ mice further result in fewer migratory neuroblasts and tyrosine hydroxylase-immunoreactive mature interneurons in the olfactory bulb compared to the wild-type (WT) mice (143). Similarly, the proliferation-promoting effects of CCK on neurons were confirmed by Reisi et al., who showed that intraperitoneal injection of CCK in male Wistar rats promotes neurogenesis, as evidenced by significantly enhanced Ki-67 positive cells in the granular layer of hippocampal dentate gyrus than those treated with placebo (146).

Mechanism of Proliferative Actions of Gastrin and CCK

Physiologically, two sources of gastrin, originating from mucosal G cells and gastrointestinal tumors, have been suggested to act in different fashions to promote the pathogenesis and development of certain types of cancer. However, the signaling pathways mediating gastrin- and CCK-induced growth-promoting effects are still not fully understood. Since the processes of cellular proliferation are regulated by a complex signaling network of phosphorylation events initiated by the interaction of growth factors with their specific cellular receptors (147), we depicted the critical pathways that might be responsible for gastrin- or CCK-induced trophic effects in **Figure 1**, based on results obtained from various cell models. These include Gq-mediated phosphatidyl inositol turnover, Ca^{2+} mobilization, and PKC phosphorylation, Gs-mediated intracellular cAMP accumulation, $G\alpha_{12/13}$-mediated Rho-dependent tyrosine phosphorylation of FAKs, activation of MAPK, Src, and PI3K/AKT, as well as transactivation of EGFR, which ultimately results in the regulation of target genes and contribute to proliferative actions of gastrin or CCK (5, 85, 148).

Interestingly, heterodimerization of CCK2R and CCK1R has been also implicated in promoting CCK-induced cell

proliferation. In CCK1R-tansfected COS cells, the presence of CCK1R oligomeric complexes was demonstrated by Cheng and Miller in bioluminescence resonance energy transfer and co-immunoprecipitation experiments (149). CCK occupation of CCK1R induces the dissociation of those complexes in a concentration-dependent but phosphorylation-independent manner (149). The same group further showed that heterodimers composed of CCK2R and CCK1R display novel functional and regulatory properties with increased intracellular Ca^{2+} mobilization and delayed receptor internalization in response to CCK stimulation compared to these in cells only expressing individual receptor (150). It should be noted that CCK1R/CCK2R heterodimer-expressing COS cells tend to have enhanced CCK-induced cell proliferation responses, indicating a stimulant role of heterodimerization on the trophic effects of CCK (150).

GASTRIN AND CCK IN APOPTOSIS

Gastrin and CCK Effects *in vitro*

Compared to the well-described proliferative actions of gastrin and CCK, their involvement in the regulation of apoptosis is, however, poorly understood. To date, whether gastrin and CCK exert apoptotic or anti-apoptotic effect is still of debate and might be dependent on the exact physiological and pathological conditions.

Using flow cytometry and terminal deoxynucleotidyl transferase-mediated dUTP-FITC nick end labeling (TUNEL) method, Todisco et al. first demonstrated that gastrin reverses serum withdrawal-induced cellular apoptosis and promotes AR42J cell survival through PI3K- and p38-dependent activation of AKT (151). The same group further showed AKT inhibits apoptosis through activation of the pro-apoptotic proteins BAD and caspase-9, and transcriptional inactivation of FOXO forkhead transcription factors (152). In MKN-45 cells, blockade of CCK2R and COX-2 by AG-041R (a CCK2R antagonist) and NS-398 (a selective COX-2 inhibitor), respectively, was shown to synergistically induce apoptosis through downregulation of the anti-apoptotic protein BCL-2 and upregulation of the pro-apoptotic Bax (95). Similarly, gastrin-induced MCL-1 expression through CCK2R/PKC/MAPK pathway was also shown to be responsible for decreased apoptosis of AGS-CCK2R cells (153). *In vitro* study in human retinal pigment epithelial cells showed that CCK suppresses peroxynitrite-triggered cell apoptosis through inhibiting the expression of Fas, Fas-associated death domain (FADD), caspase-8, and BAX (154). In addition, the level of plasminogen activator inhibitor type 2 (PAI-2), a major gastrin-targeted gene implicated as an inhibitor for cell invasion and apoptosis (155, 156), was shown to be elevated in serum and stomach of hypergastrinemic patients and gastrin-treated media (157). Furthermore, by transfecting AGS-CCK2R cells with PAI-2 promoter-luciferase construct, Dockray and his colleagues showed that gastrin dose- and time-dependently induces the expression of PAI-2 luciferase, likely through Gq/PKC/RhoA-dependent activation of transcription factors CREB and AP1 (157).

Despite the anti-apoptotic effects of gastrin and CCK discussed above, gastrin and CCK were also reported to stimulate apoptosis of various cell lines. In human colorectal cancer Lovo cells expressing endogenous CCK2R and Colo320 cells transfected with WT CCK2R (Colo320wt), 10 nM of gastrin significantly increases the number of apoptotic Lovo cells and Colo320 cells by 21 and 42%, respectively, which is completely abolished in the presence of 500 nM of CCK2R antagonist L365-260 (158). Further *In vitro* signal transduction studies showed that, in Colo320wt cells but not in Colo320 cells lacking CCK2R or expressing loss-of-function CCK2R mutant, gastrin stimulates apoptosis, induces MAPK/ERK/AP-1 cascade, and suppresses the activity of NF-κB, indicating CCK2R mediates gastrin-induced apoptosis (158). Similarly, gastrin-induced apoptosis was also demonstrated in gastric epithelial RGM-1 cells and cholangiocarcinoma Mz-ChA-1 cells (159, 160).

Gastrin and CCK Effects *in vivo*

To investigate the *in vivo* effects of gastrin on apoptosis, several hypergastrinemic rodent models, such as INS-GAS mice expressing human gastrin minigene spliced with insulin promoter in pancreatic islets, *Mastomys* rodents treated with an H_2 histamine receptor antagonist, and FVB/N mice treated with a PPI, have been utilized (160–162). Although the ambivalent actions of gastrin were reported in a number of *in vitro* studies, the majority of evidence from *in vivo* studies suggested gastrin signaling through CCK2R stimulates gastric cell apoptosis.

Moss and colleagues, using *TUNEL* technique, showed that *Mastomys* rodents treated with loxtidine, an irreversible H_2 receptor antagonist, for 8 weeks have a 1.8-fold increase in the apoptotic cells in the hyperplastic mucosa and apoptotic cells return to the control levels upon loxtidine withdraw in 10 days (161). Nevertheless, the ratio of fundic mucosal proliferative to apoptotic cells also increases in the loxtidine-treated *Mastomys* rodents compared to that of the controls (161). In addition, INS-GAS mice and gastrin-infused GAS-KO mice have significantly elevated apoptotic glandular parietal cells, extraglandular mesenchymal cells and infiltrating immune cells, along with increased expression of proapoptotic BAX and decreased expression of BCL-2 compared to the corresponding controls (160). Sustained *H. felis* infection of INS-GAS mice results in exacerbated hypergastrinemia, increased apoptosis, as well as accelerated progression to atrophy (160). Treatment of *H. felis*-infected INS-GAS mice with YF476 (a highly specific CCK2R antagonist) and/or loxtidine for 6 months demonstrated that both agents have equivalent suppressing effects on gastric apoptosis and atrophy, and combination of both drugs exert more profound inhibitory effects on gastric cell apoptosis (160). Using hypergastrinemic mice models including INS-GAS and FVB/N mice treated with omeprazole, Przemeck et al. showed that hypergastrinemia renders gastric epithelial cells more susceptible to induction of apoptosis by 12Gy γ-radiation or *H. pylori* infection, and in both cases these effects are suppressed by CCK2R antagonist YM022 (162).

Similar results were observed in gastric corpus biopsies obtained from *H. pylori*-infected humans with moderate hypergastrinemia (162). One possible mechanism by which

gastrin-induced apoptosis results in gastrointestinal cancers was proposed by Houghton et al., through establishment of the *Helicobacter felis*/C57BL/6 mouse model of gastric cancer, who demonstrated that chronic *Helicobacter felis*-induced hypergastrinemia stimulates the apoptosis of gastric stem cells, followed by recruitment and repopulation of bone marrow–derived cells in the gastric mucosa. Subsequently, the bone marrow–derived cells progress through metaplasia and dysplasia to intraepithelial cancer since they are more susceptible to development of malignancy than the originally inhabited gastric epithelial stem cells (163).

In contrast, CCK has been shown to suppress neuronal apoptosis in several animal models. The pilot study of Sugaya et al. demonstrated the ability of CCK to protect the degeneration of cholinergic neurons in a basal forebrain-lesioned rat model, as evidenced by preserved choline acetyltransferase activity and acetylcholine release (164). Similarly, in cultured rat cortical neurons, CCK was shown to inhibit glutamate-induced neuronal death in a dose-dependent manner at concentrations of 1–100 nM. Furthermore, CCK2R was suggested to mediate CCK-induced neuronal protection since this effect was antagonized by the CCK2R antagonist L-365260 but not by the CCK1R antagonist L-364718 (165). In addition, Reisi et al. showed that intraperitoneal injection of CCK-8S, the octapeptide that can rapidly cross the blood-brain barrier and spread across the brain, into male Wistar rats inhibits neuronal apoptosis in the hippocampus, as evidenced by reduced number of TUNEL-positive cells in granular layer of hippocampal dentate gyrus (146). Interestingly. the pilot studies of Lavine et al. demonstrated that CCK is up-regulated and expressed in the pancreatic islet β-cells of obese mice, and whole-body deletion of *Cck* results in reduced islet size and β-cell mass through increased β-cell apoptosis (166). In cultured β-cells or isolated islets, CCK also functions as a paracrine or autocrine factor to protect β-cells from cytokine- or ER stress-stimulating agent-induced apoptosis (166). Similar anti-apoptotic effects of islet-derived CCK were observed in lean transgenic mice that endogenously express CCK in the β-cells (167).

Mechanism of Gastrin- and CCK-Mediated Effects in Apoptosis

Due to lack of evidence, the exact roles of gastrin and CCK in the regulation of apoptosis are still of debate. Some *in vitro* studies demonstrated that gastrin and CCK exert an anti-apoptotic effect through PI3K/AKT- and p38/AKT-dependent activation of BAD and caspase-8, CCK2R/COX-2-dependent and Fas/FADD/caspase-8-dependent upregulation of BCL-2 and downregulation of BAX, CCK2R/PKC/MAPK-dependent upregulation of MCL-1, as well as PKC/RhoA/CREB/AP1-dependent activation of PAI-2. However, other studies showed gastrin induces apoptosis via activation of MAPK/ERK/AP-1, blockade of NF-κB, and Ca^{2+}-dependent activation of PKC-α. Recent *in vivo* studies showed that CCK protects central neurons and pancreatic β-cells from apoptosis in autocrine and/or paracrine manners, whereas the underling mechanisms remain elusive. Further investigations are needed to elucidate gastrin- and CCK-induced effects in apoptosis in the context of cell types and animal models.

IMAGING AND THERAPEUTIC PERSPECTIVES

It has been well-recognized that both the expression of CCK2R and CCK1R are increased in numerous human NETs over the corresponding normal tissues, suggesting that both receptors might be utilized as molecular targets for localization of certain adenocarcinomas by radiopeptide imaging *in vivo*, and more recently, for treatment by peptide receptor radiation therapy (168, 169). Indeed, inspired by somatostatin receptor scintigraphy, the diagnostic gold standard procedure for the detection of several tumor entities, Gotthardt et al. developed a novel imaging method, gastrin receptor scintigraphy (GRS), for the detection of metastases of medullary thyroid carcinoma (MTC) (170). By comparing different detection methods in 26 patients with metastasized MTC, it was shown that GRS combined with computed tomography is the most effective in MTC detection, with a tumor detection rate of 96.7% (170). In a 60-patient cohort of carcinoids and other NETs the same group suggested that GRS should be performed in selected patients since it may provide additional information in NET patients with equivocal or absent somatostatin uptake (171).

In this scenario, the search of radiolabeled CCK2R ligands with good tumor-to-kidney pharmacodynamics is of great importance in clinical settings. Thirty-four radiolabeled candidate compounds derived from gastrin were screened by measuring tumor and kidney uptake in several pancreatic xenograft nude mouse models, and the peptide with sequence DOTA-HHEAYGWMDF-NH_2 showed the highest tumor-to-kidney ratio with saturable uptake in target organs and low uptake by non-target tissues, indicating a promising candidate for peptide receptor radiation therapy (172).

Accumulating evidence showed that gastrin signaling via CCK2R stimulates the growth of gastrointestinal cancer cells *in vitro* and *in vivo*, indicating blockade of CCK2R pathway might present a promising strategy for the treatment of gastrointestinal carcinoma. Indeed, in xenografted nude mice transplanted with the mouse colon adenocarcinoma cell line MC-26, proglumide, a weak CCK2R inhibitor, suppresses growth of MC-26 colon cancer and prolongs survival in tumor-bearing mice (173). In addition, decreased mean tumor area, mean tumor weight, and tumor DNA and RNA contents were also observed in proglumide-treated group compared to the control group (173). However, the beneficial effects of proglumide on survival from gastric carcinoma were abolished in a randomized, controlled study of proglumide in 110 gastric tumor patients (174). The authors proposed that more specific and potent CCK2R antagonists, in combination with agents that block gastrin secretion such as somatostatin analogs or prostaglandin analogs, might exert a greater benefit on survival in humans (174).

Furthermore, Watson et al. developed a gastrin-specific monoclonal antibody G17DT, an immunogen composed of the amino terminal portion of G17 linked to a diphtheria toxoid

(175). The colon tumor-xenografted rats treated with G17DT were shown to have significantly reduced median cross-sectional tumor area and weights, and increased degree of necrosis compared to control rats (175). Similar survival-promoting effects were observed in severe combined immune deficient mice xenografted with two human gastric cancer lines MGLVA1 cells and ST16 cells (176). In the MGLVA1asc-xenografted mice, the enhancement in survival induced by G17DT was not significantly different from that achieved by treatment with 5-fluorouracil/leucovorin (177). Mice treated with a combination therapy with G17DT and 5-fluorouracil/leucovorin benefit more in survival compared to those treated with G17DT or 5-fluorouracil/leucovorin alone, indicating additive effects of both treatments (177).

Due to the positive results of G17DT achieved in multiple gastrin-sensitive tumor models, the same group further investigated the therapeutic effectiveness of G17DT in clinical trials. G17DT was shown to elicit functional antibodies against gastrin with safe and well-tolerated profile in 52 patients with gastric carcinomas, with the exception that two patients suffered significant adverse reactions (178). In another open-label, multinational, and multicenter phase II study, sixty-five of 94 advanced gastric cancer patients were successfully vaccinated with G17DT in terms of anti-gastrin antibody production and showed longer time-to-progression and median survival compared to control patients (179). A further international multicenter randomized controlled Phase III clinical trial consisting of 154 patients (79 G17DT and 75 placebo) with advanced pancreatic cancer confirmed improved survival of patients in the G17DT group through an intention-to-treat analysis (180). Therefore, G17DT represents a promising therapeutic option for gastrointestinal malignancy.

CONCLUSIONS

Since the discovery of gastrin and CCK a century ago as digestion-related gastrointestinal peptides, our understanding of these peptides have considerably improved. Extensive investigations have demonstrated the expression of gastrin, CCK, and their receptors in a variety of tumor cells and tissues and their involvement in the regulation of cell proliferation and apoptosis, as well as the pathogenesis of cancer. Indeed, a complex network of signaling pathways, including Gq/PLC/Ca^{2+}/PKC, Gs/AC/cAMP, and NO/cGMP cascades, activation of kinases such as MAPK, PI3K/AKT, Src, and FAKs, as well as transactivation of the EGFR, have been suggested to contribute to the trophic effects of these two peptides. Finally, diagnosis and treatment approaches targeting peptides and CCK2R, such as receptor scintigraphy and radiopharmaceuticals, have been utilized in tumor imaging and/or therapy *in vitro*, *in vivo*, and in clinical trials. However, it should be noted that the CCK2R- and CCK1R-mediated signal transduction varies in the context of cell types, suggesting that cautions should be taken in future investigations attempting to target the gastrin and CCK system for the treatment of certain types of cancer.

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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Galanin System in Human Glioma and Pituitary Adenoma

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Expression of neuropeptides and their corresponding receptors has been demonstrated in different cancer types, where they can play a role in tumor cell growth, invasion, and migration. Human galanin (GAL) is a 30-amino-acid regulatory neuropeptide which acts through three G protein-coupled receptors, GAL_1-R, GAL_2-R, and GAL_3-R that differ in their signal transduction pathways. GAL and galanin receptors (GALRs) are expressed by different tumors, and direct involvement of GAL in tumorigenesis has been shown. Despite its strong expression in the central nervous system (CNS), the role of GAL in CNS tumors has not been extensively studied. To date, GAL peptide expression, GAL receptor binding and mRNA expression have been reported in glioma, meningioma, and pituitary adenoma. However, data on the cellular distribution of GALRs are sparse. The aim of the present study was to examine the expression of GAL and GALRs in different brain tumors by immunohistochemistry. Anterior pituitary gland ($n = 7$), pituitary adenoma ($n = 9$) and glioma of different WHO grades I–IV ($n = 55$) were analyzed for the expression of GAL and the three GALRs with antibodies recently extensively validated for specificity. While high focal GAL immunoreactivity was detected in up to 40% of cells in the anterior pituitary gland samples, only one pituitary adenoma showed focal GAL expression, at a low level. In the anterior pituitary, GAL_1-R and GAL_3-R protein expression was observed in up to 15% of cells, whereas receptor expression was not detected in pituitary adenoma. In glioma, diffuse and focal GAL staining was noticed in the majority of cases. GAL_1-R was observed in eight out of nine glioma subtypes. GAL_2-R immunoreactivity was not detected in glioma and pituitary adenoma, while GAL_3-R expression was significantly associated to high-grade glioma (WHO grade IV). Most interestingly, expression of GAL and GALRs was observed in tumor-infiltrating immune cells, including neutrophils and glioma-associated macrophages/microglia. The presence of GALRs on tumor-associated immune cells, especially macrophages, indicates that GAL signaling contributes to homeostasis of the tumor microenvironment. Thus, our data indicate that GAL signaling in tumor-supportive myeloid cells could be a novel therapeutic target.

Keywords: galanin receptor, neuropeptide, brain tumor, glioma, pituitary adenoma, macrophage

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INTRODUCTION

Malignant brain tumors are the most common cause of cancer-related deaths in adolescents and young adults aged 15–39 and the most common cancer occurring among 15–19 year olds (1). Due to the diffuse infiltration into the brain by various brain cancer types, such as glioma (i.e., astrocytoma and oligodendroglioma; WHO grade I–IV), surgical intervention is difficult and often limited (2). Consequently, there is an urgent need to understand tumor biology and subsequently identify new drug targets for the treatment of brain tumors.

Possible new drug candidates might be found in the group of neuropeptides. Neuropeptide expression has been shown in many different cancer types, and neuropeptide expression levels correlate with tumor differentiation or aggressive behavior. Thus, neuropeptides could be useful for tumor imaging and as biomarkers for prognosis. More importantly, neuropeptides are involved in tumor cell growth, invasion, and migration (3–5), supporting their potential in developing novel anti-tumor treatment strategies.

Human galanin (GAL) is a 30-aa regulatory neuropeptide which plays a role in several physiological processes. Its functions are mediated by the G protein-coupled receptors GAL_1-R, GAL_2-R, and GAL_3-R that differ in their signal transduction pathways. GAL_1-R and GAL_3-R predominantly couple to Gi/o, leading to a reduction of cAMP and consequently an inactivation of the protein kinase A (PKA). GAL_2-R signals via multiple classes of G proteins, but preferably via Gq/11, which results in the activation of the protein kinase C (PKC). GALRs show sequence homologies, particularly in the transmembrane regions. GAL_1-R and GAL_3-R show 33% sequence homology, whereas GAL_2-R and GAL_3-R show 54% sequence homology (6). Besides species-specific expression patterns of GAL and galanin receptors (GALRs), expression is also tissue-specific. GAL is expressed in neuronal and endocrinal tissues at highest levels. In addition, GALRs are expressed in different tissues, with GAL_1-R mRNA in particular being strongly expressed in the brain. GAL_2-R mRNA is less abundant and restricted to certain brain regions, whereas GAL_3-R mRNA is more restricted to peripheral tissues (6). Recently, expression of GAL and GALRs in human immune cells such as neutrophils and macrophages was also reported (7).

Human pheochromocytoma was the first tumor in which GAL was identified (8, 9). Later, GAL-like immunoreactivity was detected in other neuroendocrine tumors, including human pituitary adenoma, particularly associated with adrenocorticotrophic hormone-secreting cells (10–16), and gangliocytoma (14, 17), paraganglioma (18, 19), and neuroblastoma (20). GAL has also been detected in a variety of non-neuroendocrine human tumors of different origin, including glioblastoma and other brain tumors (21), melanoma (22), head and neck squamous cell carcinoma (HNSCC) (23), basal cell carcinoma (24), colon cancer (25–27) and embryonic carcinoma (28). Interestingly, the majority of these tumors exhibited significantly higher GAL levels than corresponding non-cancerous tissue (22, 23, 25, 27, 28). In colon cancers, GAL mRNA levels correlated with tumor size and stage (25), for which a significant correlation between high GAL expression and shorter disease-free survival in colon cancer patients was observed (27).

In humans, GALRs were first discovered in pituitary tumors (29) and subsequently identified in pheochromocytoma (30), neuroblastoma (20), glioma (21), prostate carcinoma (30), colon carcinoma (27), and HNSCC (31). GAL_1-R mRNA is the most abundantly expressed GALR mRNA in human meningioma, glioblastoma (21) and neuroblastoma (32). Elevated GAL_1-R mRNA expression is associated with increased malignancy (33). Increased GAL_1-R mRNA expression was also observed in human pituitary adenoma relative to levels in normal human pituitary gland (34), suggesting cancer-promoting properties for GAL_1-R at least in these tumors. Furthermore, activation of GAL_1-R induces cell-cycle arrest and suppresses proliferation of HNSCC cell lines (31, 35, 36). Anti-proliferative effects via GAL_1-R signaling have also been observed in human SH-SY5Y neuroblastoma cells transfected with GAL_1-R (37).

In contrast, the presence of GAL_2-R mRNA is less common in human glioma (21) and neuroblastoma (20). GAL_2-R mRNA expression is low in the majority of human pituitary adenomas compared to levels in normal human pituitary (34). However, elevated GAL_2-R mRNA expression was observed in human pheochromocytoma (38).

It is noteworthy that transfection of GAL_2-R into human SH-SY5Y neuroblastoma cells and into human HNSCC cells led to suppressed cell proliferation and induction of caspase-dependent apoptosis (36–40). On the other hand, in small cell lung cancer, activation of GAL_2-R exerted growth-promoting effects (41, 42).

The impact of GAL_3-R signaling on the biological activity of cancer cells is less well-studied. GAL_3-R expression was detected in neuroblastoma (32, 33) and glioma (21). Analysis of human HNSCC revealed significantly increased GAL_3-R expression in the tumors compared to normal tissue (23). Similarly, GAL_3-R mRNA expression was detected in human pituitary adenoma associated with tumor relapse, whereas it was absent in post-mortem pituitary glands (34).

To date, GAL-binding studies have been used to deduce the presence of GALRs in human glioma, meningioma (21) and pituitary adenoma (29), but no receptor subtype has been identified at the cellular level, except indirectly from mRNA expression analyses in tissue extracts (21, 34). Thus, information on the cellular distribution of GALRs has been missing due to a lack of specific GAL receptor antibodies. Recently, we were able to identify specific anti-human GALR- specific antibodies, which now allow us to determine the distribution of the three GALRs at the cellular level (43).

The aim of the present study was to elucidate the expression of GAL and GALRs in different human brain tumors by immunohistochemistry (IHC) with carefully validated antibodies.

MATERIALS AND METHODS

Ethics Statement

Experiments were conducted in accordance with the Helsinki Declaration of 1975 (revised 1983) and the guidelines of

the Salzburg State Ethics Research Committee (AZ2 09-11-E1/823-2006), being no clinical drug trial or epidemiological investigation. In accordance with the Upper Austrian Ethics Committee, upon hospital admission, patients signed an informed consent document concerning the surgical intervention, and agreed to the use of the surgically removed tumor tissue for research purposes. Furthermore, the study did not extend to examination of individual case records. Patient anonymity was ensured at all times. Cancer tissues were derived from surgery. Pituitary glands were obtained post mortem from patients with no signs of brain tumors who died due to either cardiorespiratory failure or brain hemorrhage. Demographics of individual patients are provided in **Supplementary Tables 1–3**.

Patients and Material

Formalin-fixed paraffin-embedded (FFPE) tumor tissue of glioma and pituitary adenoma as well as anterior pituitary glands were provided by the Division of Neuropathology, Neuromed Campus, Kepler University Hospital, Linz, Austria.

In total, 55 glioma and 9 pituitary adenoma samples were analyzed for the expression of GAL and GALRs by IHC. Detailed information on tumor subtypes and WHO grades, including astrocytic tumors ($n = 37$), oligodendroglial tumors ($n = 15$) and mixed neuronal-glial tumors ($n = 3$), and age of the patients is provided in **Table 1** and **Supplementary Tables 2, 3**. Data on 7 anterior pituitary glands used for antibody validation are also included (**Supplementary Table 1**). The neuropathology diagnosis was based on the diagnostic criteria outlined in the revised 4th edition of the WHO Classification of tumors of the CNS (44). Briefly, the mutation status of the *IDH1* and *IDH2* genes was assessed for astroglioma and oligodendroglioma; 1p19q co-deletion was determined for oligodendroglioma using multiplex ligation-dependent probe amplification. Cases with a former diagnosis of oligoastrocytoma were re-evaluated using the above-mentioned molecular diagnostic parameters. Two cases of oligoastrocytoma could not be assigned to astroglioma or oligodendroglioma and are therefore described separately. Their data are not included in the statistics.

Immunohistochemistry

For IHC analysis, 4 µm FFPE tissue sections were stained as described previously (45) using the Envision+ System-HRP (DAB) Kit (DAKO, Glostrup, Denmark). After drying for 1 h at 60°C, sections were deparaffinized and rehydrated. Epitope retrieval was performed with EDTA-Tris buffer (1 mM EDTA, 10 mM Tris, pH 9) for 40 min at 95°C. After blocking endogenous peroxidases with "Peroxidase blocking solution" (DAKO), the primary antibody diluted in "Antibody Diluent with Background Reducing Components" (DAKO) was added (40 min, 37°C). The following polyclonal antibodies were used: anti-GAL (Peninsula/Bachem, San Carlos, CA, USA, T-4325, LOT: A14907, rabbit, 1:300), anti-GAL_1-R (GeneTex Inc., Irvine, CA, USA, GTX108207, LOT: 39771, rabbit, 1:200), anti-GAL_2-R [Proteintech Group Inc., Rosemont, IL, USA, customized, LOT: S4510-1, rabbit, 1:400; (45)] and anti- GAL_3-R (GeneTex Inc., Irvine, CA, USA, GTX108163, LOT: 39764, rabbit, 1:500). The specificity of the antibodies against human GALRs was recently demonstrated (43, 45). Subsequently, the anti-rabbit secondary antibody "Envision+HRP-labeled polymer" (DAKO) was added for 30 min at RT. For visualization, "Envision+Liquid DAB+Chromogen" (DAKO) was applied (10 min, RT). Mayer's hemalum solution (Merck KGaA, Darmstadt, Germany) was used for counterstaining (3–5 min). Slides were immersed in 0.75% HCl in ethanol and rinsed under running tap water (10 min). After dehydration, the slides were mounted with Histokitt (Karl Hecht GmbH & Co KG, Sondheim, Germany). Digital micrographs were taken with a Moticam 5+ camera using Motic Image Plus 2.0 software (Motic, Wetzlar, Germany).

For each round of IHC staining, appropriate control sections were included as quality control. Human skin sections were used as positive controls for GAL [epidermis, sweat glands (46, 47)] and GAL_3-R [blood vessels (48)]. The cell line SH-SY5Y transfected with human GAL_1-R or human GAL_2-R was used as a positive control for GAL_1-R and GAL_2-R staining [**Supplementary Figures 1, 2**; (43, 45)]. Furthermore, as a control, the primary antibody was omitted.

The percentage of stained tumor cells was estimated, excluding adjacent normal appearing tissue, as well as necrotic or hemorrhagic areas. If a section contained <10 positive-stained cells, the staining was regarded as negative. The staining intensity of tumor cells was rated from negative (0) to strong (3). IHC staining of non-tumor cells, vessels and immune cells such as neutrophils and glioma-associated macrophages/microglia (GAMs) was also evaluated. The IHC analysis was performed by two independent observers.

RT-PCR Analysis

RNA was isolated from frozen tissue with Tri Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. Two micrograms of human RNA were used to generate cDNA by using maxima reverse transcriptase (Thermo Fisher, Waltham, MA, USA) following the manufacturer's protocol. Expression levels were quantified via qPCR using SYBR green SuperMix (BioRad, Hercules, CA, USA). The amplification was performed for 40 cycles (97°C for 15 s, 63°C for 30 s, and 72°C for 10 s) with specific primers for the genes of interest (**Supplementary Table 4**).

Relative expression levels of all genes were calculated as differences between the threshold cycle (Ct) of the gene of interest and Ct of the human housekeeping gene ribosomal protein L27 (RPL27).

Statistical Analysis

Cramer-V was used to compare the expression of GAL and GALRs among 55 glioma cases with astrocytic, oligodendroglial, and mixed neuronal-glial tumor subclasses as appropriate. Cramer-V and Fisher's exact test were used to compare the expression of GAL and GALRs in glioma of different WHO grades. $P < 0.05$ was considered significant. All analyses were performed using SPSS 24.0 (SPSS Inc., Chicago, IL., USA).

TABLE 1 | Information on tumor samples (incl. WHO classification and grade, sample size (n), and patient age range), positive-stained samples (%), as well as the range of positive-stained cells in (%) and the range of staining intensity.

Tumor type	WHO grade	*n*	Median age [age range] (years)	Diffuse GAL staining	Focal GAL staining	GAL_1-R	GAL_2-R	GAL_3-R
				positive-stained samples (%) range of positive-stained cells (%), intensity range (0–3)				
Pilocytic astrocytoma	I	5	16 [3–20]	100% 0–2	60% <1–18%, 1–3	20% <1%, 1	0%	20% <1%, 2–3
Diffuse astrocytoma	II	7	39 [4–76]	100% 0–2	57% 2–40%, 1–3	0%	0%	0%
Anaplastic astrocytoma	III	7	33 [4–61]	100% 0–2	86% <1–65%, 1–2	43% <1%, 1	0%	0%
Glioblastoma multiforme	IV	8	62 [21–75]	100% 0–2	75% <1–30%, 1–2	38% <1–8%, 1–2	0%	63% <1–7%, 1–3
Gliosarcoma	IV	6	55 [40–68]	100% 0–2	50% 15–70%, 1–2	33% <1–1%, 1	0%	50% <1–2%, 1–2
Giant cell glioblastoma	IV	4	42 [24–76]	100% 0–1	100% 35–80%, 1–2	50% <1%, 1–2	0%	0%
Oligodendroglioma	II	9	38 [20–76]	89% 0–2	78% <1–10%, 1–2	22% <1%, 1–2	0%	11% <1%, 1–2
Anaplastic oligodendroglioma	III	6	36 [31–37]	67% 0–2	67% <1–30%, 1–3	17% 1%, 1–2	0%	17% <1%, 1–2
Ganglioglioma	I	3	21 [4–21]	67% 0–2	67% <1–6%, 2–3	67% <1%, 1	0%	67% <1–3%, 1–2
Pituitary adenoma		9	57 [27–74]	89% 0–2	11% 2%, 2–3	0%	0%	0%
Anterior pituitary gland		7	78 [61–92]	100% 1–2	100% 2–40%, 3	100% 7–15%, 3	0%	100% <1–5%, 2–3

RESULTS

Antibody Validation on Human Anterior Pituitary Glands

Although the antibodies used in the present studies had been validated on peripheral tissues and overexpressing cell lines, we first validated the IHC protocol for healthy brain tissue, processed the same way as the tumor tissues, and compared the results to mRNA expression data. From seven healthy anterior pituitary glands, half the tissue was fixed in formaldehyde and processed for IHC analysis whereas the other half of the gland was fresh frozen for subsequent mRNA expression analysis.

IHC analysis revealed very strong focal intracellular GAL-immunoreactivity (2–40% of cells) in the pituitary glands. The remaining cells showed diffuse staining for GAL. This diffuse staining might be due to low expression levels and also to intercellular GAL secreted by high- GAL-expressing cells (**Figure 1A**). The high expression levels of the GAL peptide were also confirmed by RT-PCR analysis (**Figure 2**).

GAL_1-R was the most prominent receptor in the anterior pituitary gland, with 7–15% positive membrane-associated cellular staining (medium to high staining intensity). GAL_2-R-immunoreactivity was not detectable in anterior pituitary gland, whereas <1–5% of cells showed membrane-associated GAL_3-R-immunoreactivity (medium to high staining intensity; **Figure 1**, **Table 1**, **Supplementary Table 1**).

The IHC staining results correlate well with mRNA expression, with GAL_1-R being the most prominent galanin receptor at the mRNA level, followed by GAL_2-R and GAL_3-R, which on average showed the same ΔCt values (**Figure 2**).

Expression of GAL and GALRs in Pituitary Adenoma

Out of 9 pituitary adenomas, only one, a null-cell tumor (case 7), was positive for GAL, showing medium to strong focal GAL-immunoreactivity in 2% of the tumor cells (**Figure 3A**). Three pituitary adenomas [cases 3 (FSH), 5 (null cell), 6 (STH, prolactin)] had <10 GAL-positive stained cells and the remaining five were negative for focal GAL staining. Eight pituitary adenomas displayed diffuse GAL-immunoreactivity with low to medium intensity. Case 1 (prolactin) remained completely negative for diffuse and focal GAL staining. None of the GALRs was detectable in pituitary adenoma by IHC staining (**Figures 3B–D**, **Table 1**, **Supplementary Table 2**).

Expression of GAL and GALRs in Gliomas

Overall, the cellular heterogeneity of glioma subtypes was reflected by a heterogeneous pattern of expression of GAL and GALRs (**Figures 4–6**, **Table 1**). The majority of gliomas showed focal GAL-immunoreactivity (71% of cases) and regions with diffuse GAL-staining (93% of cases). The proportion of GAL-positive stained cells ranged from <1 to 80%.

The most prominent receptor expressed in glioma was GAL_1-R (29% of cases), followed by GAL_3-R (24% of cases). GAL_2-R

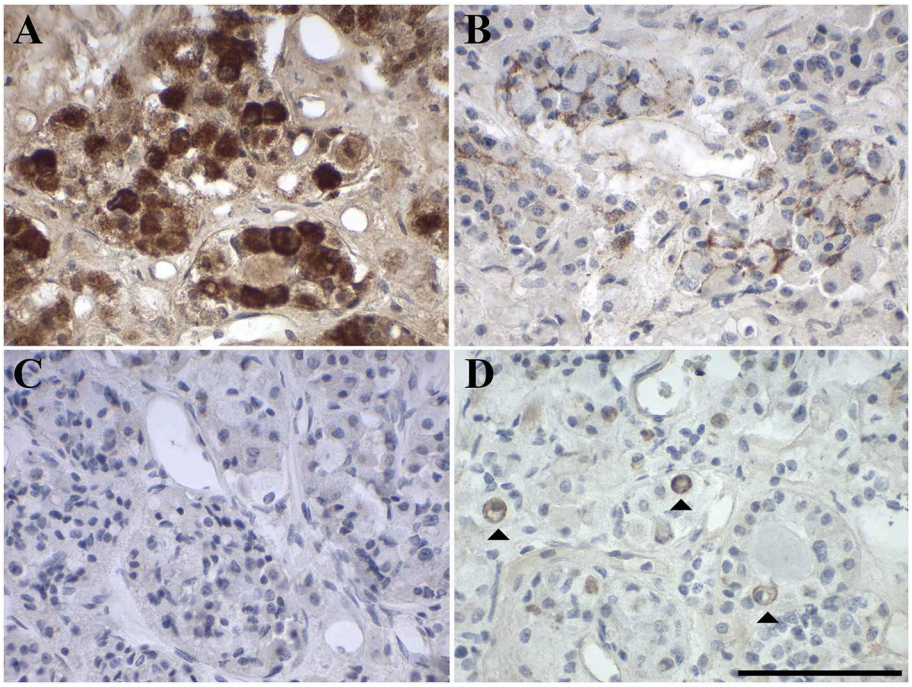

FIGURE 1 | Representative images of immunohistochemical staining of **(A)** GAL, **(B)** GAL_1-R, **(C)** GAL_2-R, and **(D)** GAL_3-R in human anterior pituitary gland (case 1). [scale bar: 100 μm].

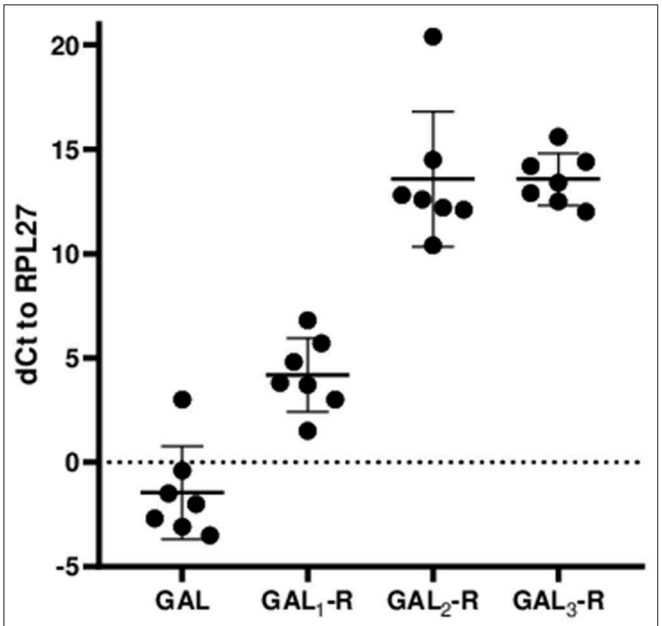

FIGURE 2 | Relative mRNA expression levels of GAL and GALRs in anterior pituitary glands are shown as ΔCt values relative to the human housekeeping gene hRPL27. The values represent mean ± SD ($n = 7$).

was not detectable by IHC in glioma. The proportion of GAL_1-R-stained cells as well as the proportion of GAL_3-R-stained cells in the tissue sections was very low (mainly <1% of tumor cells).

Statistical testing revealed significant correlations between astrocytic, oligodendroglial and mixed neuronal-glial tumors in diffuse GAL staining ($p = 0.009$; Cramer-V). For focal GAL ($p = 0.963$; Cramer-V) as well as GAL_1-R ($p = 0.264$; Cramer-V) and GAL_3-R ($p = 0.137$; Cramer-V), no significant correlations between astrocytic, oligodendroglial, and mixed neuronal-glial tumors were observed.

Furthermore, correlations between GAL and GALR expression and different WHO grades I–IV were tested. The only significant correlation was found for GAL_3-R and WHO grades ($p = 0.015$; Cramer-V): 13 of 55 samples were positive for GAL_3-R, with 23% of GAL_3-R positive samples being WHO grade I, 8% WHO grade II, 8% WHO grade III (39% WHO I–III) and 61% WHO grade IV, indicating that the significant correlation is between GAL_3-R and WHO IV. Further testing of GAL_3-R against WHO grade IV and non-WHO grade IV confirmed this presumption ($p = 0.018$; Fisher's exact test).

Expression of GAL and GALRs in Astrocytic Tumors

GAL-immunoreactivity showed substantial differences between and within different subtypes of 37 astrocytic tumors (WHO grade I–IV). In 70% of all subtypes, cases of focal GAL-immunoreactivity were observed, although the percentage of GAL-positive cells varied. Furthermore, in all astrocytic tumors, areas with diffuse GAL-staining were noticed (**Figures 4**, **5**, **Table 1**, **Supplementary Table 3**). A small proportion of astrocytic tumors revealed expression of GAL_1-R and GAL_3-R, but only at low levels and in a small subset of tumor cells. Interestingly, GAL_2-R-immunoreactivity was not detectable in astrocytic tumors.

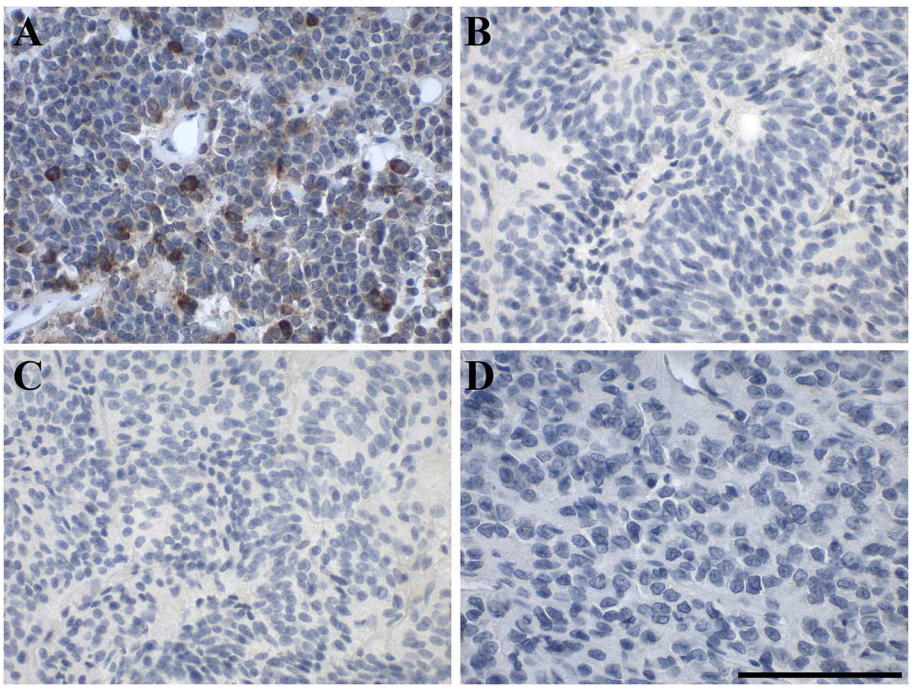

FIGURE 3 | Representative images of immunohistochemical staining of in human pituitary adenoma. **(A)** GAL (case 7), **(B)** GAL_1-R (case 5), **(C)** GAL_2-R (case 5), and **(D)** GAL_3-R (case 8). [scale bar: 100 μm].

Sixty percent of pilocytic astrocytomas (WHO grade I) displayed focal GAL-immunoreactivity (<1–18% GAL-positive tumor cells; **Figure 4A**, **Table 1**, **Supplementary Table 3**). In 57% of diffuse astrocytomas (WHO grade II), focal GAL-immunoreactivity (2–40% of tumor cells) was detected (**Figure 4D**). Also, 86% of anaplastic astrocytomas (WHO grade III) contained focal GAL-immunoreactivity (<1–65% of tumor cells; **Figure 4G**). In glioblastoma multiforme (WHO grade IV), GAL-positive cell staining was observed in 75% of cases (<1–30% of tumor cells, **Figure 5A**). In gliosarcoma (WHO grade IV), only half of the samples displayed focal GAL-immunoreactivity (15–70% of tumor cells, **Figure 5D**), whereas all giant cell glioblastomas (WHO grade IV) revealed focal GAL-immunoreactivity (35–80% of tumor cells; **Figure 5G**).

Only one out of 5 pilocytic astrocytoma (WHO grade I) showed GAL_1-R-immunoreactivity in some tumor cells (<1% of tumor cells), with low staining intensity (**Figure 4B**). GAL_1-R-immunoreactivity was not detectable in diffuse astrocytoma (WHO grade II; **Figure 4E**). About 43% of anaplastic astrocytomas (WHO grade III) revealed some weakly stained GAL_1-R-positive cells (<1% of tumor cells; **Figure 4H**). In 38% of glioblastoma multiforme (WHO grade IV), GAL_1-R-immunoreactivity was detected (<1–8% of tumor cells; **Figure 5B**). One third of gliosarcoma (WHO grade IV) and half of giant cell glioblastoma (WHO grade IV) samples were GAL_1-R-positive (≤1% of tumor cells; **Figures 5E,H**).

A single pilocytic astrocytoma (WHO grade I) showed substantial GAL_3-R expression in some tumor cells (<1% of tumor cells; **Figure 4C**). Tumor cell-associated GAL_3-R-immunoreactivity was not detectable in diffuse astrocytoma (WHO grade II), anaplastic astrocytoma (WHO grade III) and giant cell glioblastoma (WHO grade IV; **Figures 4F,I**, **5I**). In contrast, 63% of glioblastoma multiforme samples (WHO grade IV) and 50% of gliosarcomas (WHO grade IV) showed sparse GAL_3-R-immunoreactivity (<1–7% of tumor cells; **Figures 5C,F**).

Expression of GAL and GALRs in Oligodendroglial Tumors

IHC analysis of 15 human oligodendroglial tumors (WHO grades II and III) revealed focal and diffuse GAL-immunoreactivity in the majority of cases. (**Figure 6**, **Table 1**, **Supplementary Table 3**). Receptor expression was generally low and found only in a subset of tumor cells. Tumor cell-associated GAL_2-R-immunoreactivity was not detectable in oligodendroglial tumors.

In more detail, 78% of oligodendrogliomas (WHO grade II) showed focal GAL-immunoreactivity (<1–10% of tumor cells) and 89% contained areas of diffuse GAL staining (**Figure 6A**). Sixty percent of anaplastic oligodendrogliomas (WHO grade III) showed focal (<1–30% of tumor cells) and diffuse GAL-staining (**Figure 6D**).

GAL_1-R-immunoreactivity was detectable in 22% of oligodendrogliomas (WHO grade II; <1% of tumor cells; **Figure 6B**). Anaplastic oligodendroglioma (WHO grade III) revealed sparse tumor cell-associated GAL_1-R-immunoreactivity in 17% of samples (<1% of tumor cells; **Figure 6E**).

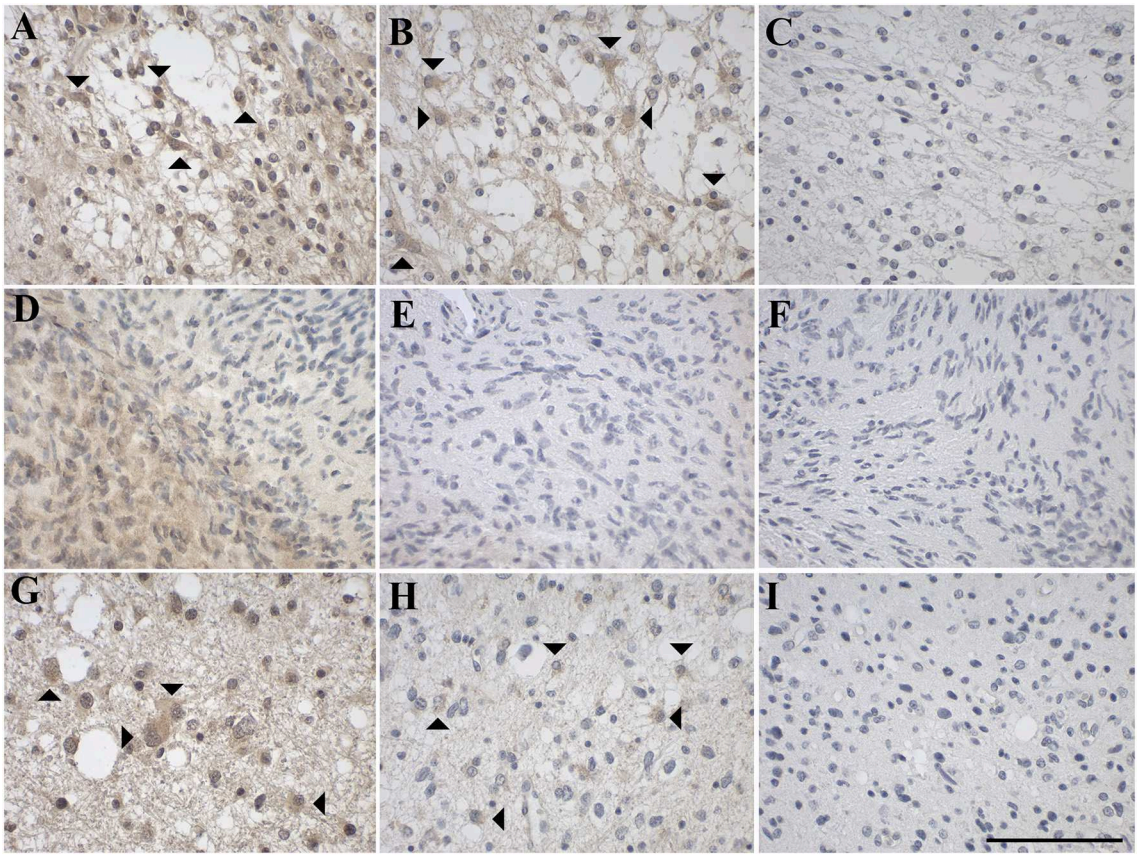

FIGURE 4 | Representative images of immunohistochemical staining of low-grade astrocytic tumors with **(A–C)** pilocytic astrocytoma (WHO grade I; case 1), **(D–F)** diffuse astrocytoma (WHO grade II; case 8), and **(G–I)** anaplastic astrocytoma (WHO grade III; case 12). GAL immunoreactivity is shown in **(A,D,G)**; GAL_1-R immunoreactivity in **(B,E,H),** and GAL_3-R immunoreactivity in **(C,F,I)**. Arrow heads indicate positive-stained glial cells. [scale bar: 100 μm].

Tumor cell-associated GAL_3-R-immunoreactivity was detectable in 11% of oligodendrogliomas (WHO grade II; <1% of tumor cells; **Figure 6C**). Sparse GAL_3-R-immunoreactivity was detected in 17% of anaplastic oligodendrogliomas (WHO grade III; <1% of tumor cells; **Figure 6F**).

Expression of GAL and GALRs in Mixed Neuronal-Glial Tumors

IHC analysis of three gangliogliomas (WHO grade I; **Figure 7**, **Table 1**, **Supplementary Table 3**) revealed focal (<1 and 6% of tumor cells) as well as diffuse GAL staining (**Figure 7A**) in two of the three cases. Interestingly one case was completely negative for focal and diffuse GAL staining.

GAL_1-R-immunoreactivity was detectable in 67% of gangliogliomas (WHO grade I; ≤1% of tumor cells (**Figure 7B**).

Tumor cell-associated GAL_3-R-immunoreactivity was detectable in 67% of gangliogliomas (WHO grade I; <1–3% of tumor cells; **Figure 7C**).

Expression of GAL and GALRs in Oligoastrocytic Tumors

IHC analysis of one oligoastrocytoma (OA, NOS; WHO grade II) and one anaplastic oligoastrocytoma (OAA, NOS; WHO grade III; **Supplementary Table 3**) revealed focal (20 and 90% of tumor cells) as well as diffuse GAL staining.

GAL_1-R-immunoreactivity was detectable in both cases (WHO grade II and III; <1% of tumor cells).

Tumor cell-associated GAL_2-R and GAL_3-R-immunoreactivity was not detectable.

Expression of GAL and GALRs in Tumor-Infiltrating Immune Cells

GAL and GALRs were also detected in infiltrating immune cells such a neutrophils and GAMs (**Supplementary Tables 2, 3**). GAL-immunoreactivity was sparse in neutrophilic granulocytes in glioma (5% of 57 cases) and was only observed in subpopulations (**Figure 8**). In 18% of glioma samples, GAMs were GAL positive (**Figure 9**). GAL-immunoreactivity was absent in tumor-associated immune cells in pituitary adenoma.

GAL_1-R-immunoreactivity in GAMs was observed in 16% of glioma samples but was absent in neutrophils. Although GAL_2-R-immunoreacitivity was undetectable in tumor cells of glioma and pituitary adenoma, 27% of glioma samples, particularly astrocytoma (WHO grade I-III), and 44% of

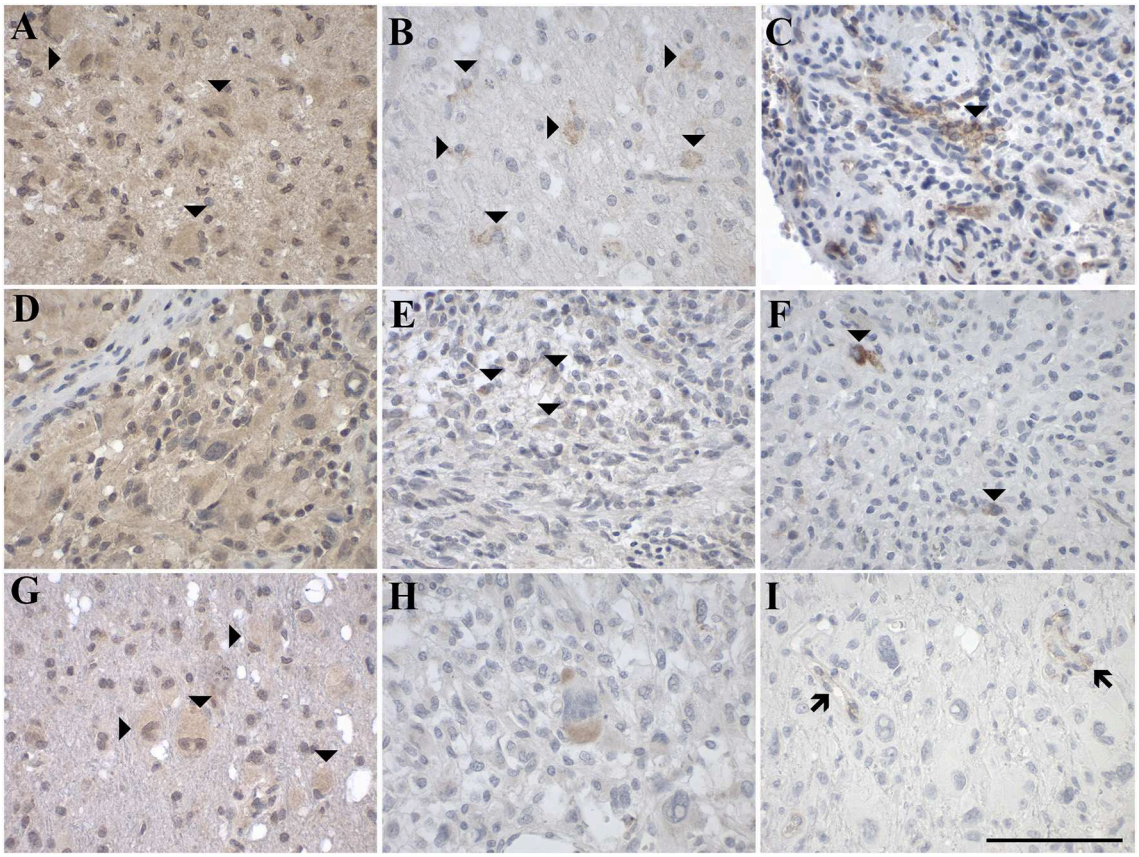

FIGURE 5 | Representative images of immunohistochemical staining of high-grade astrocytic tumors with **(A–C)** glioblastoma multiforme (WHO grade IV; case 18, 17, 20), **(D–F)** gliosarcoma (WHO grade IV; case 27, 25, 27), and **(G–I)** giant cell glioblastoma (WHO grade IV; case 31, 30, 31). GAL immunoreactivity is shown in **(A,D,G)**; GAL_1-R immunoreactivity in **(B,E,H),** and GAL_3-R immunoreactivity in **(C,F,I)**. Arrow heads indicate positive-stained cells whereas the arrows point out GAL_3-R-positive blood vessels. [scale bar: 100 μm].

pituitary adenomas revealed a small proportion of GAL_2-R-positive neutrophilic granulocytes (**Figure 8C**). GAL_2-R-positive GAMs were observed in one glioblastoma multiforme (WHO grade IV), one ganglioglioma (WHO grade I) and one anaplastic oligoastrocytoma (OAA, NOS; WHO grade III; **Figure 9C**).

GAL_3-R was much more abundant in tumor-associated GAMs. In 35% of glioma samples, particularly high-grade glioma (WHO grade IV), GAL_3-R-positive stained GAMs were identified (**Figures 9D**, **10**). Forty nine percent of glioma samples and two pituitary adenomas revealed GAL_3-R neutrophilic granulocytes (**Figure 8D**). GALR-positive GAMs were mainly localized near blood vessels or areas with necrotic tissue (**Figure 10**). GALR-positive neutrophilic granulocytes were mostly observed in hemorrhagic areas.

In addition to the presence of GAL_3-R in immune cells, endothelial cells of blood vessels in glioma and pituitary adenoma showed frequent GAL_3-R-immunoreactivity (**Figure 5I**, **Supplementary Tables 2, 3**). The other two receptors were not detected around blood vessels.

DISCUSSION

In this study, we show for the first time the cellular distribution of GALR proteins in human glioma, pituitary adenoma, and anterior pituitary gland.

Our findings correlate with our previous study, where GAL staining in glial cell bodies was found in 18 out of 20 (90%) human brain tumors, including glioblastoma multiforme (WHO grade IV), meningioma (WHO grade I-II), and gliosarcoma (WHO grade IV) (21). Presence of GALRs indicated by GAL binding occurred in 6 out of 15 gliomas (40%). In the present study, in 12 out of 18 (67%) gliomas (WHO grade IV), GALRs were detectable by IHC staining. The frequency of GALR-immunoreactivity in tumor cells was usually below 10% and the intensity of IHC staining was mainly low to medium, indicating low expression levels of the GALRs. Such low amounts of GALRs might be undetectable by GAL-binding studies. Due to the use of photoemulsions in binding studies using autoradiography, identification of the underlying cell type is not possible and therefore allocation of the binding to tumor cells, stroma or tumor-associated immune cells is not possible. Thus, we provide

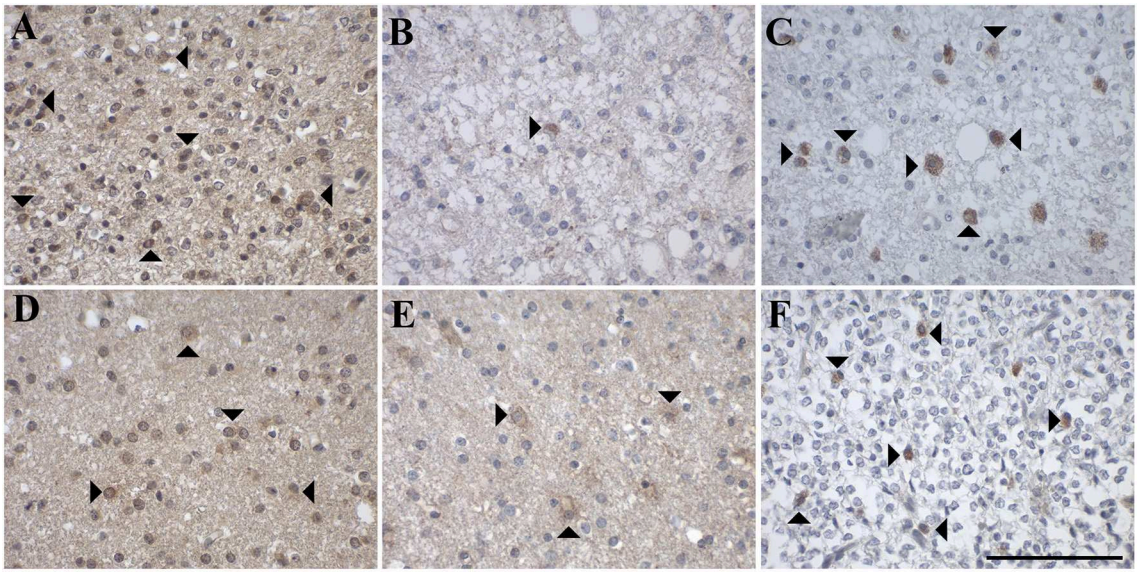

FIGURE 6 | Representative images of immunohistochemical stainings of oligodendroglial tumors with **(A–C)** oligodendroglioma (WHO grade II; case 37, 52, 52) and **(D–F)** anaplastic oligodendroglioma (WHO grade III; case 44, 44, 57). GAL immunoreactivity is shown in **(A,D)**; GAL_1-R immunoreactivity in **(B,E),** and GAL_3-R immunoreactivity in **(C,F)**. Arrow heads indicate positive-stained cells. [scale bar: 100 μm].

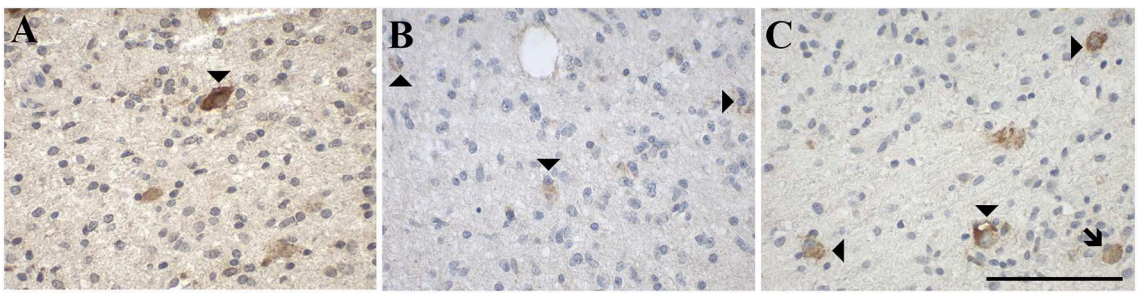

FIGURE 7 | Representative images of immunohistochemical stainings of ganglioglioma (WHO grade I), a mixed neuronal-glial tumor, which show **(A)** GAL immunoreactivity (case 34), **(B)** GAL_1-R immunoreactivity (case 36), and **(C)** GAL_3-R immunoreactivity (case 34). Arrow heads indicate positive-stained cells whereas the arrow points out a GAL_3-R positive GAM. [scale bar: 100 μm].

here the first evidence of GALR expression in tumor-infiltrating immune cells.

Previous RT-PCR analysis of glioblastoma multiforme (WHO grade IV) revealed that GAL_1-R is the most prominent receptor, followed by GAL_3-R and GAL_2-R (21). This is in accordance with our study, which revealed GAL_1-R and GAL_3-R but not GAL_2-R immunoreactivity. Low levels of the GALRs might be detectable by RT-PCR analysis but not IHC. This is also evident from our data in pituitary glands, where we detected all three GALRs by RT-PCR but only GAL_1-R and GAL_3-R by IHC staining.

Anti-proliferative effects of GAL and GAL_1-R have been reported for HNSCC (35, 36, 40, 49). As we observed only minor amounts of GALR-positive tumor cell populations in glioma, downregulation of these receptors may be a survival mechanism of glioma cells to ensure proliferation. In accordance with this hypothesis, GAL suppressed proliferation of human U251 and T98G glioma cells via GAL_1-R signaling (49).

Several studies reported GAL-like immunoreactivity in pituitary adenoma (11–16). The percentage of GAL-positive cases was dependent on the type of pituitary adenoma. Mainly ACTH-secreting pituitary adenomas were GAL-positive, whereas growth hormone- and prolactin-secreting as well as non-functioning pituitary adenomas showed lower frequencies of GAL-immunoreactivity. In ACTH-secreting adenoma, GAL seems to serve as a biomarker, with GAL levels being inversely correlated with tumor volume. Additionally, GAL-positive corticotroph adenomas were associated with a higher cure rate in patients (15), suggesting clinical relevance for GAL in this brain tumor type. In our study, 8 of 9 cases of pituitary adenoma had at least some areas with diffuse GAL-immunoreactivity. Our data on the expression of GAL in pituitary adenoma

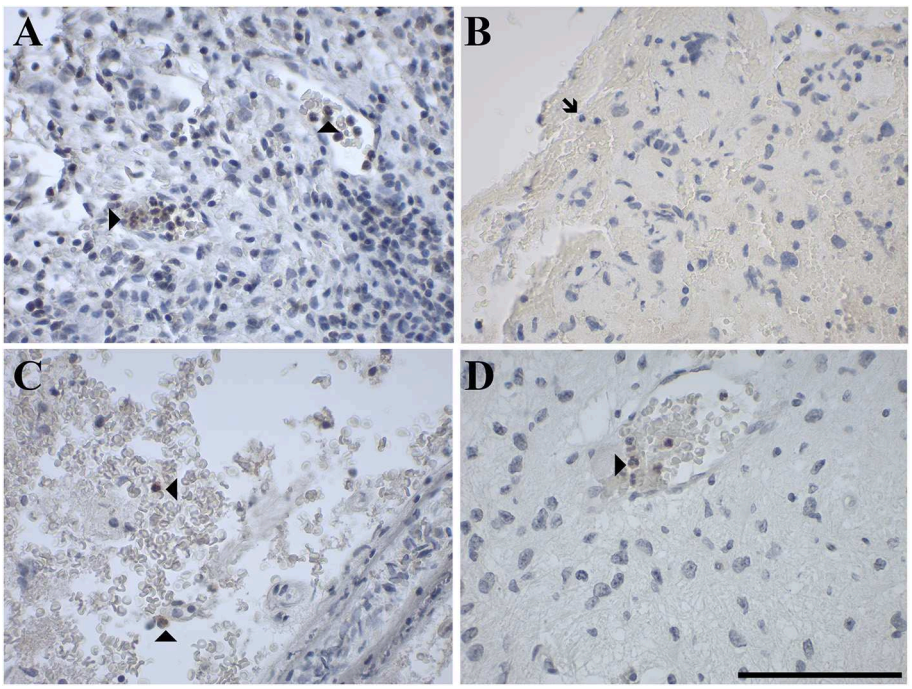

FIGURE 8 | Representative images of immunohistochemical staining of neutrophil granulocytes stained positive for **(A)** GAL in a gliosarcoma (WHO grade IV; case 29), negative for **(B)** GAL_1-R in a gliosarcoma (WHO grade IV; case 29) and positive for **(C)** GAL_2-R in an anaplastic astrocytoma (WHO grade III; case 15) and **(D)** GAL_3-R in a pilocytic astrocytoma (WHO grade I; case 5). Arrow heads indicate positive-stained neutrophil granulocytes, the arrow indicates a negative neutrophil granulocyte. [scale bar: 100 μm].

(**Supplementary Table 2**) correlate with previous published data, where 77% of patients with Cushing's disease (ACTH-secreting), 25% of patients with acromegaly (growth hormone-secreting), 13% of prolactinomas and 34% of non-functioning tumors expressed GAL (**Supplementary Table 5**).

Corresponding to observations in glioma samples, receptor downregulation might also be a possible mechanism in pituitary adenoma to escape anti-proliferative effects of GAL. While all pituitary gland samples expressed GAL_1-R and GAL_3-R, GALR expression was absent in pituitary adenoma. Furthermore, it also seemed that the tumor itself reduced GAL expression, as only 11% of pituitary adenoma showed focal GAL staining, in contrast to 100% of healthy pituitary glands. However, it cannot be ruled out that the observations in pituitary glands and pituitary adenoma are also age-related effects, as the median age of the groups was different.

In general, we observed focal as well as diffuse GAL-like immunoreactivity in human brain tumors. Diffuse GAL staining is representative of secreted GAL peptide being present extracellularly. However, it cannot be determined whether the secreted GAL originates either from the cells in the near vicinity showing focal GAL staining or from other brain regions. Additionally, it is unclear whether the secreted GAL originates from the tumor itself or from adjacent healthy tissue, for example, the pituitary which was shown to exhibit high GAL mRNA levels as well as medium to strong diffuse and focal GAL staining.

The expression of the galanin system analyzed by RT-PCR revealed case-dependent expression patterns of GAL and GALR mRNA (34). As already discussed above, RT-PCR analysis is more sensitive than IHC and this could partially explain why GALRs were not detectable in tumor cells of our pituitary adenomas by IHC analysis. As RNA is isolated from whole tumor extracts, expression of the receptors in non-tumor cells will also be detected. The level of GALRs expressing immune-infiltrating cells might at least partially account for the case differences in GALR mRNA expression levels in pituitary adenoma (34). Furthermore, GAL-immunoreactivity was much lower in pituitary adenoma as in the anterior pituitary gland. This is in accordance with GAL mRNA expression data, which showed lower GAL expression levels in 10 out of 13 pituitary adenomas (34).

The expression of GAL_3-R in small blood vessels is in agreement with the expression of GAL_3-R in the skin vasculature (48).

GAMs are key drivers of the local immunosuppressive microenvironment that promotes tumor progression and tumor resistance to immunomodulating therapeutics. Together with other myeloid cells, such as dendritic cells and neutrophils, GAMs actively shape glioma development and the glioma microenvironment, modulate the anti-tumoral immune response, and support angiogenesis, tumor cell invasion and proliferation (50). Therefore, it could also be possible that GAL is

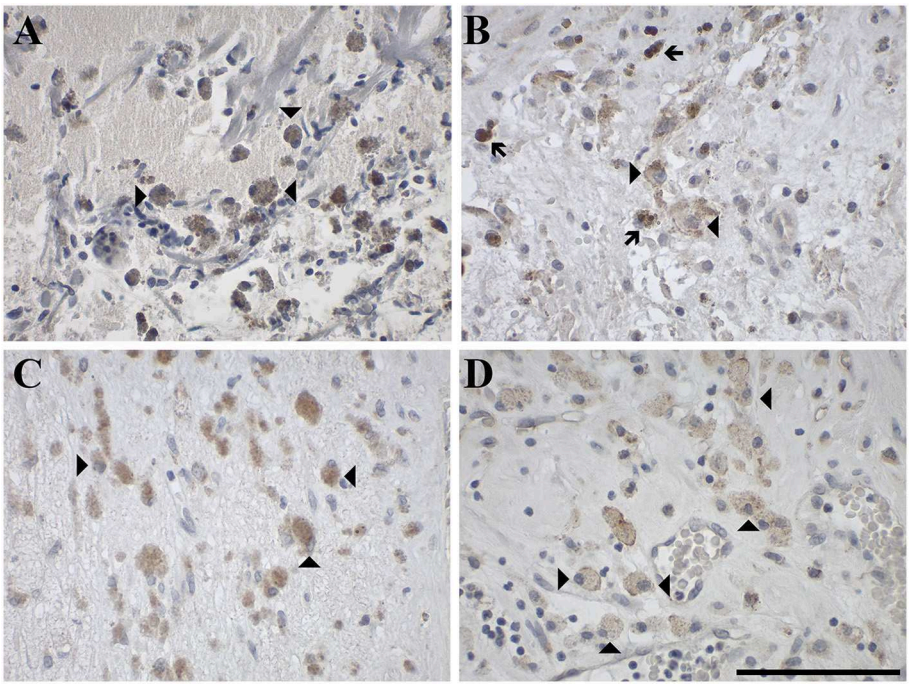

FIGURE 9 | Representative images of immunohistochemical staining of GAMs stained positive for **(A)** GAL in an oligodendroglioma (WHO grade II; case 51), **(B)** GAL_1-R in an anaplastic oligoastrocytoma, NOS III (case 54), **(C)** GAL_2-R in an anaplastic oligoastrocytoma, NOS III (case 54) and **(D)** GAL_3-R in a gliosarcoma (WHO grade IV; case 25). Arrow heads indicate positive-stained GAMs and arrows indicate hemosiderophages, which contain brown granules due to hemosiderin. [scale bar: 100 μm].

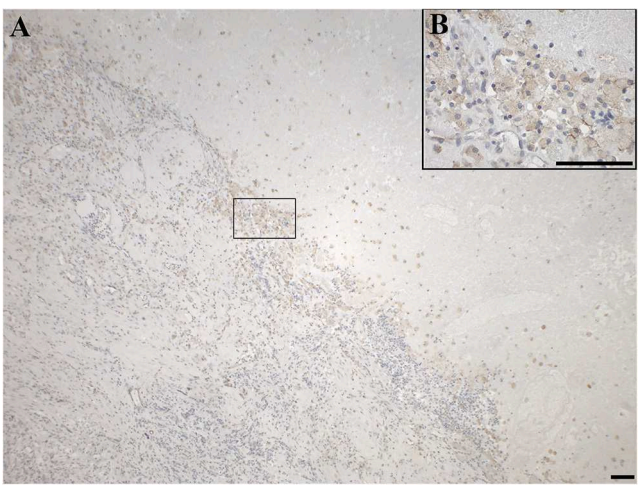

FIGURE 10 | Immunohistochemical staining for GAL_3-R of a gliosarcoma (WHO grade IV; case 25), showing positive GAMs at the junction of tumor tissue and necrotic tissue in **(A)** 4 × and **(B)** 40 × magnification. [scale bar: 100 μm].

secreted by the tumor cells showing focal GAL staining to boost tumor-supporting properties of the GAMs.

GAL and GALR mRNA expression has already been reported for immune cells isolated from peripheral blood. Macrophages express and secrete substantial amounts of galanin (7). However, GAMs showed no detectable GAL-immunoreactivity. In contrast, the majority of GAMs were GAL_3-R positive. This high proportion of GAL_3-R-immunoreactivity in GAMs is in contrast to the expression of GALRs in peripheral macrophages. A xanthelasma, also referred to as xanthoma, is a cluster of foam cells in the connective tissue of the skin. The foam cells are formed by macrophages accumulating lipids by phagocytosis (51). We reported membrane-associated GAL_1-R as well as GAL_2-R staining on some macrophages in the xanthelasma deposits (7). To our knowledge, there are no other studies available on the expression of GALRs in tumor-associated macrophages and therefore it is not known if the expression of GALRs is restricted to GAMs or if this is also the case in tumor-associated macrophages of other tumor entities. In addition, we are not aware of any study reporting the expression of the GAL system, especially GAL_3-R, in tumor-associated neutrophilic granulocytes.

Interestingly, in our previous studies we observed that GAL can have pro- and anti-inflammatory properties on macrophage function depending on their differentiation and polarization status (7). Therefore, it could be possible that GAL also induces tumor-suppressing functions in GAL_3-R positive GAMs. Regarding immunity and inflammation, we reported recently that GAL_3-R signaling has both pro- and anti-inflammatory properties (48, 52).

Currently, possible treatment strategies targeting GALR subtypes are still hampered by the lack of single-subtype specific agonists or antagonists [for review see (6)]. Most available selective ligands are peptidergic compounds, which makes their clinical application problematic due to peptide degradation. Furthermore, a GAL_3-R-specific non-peptidergic antagonist is available, but we showed non-GALR-mediated toxicity of this compound (53). Therefore, there is a need to develop novel selective, stable and non-peptidergic GALR ligands.

In conclusion, our data indicate that GALR signaling could influence the behavior of tumor cell-associated immune cells. Future studies should focus on the characterization of the immune cell subtypes expressing GALRs in glioma, for example by using markers to differentiate GAMs into resident microglia and bone marrow-derived macrophages. Based on the size and shape of the cells and their nuclei, GALR-positive GAMs resemble the bone marrow-derived macrophage type. Recently it has been shown that GAL is able to influence immune cell behavior by modulating cytokine expression and release, as demonstrated in human neutrophils, natural killer cells, monocytes and macrophages (7, 48, 54, 55). Furthermore, galanin down-regulates microglial tumor necrosis factor-alpha production and induces microglial migration (56, 57). Thus, strategies targeting tumor-supportive myeloid cells represent an encouraging novel therapeutic approach and could also be considered for the GAL system.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SF and JL designed and performed experiments. SF, JL, SB, and TR analyzed data. SF, SB, and BK contributed to drafting the manuscript. SW collected and/or provided human patient samples, rendered the neuropathology diagnoses, co-edited the manuscript, and critically discussed the data. BK obtained resources for the study, designed experiments, critically discussed the data, and co-edited the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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Involvement of Secretin in the Control of Cell Survival and Synaptic Plasticity in the Central Nervous System

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With emerging evidence showing a wide distribution of secretin (SCT) and its receptor (SCTR) in the central nervous system (CNS), the putative neuropeptide role of SCT has become more appreciated since the disruption of SCT/SCTR axis affects various neural functions. This mini review thus focuses on the effects of SCT on cell survival and synaptic plasticity, both of which play critical roles in constructing and maintaining neural circuits with optimal output of behavioral phenotypes. Specifically, SCT-dependent cellular and molecular mechanisms that may regulate these two aspects will be discussed. The potential complementary or synergistical mechanisms between SCT and other peptides of the SCT superfamily will also be discussed for bridging their actions in the brain. A full understanding of functional SCT/SCTR in the brain may lead to future perspectives regarding therapeutic implications of SCT in relieving neural symptoms.

Keywords: secretin, cell survival, neural development, synaptic plasticity, learning and memory

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INTRODUCTION

The gastrointestinal functions of secretin (SCT) has been recognized for more than one century since it was initially noted for its role in facilitating pancreatic exocrine secretion of bicarbonate-rich fluid (Bayliss and Starling, 1902). However, the central importance of SCT has been gradually appreciated only during the last four decades. One piece of the pioneering proof in 1979 suggested that SCT could exert a strong stimulatory effect on cyclic adenosine monophosphate (cAMP) in neuroblastoma glioma hybrid cells (Propst et al., 1979). Later in the same year, another study further identified SCT-like bioactivity in extracts of porcine brain, thus for the first time implying the existence of SCT in the central nervous system (CNS) (Mutt et al., 1979). Since then a growing number of studies have expanded the gene expression map of SCT and its receptor (SCTR) in the brain. To date, SCT and SCTR have been found to be expressed from forebrain to hindbrain structures including cerebral cortex, hippocampus, central amygdala (CeA), thalamus, hypothalamus, pons, cerebellum, medulla oblongata and nucleus of the solitary tract (NST) [reviewed in Wang R. et al. (2019)]. In a species-dependent manner, SCT and SCTR have been recognized in human (Carlquist, 1985; Chow, 1995), mouse (Lan et al., 1994; Vassilatis et al., 2003), rat (Gossen et al., 1989; Ishihara et al., 1991), rabbit (Gossen et al., 1990; Svoboda et al., 1998), and many other mammalian species (Nilsson et al., 1980; Mats et al., 1981; Shinomura et al., 1987; Buscail et al., 1990; Bounjoua et al., 1991). Notably, the amino acid sequence of mature SCT and SCTR peptides is well-conserved across these species. Taken together, the wide distribution

of SCT and SCTR throughout the brain and the high degree of sequence conservation among species suggest their biological significance. The key question is what roles does SCT have in the CNS under normal and pathological conditions? To further elaborate the neurological functions of SCT, researchers have been working on the development of SCT and SCTR gene knockout mouse models. Using those models in multiple biological and/or behavioral tests thus provides strong genetic support for the functional diversity of SCT in the CNS [reviewed in Zhang and Chow (2014)], which primarily includes the role of hippocampal SCT in social recognition and spatial memory, regulation of water homeostasis (Chu et al., 2007, 2009) and food intake by hypothalamic SCT (Cheng et al., 2011), and cerebellar SCT-mediated motor coordination and motor learning (Zhang et al., 2014). Moreover, SCT has been implicated in certain neurodevelopmental disorders such as autism and schizophrenia (Alamy et al., 2004; Toda et al., 2006). In this mini review, we will discuss current evidence for its specific effects on cell survival and synaptic plasticity in the CNS.

EFFECTS OF SCT ON CELL SURVIVAL AND NEURAL DEVELOPMENT

The well-coordinated interplay between neuronal death and survival constitutively occurs during development of the CNS. On one hand, apoptosis is required to maintain the adequate neuronal population by eliminating excess neurons, achieving a "quality-control" process to remove developmental errors. On the other hand, survival of neural progenitor cells and new-born neurons is required to maintain normal neurogenesis and further neural plasticity within the adult brain (Meier et al., 2000). One of the most extensively studied effects of SCT resides in its neuroprotective potency against apoptosis and in favor of cell survival. Physiologically, SCT deficiency results in excess apoptosis in the dentate gyrus (DG) of hippocampus (Jukkola et al., 2010) and the external granular layer (EGL) of cerebellum during early postnatal development (Wang et al., 2017). Using *in situ* Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay, more apoptotic cells were found in these two subregions of SCT knockout mice where neural progenitor cells reside and undergo intensive proliferation. However, when the proliferation of neural progenitor cells was examined, there was no significant difference in the number of 5-ethynyl-2′-deoxyuridine (EdU)-incorporated new-born neurons between SCT-deficient and wild-type mice (Wang et al., 2017). Under pathological conditions such as ethanol exposure at early postnatal age, the number of apoptotic cells in the EGL of cerebellum as well as in the striatum was obviously increased in both SCTR knockout and wild-type mice, but the increase was much more significant with SCTR deficiency (Hwang et al., 2009). These findings thus indicate that SCT and SCTR are necessary for the survival, but not proliferation, of neuronal progenitors in both physiological and pathological CNS.

In addition, the survival of new-born neurons also requires intact SCT/SCTR signaling. In the hippocampal DG, the total number of EdU-labeled new-born cells surviving after 3 weeks was remarkably reduced in SCT-deficient mice (Jukkola et al., 2010). In the cerebellum, newly generated granular cells in the EGL progressively migrate inward to reside within the destined positions of the internal granular layer (IGL) where further maturation follows. Similar phenotypes also occur in the cerebellar IGL where s higher number of apoptotic cells was found in SCT knockout mice than that in their wild-type littermates (Wang et al., 2017). Based on current knowledge, however, it is still unclear whether the poor survival rate of granular cells is due to the lack the neurotrophic factors that are needed for survival, or due to the deficits for their inability to establish appropriate synaptic projections with target neurons as a consequence of their premature migration (Wang et al., 2017). It is worth noting that the density of cerebellar Purkinje cells also decreased under SCT deprivation. Such phenotype appears to depend on a cell-autonomous effect of SCT as the conditional knockout of SCT in Purkinje cells gave rise to a comparable reduction of Purkinje cell density (Wang et al., 2017).

During the later stage of neural development, intact dendritic arborization is equally necessary to ensure optimal structure and functionality of the CNS (Valnegri et al., 2015). We recently found prominently impaired dendritic arborization as displayed by fewer branches and shorter lengths in Purkinje cells of SCT knockout mice. The density of their dendritic spines was also dramatically decreased in SCT knockout mice, suggesting a neurotrophic role of SCT in the cerebellum (Wang et al., 2017). However, SCT or SCTR deprivation did not affect dendritic morphology in hippocampal CA1 pyramidal neurons, whilst SCTR deficiency did reduce dendritic spines in the first order apical dendritic branches of those pyramidal neurons (Nishijima et al., 2006; Yamagata et al., 2008). We thus consider the possibility that SCT may exert a preferential or specific influence over the dendritic and spine development across different brain regions. Moreover, such impairments in dendritic arborization are thought to disrupt its wiring with presynaptic boutons, thus adversely affecting synaptic transmission and plasticity, leading to behavioral deficits.

Although the data reviewed above indicate the necessary role of intact SCT/SCTR axis in the CNS for cell survival and neural development, the understanding for its molecular mechanisms is far from complete. In general, SCT binding triggers two distinct signaling pathways *via* activation of adenylyl cyclase (AC) and phospholipase C (PLC). As the downstream effector, AC initiates an intracellular accumulation of the secondary messenger cAMP and the subsequent activation of cAMP-dependent protein kinase A (PKA), while PLC catalyzes the production of two secondary messengers, inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG) to induce Ca^{2+} release from endoplasmic reticulum and to activate protein kinase C (PKC), respectively. We thus believe that the contribution of SCT/SCTR signaling to neuronal survival and development is probably associated with those molecular pathways. Our recent studies have proposed a schematic diagram revealing the signaling pathways involved in neuroprotective effect of SCT in the cerebellum (Wang et al., 2017; Wang L. et al., 2019). Using *ex vivo* cerebellar slice culture combined with pharmaceutical manipulation, we found that SCT induced phosphorylation of cAMP response element binding

protein (CREB) largely by cAMP/PKA signaling pathway (Wang et al., 2017). As the common downstream target effector of multiple survival pathways including PI3K/Akt, MAPK/ERK, and cAMP/PKA pathways, CREB serves as one transcription factor to up-regulate anti-apoptotic proteins such as Bcl-2 and Bcl-xL (Finkbeiner, 2000). Further examinations found that SCT-induced CREB activation was also dependent on extracellular signal regulated kinase 1/2 (ERK1/2) but not Akt (Protein Kinase B), and that only concurrent suppression of both PKA- and ERK-dependent pathways can effectively abolish the anti-apoptotic effect of SCT (Wang et al., 2017). A later study also showed that PKA- and ERK-dependent CREB signaling contributed to the effect of SCT on mediating Bcl-2 and Bcl-xL expression *via* a synergistical manner (Wang L. et al., 2019). Consistently, the activity of those critical signaling molecules were all strikingly reduced in the cerebellum of SCT-deficient mice (Wang et al., 2017; Wang L. et al., 2019). Here in terms of SCT-induced ERK1/2 phosphorylation, it was also partially inhibited by the presence of PKA inhibitor, suggesting the participation of both cAMP/PKA-dependent and -independent signaling pathways. These results thus add more complexity for elucidating the mechanisms underlying neuroprotection of SCT. In addition to the cerebellum, the cAMP/PKA/CREB pathway has also been found to be involved in the neural actions of SCT within the hypothalamus (Mak et al., 2019) and CeA (Pang et al., 2015). Therefore, we may expect that such molecular mechanisms also play a role for anti-apoptotic effects of SCT in many other areas of the CNS.

So far, few researchers have been working on the mechanisms underlying SCT's neurotrophic effects. One early *in vitro* study demonstrated that SCT promoted both the number and length of neurites in cultured pheochromocytoma PC12 cells through PKA-ERK1/2 pathway (Kim et al., 2006). Notably, CREB is also known to mediate dendritic morphogenesis through transcriptional activation (Redmond et al., 2002). Therefore, it is possible that SCT stimulates dendrite growth and spine formation through similar signaling pathways as proposed above, although evidence is warranted for supporting this notion. In summary, our current findings illustrate that diverse molecular mechanisms synergistically contribute to SCT's neuroprotective role in the cerebellum (**Figure 1A**), providing clues for understanding potential signaling pathways by which SCT controls neural functions. Further studies are required to investigate how these pathways interact and converge to modulate specific roles of SCT.

EFFECTS OF SCT ON SYNAPTIC PLASTICITY AND MEMORY

Long-term potentiation (LTP) and long-term depression (LTD) are two critical processes that underlie long-term synaptic plasticity. Both are long-lasting changes in synaptic strength resulting from specific patterns of synaptic activity, and are considered as putative synaptic mechanisms contributing to learning and memory (Citri and Malenka, 2008). To date, only limited research has been conducted to investigate the role of SCT/SCTR signaling in regulating synaptic plasticity, although animal experiments have clearly shown its essential role in learning and memory. SCTR-dependent LTP was first

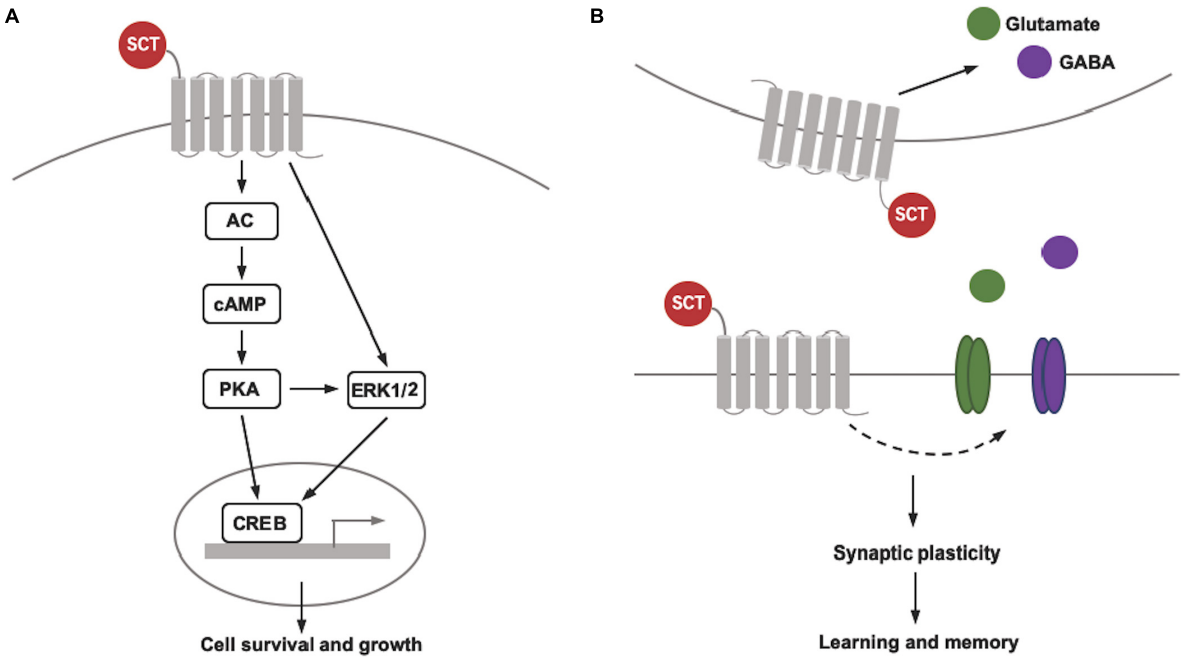

FIGURE 1 | Schematic diagram showing SCT's pleiotropic actions on cell survival and synaptic plasticity. **(A)** SCT/SCTR signaling pathway that contributes to the cell survival and growth. **(B)** Bidirectional regulation of synaptic plasticity of SCT *via* different pre- and post-synaptic mechanisms.

noted at the hippocampal Schaffer collateral to CA1 pyramidal neuron (SC-CA1) synapse (Nishijima et al., 2006), a neural circuit that has been well-studied as a key component for hippocampal-dependent memory encoding. Utilizing the SCTR-knockout mouse model, the authors found that a high-frequency stimulation (two trains at 100 Hz for 1 s separated by 20 s) at the SC-CA1 synapse failed to induce an apparent LTP of population excitatory postsynaptic potentials (pEPSPs). In specific, both the induction and maintenance of LTP were significantly impaired in SCTR-deficient mice (Nishijima et al., 2006). A consistent phenotype of LTP deficit was later obtained in SCT-knockout mice, which also showed a remarkable decrease in LTP induction and maintenance compared to their wild-type controls (Yamagata et al., 2008). In conjunction with the prominent expression of SCT and SCTR in the hippocampal CA1 region (Nishijima et al., 2006; Yamagata et al., 2008), these findings collectively indicate that intact SCT/SCTR signaling is needed to induce normal LTP in the CA1 area of the hippocampus. More importantly, as a consequence of LTP dysfunction at the SC-CA1 synapse, SCT-knockout and SCTR-knockout mice exhibited behavioral deficits of spatial learning in the water maze task and social recognition memory in the partition test (Nishijima et al., 2006; Jukkola et al., 2010), highlighting the functional significance of SCT/SCTR signaling-mediated synaptic plasticity.

The effect of SCT on synaptic plasticity and memory has also been implicated in the cerebellum. In rats, infusion of SCT into the cerebellar cortex facilitated the acquisition of delay eyeblink conditioning (EBC), a classical cerebellum-dependent motor learning behavior, while intracerebellar infusion of SCTR antagonist exerted the opposite effect and neither of the infusions significantly affected the extinction phase of delay EBC (Williams et al., 2012; Fuchs et al., 2014). These two separate studies from the same research group demonstrate the activation of SCTR in the cerebellum by both exogenous and endogenous SCT during the learning process of EBC. Moreover, motor learning deficits have been observed in mice lacking SCT or SCTR. In particular, when SCT gene is specifically deleted from cerebellar Purkinje neurons, significant learning deficits in the accelerating rotarod test were observed in those transgenic mice (Zhang et al., 2014). These findings from different mouse models thus add profound evidence for the functional role of cerebellar SCT in motor skill learning. To provide mechanistic explanations, further studies are still needed to directly investigate the effect of SCT on synaptic plasticity of cerebellar circuits, which can be linked to these behavioral changes. It has been growingly believed that different forms of plasticity in the cerebellar cortex operating in a distributed and synergistic manner underlie motor learning (Gao et al., 2012). For example, as supported by a recent study, EBC is dependent on both LTD at the parallel fiber-Purkinje cell (PF-PC) synapse and feed-forward inhibition of molecular layer interneuron-Purkinje cell (MLI-PC) transmission with both mechanisms compensating for each other's disruption (Boele et al., 2018). As the presynaptic modulation, SCT may induce endogenous release of glutamate from the cerebellum and facilitate GABA release from presynaptic basket cell terminals onto postsynaptic Purkinje cells (Yung et al., 2001; Lee et al., 2005). Meanwhile, on the postsynaptic side, SCT potentiate the inhibition of Purkinje cells by reducing surface expression of Kv1.2 at basket cell-Purkinje cell synapses and in Purkinje cell dendrites (Williams et al., 2012; Fuchs et al., 2014). In addition, SCT-induced glutamate release and surface Kv1.2 reduction may also facilitate PF-PC LTD. These findings suggest that SCT has potential in mediating different forms of cerebellar cortical plasticity.

In contrast to the improvement of hippocampus- and cerebellum-related memory, SCT suppresses conditioned fear memory as demonstrated by the decreased magnitude of conditioned fear-induced startle response in rats following

TABLE 1 | Cellular distribution of SCT/SCTR and their effects on cell survival and synaptic plasticity in specific brain regions: implications for learning and memory.

	Hippocampus	Cerebellum	Amygdala
SCT/SCTR expression	SCT in dentate gyms (DG), hilus, molecular layer (Yamagata et al., 2008); SCTR in CA1 (Nishijima et al., 2006)	SCT in Purkinje neuron, deep cerebellar nuclei (DCN) (Yung et al., 2001; Zhang et al., 2014); SCTR in Purkinje neuron, basket cell, granular cell progenitor (GCP, during postnatal development) (Yung et al., 2001; Wang et al., 2017)	SCT and SCTR in central nucleus of the amygdala (CeA) (Nozaki et al., 2002; Yang et al., 2004)
Cell survival and neural development	Reduced survival of neural progenitor cells and new-born neurons in the DG of SCT knockout mice (Jukkola et al., 2010); Reduced number of dendritic spines in CA1 pyramidal neurons of SCTR knockout mice (Nishijima et al., 2006)	Increased apoptosis in the external granular layer (EGL) and internal granular layer (IGL) of SCT knockout mice (Wang et al., 2017); Significant ethanol-induced apoptosis in the EGL of SCTR knockout mice (Hwang et al., 2009); Reduced Purkinje cell number in SCT knockout and Purkinje cell-specific SCT knockout (Pur-Sct$^{-/-}$) mice (Zhang et al., 2014; Wang et al., 2017) Impaired dendritic arborization and reduced spine density in the Purkinje neurons of SCT knockout mice (Wang et al., 2017)	N/A
Synaptic plasticity	Decreased LTP induction and maintenance at the Schaffer collateral-CA1 (SC-CA1) synapse in SCT and SCTR knockout mice (Nishijima et al., 2006; Yamagata et al., 2008)	Putative SCT-induced LTD at the parallel fiber-Purkinje cell (PF-PC) synapse (Lee et al., 2005; Williams et al., 2012)	N/A
Behavioral phenotypes	Impaired spatial learning ability in SCT knockout mice (Jukkola et al., 2010); Impaired spatial learning and social recognition behaviors in SCTR knockout mice (Nishijima et al., 2006)	SCTR-dependent acquisition of delay eyeblink conditioning (EBC) (Williams et al., 2012; Fuchs et al., 2014) Motor learning deficits in SCT knockout, SCTR knockout and Pur-Set$^{-/-}$ mice (Zhang et al., 2014)	SCT-inhibited conditioned fear memory (Myers et al., 2004)

peripheral administration (Myers et al., 2004). Such inhibition of fear conditioning by SCT was thought to depend upon amygdala, a brain site with a critical role in the acquisition and expression of conditioned fear memory. Using an *in vitro* autoradiography technique, one previous study has reported moderate SCT binding in the CeA (Nozaki et al., 2002). SCT and SCTR mRNA expression in the CeA was also detected by quantitative real-time PCR (Yang et al., 2004). As functional evidence, both peripheral and central injection of SCT induced intensive expression of the immediate-early gene c-Fos in the CeA of rats (Goulet et al., 2003; Welch et al., 2003). More specifically, local microinjection of SCT into the CeA has been recently revealed to modulate spontaneous firing of CeA neurons (Pang et al., 2015). In particular, consistent with these animal data, intravenous administration of SCT into human clearly increased the amygdala activation in response to fear stimuli (Yurgelun-Todd et al., 2008), supporting the idea that SCT may modulate amygdala activity and synaptic plasticity during fear learning and memory. Taken together, SCT has emerged as a pleiotropic neuropeptide to regulate the bidirectional long-term synaptic plasticity and thereby regulate learning and memory functions (**Figure 1B**). However, it is still in need of more research to clarify the specific role of SCT in controlling different forms of plasticity and to illustrate their underlying cellular and molecular mechanisms.

CONCLUSION AND FUTURE PERSPECTIVES

Some striking results on the central roles of SCT have been obtained in the past 40 years. Here we mainly reviewed the involvement of SCT in the control of cell survival and synaptic plasticity and thereby in the regulation of neural development and memory process as summarized in **Table 1**. The phenotypes of SCT and SCTR knockout mice are generally consistent despite of the differences of their cell-specific expression within brain areas, suggesting SCT's pleiotropic actions on cell survival and synaptic plasticity are exerted by specifically binding to SCTR. Future studies should be performed to explore the cell-autonomous and non-autonomous mechanisms of SCT so as to gain a more comprehensive understanding on SCT's functional profiles.

In phylogenetic analysis, SCT is categorized into a peptide superfamily, which also consists of vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP) and many other members with particular importance in the CNS. Interestingly, SCT and these neuropeptides have been found to share some overlapping neural functions. For example, both PACAP and VIP can act as a powerful neuroprotective factor and promote cell survival through cAMP signaling pathways with direct modulation on Bcl-2 (Gutiérrez-Cañas et al., 2003; Castorina et al., 2008). Additionally, PACAP-deficient and PAC1 receptor-deficient mice also showed reduced hippocampal LTP and impaired hippocampus-dependent recognition memory and associative learning (Otto et al., 2001; Matsuyama et al., 2003; Takuma et al., 2014). Therefore, we propose that SCT may work with different neuropeptides in a complementary or synergistical manner to fine-tune the behavioral output of neural circuits across different brain regions. Indeed, a recent study has found that receptors of SCT and glucagonlike peptide-1 (GLP-1), another member of SCT superfamily, are able to form heteromer in cells coexpressing these two receptors. The heteroreceptor complexes mediated cell responses to SCT by reducing intracellular calcium and inducing the cointernalization of both receptors, and as a result may also bring functional alterations to stimulatory actions of GLP-1 (Harikumar et al., 2017). This illustrates that SCT and GLP-1 can achieve some combinational effects *via* heterodimerization of their receptors. Meanwhile, while sharing functional similarities on stimulating insulin secretion, SCT and GLP-1 have the opposite roles in the regulation of water intake (Lee et al., 2010; McKay et al., 2014). Some consideration should be still given to the distinct actions and mechanisms of each peptide. Combination of the beneficial effects of SCT and its cousin peptides might hopefully improve multiple biological activities and thereby achieve optimal therapeutic outcomes.

Both neuronal loss and deficits in long-term synaptic plasticity are pathological features in various neurodegenerative disorders such as Alzheimer' s disease, Parkinson' s disease, and Huntington's disease. With the potency of promoting cell survival and modulating synaptic plasticity, SCT or its analogs may serve as a therapeutic agent targeting those neurological diseases. Future studies can be performed to test the effects of SCT infusion or pharmacological activation of SCTR in animal models with brain pathologies and cognitive disability. The elucidation of the cellular and molecular mechanisms underlying SCT neural functions may provide insights for precise intervention and pharmaceutical development.

AUTHOR CONTRIBUTIONS

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Activation of the VPAC2 Receptor Impairs Axon Outgrowth and Decreases Dendritic Arborization in Mouse Cortical Neurons by a PKA-Dependent Mechanism

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Clinical studies have shown that microduplications at 7q36.3, containing *VIPR2*, confer significant risk for schizophrenia and autism spectrum disorder (ASD). *VIPR2* gene encodes the VPAC2 receptor for vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP). Lymphocytes from patients with these mutations exhibited higher *VIPR2* gene expression and VIP-induced cAMP responsiveness, but mechanisms by which overactive VPAC2 signaling may lead to these psychiatric disorders are unknown. We have previously found that repeated administration of a selective VPAC2 receptor agonist Ro25-1553 in the mouse during early postnatal development caused synaptic alterations in the prefrontal cortex and sensorimotor gating deficits. In this study, we aimed to clarify the effects of VPAC2 receptor activation on neurite outgrowth in cultured primary mouse cortical neurons. Ro25-1553 and VIP caused reductions in total numbers and lengths of both neuronal dendrites and axons, while PACAP38 facilitated elongation of dendrites, but not axons. These effects of Ro25-1553 and VIP were blocked by a VPAC2 receptor antagonist PG99-465 and abolished in VPAC2 receptor-deficient mice. Additionally, Ro25-1553-induced decreases in axon and dendritic outgrowth in wild-type mice were blocked by a protein kinase A (PKA) inhibitor H89, but not by a PKC inhibitor GF109203X or a mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor U0126. PACAP38-

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induced facilitation of dendritic outgrowth was blocked by U0126. These results suggest that activation of the VPAC2 receptor impairs neurite outgrowth and decreases branching of cortical neurons by a PKA-dependent mechanism. These findings also imply that the *VIPR2*-linkage to mental health disorders may be due in part to deficits in neuronal maturation induced by VPAC2 receptor overactivation.

Keywords: VPAC2 receptor, psychiatric disorders, cortical neurons, axon, dendrite

INTRODUCTION

Accumulating evidence indicates that a number of rare copy number variants (CNVs), including both deletions and duplications, have been strongly associated with schizophrenia and neurodevelopmental disorders such as autism spectrum disorder (ASD) (Sullivan et al., 2012; Foley et al., 2017; Deshpande and Weiss, 2018; Vicari et al., 2019). Among the most highly penetrant genetic risk factors for neuropsychiatric disorders, clinical studies have shown that microduplications at 7q36.3, containing *VIPR2*, confer significant risk for schizophrenia (Levinson et al., 2011; Vacic et al., 2011; Yuan et al., 2014; Li et al., 2016; Marshall et al., 2017) and ASD (Vacic et al., 2011; Firouzabadi et al., 2017). *VIPR2* encodes VPAC2, a seven transmembrane heterotrimeric G protein-coupled receptor (Gs) that binds two homologous neuropeptides with high affinity, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP). Lymphocytes from patients with these microduplications exhibited higher *VIPR2* gene expression and VIP responsiveness (cAMP induction) (Vacic et al., 2011), demonstrating the functional significance of the microduplications. Additionally, the blood concentration of VIP, but not PACAP, was higher in children with ASD compared to healthy control subjects (Nelson et al., 2001). These suggest that overactivation of the VPAC2 receptor signaling is involved in the etiology of schizophrenia and ASD. We previously found that repeated administration of the selective VPAC2 receptor agonist Ro25-1553 in the mouse during early postnatal development caused prepulse inhibition deficits and reductions in synaptic proteins synaptophysin and postsynaptic density protein 95 (PSD-95) in the prefrontal cortex, but not in the hippocampus (Ago et al., 2015). Recently, Tian et al. (2019) have developed a conditional human *VIPR2* CNV bacterial artificial chromosome (BAC) transgenic (h*VIPR2*-BAC tg) mouse model of *VIPR2* CNV, and they reported that h*VIPR2*-BAC tg mice showed cognitive, sensorimotor gating, and social behavioral deficits and decrease in the complexity of dendritic arborization of the striatal spiny projection neurons. Additionally, VPAC2 receptor knockout mice have abnormal dendritic morphology of the prefrontal cortex neurons, but not basolateral amygdala neurons (Ago et al., 2017). These findings suggest that the VPAC2 receptor plays an important role in the regulation of the dendritic morphology and overactivation of the VPAC2 receptor might impair neural development in the brain.

Both PACAP and VIP have been known to regulate cell proliferation, differentiation, survival, maturation, neurite outgrowth, and expression of trophic factors (see review: Waschek, 1996, 2002; Muller et al., 2006; Falluel-Morel et al., 2007; Hill, 2007; Vaudry et al., 2009; Harmar et al., 2012). In particular, our and other studies have shown that PACAP and VIP promote neurite outgrowth in sympathetic precursors (Lu et al., 1998; DiCicco-Bloom et al., 2000; Nicot and DiCicco-Bloom, 2001; Suh et al., 2001), pheochromocytoma PC12 cells (Deutsch and Sun, 1992; Hernandez et al., 1995; Lazarovici et al., 1998; Vaudry et al., 2002; Sakai et al., 2004; Manecka et al., 2013), neuroblastoma NB-OK, Neuro2a and SH-SY5Y cells (Deutsch et al., 1993; Héraud et al., 2004; Monaghan et al., 2008; Kambe and Miyata, 2012) and primary cultured hippocampal neurons (Leemhuis et al., 2007; Kambe and Miyata, 2012; Ogata et al., 2015), cerebellar granule cells (Gonzalez et al., 1997; Falluel-Morel et al., 2005), dorsal root ganglion cells (White and Mansfield, 1996; White et al., 2000), and trigeminal ganglion cells (Fukiage et al., 2007). In contrast, both PACAP and VIP inhibited the induction of dendritic growth by bone morphogenetic protein-7 (BMP-7) in primary cultured sympathetic neurons (Drahushuk et al., 2002). This highlights a hierarchal reversal of action that may occur in the presence of a patterning molecule. Opposing activities of PACAP on neural precursors have also been observed in other contexts. For instance, the action of PACAP on embryonic rat cortical precursors switches from antimitotic to promitotic depending on the presence or absence of specific PACAP receptor splice variants (Nicot and DiCicco-Bloom, 2001). The switch to promitotic action appeared to occur by a mechanism that involves recruitment of the phospholipase C pathway. Overall, these findings suggest that neuropeptides PACAP and VIP can exhibit tissue- or cell-type-specific effects due to the expression level and pattern of the PACAP receptor subtypes and splice variants, and the presence or absence of growth and patterning factors. In most cases, however, the precise receptors and intracellular signaling mechanisms that account for this remain unknown.

Here we aimed to investigate the effects of the VPAC2 receptor activation by using Ro25-1553 and VPAC2 receptor-null mice on axon and dendritic outgrowth in primary cultured mouse cortical neurons. We also compare the effects of Ro25-1553 with those of VIP and PACAP. Furthermore, we examined the intracellular signaling pathways involved in the effects of Ro25-1553 and PACAP.

ANIMALS AND METHODS

Animals and Materials

The pregnant ICR (CD1) mice at 16 days of gestation were purchased from Japan SLC, Inc (Hamamatsu, Japan). The generation of VPAC2 receptor deficient mice using gene targeting

has been previously reported (Harmar et al., 2002). VPAC2 receptor homozygous knockout mice and littermate wild-type mice (C57BL/6 strain) used here were obtained by crossing with VPAC2 receptor heterozygous mice (Ago et al., 2017). Mice were housed in clear cages in groups of 3–5 animals under controlled environmental conditions (22 ± 1°C; 50 ± 10% relative humidity; a 12-h light-dark cycle, lights on at 0800 h; food and water *ad libitum*). Ro25-1553, VIP, and PACAP38 were purchased from Peptide Institute, Inc (Osaka, Japan). H89, GF109203X, and U0126 were purchased from Sigma-Aldrich (St. Louis, MO, United States). PG99-465 was purchased from Bachem (Bubendorf, Switzerland). The concentrations of the peptides and compounds used here were selected based on previous studies (Gourlet et al., 1997; Drahushuk et al., 2002; Leemhuis et al., 2007; Ogata et al., 2015). Primary neuronal cultures prepared from CD1 mice were used in most experiments (**Figures 1–4**, **6**). Wild-type and VPAC2 receptor deficient C57BL/6 mice were used to examine the VPAC2 receptor-mediated effects of Ro25-1553 (**Figure 5**).

Preparation of Primary Neuronal Cultures

Primary neuronal cultures were prepared from cerebral cortices of 16-day-old embryonic mice as previously described (Takuma et al., 2009; Kawanai et al., 2016). In brief, cerebral cortices were dissected from embryonic mice, sliced at a thickness of 0.5–1 mm with a razor blade under ice cold conditions (1–4°C), and incubated with 0.05% Trypsin-EDTA-4Na under a humidified atmosphere of 95% air/5% CO_2 at 37°C for 15 min. Tissues were then mechanically dissociated with a fire-polished Pasteur pipette in Neurobasal™ medium containing 2% B27 supplement, 2 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin. Cells were then plated at a density of 1×10^4 cells/cm^2 on glass coverslips in 24-well tissue culture plates, which were coated with 20 μg/mL of poly-L-lysine (day 0). Cell cultures were kept at 37°C in a 95% air/5% CO_2 humidified incubator. Cells were treated with Ro25-1553, VIP, or PACAP38 at 1 day *in vitro* (DIV) until 3, 7, or 14 DIV. During the experiments, the culture medium including freshly prepared compounds and peptides at the same concentration as 1 DIV was replaced every 3 days.

Morphological Analysis

Cells were fixed with 4% paraformaldehyde for 10 min at 4°C, and incubated with 0.2% Triton X-100 in Ca^{2+}-, Mg^{2+}-free phosphate buffered saline (PBS, pH 7.2) for 5 min at room temperature. After blocking with 1% bovine serum albumin (BSA)/PBS for 30 min, they were incubated with a chicken polyclonal anti-MAP2 antibody (1:5000; Cat# ab5392, Abcam, Cambridge, United Kingdom; RRID:AB_2138153) and a mouse monoclonal anti-Neurofilament H & M, Phosphorylated antibody (1:500; Cat# SMI-310, Covance Japan, Tokyo, Japan; RRID:AB_448147) at 4°C overnight. After washing with PBS, the cells were incubated with species-specific fluorophore-conjugated secondary antibodies (1:200; Alexa 488-conjugated anti-chicken IgY (Cat# A32931, RRID:AB_2762843) and Alexa 594-conjugated anti-mouse IgG (Cat# A-11005, RRID:AB_2534073), Thermo Fisher Scientific) for 2 h at room temperature. The cells were washed with PBS and mounted using the ProLong™ Gold antifade reagent (Thermo Fisher Scientific). Digitized images were obtained with an upright light microscope with a cooled CCD digital camera system (Axio Imager. M2/AxioCam MRc5; Carl Zeiss, Jena, Germany). In this study, phosphorylated neurofilaments (pNF)-positive neurites were classified morphologically as axons, and MAP2-positive and pNF-negative neurites were classified as dendrites. We exclude the cells that did not have axons or dendrites from further analysis. Additionally, a few complex overlapping neurons were not measured. Then, 10–20 neurons in each experiment were randomly selected by an observer blind to the treatment. Each experiment was independently repeated three times. The axon and all dendrites of each neuron were manually traced with Neurolucida software (Version 11; MBF Bioscience, Inc., Williston, VT, United States). The axon length, total number of dendrites (total number of terminal dendrite branches), total length of all dendrites, and dendritic complexity in each neuron were calculated from the traced neurites using Neurolucida. Dendritic complexity was assessed by measuring the lengths of dendrites and counting the number of branch points in each branch order. The dendritic complexity index was calculated from: (sum of the terminal orders + number of terminals) × (total dendritic length/number of primary dendrites) (Pillai et al., 2012). Terminal order indicates the number of sister branches emanating from the dendritic segment between a particular terminal tip and cell body.

RT-PCR

Total RNAs from cultured cells were isolated using the SV Total RNA isolation system (Promega, Madison, WI, United States) according to the manufacturer's instructions. The total RNAs were reverse transcribed with Superscript III (Thermo Fisher Scientific, Waltham, MA, United States). RT-PCR was performed with GoTaq® Green Master Mix (Promega) using Applied Biosystems™ Veriti™ 96-Well Thermal Cycler. The following primers were used: 5′-ATGAGTCTTCCCCAGGTTG-3′ (forward) and 5′-ACCGACAGGTAGTAATAATCC-3′ (reverse) for the PAC1 receptor; 5′-AGTGAAGACCGGCTACACCA-3′ (forward) and 5′-TCGACCAGCAGCCAGAAGAA-3′ (reverse) for the VPAC1 receptor; 5′-ATGGACAGCAACTCGCCTC TCTTTAG-3′ (forward) and 5′-GGAAGGAACCAACACATAA CTCAAACAG-3′ (reverse) for the VPAC2 receptor; 5′-AC CACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCA CCACCCTGTTGCTGTA-3′ (reverse) for GAPDH. PCR was performed for 40 cycles at 95°C for 30 s; 55°C for 30 s; and 72°C for 30 s.

Statistical Analysis

All results are presented as the mean ± standard deviation (SD). Statistical analyses were performed using Statview (SAS Institute Japan Ltd., Tokyo, Japan), and significant differences determined by one- or two-way ANOVA followed by the Tukey–Kramer test. The threshold for statistical significance was defined as $P < 0.05$.

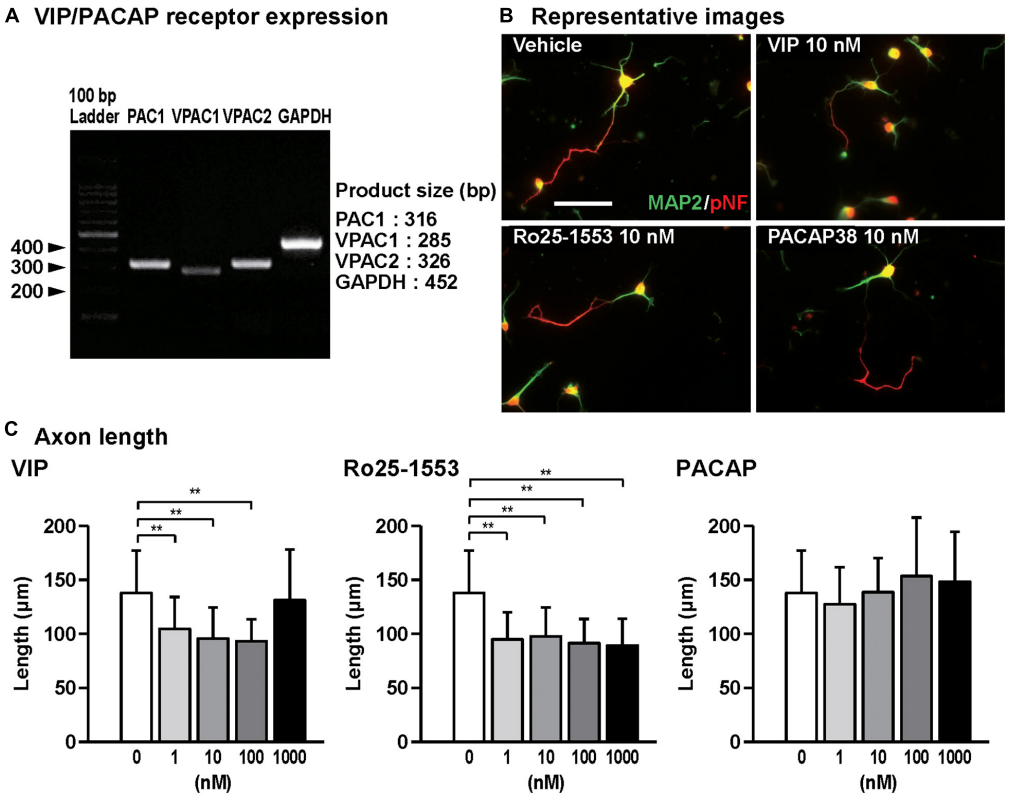

FIGURE 1 | Effects of Ro25-1553, VIP, and PACAP on axon outgrowth in cultured cortical neurons. **(A)** RT-PCR analysis showed that all VIP/PACAP receptors PAC1, VPAC1, and VPAC2 were expressed in primary cultured cortical neurons. **(B)** Representative pNF- and MAP2-immunostained images of cultured cortical neurons are shown. Cells were cultured with Ro25-1553 (10 nM), VIP (10 nM), or PACAP38 (10 nM) for 3 days *in vitro* and double-immunostained for pNF (red) and MAP2 (green). Scale bar, 50 μm. **(C)** Quantitative analysis of changes in axon length was shown. VIP ($F_{4,215}$ = 16.542, $P < 0.0001$) and Ro25-1553 ($F_{4,215}$ = 25.257, $P < 0.0001$), but not PACAP ($F_{4,215}$ = 2.383, $P > 0.05$), reduced the axon length. Values represent mean ± SD of 40–60 neurons from three independent experiments. $^{**}P < 0.01$ vs. control.

RESULTS

Figure 1A shows the gel images for RT-PCR for PAC1, VPAC1, VPAC2 receptors, and GAPDH, indicating that all VIP/PACAP receptors are expressed in primary cultured cortical neurons. Then, we examined the effects of VIP, Ro25-1553, and PACAP on axon and dendritic outgrowth in cultured cortical neurons. **Figure 1B** shows the representative pNF- and MAP2-immunostained images of primary cultured cortical neurons treated with Ro25-1553 (10 nM), a selective VPAC2 receptor agonist, VIP (10 nM), and PACAP (10 nM) for 3 days *in vitro* (DIV). Treatment with VIP (1, 10, and 100 nM) and Ro25-1553 (1–1000 nM) for 3 DIV significantly reduced axon length (**Figure 1C**). PACAP at doses used here (1–1000 nM) did not affect the axon length.

Figure 2 shows the effects of VIP on dendritic outgrowth. Primary cortical neurons were cultured with VIP for 14 DIV. Representative MAP2-immunostained images of neurons treated with different concentrations of VIP at 7 and 14 DIV are shown. With longer periods in culture, overall increase in dendrite extension and branching were observed. Quantitative morphological analysis revealed that VIP at doses of 1–100 nM, but not 1000 nM, decreased the total numbers and length of dendrites, and dendritic complexity for 3 to 14 DIV.

Figure 3 shows the effects of Ro25-1553 on dendritic outgrowth. Primary cortical neurons were cultured with Ro25-1553 for 14 DIV. Representative MAP2-immunostained images of neurons treated with different concentrations of Ro25-1553 at 7 and 14 DIV are shown. Quantitative morphological analysis revealed that Ro25-1553 at doses of 1–1000 nM decreased the total numbers and length of dendrites, and dendritic complexity for 3 to 14 DIV.

Figure 4 shows the effects of PACAP on dendritic outgrowth. Primary cortical neurons were cultured with PACAP for 7 DIV. Representative MAP2-immunostained images of neurons treated with different concentrations of PACAP at 7 DIV are shown. Quantitative morphological analysis revealed that PACAP (1–1000 nM) dose-dependently increased the total numbers and length of dendrites, and dendritic complexity for 3 to 7 DIV.

Figure 5 shows the involvement of the VPAC2 receptor in the inhibitory effects of Ro25-1553 and VIP on axon and dendritic outgrowth. Pretreatment with PG99-465 (100 nM), a VPAC2 receptor antagonist, blocked Ro25-1553 (10 nM)- and VIP (10 nM)-induced reductions in axon length, total numbers

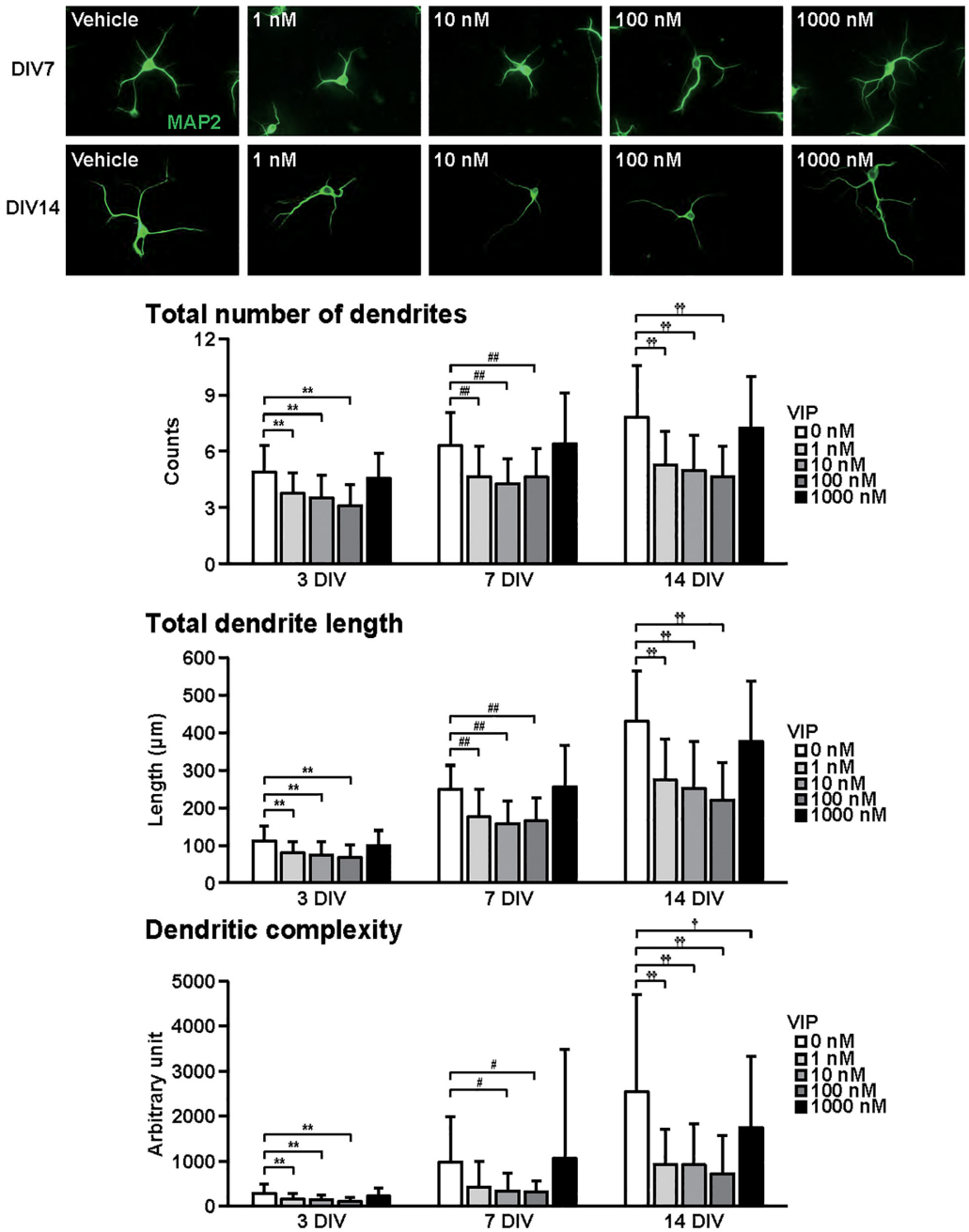

FIGURE 2 | Effects of VIP on dendritic outgrowth in cultured cortical neurons. Representative MAP2 (green)-immunostained images of neurons cultured with VIP (1–1000 nM) for 7 to 14 days *in vitro* (DIV) are shown. Quantitative analysis of dendritic morphology at 3 (**Figure 1B**), 7, and 14 DIV revealed that VIP significantly decreased the total numbers (main effects: $F_{2,805} = 75.487$, $P < 0.0001$ for time, $F_{4,805} = 52.424$, $P < 0.0001$ for treatment; interaction: $F_{8,805} = 2.556$, $P < 0.01$) and length (main effects: $F_{2,805} = 374.760$, $P < 0.0001$ for time, $F_{4,805} = 51.272$, $P < 0.0001$ for treatment; interaction: $F_{8,805} = 8.563$, $P < 0.0001$) of dendrites, and dendritic complexity (main effects: $F_{2,805} = 76.423$, $P < 0.0001$ for time, $F_{4,805} = 20.616$, $P < 0.0001$ for treatment; interaction: $F_{8,805} = 5.856$, $P < 0.0001$). Values represent mean ± SD of 40–60 neurons from three independent experiments. $^{**}P < 0.01$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. control.

and length of dendrites, and dendritic complexity (**Figure 5A**). Additionally, Ro25-1553-induced reductions in axon length, total numbers and length of dendrites, and dendritic complexity were abolished in cortical neurons derived from VPAC2 receptor knockout mice (**Figure 5B**).

Figure 6 shows the signaling pathway involved in the effects of Ro25-1553 and PACAP on neurite outgrowth. Pretreatment with a PKA inhibitor H89 (1 μM), but not a PKC inhibitor GF109203X (1 μM) or a MEK inhibitor U0126 (1 μM), blocked Ro25-1553 (10 nM)-induced reductions in axon length, total

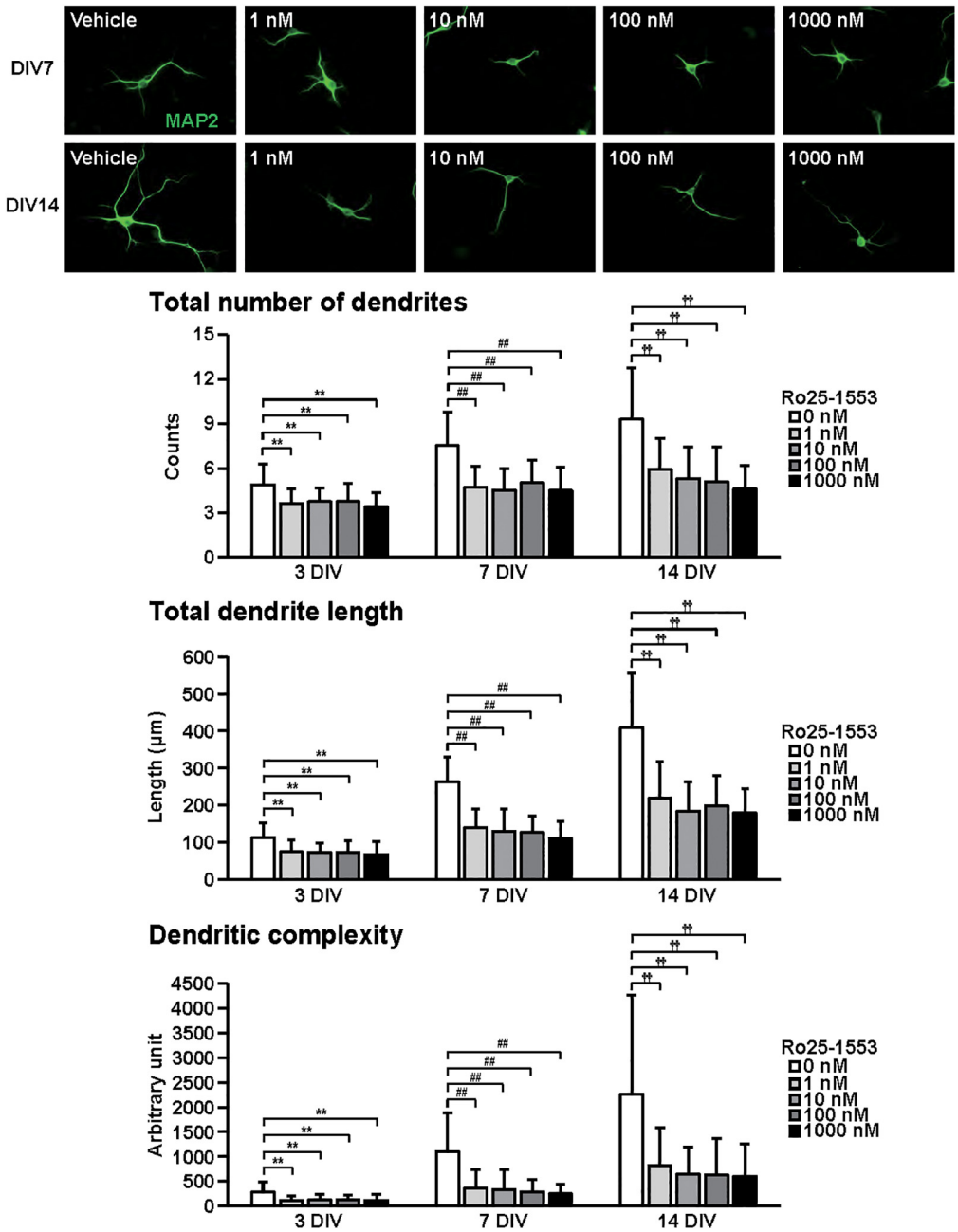

FIGURE 3 | Effects of Ro25-1553 on dendritic outgrowth in cultured cortical neurons. Representative MAP2 (green)-immunostained images of neurons cultured with Ro25-1553 (1–1000 nM) for 7 to 14 days *in vitro* (DIV) are shown. Quantitative analysis of dendritic morphology at 3 (**Figure 1B**), 7, and 14 DIV revealed that Ro25-1553 significantly decreased the total numbers (main effects: $F_{2,805} = 83.382$, $P < 0.0001$ for time, $F_{4,805} = 75.612$, $P < 0.0001$ for treatment; interaction: $F_{8,805} = 7.531$, $P < 0.0001$) and length (main effects: $F_{2,805} = 323.212$, $P < 0.0001$ for time, $F_{4,805} = 122.621$, $P < 0.0001$ for treatment; interaction: $F_{8,805} = 17.981$, $P < 0.0001$) of dendrites, and dendritic complexity (main effects: $F_{2,805} = 91.541$, $P < 0.0001$ for time, $F_{4,805} = 49.193$, $P < 0.0001$ for treatment; interaction: $F_{8,805} = 11.777$, $P < 0.0001$). Values represent mean ± SD of 40–60 neurons from three independent experiments. $^{**}P < 0.01$, $^{\#\#}P < 0.01$, $^{\dagger\dagger}P < 0.01$ vs. control.

numbers and length of dendrites, and dendritic complexity (**Figure 6A**). On the other hand, pretreatment with U0126, but not H89 or GF109203X, blocked PACAP (100 nM)-induced increases in total numbers and length of dendrites, and dendritic complexity (**Figure 6B**).

DISCUSSION

The present study confirmed that PAC1, VPAC1, and VPAC2 receptors are expressed in primary cultured mouse cortical neurons as observed *in vivo* mouse cortex (Marzagalli et al.,

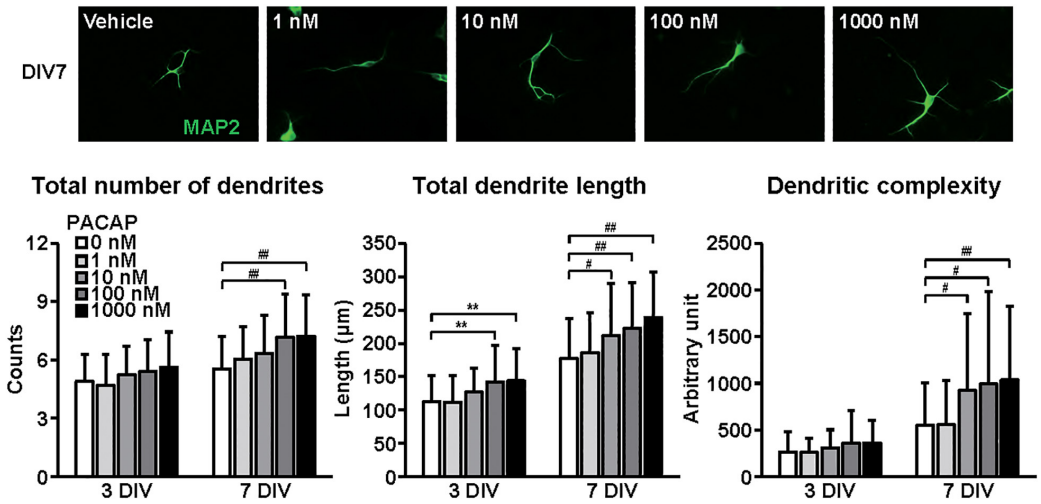

FIGURE 4 | Effects of PACAP on dendritic outgrowth in cultured cortical neurons. Representative MAP2 (green)-immunostained images of neurons cultured with PACAP (1–1000 nM) for 7 days *in vitro* (DIV) are shown. Quantitative analysis of dendritic morphology at 3 (**Figure 1B**) and 7 DIV revealed that PACAP dose-dependently increased the total numbers (main effects: $F_{1,510} = 63.825$, $P < 0.0001$ for time, $F_{4,510} = 9.171$, $P < 0.0001$ for treatment; interaction: $F_{4,510} = 1.661$, $P > 0.05$) and length (main effects: $F_{1,510} = 231.606$, $P < 0.0001$ for time, $F_{4,510} = 12.279$, $P < 0.0001$ for treatment; interaction: $F_{4,510} = 0.957$, $P > 0.05$) of dendrites, and dendritic complexity (main effects: $F_{1,510} = 93.016$, $P < 0.0001$ for time, $F_{4,510} = 6.279$, $P < 0.0001$ for treatment; interaction: $F_{4,510} = 2.822$, $P < 0.05$). Values represent mean ± SD of 40–60 neurons from three independent experiments. $^{**}P < 0.01$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control.

2016; Hirabayashi et al., 2018), indicating that Ro25-1553 and endogenous ligands VIP and PACAP could act through all VIP/PACAP receptors. We demonstrated that low concentrations (1–100 nM) of VIP and the VPAC2 receptor agonist Ro25-1553 reduced axon and dendritic outgrowth of cortical neural precursors measured at 3, 7, and 14 DIV. It is unlikely that the effects of Ro25-1553 and VIP on the numbers and complexity of axons and dendrites were due to general toxicity because the inhibitory effects of Ro25-1553 were abolished in VPAC2 receptor knockout mice (**Figure 5B**), and neither affected cell numbers based on the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (data not shown). Interestingly, the effect of VIP was lost at higher concentration (1 μM), demonstrating a classical bell-shaped curve. This may be due to the fact that high dose of VIP can activate the PAC1 receptor (Drahushuk et al., 2002; Ogata et al., 2015), thereby producing an opposing effect which cancels the inhibitory action. On the other hand, PACAP, which binds with high affinity to PAC1 and VPAC2 receptors, promoted dendritic outgrowth of cortical neurons. In this context, the balance between VPAC2 receptor- and PAC1 receptor-mediated signals might be important to regulate the neurite outgrowth. However, we do not yet determine precisely why and how PACAP exhibits the opposite effect. To clarify this point, future studies on the effects of different concentrations of VIP and PACAP alone and in combination in either VPAC2- or PAC1-receptor knockout mice will be needed. Previously, Leemhuis et al. (2007) reported that VIP (1 nM) at 3 DIV for 18 h caused the increases in axon and dendritic outgrowth in cultured rat hippocampal neurons. This contrasts with the results of the present study using cortical neurons, again demonstrating that effects of these peptides are cell-type specific. Importantly, the effects of Ro25-1553 and VIP were blocked in the present study by pretreatment with a VPAC2 receptor antagonist PG99-465. Furthermore, we demonstrated the high specificity of the VPAC2 receptor agonist Ro25-1553, which was abolished in cortical neurons derived from VPAC2 receptor knockout mice. Overall, the results suggest that the activation of the VPAC2 receptor delays or limits the maturation in mouse cortical neurons. In a previous study, we also found that adult VPAC2 receptor knockout mice show abnormal dendritic morphology in the prelimbic and infralimbic cortices, but not basolateral amygdala (Ago et al., 2017). Loss of the VPAC2 receptor reorganized apical and basal dendritic arbors of prelimbic cortex neurons and apical, but not basal, dendritic arbors of infralimbic cortex neurons. In the prelimbic cortex neurons, the amount of apical dendritic material distal to the soma was decreased in VPAC2 receptor knockout mice, while proximal dendritic material was increased. In the infralimbic cortex, the amount of apical dendritic material proximal to the soma was increased in VPAC2 receptor knockout mice, while other indices of morphology did not differ. Although the present experimental design does not distinguish these regions, the findings suggest that the VPAC2 receptor plays an important role in regulating the development of dendritic morphology in the cerebral cortex. Like for PAC1 receptors, several splice variants of VPAC2 receptors in the mouse and human have been reported (Grinninger et al., 2004; Bokaei et al., 2006; Huang et al., 2006; Miller et al., 2006; Dickson and Finlayson, 2009) and there is a difference in the VPAC2 receptor expression level among brain regions (Sheward et al., 1995; Vertongen et al., 1997; Joo et al., 2004; Kalló et al., 2004; Marzagalli et al., 2016; Tian et al., 2019). These might explain at least partly the difference in the response to VIP in different neural cell types, but exact mechanisms remain

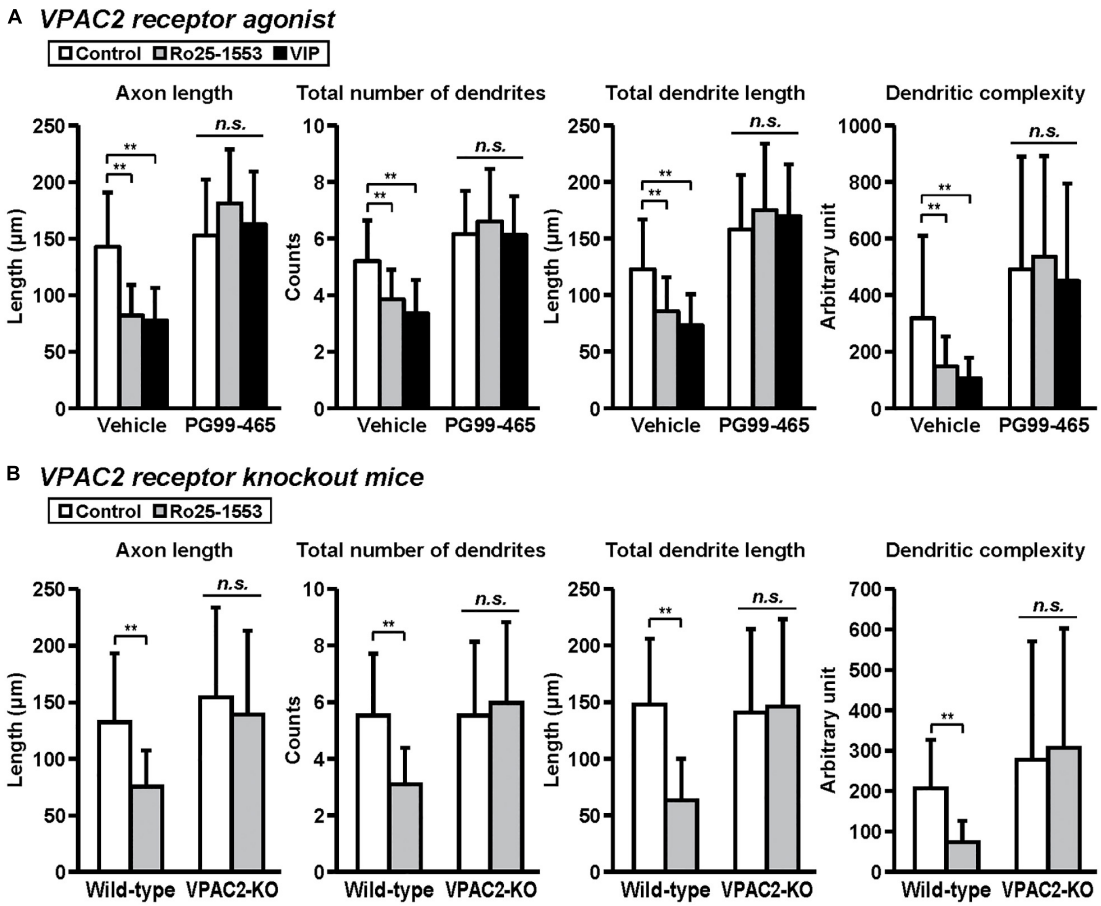

FIGURE 5 | VPAC2 receptor-mediated reductions in axon and dendritic outgrowth in cultured cortical neurons. Primary cortical neurons were cultured with Ro25-1553 (10 nM) or VIP (10 nM) for 3 days *in vitro* and double-immunostained for pNF and MAP2. **(A)** PG99-465 (100 nM) was treated 30 min before the treatment with Ro25-1553 or VIP. PG99-465 blocked Ro25-1553- and VIP-induced reductions in axon length (main effects: $F_{2,215} = 8.338$, $P < 0.001$ for Ro25-1553/VIP, $F_{1,215} = 129.957$, $P < 0.0001$ for PG99-465; interaction: $F_{2,215} = 24.711$, $P < 0.0001$), total numbers (main effects: $F_{2,215} = 8.231$, $P < 0.001$ for Ro25-1553/VIP, $F_{1,215} = 127.826$, $P < 0.0001$ for PG99-465; interaction: $F_{2,215} = 10.525$, $P < 0.0001$) and length (main effects: $F_{2,215} = 3.701$, $P < 0.05$ for Ro25-1553/VIP, $F_{1,215} = 160.683$, $P < 0.0001$ for PG99-465; interaction: $F_{2,215} = 11.789$, $P < 0.0001$) of dendrites, and dendritic complexity (main effects: $F_{2,215} = 3.744$, $P < 0.05$ for Ro25-1553/VIP, $F_{1,215} = 61.073$, $P < 0.0001$ for PG99-465; interaction: $F_{2,215} = 3.003$, $P < 0.05$). **(B)** Primary cortical neurons were prepared from VPAC2 receptor knockout (VPAC2-KO) mice and littermate wild-type mice. Ro25-1553-induced reductions in axon length (main effects: $F_{1,156} = 12.655$ $P < 0.001$ for treatment, $F_{1,156} = 17.717$, $P < 0.0001$ for genotype; interaction: $F_{1,156} = 4.182$, $P < 0.05$), total numbers (main effects: $F_{1,156} = 7.274$, $P < 0.01$ for treatment, $F_{1,156} = 15.414$, $P < 0.001$ for genotype; interaction: $F_{1,156} = 15.414$, $P < 0.001$) and length (main effects: $F_{1,156} = 15.814$, $P < 0.001$ for treatment, $F_{1,156} = 14.262$, $P < 0.001$ for genotype; interaction: $F_{1,156} = 20.342$, $P < 0.0001$) of dendrites, and dendritic complexity (main effects: $F_{1,156} = 2.175$, $P > 0.05$ for treatment, $F_{1,156} = 19.468$, $P < 0.0001$ for genotype; interaction: $F_{1,156} = 5.576$, $P < 0.05$) were abolished in cortical neurons derived from VPAC2-KO mice. Values represent mean ± SD of 40 neurons from three independent experiments. $^{**}P < 0.01$ vs. control, *n.s.*; not significant.

unknown. It would be intriguing to examine whether activation of the VPAC2 receptor affects axon and dendritic outgrowth in different cell types.

Regarding the mechanism underlying the inhibitory effects of Ro25-1553 on cortical neuronal maturation, we found that a PKA inhibitor H89, but not a PKC inhibitor GF109203X or a MEK inhibitor U0126, prevented Ro25-1553-induced impairment of axon and dendritic outgrowth. This suggests that activation of the cAMP/PKA signaling pathway is involved in the VPAC2 receptor-induced impairment of axon and dendritic outgrowth. On the other hand, the opposing stimulatory effects of PACAP on dendritic outgrowth were blocked by U0126, but not H89 or GF109203X. Interestingly, PACAP38-induced ERK phosphorylation and neuritogenesis was MEK-dependent and PKA-independent in PC12-derived Neuroscreen-1 cells (Emery and Eiden, 2012). On the other hand, cAMP responsive element binding (CREB) phosphorylation induced by PACAP was blocked by H89 in these cells. These suggest that PACAP can stimulate two distinct and independent cAMP pathways. In contrast, Kambe and Miyata (2012) showed that PACAP at doses of 1 and 10 nM, but not 0.1 nM, increased neurite outgrowth in primary hippocampal neurons, and this effect was blocked by H89. Currently, the reason for the discrepancy about the involvement of PKA-mediated signaling in the effects of PACAP on neurite outgrowth is unknown. Together, these findings suggest that PACAP and VIP might have the diverse activities on

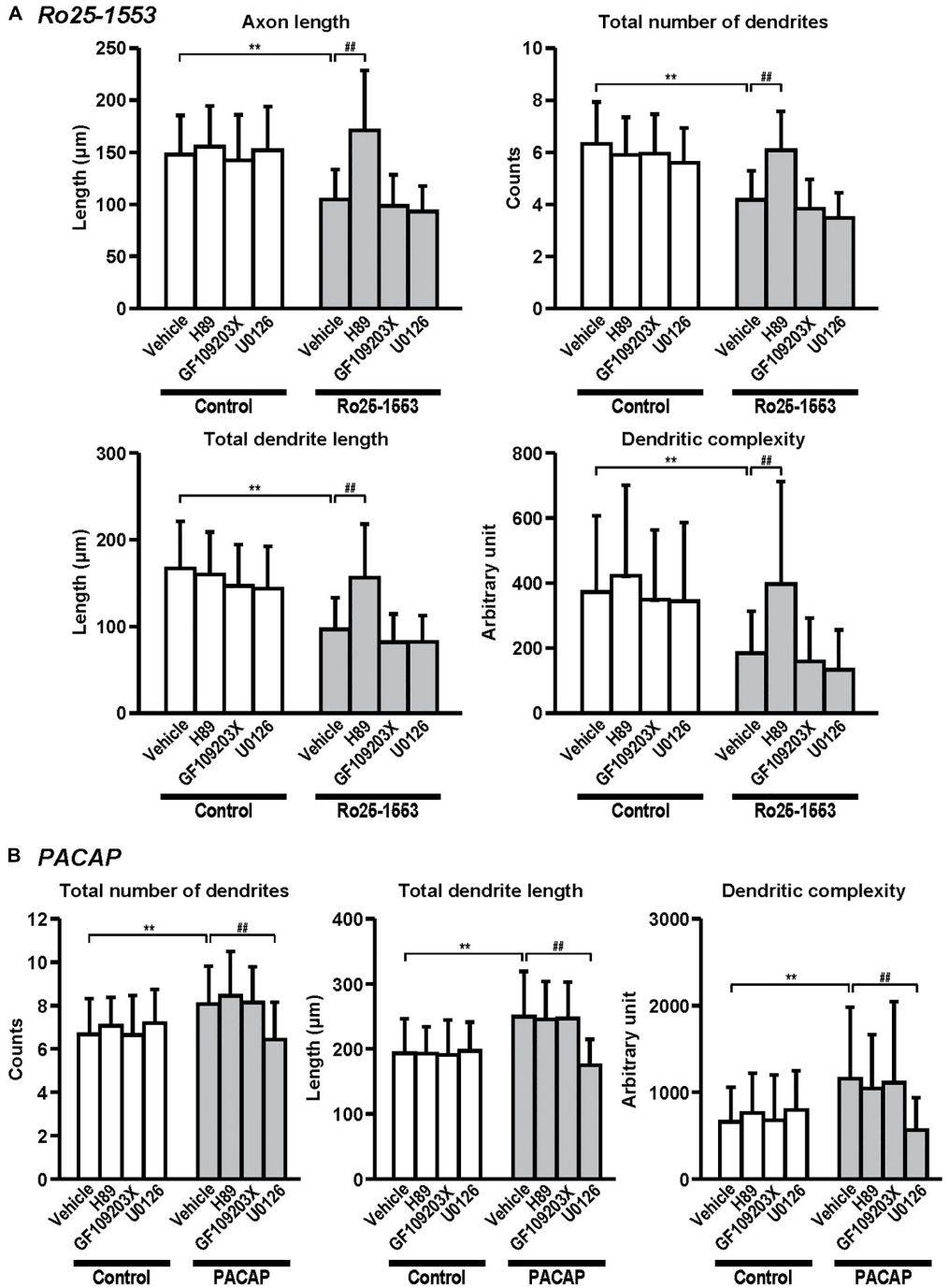

FIGURE 6 | Differential signaling pathways in the effects of Ro25-1553 and PACAP on axon and dendritic outgrowth. Primary cortical neurons were cultured with Ro25-1553 (10 nM) for 3 days *in vitro* (DIV) **(A)** or PACAP (100 nM) for 7 DIV **(B)** and double-immunostained for pNF and MAP2. H89 (1 μM), GF109203X (1 μM), or U0126 (1 μM) was treated 30 min before the treatment with Ro25-1553 or PACAP. H89, but not GF109203X or U0126, blocked Ro25-1553-induced reductions in axon length (main effects: $F_{1,312} = 55.022$, $P < 0.0001$ for Ro25-1553, $F_{3,312} = 21.421$, $P < 0.0001$ for inhibitors; interaction: $F_{3,312} = 14.153$, $P < 0.0001$), total numbers (main effects: $F_{1,312} = 107.531$, $P < 0.0001$ for Ro25-1553, $F_{3,312} = 17.086$, $P < 0.0001$ for inhibitors; interaction: $F_{3,312} = 14.788$, $P < 0.0001$) and length (main effects: $F_{1,312} = 93.817$, $P < 0.0001$ for Ro25-1553, $F_{3,312} = 16.605$, $P < 0.0001$ for inhibitors; interaction: $F_{3,312} = 9.139$, $P < 0.0001$) of dendrites, and dendritic complexity (main effects: $F_{1,312} = 39.122$, $P < 0.0001$ for Ro25-1553, $F_{3,312} = 10.089$, $P < 0.0001$ for inhibitors; interaction: $F_{3,312} = 3.091$, $P < 0.05$). On the other hand, U0126, but not H89 or GF109203X, blocked PACAP-induced increases in total numbers (main effects: $F_{1,352} = 23.759$, $P < 0.001$ for PACAP, $F_{3,352} = 4.738$, $P < 0.01$ for inhibitors; interaction: $F_{3,352} = 9.465$, $P < 0.0001$) and length (main effects: $F_{1,352} = 41.061$, $P < 0.001$ for PACAP, $F_{3,352} = 9.241$, $P < 0.0001$ for inhibitors; interaction: $F_{3,352} = 12.141$, $P < 0.0001$) of dendrites, and dendritic complexity (main effects: $F_{1,352} = 14.664$, $P < 0.001$ for PACAP, $F_{3,352} = 2.967$, $P < 0.05$ for inhibitors; interaction: $F_{3,352} = 6.756$, $P < 0.001$). Values represent mean ± SD of 40–45 neurons from three independent experiments. $^{**}P < 0.01$ vs. control, $^{\#\#}P < 0.01$ vs. vehicle.

neurite outgrowth via the distinct signaling pathway in different brain regions.

The impetus for our studies were the series of reports showing that micro-multiplications of the *VIPR2* gene is associated with schizophrenia (Levinson et al., 2011; Vacic et al., 2011; Yuan et al., 2014; Li et al., 2016; Marshall et al., 2017) and ASD (Vacic et al., 2011; Firouzabadi et al., 2017), and that these mutations were associated in patients with heightened VIP-induced cAMP responsiveness (Vacic et al., 2011). h*VIPR2*-BAC tg mice were recently shown to exhibit multiple psychiatric disorder-related behavioral phenotypes and early postnatal striatal developmental deficits that manifested as the elevated cAMP/PKA signaling, increased striatal excitatory inputs, and striatal dendritic maturation deficit (Tian et al., 2019). We also found that pharmacological activation of the VPAC2 receptor in the mouse during early postnatal development caused prepulse inhibition deficits and reductions in synaptic proteins synaptophysin and PSD-95 in the prefrontal cortex (Ago et al., 2015). Impairments of dendritic and synaptic density in pyramidal neurons across multiple brain regions, such as changes in dendritic arborization, dendritic spine number/type, and morphology, have been observed in schizophrenia (Moyer et al., 2015). In particular, reduced length of basilar dendrites and reduced dendritic number have been found in layer 3 in the prefrontal cortical areas (Brodmann area (BA) 10, BA 11, and BA 46), anterior cingulate cortex (BA 32) of schizophrenic patients (Glantz and Lewis, 2000; Kalus et al., 2000; Broadbelt et al., 2002; Black et al., 2004; Konopaske et al., 2014). VIP has been known to be highly expressed in the layer 2/3 inhibitory interneurons, and thus VIP neurons control neocortical activity (Tremblay et al., 2016). Taken together, the present *in vitro* study suggests that the activation of the VPAC2 receptor directly disrupts cortical neuronal maturation and implies that the *VIPR2* linkage can be explained in part by impaired neuronal maturation due to overactivity of VPAC2 receptors at a time of brain development when neural circuits involved in cognition and social behavior are being established and/or that VPAC2 receptor overactivity disrupts ongoing synaptic plasticity in the adult brain.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on reasonable request to the corresponding author.

ETHICS STATEMENT

All animal studies were approved by the Animal Research Committee at University of California, Los Angeles (UCLA) and the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences, Osaka University, and the Graduate School of Biomedical and Health Sciences, Hiroshima University. All experimental procedures were conducted in accordance with the guidelines of the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). Every effort was made to minimize animal suffering, and to reduce the number of animals used.

AUTHOR CONTRIBUTIONS

ST, TK, RY, LC, TM, MY, SA, AH-T, and YA performed the experiments and analyzed the data. TN, KY, NH, SN, KT, JW, HH, and YA supported the study, designed study, and wrote the manuscript. HH and YA reviewed and approved the manuscript and held all the responsibilities related to this manuscript. All authors reviewed and approved the manuscript.

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REVIEW
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Protective Effects of PACAP in Peripheral Organs

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Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuropeptide widely distributed in the nervous system, where it exerts strong neuroprotective effects. PACAP is also expressed in peripheral organs but its peripheral protective effects have not been summarized so far. Therefore, the aim of the present paper is to review the existing literature regarding the cytoprotective effects of PACAP in non-neuronal cell types, peripheral tissues, and organs. Among others, PACAP has widespread expression in the digestive system, where it shows protective effects in various intestinal pathologies, such as duodenal ulcer, small bowel ischemia, and intestinal inflammation. PACAP is present in both the exocrine and endocrine pancreas as well as liver where it reduces inflammation and steatosis by interfering with hepatic pathology related to obesity. It is found in several exocrine glands and also in urinary organs, where, with its protective effects being mainly published regarding renal pathologies, PACAP is protective in numerous conditions. PACAP displays anti-inflammatory effects in upper and lower airways of the respiratory system. In the skin, it is involved in the development of inflammatory pathology such as psoriasis and also has anti-allergic effects in a model of contact dermatitis. In the non-neuronal part of the visual system, PACAP showed protective effects in pathological conditions of the cornea and retinal pigment epithelial cells. The positive role of PACAP has been demonstrated on the formation and healing processes of cartilage and bone where it also prevents osteoarthritis and rheumatoid arthritis development. The protective role of PACAP was also demonstrated in the cardiovascular system in different pathological processes including hyperglycaemia-induced endothelial dysfunction and age-related vascular changes. In the heart, PACAP protects against ischemia, oxidative stress, and cardiomyopathies. PACAP is also involved in the protection against the development of pre-senile systemic amyloidosis, which is presented in various peripheral organs in PACAP-deficient mice. The studies summarized here provide strong evidence for the cytoprotective effects of the peptide. The survival-promoting effects of PACAP depend on a number of factors which are also shortly discussed in the present review.

Keywords: PACAP, cytoprotection, periphery, apoptosis, ischemia

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INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP) was discovered more than 30 years ago by Arimura et al. (1). The discovery was based on the ability of the hypothalamus-derived peptide to increase cAMP levels in cultured pituitary cells. Several studies following its isolation showed that PACAP exerts several distinct effects in the hypothalamo-hypophyseal system and other central regulatory pathways (2–7). PACAP belongs to the glucagon/secretin/vasoactive intestinal peptide family of peptides and it exists in two forms, with 38 and 27 amino acids. PACAP acts on G protein coupled receptors. The specific PAC1 receptor only binds PACAP, while the VPAC1 and VPAC2 receptors also bind vasoactive intestinal peptide with similar affinity (8–12). Early studies already pointed out the robust neuroprotective effects of PACAP *in vitro* and *in vivo* through a combination of antiapoptotic, antiinflammatory, and antioxidant effects (8–12). Neuroprotective actions have been shown, among others, in cerebellar granule cells, neuroblastoma cells, cortical neurons, and ganglionic cells against different toxic substances and harmful stimuli, mainly through the PAC1 receptors (9). *In vivo*, numerous animal models have been used to establish the potential neuroprotective effects of PACAP in pathological conditions (8–12). Although originally isolated in the central nervous system and early studies showed highest concentration in the brain, PACAP has a very widespread occurrence also in peripheral organs. Numerous studies have provided proof that PACAP exerts protective effects not only in the nervous system but also in many peripheral cell types and organs. The neuroprotective effects have been reviewed several times (8–16) but the peripheral protective effects of the neuropeptide have not been summarized in a review so far. Therefore, the aim of our present paper is to review the general cytoprotective effects of PACAP in non-neuronal cell types, peripheral tissues and organs (summarized in **Figure 1**, **Tables 1**, **2**).

DIGESTIVE SYSTEM

Intestines

PACAP has widespread expression in the gastrointestinal system (98–101). PACAP acts on different intestinal processes including motility (102), intestinal secretion of growth factors (103), and activity of interstitial Cajal cells (104). *In vitro* investigations of PACAP in small intestine were carried out using INT407 cells originally obtained from human embryonal jejunum and ileum (17). PACAP showed protective effects against oxidative stress, but it was not effective in $CoCl_2$-induced *in vitro* hypoxia. Surprisingly, if cells were exposed to gamma irradiation, PACAP acted negatively on clone-forming ability, but this might be due to a function in reducing the number of damaged cells (17). Furthermore, *Adcyap1* small interfering RNA transfection led to higher vulnerability in INT407 cells suggesting a protective role of endogenously present PACAP (17).

In vitro experiments using HCT-8 human colonic tumor cells revealed proliferation-enhancing effect of PACAP (105). The authors detected PACAP and specific PAC1 receptor in the HCT-8 cell line. In addition, PACAP-38 was shown to suppress Fas receptor, suggesting a possible role of PACAP in cell survival (105). Lelievre et al. tested the effect of PACAP-27 in four human colonic adenocarcinoma cell lines (HT29, SW403, DLD-1, Caco-2). They found that long-term treatment with PACAP or VIP reduced cell proliferation (106). Bacterial adhesion plays a crucial role in gastrointestinal infections. Illes et al. (107) examined the effect of PACAP on bacterial adhesion in small and large intestinal cell lines. PACAP influenced colony numbers of investigated bacteria neither in small intestinal INT407 nor in large intestinal Caco-2 cells. On the other hand, PACAP was able to act on expression of certain cytokines: it induced IL-8 and CXCL-1 activation (107).

Previous studies aimed to investigate the possible effect of PACAP in different models of intestinal pathologies. Protective effect of PACAP in a rat model of duodenal ulcer was investigated by Yagi et al. (54). Rats treated with mepirizole showed increased gastric acid secretion and hemorrhagic lesions in proximal duodenum. The applied intravenous PACAP-27 treatment led to increased HCO_3- secretion thus it could significantly reduce the severity of duodenal lesions with no effect on gastric acid secretion (54). Other studies have targeted to explore the effect of PACAP in ischemia/reperfusion. Ferencz et al. (55–57) described protective effect of PACAP in a rat model of small bowel autotransplantation, modeling cold ischemia injury. Small bowel was removed and stored in standard preservation solution with or without additional PACAP. The histological damage caused by cold ischemia was ameliorated by PACAP: changes in mucous layer were reduced and crypt morphology was better preserved (55). Besides preserving the morphology, authors found that PACAP did not change lipid peroxidation but kept the endogenous scavanger capacity. Effects of endogenously present PACAP against cold ischemic injury were investigated using PACAP deficient mice (58). Cold preservation injury was established with removing small bowel from PACAP deficient and wild type animals. Histological analysis showed more severe destruction of mucous, submucous layers, and crypts in PACAP deficient mice compared to wild type animals (58). Furthermore, the effect of endogenous PACAP was also tested in warm intestinal ischemia (59). Warm intestinal ischemia was evoked by occlusion of the superior mesenteric artery. The intestinal injury indicated by tissue damage was more severe in case of mice lacking PACAP. Oxidative stress markers, like malondialdehyde have also shown significant differences between PACAP deficient and wild type animals (59).

Protective actions of PACAP were also studied in small and large intestinal inflammations (98). Heimesaat et al. (60) described protective effect of PACAP against Toxoplasma gondii-induced acute ileitis. Both PACAP prophylaxis and treatment were effective, mice obtaining PACAP prophylaxis or treatment showed a higher survival rate (60). Authors have extended their experiments in order to investigate whether PACAP could alleviate subacute ileitis induced by low-dose Toxoplasma gondii in mice having human gut microbiota (61). PACAP treatment led to less distinct apoptotic responses in ileal and colonic epithelia. Furthermore, not only intestinal but extraintestinal

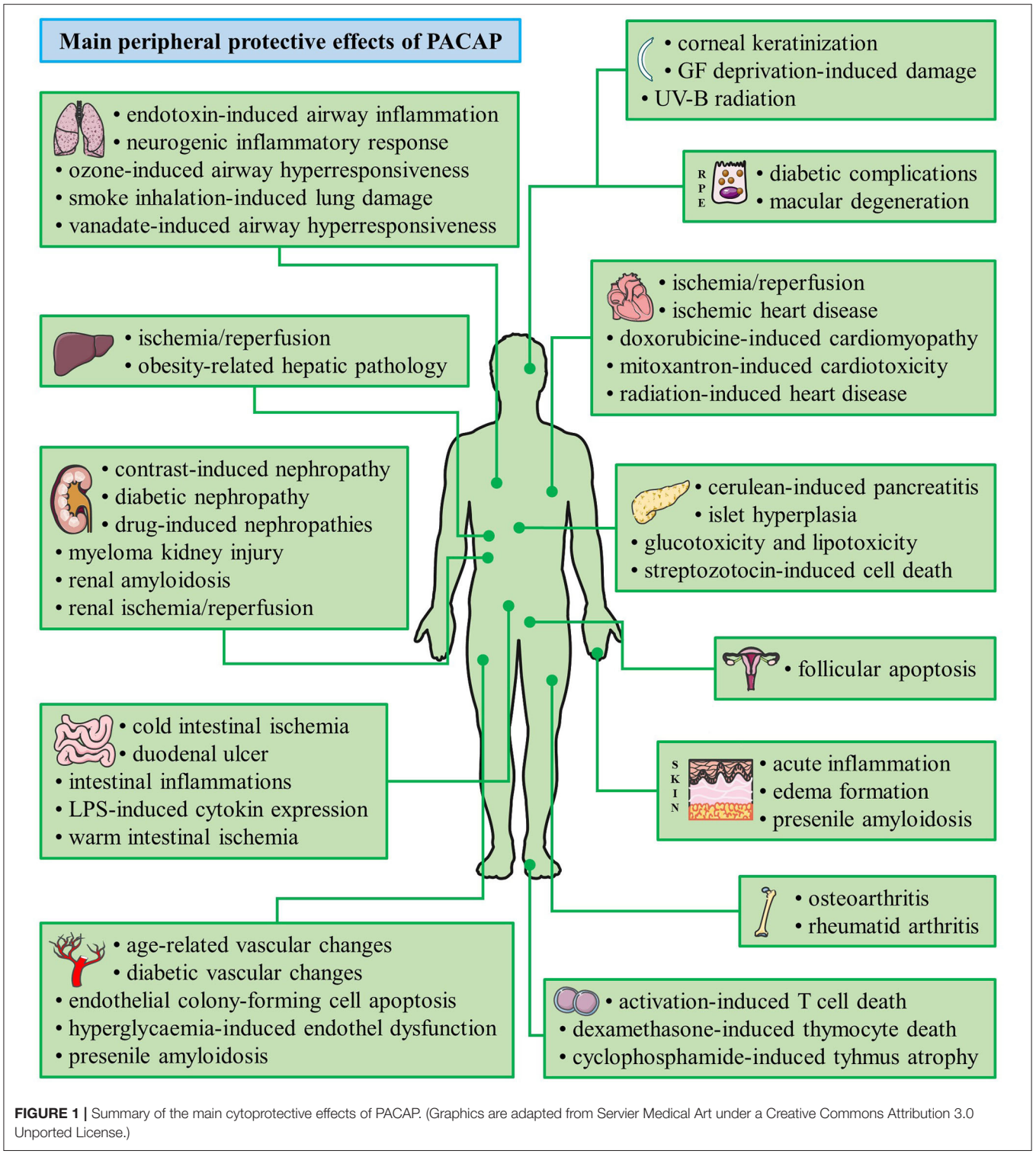

FIGURE 1 | Summary of the main cytoprotective effects of PACAP. (Graphics are adapted from Servier Medical Art under a Creative Commons Attribution 3.0 Unported License.)

sequelae of low-dose Toxoplasma gondii infection were suppressed (61).

Effect of PACAP in large intestinal inflammation can be presumed from changes of PACAP immunoreactivity in different large intestinal pathologies in the pig (108). A considerable upregulation of PACAP mRNA level and downregulation of VPAC1 receptor were detected in transient receptor potential Ankyrin type 1 (TRPA1) knockout mice in DSS-induced colitis (109). In addition, PACAP expression was significantly reduced in transient receptor potential cation channel subfamily V

TABLE 1 | *In vitro* studies showing protective effects of PACAP.

Damaging insult/disease model	Cell line	References
INTESTINES		
Oxidative stress	Human embryonic intestinal cells	(17)
PANCREAS		
Streptozotocin-induced cell death	Rat insulinoma	(18)
Cytokine-induced apoptosis	Mouse insulinoma	(19)
Gluco- and lipotoxicity	Mouse pancreatic beta cells	(20)
LIVER		
Oxidative stress	Mouse hepatocytes	(21)
Tumor necrosis factor-alpha/actinomycin D-induced apoptosis	Mouse hepatocytes	(21)
KIDNEY		
Oxidative stress	Rat kidney cells	(22)
Mineral oil evoked hypoxia	Mouse proximal tubular cells	(23)
Oxidative stress	Mouse kidney cells	(24)
CoCl2-induced hypoxia	Mouse kidney cells	(25)
Cisplatin toxicity	Mouse proximal tubular cells	(26)
Cisplatin toxicity	Human proximal tubular cells	(27)
Gentamicin toxicity	Human proximal tubular cells	(28)
Cyclosporine A toxicity	Human proximal tubular cells	(29)
Radiocontrast media toxicity	Human proximal tubular cells	(30)
Myeloma kappa-light chain toxicity	Human proximal tubular cells	(31)
Lipopolysaccharide-induced inflammation	Mouse podocytes	(32)
RESPIRATORY TRACT		
Cigarette smoke	Rat alveolar cells	(33)
CORNEA AND PIGMENT EPITHELIAL CELLS		
Hyperosmotic and oxidative stress (diabetic macular edema)	Human (adult) RPE cells	(34, 35)
UV-B exposure	Human corneal endothelial cells	(36)
Oxidative stress	Human (adult) RPE cells	(37, 38)
Hyperosmotic and oxidative stress-induced neovascularisation	Human (adult) RPE cells	(39)
Growth factor deprivation	Human corneal endothelial cells	(40)
Increased permeability (macular edema)	Human (adult) RPE cells	(41)
IMMUNE CELLS AND THYMUS		
UV irradiation	Peripheral T cells and T cell hybridomas	(42, 43)
Glucocorticoid-induced apoptosis	Rat thymocytes	(44)
SKELETAL SYSTEM: CARTILAGE AND BONE		
Oxidative stress	Chicken chondrogenic cells	(45)
Osteoarthritis	Rat chondrocytes	(46)
Oxidative and mechanical stress	Chicken chondrogenic cells	(47)
CARDIOVASCULAR SYSTEM: VESSELS AND HEART		
Oxidative stress6	Mouse hemangioendothelioma	(48)
TNF-α-induced apoptosis	Human endothelial cells	(49)
Ischemia/reperfusion	Rat cardiomyocytes	(50, 51)
Oxidative stress	Rat cardiomyocytes	(52, 53)

RPE, retinal pigment epithelium; TNF, tumor necrosis factor.

member 1 (TRPV-1) knockout mice, which together with reduced expression of VIP can contribute to local pro-inflammatory environment in these animals (110). Horvath et al. (98) investigated the possible role of PACAP in human inflammatory bowel diseases. PACAP expression was significantly higher in samples obtained from patients suffering from ulcerative colitis, while this increase could be suppressed by antibiotic therapy. Role of endogenously present PACAP was tested in dextran sulfate sodium (DSS)-induced colitis by two research groups (62, 63). Azuma and colleagues found, based on the histological analysis and the determination of disease activity index of PACAP knockout mice, that mice lacking PACAP had a significantly higher vulnerability than wild type controls (62). Investigations of Nemetz and coworkers

TABLE 2 | *In vivo* studies showing protective effects of endogenous or exogenous PACAP.

Damaging insult/disease model	Species	Exogenous or endogenous PACAP	References
INTESTINES			
Mepirizole-induced duodenal ulcer	Rat	Exogenous	(54)
Small bowel cold ischemia	Rat	Exogenous	(55–57)
Small bowel cold ischemia	Mice	Endogenous	(58)
Small bowel warm ischemia	Rat	Exogenous	(56)
Small bowel warm ischemia	Mice	Endogenous	(59)
T. gondii-induced acute and subacute ileitis	Mice	Exogenous	(60, 61)
Dextran sulfate sodium-induced colitis	Mice	Endogenous	(62)
Inflammation-associated colorectal cancer	Mice	Endogenous	(63)
PANCREAS			
Cerulein induced-acute panreatitis	Mice	Endogenous	(64)
LIVER			
Warm liver ischemia	Mice	Both	(21, 65)
Obesity-induced liver steatosis	Mice	Exogenous	(66)
SALIVARY AND OTHER EXOCRINE GLANDS			
Salivary gland apoptosis	Snail	Exogenous	(67)
URINARY SYSTEM			
Warm renal ischemia	Mice	exogenous	(23, 68)
Warm renal ischemia	Rat	exogenous	(69–71)
Cisplatin-induced acute kidney injury	Mice	Exogenous	(26, 27)
Gentamicin-induced nephrotoxicity	Rat	Exogenous	(72)
Cyclosporine-A-induced nephrotoxicity	Mice	Exogenous	(29)
Contrast agent-induced nephropathy	Mice	Exogenous	(30)
Myeloma nephropathy	Rat	Exogenous	(31)
Streptozotocin-induced nephropathy	Rat	Exogenous	(73, 74)
Presenile kidney amyloidosis	Mice	Endogenous	(75)
Nephrotic syndrome	Zebrafish	Exogenous	(76)
RESPIRATORY TRACT			
Tracheal neurogenic inflammatory response	Rat	Exogenous	(77, 78)
LPS-induced subacute inflammation	Mice	Endogenous	(79)
Ozone-induced airway hyperresponsiveness	Rat	Exogenous	(80)
Ammonium vanadate-induced airway hyperresponsiveness	Guinea pig	Exogenous	(81)
Smoke inhalation-induced lung injury	Mice	Exogenous	(82)
SKIN			
Neurogenic skin edema	Mice	Endogenous	(83)
Oxazolone-hypersensitivity skin reaction	Mice	Endogenous	(84)
Presenile skin amyloidosis	Mice	Endogenous	(75)
Cornea and retinal pigment epithelial cells			
Corneal keratinization	Mice	Both	(85, 86)
Physical corneal injury	Mice	Exogenous	(87, 88)
Physical corneal injury	Rabbit	Exogenous	(88)
RPE cells in diabetic retinopathy	Rat	Exogenous	(89)
IMMUNE CELLS AND THYMUS			
S. aureus enterotoxin B-induced T cell death	Mice	Exogenous	(42, 43)
Cyclophosphamide-induced thymus atrophy	Mice	Exogenous	(90)
CARTILAGE AND BONE			
Disturbed callus formation	Mice	Endogenous	(91)
Serum transfer-induced immune arthritis	Mice	Endogenous	(92)
CARDIOVASCULAR SYSTEM			
Ischemia/reperfusion	Pig	Exogenous	(93)
Diabetic vascular complications	Mice	Exogenous	(94)
Presenile vessel amyloidosis	Mice	Endogenous	(75)
Doxorubicin-induced cardiomyopathy	Mice	Endogenous	(95)
Mitoxantrone-induced cardiomyopathy	Mice	Exogenous	(96)
Irradiation-induced heart diseases	Mice	Exogenous	(97)

RPE, retinal pigment epithelium; TNF, tumor necrosis factor.

supported these findings. Mice lacking PACAP displayed more severe symptoms of colitis and significantly stronger colonic inflammation. Moreover, 60% of DSS-treated PACAP deficient mice developed aggressive-appearing colorectal cancer (63). An altered microbiota composition can also be in the background of the increased vulnerability of PACAP knockout mice, as investigations of intestinal microbiota composition in wild type and PACAP deficient mice showed that Bifidobacteria were virtually absent in PACAP deficient mice, even when they were still breastfed (111).

Pancreas

PACAP is present in both the exocrine pancreas and in the endocrine islets of Langerhans and it is thought to be a potent intra-pancreatic regulator of beta cells under physiological and pathological conditions (112–114). Interestingly, cerulein-induced pancreatitits was aggravated in PACAP deficient mice (64), but pancreatic beta cells derived from rat insulinoma, key elements in pathogenesis in diabetes mellitus, were prevented from streptozotocin-induced cell death (18). Han and Wu (19) found that Adcyap1 overexpression reduced cytokine-induced apoptosis in a mouse insulinoma cell line. Moreover, pancreatic islets prepared from PACAP knockout and wild type mice showed significant differences in defense against glucotoxicity and lipotoxicity (20). Pancreatic islets cultured with high glucose or palmitate displayed severely impaired glucose-induced first phase Ca^{2+} increase and insulin secretion in PACAP deficient mice, but not in wild type animals (20). PACAP overexpression in KKAγ mice suffering from diabetes type II attenuated hyperinsulinaemia and islet hyperplasia without alteration of plasma glucose, glucose tolerance and insulin tolerance (115). This was observed both in animals on normal diet and in mice kept on high-fat diet suggesting that PACAP regulates abnormal increase in islet mass and hyperinsulinaemia in type II diabetes (115, 116).

Liver

Few data indicate that PACAP is also protective in some pathological liver conditions. Although no effects on survival were reported in normal or tumorous human hepatocyte cells *in vitro* exposed to oxidative stress (22), PACAP showed protection in mouse hepatocytes *in vitro* exposed to oxidative stress by H_2O_2 or subjected to apoptosis-inducing TNF-α/actinomycin D treatment (21). *In vivo* protection was also described in ischemia/reperfusion liver injury (21). Pretreatment with PACAP27 or 38 1 h before the onset of ischemia diminished serum alanine aminotransferase levels, reduced the accumulation of neutrophils and macrophages, suppressed inflammatory chemokines (CXCL-1, CCL-2, CXCL-10) and cytokines (TNF-α, IL-1β, IL-6, and IFN-β). The histological structure of the liver was better preserved after PACAP treatment: necrosis and apoptosis was reduced, caspase-3 activity was decreased along with increased antiapoptotic molecule expression of bcl-2 and bcl-xL via cAMP/PKA activation. The phosphorylation, thus activation, of IκBα/NF-κB p-65 proteins was reduced and toll-like receptor four immune response was inhibited (21).

The role of endogenous PACAP in liver protection was examined in PACAP deficient mice. Ischemia/reperfusion injury was augmented in animals lacking endogenous PACAP: serum alanin aminotransferase levels were increased and more severe tissue damage, indicated by edema, hemorrhage, congestion, and hepatocellular necrosis, was observed compared to wild type mice (21). In wild type mice, ischemic injury followed by reperfusion led to a transient drop in endogenous PACAP mRNA expression followed by a progressive increase. Similarly, ischemic reperfusion injury triggered changes in the receptor expression: VPAC receptor expression was also increased after an initial drop, while PAC1 receptor expression was increased from the onset of the ischemic period (21). A follow-up study demonstrated that the protective effects of PACAP in hepatic ischemia/reperfusion are partially mediated by induction of Yes-associated protein, a cellular modulator of tissue regeneration (65). The ischemia-induced induction of this protein was absent in mice lacking endogenous PACAP, while PACAP substitution enhanced its expression.

A recent paper showed that PACAP can alleviate inflammation and steatosis, thus it can be protective in obesity-related hepatic pathology and can ameliorate glucose and lipid metabolism (66). These effects were mainly mediated by the specific PAC1 receptor, involving Fas apoptosis inhibitory molecule (FAIM), proven both *in vitro* and *in vivo*. Lower PACAP expression was found in leukocytes isolated from obese human patients, and levels were lower in livers of obese mice. PACAP treatment of obese mice not only increased FAIM levels, but also decreased serum triglycerides and total cholesterol and reduced body weight (66). Liver triglycerides were also reduced after PACAP treatment. Systemic inflammatory markers were decreased, including MCP-1, IL-6, and TNF-α. Examining fat tissue revealed that PACAP treatment reduced the size of adipocytes and attenuated liver steatosis. Altogether, these data indicate that PACAP ameliorates hepatic metabolism and inflammation in obesity (66). An indirect mechanism has also been suggested to play a role in the regeneration-stimulating effect of PACAP: the PACAP-regulated selenoproteine expression is strongly stimulated in liver cells during the regenerative process that occurs after partial hepatectomy (117).

Salivary and Other Exocrine Glands

Occurrence of PACAP and receptors has been shown in several exocrine glands and their secretions, including salivary, mammary and lacrimal gland (85, 114, 118–121). Among others, PACAP enhances salivary and lacrimal gland secretion, increases salivary gland blood flow, and is implicated in breast cancer growth (85, 118, 122–125). In contrast to the general cytoprotective effects of PACAP, in MCF-7 breast cancer cell line PACAP induced reactive oxygen species through H_2O_2 production, induced calcium release, and promoted apoptosis by increasing Bax and decreasing bcl-2 expression (126). The evolutionarily conserved nature of the antiapoptotic effect has been proven in molluscan salivary gland (67). PACAP induced a significant elevation of cAMP level in salivary gland extracts and attenuated the apoptosis-inducing effect of dopamine and colchicine, shown by the reduced caspase-positive cells (67).

URINARY SYSTEM

Widespread distribution of PACAP and its receptors has been described in the kidney and lower urinary tract (127, 128), where PACAP plays distinct roles in the micturition pathways, blood supply, hormone production and inflammation (128–130). Direct protective effects of PACAP have mainly been described in the kidney, its nephroprotective actions have been widely studied. Its protective effects could be observed in different models of renal pathological conditions (131). It shows protective effects both *in vitro* and *in vivo*. *In vitro* data are available proving the renoprotective effect of PACAP in different models of cellular damage. It was shown to decrease the cell survival worsening effect of oxidative stress in primary renal cell cultures (22). In addition, Li et al. described its protection against mineral oil evoked *in vitro* hypoxia in proximal tubule epithelial cells obtained from wild type and MyD88 deficient mice (23). Furthermore, its endogenous action against oxidative stress and hypoxia can also be observed using PACAP deficient mice, responding with higher vulnerability to oxidative stress leading to decreased survival rate (24). Similarly, this susceptibility could also be detected when renal cells were exposed to $CoCl_2$-evoking *in vitro* hypoxia (25). Experiments investigating the effect of PACAP against proteinuria mimicking albumin treatment in human proximal tubule cells showed that PACAP could not influence cell viability either positively or negatively (132). It was not able to change the increased TGF-β1 expression either.

In vivo observations were obtained from a series of experiments modeling renal ischemia/reperfusion injury. The first experiments proving PACAP's renoprotective effect in ischemia/reperfusion were performed by Riera et al. (69). They found that continuous PACAP infusion improved renal function, attenuated morphological damage, and influenced inflammatory cellular infiltration. In addition, Szakaly et al. performed experiments, in which PACAP was able to ameliorate tubular damage and the level of oxidative stress, thus to decrease mortality of rats that underwent ischemia/reperfusion (70). PACAP was shown to reverse the cytokine expression profile after ischemic injury (133). Such renoprotective effect was also detected in mice (23, 26, 27). Khan et al. (68) performed experiments to investigate the involvement of toll-like receptors. Dozens of toll-like receptor genes changed after ischemia, while PACAP was able to reverse these changes. Gender-dependence was investigated in a recent study comparing renoprotective effect of PACAP in male and female rats (71). Tubular alteration was markedly less severe in female rats. Female animals showed better results in both PACAP-treated and vehicle-treated experimental groups indicating the presence of several additional protective factors in females.

Actions of PACAP have also been studied in different models of drug-induced nephropathies. PACAP was able to diminish the nephrotoxic effect of gentamicin both *in vitro* and *in vivo*. *In vitro* experiments showed its cell survival enhancing effect in human proximal tubule cell line (HK-2) assessed by cytotoxicity assay (28). In the same experimental model, it could counteract the downregulating effect of gentamicin on dipeptidyl peptidase IV and vascular endothelial growth factor (28). In accordance with *in vitro* data, *in vivo* investigations have also explored its protective effect in gentamicin-induced nephropathy. Tubular damage caused by the accumulation of gentamicin could be attenuated by repeated intravenous administration of PACAP in rats, as indicated by the decreased TNF-α production (72). Chemotherapeutic agents, like cisplatin, can also lead to renal injury (134). *In vitro* studies using human HK-2 cells proved that PACAP protected against cisplatin-induced injury, decreased the cisplatin-induced TNF-α activation, influenced signaling pathways activated by cisplatin (27). Li et al. examined primary mouse renal proximal tubular epithelial cell culture (26). In accordance with results in human cells, PACAP's protective effect was detectable. *In vitro* results were supported by *in vivo* renoprotection in a mouse model of cisplatin-induced nephrotoxicity (26). Mice treated with PACAP showed less severe decrease of renal function. Furthermore, PACAP treatment was able to alleviate morphological damage and reverse the cisplatin-induced p53 activation. A further drug, cyclosporine A has also been widely studied. Cylosporine A, a potent immunosuppressant used for preventing allograft rejection and in treatment of autoimmune diseases, can also lead to impaired renal function (135). Khan et al. (29) investigated the effect of PACAP against cyclosporine A both *in vitro* and *in vivo*. PACAP was able to improve morphological changes and attenuate TGF-β activation caused by cyclosporine A treatment in HK-2 cells. *In vitro* data were further supported by their *in vivo* findings. PACAP could improve the impaired renal function with normalizing serum creatinine level. In addition, tubulointerstitial damage was diminished and changes in cell junctional markers were restored by PACAP treatment (29). Protective effect of PACAP against contrast-induced nephropathy was also tested by Khan et al. (30). PACAP could decrease the proliferation-inhibiting effect of both ionic and non-ionic contrast media in HK-2 cells. If added prior to urografin, it was able to enhance cell survival of HK-2 cells. Furthermore, PACAP decreased the elevated kidney injury molecule-1 (KIM-1) expression evoked by contrast medium (30). *In vivo* findings complete the *in vitro* data. Mice pretreated with PACAP before contrast agent did not show severe tubular damage, apoptosis, increased oxidative stress or inflammatory reactions unlike animals receiving only contrast agent. A kidney biomarker assay further supported these data, as numerous markers associated with kidney injury were decreased in PACAP-treated mice (30).

Arimura et al. studied the actions of PACAP in myeloma kidney injury both *in vitro* and *in vivo* (31). *In vitro* myeloma kidney injury was generated with kappa light chains isolated from the urine of a patient suffering multiple myeloma. Human renal proximal tubule cells exposed to kappa light chains were protected by PACAP: it could mitigate the cellular injury and elevated expression of IL-6 and TNF-α. *In vivo* investigations in rats were in accordance with *in vitro* results. Rats receiving both PACAP and myeloma light chain showed reduced cytokine activation compared to animals treated only with light chain (31). In addition, effectiveness of PACAP was also examined in a single patient case study, in which it was shown to reduce free lambda light chains in urine indicating its possible therapeutic use in the future (136). Another target of investigations exploring

renoprotective effect of PACAP is diabetic nephropathy, the leading cause of renal insufficiency (137). Sakamoto et al. used podocytes to model inflammation in diabetic nephropathy (32). PACAP was able to reduce the lipopolysaccharide-induced proinflammatory cytokine activation, ERK phosphorylation and NF-κB transnuclear localization. *In vivo* studies revealed protection in streptozotocin-induced diabetes in rats (73). Intraperitoneal PACAP treatment led to less severe morphological changes and reduced proinflammatory cytokine activation (73). Li et al. (72) applied continuous PACAP infusion for 2 weeks and they found that PACAP reduced the diabetic changes like proteinuria and glomerular enlargement. Molecular mechanism of PACAP-exerted protective effects was also examined in streptozotocin-induced diabetes (74). Results showed that PACAP could decrease the activation of apoptotic signaling pathways.

Investigating PACAP deficient mice, it was revealed that animals lacking endogenously present PACAP suffer from presenile systemic amyloidosis (75). Severe amyloidosis can be observed in several organs including the kidney. Kidney was one of the most affected organs, amyloid deposits were found in renal corpuscles. Level of renal function was also in accordance with amyloid deposits: serum creatinine level was increased in aging PACAP-deficient mice (75, 138).

PACAP suppression experiments in zebrafish of Eneman et al. revealed that nephrin depletion, a model of nephrotic syndrome is associated with adcyap1a and vip downregulation (76). Using adcyap1a and adcyap1b morpholinos the authors decribed more severe sequelae of nephrin depletion. In addition, administration of human PACAP38 could rescue the phenotype of zebrafish embryos injected with PACAP morpholino, but it was not able to save them in case of nephrin depletion. Nephrotic syndrome was also modeled by adriamycin exposure, when only adcyap1a gene was downregulated. Furthermore, nephrotic fishes showed reduced protein expression of PACAP. PACAP morpholinos worsened the change in phenotype induced by adriamycin exposure, which could be attenuated by addition of human PACAP38 (76).

RESPIRATORY TRACT

Although the role of PACAP in the respiratory tract is not as widely studied as that of VIP, PACAP and is receptors occur in the entire length of the respiratory tract (139–141) and several effects have been described in airway smooth muscle contraction and mucous secretion (142–144). It has been reported that PACAP increases allergic reactions in the human nasal mucosa by increasing resistance and plasma leakage (145), but PAC1 receptor is implicated in anti-inflammatory reactions and mediates alleviation of bronchial hyperreactivity (146). Exogenous PACAP diminished both capsaicin- and electric field stimulation-evoked sensory neuropeptide release in a concentration-dependent manner in trachea preparations (77, 78), showing that PACAP is able to diminish neurogenic inflammatory response *in vivo*. The protective role of PACAP has also been demonstrated in a lung inflammation model of mice (79). In endotoxin-induced subacute airway inflammation, airway hyperreactivity, histopathological changes, and myeloperoxidase activity were markedly higher in mice lacking endogenous PACAP, pointing to the anti-inflammatory role of endogenous PACAP in the lungs (79). In another rat model, in vanadate-induced airway hyperresponsiveness, PACAP inhalation alleviated the increase in bronchial resistance, reduced the increased inflammatory chemokine, and cytokine release and improved the antioxidant status, also pointing to the potential of PACAP treatment in inflammatory and allergic respiratory conditions (80). Similar results were found in ozone-induced airway hyperresponsiveness, which was suppressed by PACAP without affecting plasma extravasation (81). Furthermore, Yu et al. (82) have described that PACAP, bound to a traversing-enhancing TAT peptide, can alleviate smoke inhalation-induced condition. They found that both PACAP and PACAP-TAT decreased mortality, led to a body-weight increase, alleviated edema and vascular permeability increase, and decreased oxidative stress as indicated by reduced myeloperoxidase activity, interleukin-6, and malondyaldehyde levels while increased catalase levels in the lungs of mice that were exposed to repeated smoke inhalation (82). PACAP and PACAP-TAT treatments also resulted in decreased cell infiltration and bronchial epithelial hyperplasia. These data indicate that PACAP can alleviate smoke inhalation-induced damage of the lungs. These data confirmed earlier results showing that PACAP protected rat alveolar L2 cells from cytotoxicity of cigarette smoke by reducing caspase activity resulting in reduced apoptotic cell death (33). PACAP has also been implicated in lung cancer cell growth (147). PACAP stimulates colony formation and nuclear oncogene expression in NCI-N417 lung cancer cells, while PACAP antagonist treatment slows down small cell lung cancer growth (148, 149) and lower levels of PACAP were described in human lung cancer biopsies in comparison with neighboring healthy tissue (150). PACAP induces vasodilation, including pulmonary vessels. The absence of its specific receptor PAC1 causes pulmonary hypertension and right heart failure after birth (151). These findings demonstrate the crucial importance of PAC1-mediated signaling for the maintenance of normal pulmonary vascular tone during early post-natal life (151).

SKIN

The presence of PACAP and receptors has been shown in the skin (83, 152, 153). Potent vasodilatory and edema-building effects have been attributed to cutaneous PACAP treatment soon after its discovery (154, 155). Recent studies have shown that PACAP stimulates sweat gland activity (156). PACAP in the skin is implicated as a protective factor in the development of inflammatory dermatological conditions, such as psoriasis (153, 157) and neurogenic skin inflammation (83). Although both stimulatory and inhibitory actions on skin edema formation have been described, PACAP deficient mice show increased delayed-type of hypersensitivity reaction induced by oxazolone (84). Mice lacking endogenous PACAP had increased edema

formation, moderately enhanced cellular inflammatory reactions, and increased levels of the inflammatory cytokine monocyte chemoattractant protein-1 levels (84). These results point to the anti-allergic effects of PACAP in a model of contact dermatitis. We have already mentioned above the presenile appearance of a systemic type of amyloidosis in PACAP deficient mice (75, 138). The amyloid deposition was markedly present in the skin, under the epidermis. In the skin, the main location of the deposits was the dermal papillary layer, continuous with the homogeneous mass in the connective tissue surrounding appendages (hair follicles and sebaceous and sweat glands) and vessel walls. While in wild type mice occurrence of amyloidosis was observed in 57% of the animals at old age, and only 14% young mice, already in 67% of young PACAP KO mice and nearly 90% of old PACAP KO mice exhibited amyloid deposits (75) in the skin. This shows that PACAP deficiency can be an attributing factor in skin aging.

NON-NEURONAL PARTS OF THE VISUAL SYSTEM: CORNEA AND RETINAL PIGMENT EPITHELIAL CELLS

The effects of PACAP in the visual system have been widely investigated and numerous reports have shown the effects of PACAP in the neural parts, especially in the retina. Both *in vitro* and *in vivo* studies have demonstrated that PACAP is a potent neuroprotective agent in different retinal pathologies. These effects have been reviewed in several papers (86, 158, 159). Other, non-neuronal parts of the eye have also been investigated for the actions of PACAP. Among others, PACAP influences sphincter and dilator pupillary muscle contractility (160), increases cAMP in the ciliary epithelium (161) and is involved in ocular inflammatory reactions (162). Regarding cytoprotective effects, several studies have described that PACAP protects the cornea. The cornea consists of 5 layers, the outer epithelium building the barrier toward the outside and the inner endothelium serves as a barrier toward the aqueous humor. Both inner and outer epithelial layers play an important role in maintaining the hydration and transparency of the main stromal layer, which is separated from the outer and inner epithelial layers by outer and inner limiting membranes, respectively. PACAP receptors have been described on the surface of the cornea (85). It is thought that the peptide produced by the lacrimal gland and present in the lacrimal fluid can act on the surface of the cornea and can play a role in the regeneration of the corneal surface epithelial cells. Indeed, PACAP eye drops prevent corneal keratinization in PACAP deficient mice (85). A PACAP-derived peptide has been shown to promote corneal wound healing (87, 88). PACAP given in form of eye drops not only acts directly on the surface but also passes ocular barriers and is able to induce protective effects in the retina (163, 164).

Recent studies have reported that PACAP is protective in corneal endothelial cells, where PACAP and all three receptors are expressed (34, 35). In isolated human corneal endothelial cells PACAP protected growth factor deprivation-induced decrease in cellular viability and restored transepithelial electrical resistance (34). Furthermore, PACAP increased the expression of tight junction proteins and stimulated corneal endothelial repair demonstrated in a wound healing assay (34). Noteworthy, PACAP exerted a protective effect on corneal endothelium against ultraviolet B (UV-B) radiation, by reducing the activation of apoptotic pathway through a down-regulation of bax and cleaved caspase-3 and up-regulation of bcl-2 protein. Moreover, PACAP preserved corneal endothelium integrity following UV-B exposure by increasing transepithelial electrical resistance and the expression of ZO-1 and claudin-1 proteins (36). The same authors investigated the involvement of epidermal growth factor receptor involvement in PACAP-induced protection of corneal endothelial cells (35). They found that PACAP, through PAC1 receptor, induced epidermal growth factor receptor phosphorylation and MAP kinase/ERK1/2 activation. These results are also in accordance with data obtained in retinal pigment epithelial cells where PACAP restored cell viability in pigment epithelial cells exposed to different stressors, induced ERK and epidermal growth factor receptor phosphorylation and ameliorated junctional protein damage (37–40).

The retinal pigment epithelium is the outermost, non-neural layer of the retina. The apical surface of the cells is in contact with the outer segments of the photoreceptor cells, while the basolateral surface attaches to the Bruch's membrane, which separates the pigment cells from the choriocapillary layer. Tight junctions interconnecting retinal pigment cells are the main components of the outer blood-retina barrier, which has essential roles in the maintenance of the retinal homeostasis. Dysfunction of the tight junctions has been observed in diabetes and ischemia, leading to leakage of macromolecules into the retina, contributing to the development of retinopathies (165). Retinal pigment epithelial cells are likely to undergo hyperosmotic stress during the development of macular edema, resulting in reduction in aquaporin-4 expression (166). Moreover, hyperosmolarity was observed to induce transcription of bFGF and HB-EGF genes and secretion of bFGF from the pigment cells (167). One of the most important factors secreted by the pigment epithelial cells is vascular endothelial growth factor, VEGF (168–172). Overexpression of VEGF is one of the major inducers of age related macular degeneration and proliferative diabetic retinopathy, which are among the leading causes of blindness worldwide (173).

All three PACAP receptors (PAC1, VPAC1, and VPAC2) mRNAs were detectable in the pigment epithelium (174). According to Mester et al. (37) adult retinal pigment epithelial cells (ARPE-19) exposed to H_2O_2 could be rescued with PACAP in a dose-dependent manner. In a subsequent study it was also proved that PACAP could inhibit the expression of pro-apoptotic factors (Bad, Bax, and Hif1α) and elevate the levels of anti-apoptotic factors such as ERK1/2 and CREB (38). Besides its general cell protective, pro-survival, and anti-inflammatory effects, PACAP possesses protective effects on tight junctions of endothelial and epithelial cells (175). PACAP was shown to have a protective effect on the barrier properties of the cells of the outer-blood-retinal barrier in the presence of factors accounting for diabetic macular edema (34, 35, 41, 89).

PACAP could also reduce the concentration of VEGF in ARPE-19 cells. Moreover, PACAP was able to attenuate

the levels of some other pro-angiogenic proteins (uPA, angiogenin, and endothelin-1). As a conclusion, PACAP is among the emerging molecules to fight diabetic complications and macular degeneration, similarly to VEGF antagonists, antioxidants, anti-inflammatory agents, and other neuropeptides (39).

IMMUNE CELLS AND THYMUS

PACAP is a well-established modulator of innate and acquired immunity and exerts protective functions in immunological diseases, although the presence of both anti- and pro-inflammatory roles of PACAP depending on the immune status, disease, age, and pathological conditions complicate the immunological role of PACAP (176, 177). As there are several reviews on the immunomodulatory roles of PACAP (177–179), the present review summarizes only data regarding direct cellular protection in lymphatic organs and cells. Activation induced cell death in T lymphocytes is an important mechanism in peripheral tolerance, initiated by antigen reengagement, and mediated through Fas/Fas ligand (FasL) interactions. PACAP was found to inhibit this induced cell death *in vivo* and *in vitro* in peripheral T cells and T cell hybridomas (42, 43). Both forms of PACAP, PACAP27 and PACAP38, can protect CD4+CD8+ thymocytes from glucocorticoid-induced apoptosis, suggesting an involvement of PACAP in thymic T-cell maturation (44). The expression of PAC1 receptor and PACAP increased in the degenerative thymus induced by cyclophosphamide (90). The authors have also described that while high dose PACAP had no protective effects against cyclophosphamide-induced thymus atrophy, low dose PACAP promoted the thymus index, inhibited apoptosis, enhanced oxidative status, and decreased caspase activity (90).

SKELETAL SYSTEM: CARTILAGE AND BONE

Neuropeptides have important functions in the development of skeletal elements and also in regeneration processes (180, 181). Detailed analysis of PACAP receptors has been performed in chondrogenic cell cultures (45) and osteogenic cell lines (182, 183). The specific PAC1 receptor has been identified in bone and cartilage (45, 183) and the activation of PAC1 by PACAP has positive effect on cartilage and bone formation (45, 184) via the activation of protein kinase A (PKA)-regulated pathways. PACAP also positively regulates matrix expresssion of both musculoskeletal elements. Addition of PACAP to chondrogenic cell cultures increases the activation of Sox9 (SRY-related HMG-BOX gene) and regulates the expression of HAS2 and HAS3 (hyaluronan synthase) expression (45). In bone PACAP increased the activation of alkaline phosphatase (ALP) and elevated the expression of collagen type I (184).

In degenerative cartilage diseases increasing number of experiments have been performed in the last decade to prove that PACAP plays a protective role. First, it was shown that the expression of PAC1 receptor was altered in specific layers of cartilage in osteoarthritis (OA) (46) and reduced during oxidative stress (45). On the other hand, the neuropeptide has a preventive function in pathological processes. In cartilage, the antagonist PACAP6-38 behaves as an agonist and administration of both PACAP1-38 and 6-38 protected against the harmful effect of oxidative stress in high density chondrogenic cell cultures via the elevation of collagen type II, aggrecan and hyaluronic acid expression (45). PACAP elevated the activation of PP2B (protein phosphatase 2B) and subsequently triggered the activation of NFAT (Nuclear factor of activated T-cells). In oxidative stress PACAP administration prevented the phosphorylation processes of chondrogenic signaling pathways in chicken high density cultures. The administration of the neuropeptide was able to defend cartilage formation during mechanical overload via decreasing the expression of collagen type X, a characteristic sign of OA cartilage. On the other hand, it increased collagen type II expression inducing a chondrogenic phenotype formation (185). PACAP signaling also communicates with the hedgehog pathways leading to decreased expression of SHH (sonic hedgehog) and IHH (Indian hedgehog) (185). The reduced activation of hedgehog pathways kept cartilage in a prehypertrophic condition (185) suggesting an important role of PACAP in cartilage degradation processes. Furthermore, mechanical overload and presence of reactive oxygen species trigger the activation of several matrix degrading enzymes such as matrix metalloproteinases, hyaluronidases, and aggrecanases in OA. PACAP was able to reduce the activation of matrix metalloproteinases and aggrecanases in chondrogenic cell cultures exposed to forced physical stress and oxidative stress, further strengthening the preventive function of the neuropeptide in cartilage diseases (47). In OA patients concentration of PACAP is decreased (186) and the administration of PACAP with hyaluronic acid injection increased the synovial fluid PACAP concentration and prevented the cartilage degradation processes (186). Moreover, PACAP addition during anterior cruciate ligament related OA formation can be an adjuvant therapy to prevent the physiological structure of articular cartilage (187). Taken together application of PACAP as a potential therapeutic target of OA formation is likely as it was discussed by Grassel et al. (188).

Some data point to the possible importance of PACAP in bone regeneration processes. First of all, the presence of PACAP is needed for proper bone architecture formation (184). In the lack of PACAP the long bones are more fragile and their organic and inorganic extracellular matrix balance and distribution are disturbed (184). In callus formation PACAP is proven to have important function via the activation of BMP (bone morphogenetic protein) signaling pathway and balance the ALP expression (91). In mice lacking endogenous PACAP, callus formation after tibia fracture was disturbed with several alterations in compensatory pathways (91). Furthermore, PACAP has an important function in bone turnover and inflammation (92). On the other hand, the presence of PACAP inhibited osteophyte formation and had a preventive role in rheumatoid arthritis development (92).

CARDIOVASCULAR SYSTEM: VESSELS AND HEART

PACAP is a well-known vasodilator peptide (189–191). Receptors are found in vessel walls and PACAP-induced vasodilatory effects have been demonstrated in various vessels *in vitro* and *in vivo*. This perfusion-increasing potency has beneficial effects in several organs, but can also trigger migraines through this activity in meningeal arteries (192, 193). PACAP has also been described to preserve post-ischemic cerebrovascular reactivity in pigs, independent of its vasodilatory effect (93). As there are several original and review papers published in the recent years regarding this vasodilatory effects of PACAP (191–196), now we focus more on the protective effects of PACAP exerted directly on the vascular wall, especially on endothelial cells. First results showed protective effects against oxidative stress: exposure of mouse hemangioendothelioma cells to hydrogen peroxide resulted in a robust reduction of viability and an increase of apoptotic cells, while co-incubation with PACAP increased cell viability and reduced the number of apoptotic cells (48). PACAP treatment also ameliorated the reduced level of ERK phosphorylation and counteracted the increased phosphorylation of the pro-apoptotic JNK and p38 MAP kinases. PACAP also exerts a cytoprotective effect on endothelial colony forming cells exposed to TNF-α and partially rescues their proliferation potential inhibited by prolonged TNF-α exposure (49). In a more recent study, ameliorating effect of PACAP has been described in hyperglycaemia-induced endothelial dysfunction, an important factor contributing to diabetes-related vascular pathology (94). PACAP reduced the hyperglycaemia-induced elevation of fibroblast growth factor basic, matrix metalloproteinase 9 and nephroblastoma overexpressed gene proteins, implicating a protective role of PACAP in vascular complications of diabetes (94). PACAP deficient mice are susceptible to develop a systemic form of senile apolipoprotein IV-predominant amyloidosis, characterized by typical perivascular deposits in most organs (75, 138), pointing to the role of PACAP in age-related vascular changes. This has been also confirmed in a study where angiogenic capacity was examined in cerebromicrovascular endothelial cells (197). In aged cells, expression of PACAP was decreased, associated with reduced capacity to form capillary-like structures, impaired adhesiveness to collagen, and increased apoptosis (caspase3 activity). Overexpression of PACAP in aged endothelial cells resulted in increased tube-formation capacity. Treatment with recombinant PACAP also increased tube formation and inhibited apoptosis in aged cells. In young cerebromicrovascular endothelial cells, knockdown of endogenous PACAP expression impaired tube formation capacity, mimicking the aging phenotype (197).

PACAP and its receptors occur in the heart and the neuropeptide exerts various different cardiac functions. Several studies examined the potential effects of PACAP in the cardiovascular system. The presence of this polypeptide and its PAC1 receptor have been demonstrated in cardiomyocyte cell cultures, mouse heart tissue and also in human heart samples (150, 198–200). PACAP has direct positive chronotrop, inotrop, dromotrop, and vasodilatator effects and exerts robust cardioprotective actions due to its antiapoptotic and antioxidant properties (201). The cardioprotective effects of PACAP were first identified in cardiomyocyte cell cultures against different ischemia/reperfusion injuries. In these experiments, exogenous PACAP treatment lead to decreased level of pro-apoptotic factors (Bad, caspase-3) and elevated levels of anti-apoptotic proteins (cleaved caspase-8, Bcl-xl) inducing significantly increased cell viability (50, 51). Moreover, several *in vitro* experiments have already proven that PACAP provides effective protection against oxidative stress induced apoptosis due to higher Bcl-2 and phospho-Bad expression and lower caspase or Jun N-terminal kinase and p38 mitogen activated protein (MAP) kinase activation (51–53).

Besides *in vitro* researches, different animal studies also examined the cardioprotective effects of PACAP. Alston et al. detected significantly higher endogenous PACAP levels and mRNA expression in mice after myocardial infarction (202). Furthermore, examining doxorubicine induced cardiomyopathy, significantly more severe DNA damage and apoptotic cell death were detected in PACAP-deficient mice compared to wild types (95). In another study, PACAP treatment had protective effect against mitoxandron induced cardiotoxicity (96). PACAP attenuated body weight reduction of mice, prevented mitoxantrone-induced left ventricular dilation resulting in diminished functional deterioration shown by the ejection fraction and fractional shortening (96). A recent study provided both *in vitro* and *in vivo* evidence that PACAP attenuates radiation-induced heart disease, a common consequence of thoracic irradiation therapy (97). PACAP enhanced viability and colony-forming efficiency, while reduced generation of reactive oxygen species in cardiomyocytes exposed to radiation. PACAP exposure suppressed myocardial apoptosis and G2/M arrest through blunting the radiation-induced down-regulation of Bcl-2, CyclinB1 and CDC2, and inhibiting the up-regulation of Bax (97). Pre-treatment with PACAP also protected mice from radiation-induced histological damage including myocardial apoptosis and fibrosis (97).

In addition to the several *in vitro* and animal experiments, a few human studies have also been performed that raise the possibility of PACAP being cardioprotective also in the human heart. Szanto et al. examined human right auricle tissue samples from coronary artery bypass or heart valve implantation surgery. They found significantly higher PACAP concentration in patients with ischemic heart disease compared to valvular abnormalities emphasizing the protective role of PACAP in ischemic heart diseases (150). Remarkable differences were detected in plasma PACAP levels of ischemic and primary dilated cardiomyopathy patients. A significant negative correlation was observed between the severity of ischemic heart failure and plasma PACAP levels suggesting that PACAP might play an important role in the pathomechanism and progression of ischemic heart failure (203). Lack of PAC1-mediated signaling has been shown to be associated with pulmonary hypertension and right heart failure in PAC1 deficient mice, indicating the crucial importance

of PACAP in the maintenance of normal pulmonary vascular tone (151).

REPRODUCTIVE ORGANS

PACAP and its receptors have widespread and stage-specific occurrence in the gonads and their presence have been shown in several other reproductive organs (100, 204, 205). PACAP has been described as a follicle-surviving factor due to suppression of apoptosis in a dose-dependent manner in rat ovaries (206). Although PACAP has been detected in the human ovarian follicular fluid, its exact role in the human ovary is not known (207). In the testis, PACAP plays a role in spermatogenesis and is supposed to be involved in tumor growth (100, 208, 209). Interestingly, in spite of PACAP deficient mice displaying disturbed spermatogenesis and altered testicular immunity (100, 210), they also show delayed testicular aging supposedly due to the stimulatory effect of PACAP on testosterone production, which, in turn, accelerates aging due to increased oxidative stress (211). The involvement of PACAP has been described in numerous reproductive processes from fertilization to implantation (212–214). However, its direct cytoprotective effects have only been shown in a few cases. PACAP protects human trophoblast cells against oxidative stress, but has no effect against methotrexate-induced cell death and in contrast, it potentiates cell death in choriocarcinoma cells (205, 215, 216). Similar tumor cell growth inhibiting effects have been described in cervical cancer cells (217). These data demonstrate that while in many tissues PACAP is clearly a pro-survival factor, no such clear effect has been found in the reproductive tissues, where PACAP seems to play a more complex regulatory role in reproductive processes and during tumor growth.

OTHER CELL TYPES

One study described that PACAP was able to attenuate apoptosis of human hypophysis adenoma cell line HP75 induced by transforming growth factor-β1 (218). In PC-3 human prostate cancer cells PACAP inhibited apoptosis induced by serum starvation (219). PACAP alone did not influence cell survival in cultured pinealocytes, but could attenuate the toxic effect of H_2O_2. However, co-incubation of pinealocytes with PACAP promoted survival only in the dark phase, PACAP during the light phase did not result in significant differences in the percentage of living cells. This suggests that the time of day can also influence the protective effects of PACAP (220). PACAP inhibited serum depletion-induced apoptosis of megakaryocytes via VPAC1 stimulation (221).

CONTRADICTORY ASPECTS

Based on the studies summarized here PACAP is now considered as a general cytoprotective peptide. However, its cell survival-promoting effect depends on a number of factors. First of all, it depends on the type of cell and developmental stage of a cell type. Also, numerous other factors can influence whether PACAP acts as a pro-survival factor, has no effect, or even on the contrary, it induces cell death (222). For example, PACAP has been described to counteract the cytotoxic effects of cisplatin chemotherapy treatment in neurons, without affecting the toxic effects in ovarian cells, thereby not influencing the therapeutic effect of cisplatin on tumor cells (223). Glioblastoma cell invasion is inhibited by PACAP under low oxygen tension (224), while both pro- and anti-proliferative effects have been found in different glioblastoma cell lines (225, 226). As we summarized the cytoprotective effects of the peptide present in several cell types, PACAP has also been described to exert cytotoxic effects in high doses in certain tumor cells, like in retinoblastoma cells (227). In certain cells, neither pro- nor anti-survival effects could be found, like JAR cytotrophoblast cells with or without methotrexate treatment (205) or in HEP-G2 hepatocellular carcinoma cells subjected to oxidative stress (22). In contrast, in several other tumor cell line, PACAP has been shown to inhibit growth, like MCF-7 breast cancer cells (126), a negative regulator of cervical carcinoma as overexpression of the PACAP gene in cervical cancer cell lines lacking PACAP expression significantly inhibited cell growth and induced apoptosis (217), is a pro-apoptotic factor in choriocarcinoma cells (215), and inhibits the growth of myeloma, leukemia and medulloblastoma cells (228–230).

SUMMARY

In summary, PACAP has protective effects against various harmful stimuli in a wide range of tissues in the periphery. Numerous factors influence this protective effect, the detailed mapping of which awaits further investigatons.

AUTHOR CONTRIBUTIONS

DT and DR conceptualized the paper. DT and VV constructed the figure. AT, ES, TJ, GH, EF, BO, DS, GM, AD'A, and VD'A wrote parts of the review.

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REVIEW
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Differential Vulnerability of Oculomotor Versus Hypoglossal Nucleus During ALS: Involvement of PACAP

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Amyotrophic lateral sclerosis (ALS) is a progressive multifactorial disease characterized by the loss of motor neurons (MNs). Not all MNs undergo degeneration: neurons of the oculomotor nucleus, which regulate eye movements, are less vulnerable compared to hypoglossal nucleus MNs. Several molecular studies have been performed to understand the different vulnerability of these MNs. By analyzing postmortem samples from ALS patients to other unrelated decedents, the differential genomic pattern between the two nuclei has been profiled. Among identified genes, adenylate cyclase activating polypeptide 1 (ADCYAP1) gene, encoding for pituitary adenylate cyclase-activating polypeptide (PACAP), was found significantly up-regulated in the oculomotor versus hypoglossal nucleus suggesting that it could play a trophic effect on MNs in ALS. In the present review, some aspects regarding the different vulnerability of oculomotor and hypoglossal nucleus to degeneration will be summarized. The distribution and potential role of PACAP on these MNs as studied largely in an animal model of ALS compared to controls, will be discussed.

Keywords: amyotrophic lateral sclerosis, lower motor neurons, oculomotor nucleus, hypoglossal nucleus, pituitary adenylate cyclase-activating polypeptide

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an incurable and multifactorial neurodegenerative disease induced by the synergistic action of genetic and environmental factors. The ALS cases are classified into sporadic (SALS) and familial (FALS) forms. Mutations in the Cu/Zn superoxide dismutase 1 (SOD1) gene account for approximately 20% of FALS cases (Rosen et al., 1993). For the most of ALS patients, disease etiology is unknown and pathological events leading to degeneration of upper and lower motor neurons (MNs) start long before the appearance of clinical symptoms. Not all MNs undergo degeneration during the progression of ALS. In fact, somatic MNs of the oculomotor nucleus controlling eye movements are generally spared compared to the more vulnerable hypoglossal MNs innervating the tongue (Mannen et al., 1977; Reiner et al., 1995; Nimchinsky et al., 2000; Haenggeli and Kato, 2002; Hedlund et al., 2010). It has been suggested that phenotypic and genetic heterogeneity of MNs is responsible for the different susceptibility to injury (Hedlund et al., 2010).

Through microarray technology, Hedlund et al. (2010) compared the genomic pattern of oculomotor to hypoglossal nucleus. Among identified genes, adenylate cyclase activating polypeptide 1 (ADCYAP1) gene, encoding for pituitary adenylate cyclase-activating polypeptide (PACAP), is significantly up-regulated in the oculomotor as compared to hypoglossal nucleus suggesting a possible role of the peptide in the higher resistance of oculomotor MNs to death.

The involvement of PACAP in ALS has been described in several papers. Comparison of microarray datasets of sporadic ALS motor cortex and mutated SOD1 (mSOD1) G93A mice brains revealed that some genes, including those of PACAP and its receptor PAC1R, are deregulated in the same direction in both human and transgenic animals (Morello et al., 2017b).

The cellular and regional distribution of PACAP and its receptors, known as PAC1R, VPAC1, and VPAC2 receptors, has been extensively investigated in central and peripheral nervous system. In response to neurodegenerative insult, the peptide is released from MNs and exerts a neuroprotective role through autocrine and/or paracrine mechanisms mediated by cAMP/PKA-(cyclic adenosine monophosphate/protein kinase A) or PI3K (phosphoinositol three kinase) pathways (Manecka et al., 2013). Moreover, it has also been demonstrated that the trophic effect of PACAP can be exerted either directly or indirectly through EGFR trans-activation.

The present review aims to summarize findings on the role played by PACAP in the neurodegenerative process affecting lower MNs during ALS on postmortem samples of decedents with ALS and in an animal model of ALS, mainly focusing on the oculomotor and hypoglossal nuclei.

OVERVIEW ON ALS AND DIFFERENT VULNERABILITY OF OCULOMOTOR VERSUS HYPOGLOSSAL NUCLEUS MNS TO DEGENERATION

ALS is a motor neuron disease, a degenerative, progressive and paralytic disorder targeting MNs of the brain and spinal cord (Rowland and Shneider, 2001). The early stage of the disease is characterized by focal weakness progressing toward the impairment of most muscles including the diaphragm (Wijesekera and Leigh, 2009). Patients' mean survival from onset is ∼3 years and the most common cause of death is respiratory paralysis. In United States and Europe, the incidence of ALS is one case per 100,000 people per year. Its prevalence drastically increases with age (Johnston et al., 2006; Chiò et al., 2013; Robberecht and Philips, 2013).

The ALS cases are classified in sporadic (SALS) and familial (FALS) forms. The first one, without genetic inherited component, occurs in 90% of cases, while FALS cases (10%) are associated with mutations identified in distinctive genes. Among these, superoxide dismutase 1 (SOD1) was the first mutated gene to be discovered about two decades ago and it accounts for 20% of FALS cases (Rosen et al., 1993). More than 160 mutations in SOD1 gene, including G93A, A4V, H46R, and D90A have been reported. The toxic mechanism through which SOD1 mutations lead to MNs degeneration is not completely clarified. However, it is known that SOD1 mutant protein is overexpressed, misfolded and elicits various toxic effects such as increased oxidative stress, activation of microglia leading to inflammation, alteration of protein quality control due to proteasome defect, excitotoxicity due to decreased glutamate re-uptake and alteration of axonal transport (**Figure 1**; Bristol and Rothstein, 1996; Hoffman et al., 1996; Grosskreutz et al., 2010).

During the last 15 years, other FALS mutated genes have been identified, including TAR DNA binding protein (TARDBP), Fused in Sarcoma (FUS) and Chromosome 9 open reading frame 72 genes (C9orf72). TDP43, encoded by the most common mutated ALS gene TARDBP, and FUS proteins regulate RNAs expression and maturation (Neumann et al., 2006; Lagier-Tourenne et al., 2010; DeJesus-Hernandez et al., 2011). Additional ALS risk genes comprise: heterogenous nuclear ribonucleoprotein A1 (HNRNPA1) and heterogenous nuclear ribonucleoprotein A2B1 (HNRNPA2B1) both involved in pre-mRNA processing, metabolism and transport; profilin 1 (PFN1) binding to actin and affecting cytoskeleton structures; ataxin-2 (ATXN2) modulating RNA processing; chromatin modifying protein 2B (CHMP2B) involved in recycling and degradation of cell surface receptors; ubiquilin 2 (UBQLN2) regulating protein degradation; vesicle-associated membrane associated-protein B (VAPB) and the ATP-binding protein valosin-containing protein (VCP) both involved in membrane and vesicle trafficking; optineurin (OPTN), TANK-Binding Kinase 1 (TBK1) and sequestosome 1 (SQSTM1), which are implicated in normal protein autophagy (Ajroud-Driss and Siddique, 2015). Although the above mentioned FALS genes are expressed in multiple cell types, their mutation induces a selective degeneration of MNs.

Based on brain localization, MNs are classified as upper MNs located in the motor cortex and lower MNs situated in brainstem motor nuclei and in spinal cord ventral horns (**Figure 2**).

Although upper and lower MNs are both affected during the disease, lower MNs show a different susceptibility to degeneration. From disease onset, MNs localized in ventral horns of spinal cord and hypoglossal nucleus begin to degenerate, whereas MNs of oculomotor, trochlear and abducens nuclei controlling eye movements are spared (Mannen et al., 1977; Reiner et al., 1995; Nimchinsky et al., 2000; Haenggeli and Kato, 2002; Hedlund et al., 2010). In accord, during disease progression patients unable to speak continue to communicate through eye-tracking devices requiring the use of eye movements.

Typical clinical symptoms linked to upper MN degeneration comprise spasticity, uncontrolled movement and significant reduction of sensitivity (Ivanhoe and Reistetter, 2004). Lower MNs receive inputs from the upper population, sensory neurons and interneurons. Their degeneration induces loss of synaptic connectivity leading to muscular atrophy and, at the end, paralysis (Stifani, 2014).

In the present paper, we are focusing on data regarding the different vulnerability of lower MNs in oculomotor versus hypoglossal nucleus. The mechanism underlying their different death susceptibility is not fully understood, but it is clear that the

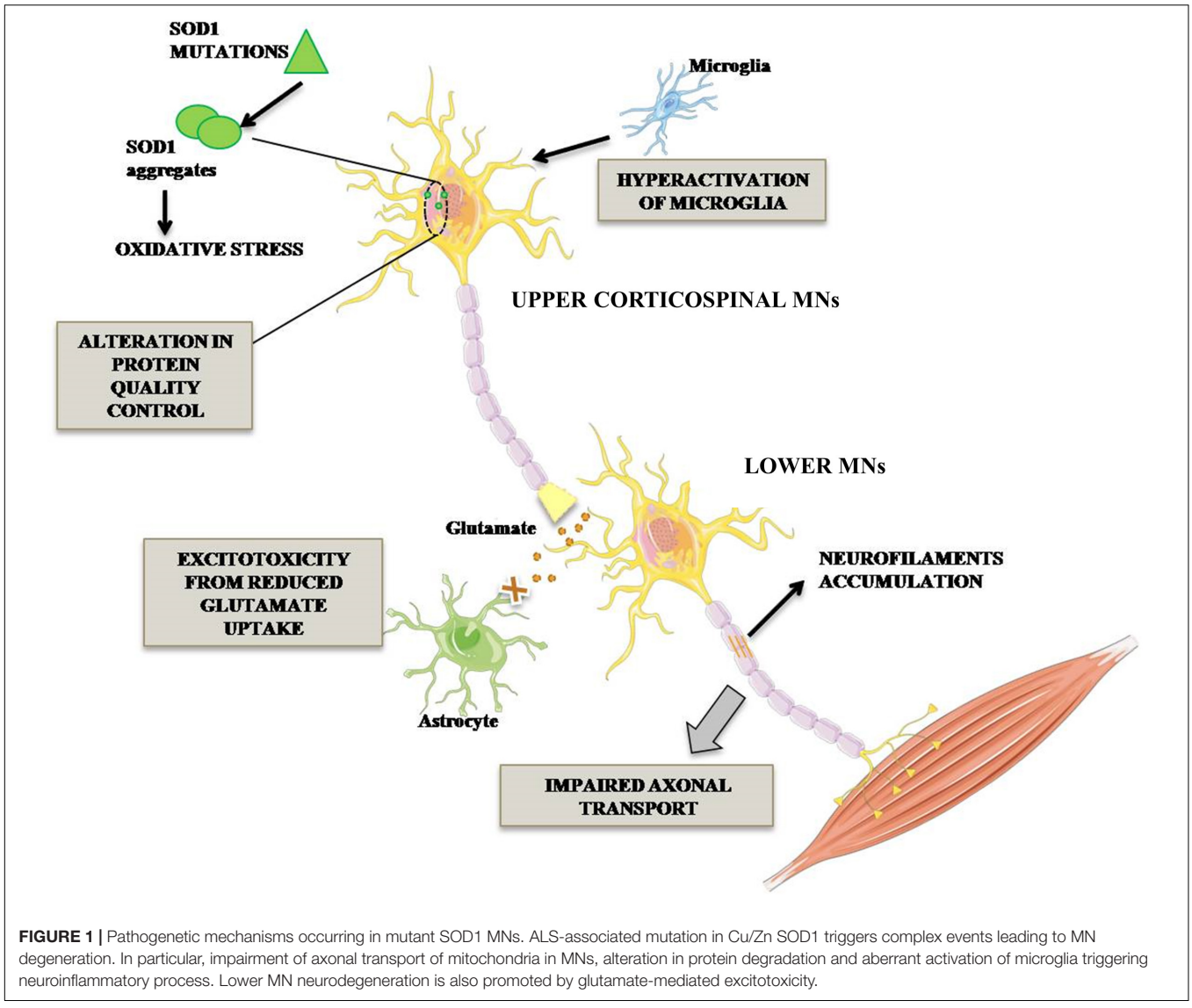

FIGURE 1 | Pathogenetic mechanisms occurring in mutant SOD1 MNs. ALS-associated mutation in Cu/Zn SOD1 triggers complex events leading to MN degeneration. In particular, impairment of axonal transport of mitochondria in MNs, alteration in protein degradation and aberrant activation of microglia triggering neuroinflammatory process. Lower MN neurodegeneration is also promoted by glutamate-mediated excitotoxicity.

resistance of some MNs to degeneration is related to anatomical specificity and their transcriptome profile.

The oculomotor nucleus, adjoining to trochlear nucleus, is situated at the level of the superior colliculus in the midbrain and it extends rostrally up to the posterior commissure. By examining its ultrastructure in rat brains, it has been demonstrated that it comprises either large MNs with abundant cytoplasm and well-developed organelles as well as small MNs with a low amount of cytoplasm. Although axo-somatic and axo-dendritic synapses are the most prevalent, axo-axonic synapses have also been identified. Fibers of the oculomotor nucleus as well as autonomic fibers of the accessory parasympathetic nucleus (Edinger-Westphal nucleus) converge into the oculomotor nerve (cranial nerve-CNIII). Somatic nerve fibers are bundled inside the nerve and are surrounded by the autonomic ones (**Figure 3**; Büttner-Ennever, 2006).

In mammals, conjugate eye movement results from combined contraction of six striated muscles inserted on the external surface of the eye bulb. Four of the six extraocular muscles (EOMs), known as medial rectus, inferior rectus, superior rectus and inferior oblique muscles are innervated by MNs of oculomotor nucleus. The superior oblique muscle is innervated by trochlear nerve MNs whereas the abducens nucleus MNs innervate the lateral rectus muscle.

EOMs comprise six different types of fibers, including those with very high mitochondrial content and strong fatigue resistance. They show a more complex pattern of innervation than almost all skeletal muscles. In particular, they have neuromuscular junctions extensively dispersed along their length from origin to muscle insertion (Harrison et al., 2007). Furthermore, they are characterized by ten distinct types of myosin heavy chain fibers within a single myofibre (Zhou et al., 2011). Moreover, EOMs contain several negative regulators of complement system showing higher vulnerability to myasthenia gravis, a neurotransmission disorder (Spencer and Porter, 1988). In EOMs, MNs form motor units of small size (i.e., MNs/muscles

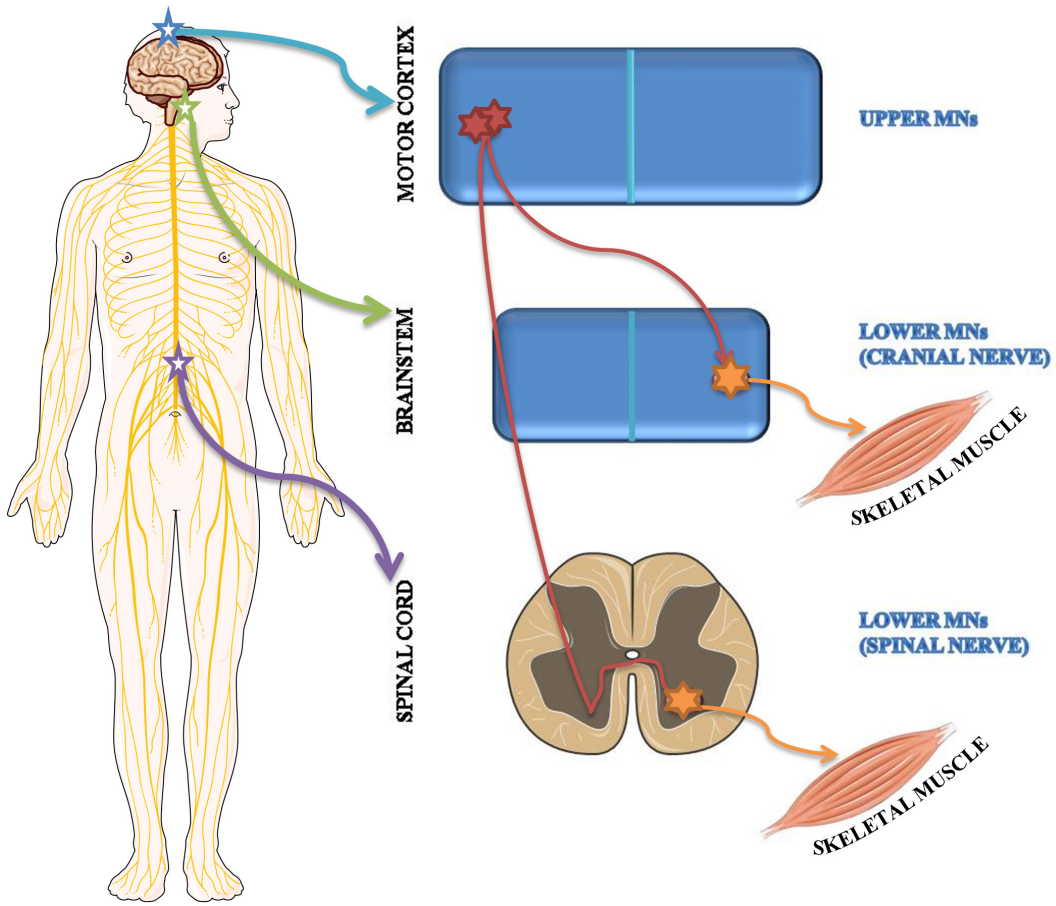

FIGURE 2 | Schematic representation of upper and lower MN localization. Upper MNs originate in the motor region of the cerebral cortex. Lower MNs are located either in some cranial nerve nuclei of the brainstem as well as in the ventral horns of the spinal cord.

fibers ratio 1:300 for EOMs; 1:2000 for large skeletal muscle), moreover, a single MN axon innervates the same muscles fibers in different regions allowing a fine regulation of EOMs contraction.

Hypoglossal nucleus is localized on the floor of the fourth ventricle in the dorsal part of the medulla oblongata. It contains a high number of MNs and inhibitory interneurons. MNs of the hypoglossal nucleus show large soma containing high levels of Nissl substance (Boone and Aldes, 1984). Their axons emerge from the preolivary fissure forming the hypoglossal nerve (CN XII). All tongue intrinsic and extrinsic muscles are innervated by XII nerve (**Figure 3**). The tongue intrinsic muscles, including superior, inferior longitudinales, transversus and verticalis muscles allow to change the organ shape. Among extrinsic muscles, genioglossus draws the tongue forward from the root, stíloglossus upward, whereas hyoglossus exerts retraction of the tongue and its side depression. The contraction of these muscles is involved in vital functions such as mastication, swallowing, suckling, vocalization and respiration.

In addition to the anatomical and functional differences, oculomotor and hypoglossal nuclei MNs have also a different genes expression pattern. By using laser capture microdissection and microarray analysis in a $SOD1^{G93A}$ rat model of ALS, Hedlund et al. (2010) showed profound differences in gene expression patterns when motor neurons of the oculomotor/trochlear complex (which do not degenerate in ALS) were compared to motor neurons of hypoglossal and the lateral motor column of the cervical enlargement of the spinal cord (which show vulnerability in ALS).

In a previous paper, Aronica et al. (2015), have analyzed whole-genome expression profile of motor cortex in sporadic ALS (SALS) and healthy patients. Unsupervised hierarchical clustering analysis allowed to discriminate controls from SALS patients, classified in SALS1 and SALS2 subtypes, each linked to differentially expressed genes and biological pathways (Morello et al., 2017a). Particularly, the most representative functional processes deregulated in SALS1 were involved in the regulation of chemotaxis, immune response, cell adhesion, signal transduction and communication. Deregulated genes in SALS2, in turn, were selectively associated with cell adhesion and cytoskeleton organization, regulation of transport and mitochondrial oxidative phosphorylation, energy metabolism and apoptotic signaling cascade (Aronica et al., 2015).

By comparing gene expression profile data, we have found that majority of genes deregulated in oculomotor/hypoglossal

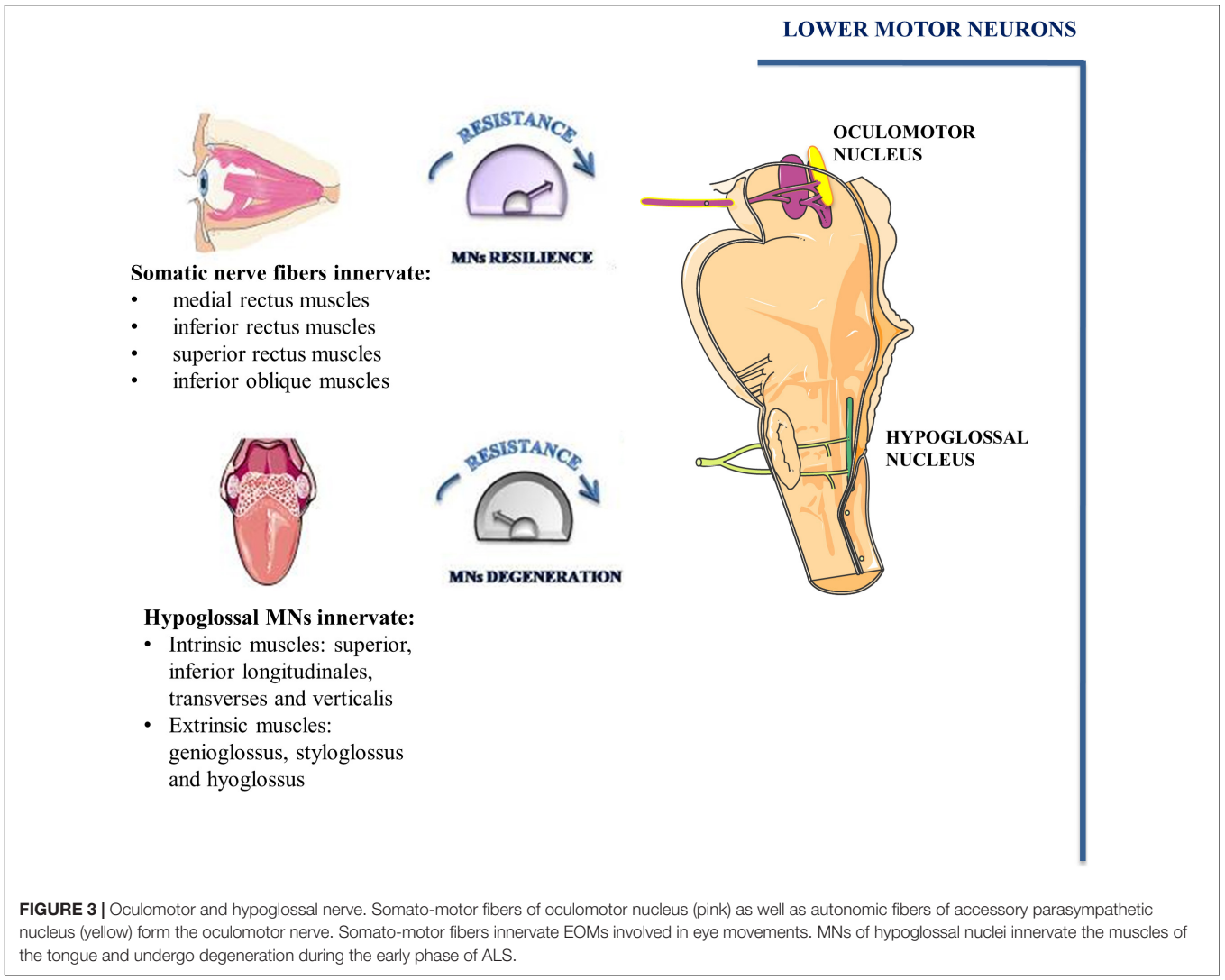

FIGURE 3 | Oculomotor and hypoglossal nerve. Somato-motor fibers of oculomotor nucleus (pink) as well as autonomic fibers of accessory parasympathetic nucleus (yellow) form the oculomotor nerve. Somato-motor fibers innervate EOMs involved in eye movements. MNs of hypoglossal nuclei innervate the muscles of the tongue and undergo degeneration during the early phase of ALS.

nuclei (127/188; **Supplementary Table**) are also deregulated in motor cortex of SALS1 and SALS2 patients (Aronica et al., 2015). Moreover, some of these genes, such as Igf2, and Gda, encode products exerting neuroprotection from glutamate-induced toxicity (Hedlund et al., 2010). The data suggest that the unique genome profile and specific protein signature could in part explain the differential vulnerability of these nuclei in ALS. Interestingly, overexpression of PACAP encoded by the ADCYAP1 in the in the oculomotor nucleus (Hedlund et al., 2010) should result in a trophic effect as demonstrated in ALS and other neurodegenerative models (Morio et al., 1996; Shintani et al., 2005; Wu et al., 2006).

PACAP AND ALS

PACAP is a neuropeptide identified for the first time in the ovine hypothalamus and belonging to VIP/secretin/glucagon family (Arimura, 1998). There are two functional isoforms: PACAP38 with 38 amino acids residues and PACAP27 including the N-terminal 27 amino acids residues of PACAP38 (Sherwood et al., 2000). It exerts different functions through activation of three distinct G-protein-coupled receptors: PAC1R, VPAC1R, and VPAC2R, whose expression is tissue and cell type-specific (Harmar et al., 2012). These receptors activate different signaling cascades including protein kinase A (PKA), protein kinase C (PKC) (Vaudry et al., 2009), MAPKs (mitogen-activated protein kinases) (Lelièvre et al., 1998) and NF-kB signaling pathways (Delgado and Ganea, 1999).

PACAP and its receptors are expressed in different tissues and organs, where they are involved in several biological processes including cell division and survival (Canonico et al., 1996; D'Agata et al., 1996; Jaworski, 2000; Maugeri et al., 2018). PACAP is also involved in learning and memory (Sacchetti et al., 2001; Adamik and Telegdy, 2005; Ciranna and Costa, 2019) and plays protective role in different neurodegenerative diseases (Reglodi et al., 2004, 2011; Waschek, 2013).

Tissue distribution studies have demonstrated that PACAP mRNA precursor is present in cerebral cortex, olfactory bulb, cingulate cortex, dentate gyrus, CA1 and CA4 subregions

of the hippocampus and cerebellum in the developing and adult rat brain (Arimura et al., 1991; Ghatei et al., 1993). PACAP-immunoreactivity was found in hypothalamic and extrahypothalamic brain sites including the preoptic area, dorsomedial and arcuate hypothalamic nuclei, pontine parabrachial nucleus, nucleus of the solitary tract and dorsal motor vagal nucleus rat brain (Légrádi et al., 1994; Hannibal, 2002). PACAP positive signal was also detected in the superficial layer of the superior colliculus, in the midline between the periaqueductal gray and the ventral tegmental area including the two oculomotor nuclei (Shioda et al., 1997). Low PACAP density immunostaining was found in the fibers and cell bodies of hypoglossal nucleus MNs (Légrádi et al., 1994).

A review paper has highlighted that PAC1R has greater expression than VPAC1-R and VPAC2-R receptors in the CNS (Basille et al., 2000). In particular, PAC1R is highly expressed in the olfactory bulb, the dentate gyrus, the supraoptic nucleus of the hypothalamus, the cerebellar cortex and hypoglossal nucleus whereas it is weakly expressed in oculomotor nucleus (Shioda et al., 1997; Skoglosa et al., 1999).

The analysis of genome expression profile ALS patients motor cortex has highlighted a significant deregulation either in PACAP and PAC1R mRNAs. In particular, PACAP is over-expressed in SALS1 and down-regulated in SALS2 motor-cortex samples, whereas PAC1R is down-regulated in both subgroups as compared to controls. To evaluate the role of PACAP in MNs degeneration, the effect of peptide has been tested in an *in vitro* model of MNs-derived from human induced pluripotent stem cells (iPSC) exposed to neurodegenerative insult (Bonaventura et al., 2018). The results have shown that PACAP and PAC1R levels are up-regulated in MNs cultured in growth factors deprived medium. Moreover, exogenous PACAP treatment effectively prevented their apoptotic death (Bonaventura et al., 2018). In accord, previous papers have suggested that endogenous PACAP promotes MNs survival following exposure to different insult through an autocrine or paracrine mechanism (Ohsawa et al., 2002; Armstrong et al., 2003; Chen and Tzeng, 2005; Tomimatsu and Arakawa, 2008; Bonaventura et al., 2018). Particularly, in response to adult rat facial nerve axotomy, a robust time-dependent increase in PACAP as well as decrease in PAC1R mRNA was observed in the facial motor nucleus as compared to the contralateral side (Zhou et al., 1999; Waschek et al., 2000). The endogenous PACAP released from the distal nerve stump binds to PAC1R expressed on Schwann cells during regeneration (Woodley et al., 2019). After nerve injury, PACAP was up-regulated and detectable in facial motor nucleus until 1 month, corresponding to the period of axon regeneration (Reimer et al., 1999). Moreover, PACAP deficiency in PACAP knock-out mice leads to a significant delay of axonal regeneration of axotomized facial nerve confirming the role of PACAP in axonal regeneration process (Armstrong et al., 2008). On the other hand, it is possible to speculate that PAC1R downregulation after nerve axotomy may correlate to increased apoptotic death of MNs of facial motor nucleus (Mattsson et al., 2006).

Therefore, it is possible to speculate that over-expression of PACAP encoding gene, ADCYAP1, in oculomotor nucleus may be implicated in its less vulnerability during ALS degeneration. In accord to this hypothesis, PACAP was found up-regulated in the axotomized facial motor nuclei of transgenic SOD1 G93A mice (Mesnard et al., 2011). Furthermore, Chung et al. (2005) demonstrated that higher resistance of ventral tegmentum compared to substantia nigra is partially due to PACAP expression.

Despite these evidences, Ringer et al. (2013), have highlighted that PACAP exerts a contradictory role during ALS progression. In fact, in the early phase of disease, it plays a neuroprotective role by promoting MNs survival, whereas in the end-stage of disease, PACAP promotes neuro-inflammation by stimulating microglial cells contributing to MNs degeneration. The dual role of PACAP on MNs and glial cells was depicted in **Figure 4**.

Apparently, conflicting data exist regarding PAC1R expression. Indeed, it is highly expressed in hypoglossal nucleus compared to oculomotor nucleus in healthy rat brains or after a moderate traumatic brain injury (Shioda et al., 1997; Skoglosa et al., 1999). However, the differential expression analysis of PACAP and PAC1R in oculomotor versus hypoglossal nucleus in ALS animal models or patients has not been performed, yet. To this regard, the expression of PAC1R could change in relation to MNs degeneration during different ALS-disease stages.

It has been hypothesized that PACAP, binding to PAC1R, activates the MAPK survival pathway directly or indirectly through EGFR trans-activation (Junier et al., 1993; Ayuso-Sacido et al., 2010; Moody et al., 2017; Maugeri et al., 2019a,b). In accord, in a meta-analysis study comparing microarray datasets of SALS motor cortex patients to mSOD1 G93A mice brains has been identified a commonly up-regulation of EGFR in both groups respect to controls (Morello et al., 2017b). Therefore, it has been speculated that the neuroprotective role played by PACAP in ALS may be direct or mediated by EGFR-phosphorylation. To this regard, it has been demonstrated that PACAP prevents epidermal growth factor (EGF) deprivation−induced cell death in NSC−34 cells expressing G93A SOD1 mutation, an *in vitro* model of ALS (Jiménez Garduño et al., 2017; Maugeri et al., 2019c). This neuroprotective effect is mediated by EGFR-phosphorylation leading to MAPK or PI3K signaling cascade activation. Moreover, PACAP modulated hypoxia-induced autophagy dysregulation through MAPK/ERK signaling activation in SOD1 G93A cells (D'Amico et al., 2020). Accordingly, high levels of ERK and AKT, two regulators of these pro-survival pathways, have been found in adult human oculomotor nucleus and may be in part responsible for the resilience of its MNs during ALS degeneration (Allodi et al., 2016).

The data shown in the present review present several limitations considering that most of the studies were performed in SOD1 G93A mice model at different disease phases (Mesnard et al., 2011; Ringer et al., 2013) and in brain samples of ALS decedents, representing the end-stage of the disease.

Although SOD1 G93A mice is the widely used animal model, reproducing motor defects similar to that observed in ALS-affected patients, there are important caveats to acknowledge. Firstly, the mSOD1 mouse model has a propensity to spontaneously delete copy number which can alter the severity

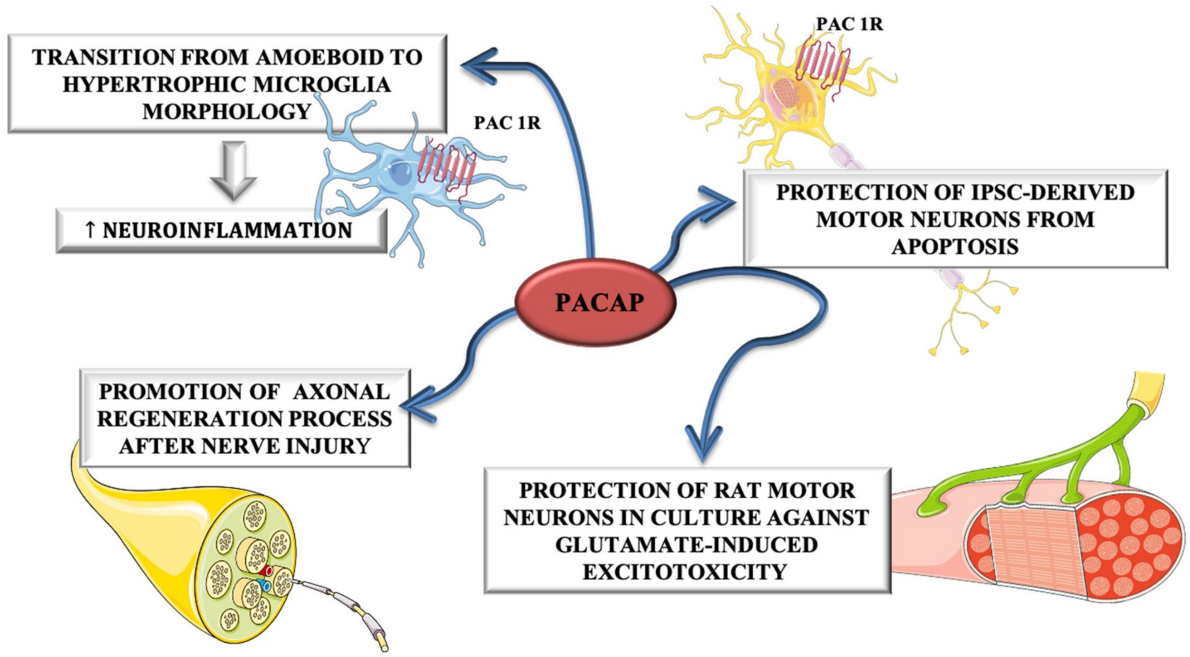

FIGURE 4 | The dual role of PACAP on MNs and glial cells. PACAP induced neuroinflammation by promoting transition from amoeboid to hypertrophic microglia morphology in SOD1(G93A) mice. On the other hand, it promoted axonal regeneration process after nerve injury and protected rat motor neurons in culture against glutamate-induced excitotoxicity and iPSC-derived motor neurons from apoptosis.

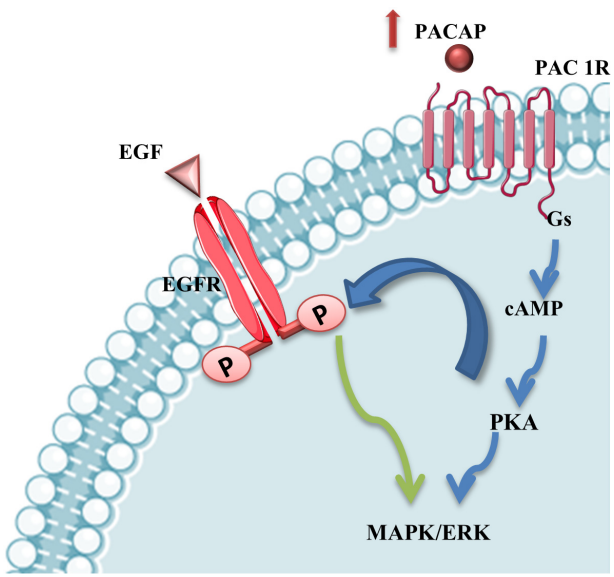

FIGURE 5 | PACAP stimulation of MAPK/ERK survival pathway. PACAP binding to PAC1R activates MAPK/ERK survival pathway directly or through EGFR phosphorylation via PKA-signaling cascade stimulation in mSOD1 MNs.

of disease presentation (Zwiegers et al., 2014; Lutz, 2018); secondly, the overexpression of human wild-type SOD1 causes axonopathy in mice, challenging the role of the mutation as the driver of pathology (Joyce et al., 2011).

Moreover, many other genes are involved in sporadic forms of ALS in addition to SOD1 G93A mutation. Therefore, to better characterize the role of PACAP and its receptor on MNs survival in ALS, new studies should be performed in other transgenic animal models.

CONCLUSION

The involvement of PACAP has been demonstrated in different neurodegenerative diseases, including MNs damage occurring in ALS. The evidences, above described, suggest that the different vulnerability of some cranial nerve motor nuclei could be also related to differential expression of PACAP in MNs. Moreover, PACAP's protective effects in MNs during ALS progression could be direct through PAC1R activation or mediated by EGFR trans-activation promoting MAPK or PI3K signaling survival cascade (**Figure 5**).

However, too much is still unknown. To better characterize the pathogenetic mechanisms involved in this progressive neurodegenerative disease, it is advisable that the research community extends studies of gene expression and transcriptomes on samples from ALS decedents obtained at autopsy and from stereotactic biopsies on living patients when available.

AUTHOR CONTRIBUTIONS

GMa and VD'A designed the research projects and wrote the manuscript. AD'A, DR, and SC contributed to the manuscript

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

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.00805/full#supplementary-material

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The Potential Roles of Ghrelin in Metabolic Syndrome and Secondary Symptoms of Alzheimer's Disease

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Although the major causative factors of Alzheimer's disease (AD) are the accumulation of amyloid β and hyperphosphorylated tau, AD can also be caused by metabolic dysfunction. The major clinical symptom of AD is cognitive dysfunction. However, AD is also accompanied by various secondary symptoms such as depression, sleep–wake disturbances, and abnormal eating behaviors. Interestingly, the orexigenic hormone ghrelin has been suggested to have beneficial effects on AD-related metabolic syndrome and secondary symptoms. Ghrelin improves lipid distribution and alters insulin sensitivity, effects that are hypothesized to delay the progression of AD. Furthermore, ghrelin can relieve depression by enhancing the secretion of hormones such as serotonin, noradrenaline, and orexin. Moreover, ghrelin can upregulate the expression of neurotrophic factors such as brain-derived neurotrophic factor and modulate the release of proinflammatory cytokines such as tumor necrosis factor α and interleukin 1β. Ghrelin alleviates sleep–wake disturbances by increasing the levels of melatonin, melanin-concentrating hormone. Ghrelin reduces the risk of abnormal eating behaviors by increasing neuropeptide Y and γ-aminobutyric acid. In addition, ghrelin increases food intake by inhibiting fatty acid biosynthesis. However, despite the numerous studies on the role of ghrelin in the AD-related pathology and metabolic disorders, there are only a few studies that investigate the effects of ghrelin on secondary symptoms associated with AD. In this mini review, our purpose is to provide the insights of future study by organizing the previous studies for the role of ghrelin in AD-related pathology and metabolic disorders.

Keywords: ghrelin, Alzheimer's disease, metabolic syndrome, depression, sleep–wake disturbances, abnormal eating behaviors

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INTRODUCTION

Alzheimer's disease (AD), characterized histopathologically by amyloid β aggregation and tau hyperphosphorylation, is the most common cause of dementia (Querfurth and LaFerla, 2010). Although AD is clinically characterized by progressive impairment of cognitive functions such as episodic memory, it is also accompanied by secondary symptoms such as depression, sleep–wake disturbances, and abnormal eating behaviors. Notably, some AD patients exhibit symptoms

of major depressive episodes such as appetite changes, insomnia, and dysphoria (Merriam et al., 1988; Novais and Starkstein, 2015; Okuda et al., 2019). In addition, subjects with mild cognitive or behavioral impairment are more likely to experience accelerated progression to AD or onset of dementia if they have a history of depression (Wilson et al., 2002). Furthermore, metabolic syndrome such as hyperglycemia, hyperinsulinemia, and hypercholesterolemia is known to be the risk factor for AD (Lane and Farlow, 2005; Nelson and Alkon, 2005; Razay et al., 2007). Psychiatric and metabolic deficits are not only symptoms of AD, but also markers of AD prognosis. Although there are drugs that effectively delay AD-related cognitive impairment, thus far, no therapeutic strategy has been established to treat the psychiatric and metabolic symptoms of AD thus far.

Ghrelin is an orexigenic hormone which regulates body weight, energy homeostasis, and metabolism through the hypothalamus, and plays an enhancing role in insulin resistance and growth hormone secretion (Pradhan et al., 2013; Muller et al., 2015; Yanagi et al., 2018). Remarkably, extensive evidence has indicated that ghrelin may alleviate AD-related pathology such as Aβ accumulation (Dhurandhar et al., 2013; Jeong et al., 2018), tau hyperphosphorylation (Kang et al., 2015), mitochondrial dysfunction (Chung et al., 2007), impaired adult neurogenesis (Moon et al., 2014), and neuroinflammation (Moon et al., 2011; Sibilia et al., 2012). Therefore, due to its potential for mitigating AD-related pathologies, ghrelin could be a possible therapeutic target for AD (Jeon et al., 2019). In addition, several studies have reported that ghrelin plays a protective role in metabolic syndrome (Broglio et al., 2004) and various psychiatric disorders, including depression (Carlini et al., 2012), sleep–wake disturbances (Yannielli et al., 2007), and abnormal eating behaviors (Overduin et al., 2012). However, the possible roles of ghrelin in AD-related metabolic syndrome and psychiatric disorders have not yet been investigated. Furthermore, although ghrelin plays a pivotal role in energy metabolism and homeostasis (Yanagi et al., 2018), the effects of ghrelin on metabolic disorders and secondary symptoms of AD remain unclear. In this review, we discuss the possibility of using ghrelin as a therapeutic target for AD by presenting evidence for the potential roles of ghrelin in the metabolic symptoms and secondary symptoms associated with AD.

THE ROLE OF GHRELIN IN METABOLIC SYNDROME AND SECONDARY SYMPTOMS OF AD

The Role of Ghrelin in AD-Related Metabolic Syndrome

Alzheimer disease is considered to be another type of diabetes, and hyperinsulinemia and hypercholesterolemia are known to promote AD pathogenesis (de La Monte and Wands, 2008; Merlo et al., 2010). Hyperinsulinemia inhibits the activity of AMP-activated protein kinase (AMPK) (Valentine et al., 2014), and inhibition of AMPK activity as a result of metabolic syndrome inactivates the pentose phosphate pathway (Saito et al., 2015). Abnormal metabolic conditions including diabetes mellitus may induce impairment of energy metabolism by increasing the production of reactive oxygen species and mitochondrial dysfunction (Bonomini et al., 2015; Bhatti et al., 2017) and may accelerate cognitive impairment by promoting abnormal release of neurotransmitters, particularly γ-aminobutyric acid (GABA) (van Bussel et al., 2016). Several studies have suggested that in neurodegenerative diseases, there exists a link between insulin and cholesterol levels (Laws et al., 1991; Nelson and Alkon, 2005). Indeed, insulin increases the activity of 3-hydroxy-3-methylglutaryl-CoA reductase, the enzyme that catalyzes an intermediate in cholesterol synthesis (Nelson and Alkon, 2005). In a previous study, individuals with type 2 diabetes mellitus exhibited decreased cholesterol absorption and increased cholesterol synthesis regardless of obesity (Simonen et al., 2002). In the case of AD, Aβ-induced metabolic imbalance involving AMPK results in tau phosphorylation and neuroinflammation (Martinez de Morentin et al., 2010; Thornton et al., 2011; Lee et al., 2013). Furthermore, AD patients suffer from insulin signaling dysfunction due to a reduction in activity of tyrosine kinase, an important effector system for insulin receptors (Frolich et al., 1999), and decreased activities of elements of insulin–PI3K–AKT signaling, which results in elevated tau phosphorylation and decreased glucose metabolism (Liu et al., 2011). In particular, apolipoprotein E (ApoE), a protein responsible for the metabolism of plasma lipids (Jones et al., 2019), is also associated with AD (Lane and Farlow, 2005). Reportedly, polymorphism of ApoE allele, especially ApoE ε4, attributes to risk of AD development by increasing Aβ and Tau aggregation, whereas ApoE ε2 exhibits protective effects on risk of AD development (Verghese et al., 2011). Moreover, ApoE ε4 induces dysregulation of cerebral metabolism by decreasing lipid and glucose metabolism (Brandon et al., 2018). Interestingly, the control of insulin and plasma glucose by ghrelin administration can vary depending on the details of administration (i.e., duration, route, and dose) (Nieminen and Mustonen, 2004; Theander-Carrillo et al., 2006; Barazzoni et al., 2007a; Goshadrou et al., 2015). In rats, acute (1 day) administration of ghrelin increased levels of insulin and fasting plasma glucose, but chronic (21 days) administration of ghrelin normalized these upregulations (Goshadrou et al., 2015). The mechanism of insulin and glucose regulation after administration of exogenous ghrelin has not yet been clearly identified. Known mechanisms through which insulin inhibits ghrelin include upregulation of the AMPK- uncoupling protein 2 (UCP2) pathway through AMPK phosphorylation and UCP2 expression (Chmielewska et al., 2010; Wang et al., 2010), and the IA-2β pathway, which inhibits glucose-stimulated insulin through induction of IA-2β (Doi et al., 2006). These two pathways independently inhibit insulin. Remarkably, ghrelin not only regulates insulin but also regulates nigrostriatal dopamine function in a UCP2-dependent manner (Andrews et al., 2009). In addition, upregulation of UCP2 has

Abbreviations: AMPK, AMP-activated protein kinase; ARC, arcuate nucleus; BDNF, brain-derived neurotrophic factor; GABA, γ-aminobutyric acid; LHA, lateral hypothalamus; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVhd, dorsal parvocellular paraventricular nucleus; ROS, reactive oxygen species; SCN, suprachiasmatic nucleus; VLPO, ventrolateral preoptic nucleus.

been demonstrated to have a protective effect in animal models of ischemic stroke and Parkinson disease (Andrews et al., 2009; Liu et al., 2009).

The concentration of ghrelin is decreased in the middle-aged and elderly people with metabolic syndrome compared to individuals of the same age who do not have metabolic syndrome, and its concentration rapidly is decreased as metabolic abnormalities intensify (Ukkola et al., 2006; Serra-Prat et al., 2009; Mora et al., 2014). Several studies have suggested that ghrelin may be involved in the metabolism of insulin and glucose. In healthy subjects, administration of acyl-ghrelin reduced insulin levels and increased glucose levels (Broglio et al., 2004). By contrast, administration of des-acyl-ghrelin improved glucose metabolism and insulin sensitivity in subjects (Benso et al., 2012). In addition, administration of acyl-ghrelin alone to growth hormone–deficient patients increases insulin and glucose levels rapidly but decreases insulin sensitivity, whereas administration of acyl-ghrelin and des-acyl-ghrelin increases insulin sensitivity (Gauna et al., 2004). Moreover, transgenic mice overexpressing des-acyl-ghrelin exhibited a reduction in white adipose tissue weight and improvement in glucose tolerance and insulin sensitivity (Zhang et al., 2008). In a previous study, obese children with metabolic syndrome exhibited decreased levels of des-acyl-ghrelin and an increased acyl-ghrelin/des-acyl-ghrelin ratio compared to obese children without metabolic syndrome (Pacifico et al., 2009). Similarly, obese individuals with normoglycemia and type 2 diabetes mellitus exhibited increased plasma levels of acyl-ghrelin and decreased levels of des-acyl-ghrelin compared to lean individuals (Rodriguez et al., 2009). Therefore, individuals with metabolic syndrome and obesity have a higher acyl-ghrelin/des-acyl-ghrelin ratio than non-obese individuals with metabolic syndrome, suggesting that excessive acyl-ghrelin levels may promote insulin resistance (Barazzoni et al., 2007b). Moreover, administration of ghrelin causes tissue-specific changes in the activity of mitochondrial oxidative enzyme, the expression of gene involved in lipid metabolism, and triglyceride content in rats, suggesting that ghrelin may be involved in the regulation of lipid distribution and metabolism (Barazzoni et al., 2005).

Patients with AD exhibited lower lean mass compared to controls. Although patients with AD and controls exhibited similar basal levels of ghrelin, the area under the curve value was lower in male patients with AD than in control males (Theodoropoulou et al., 2012). Although further evidence and investigation are required, a previous study by Yoshino et al. (2018) showed increased levels of serum acyl-ghrelin in AD subjects compared to control subjects that might be a result of changes of the ghrelin pathway in brain (Yoshino et al., 2018). Thus, further deliberate examination and interpretation should be made. Given that ghrelin-O-acyltransferase blockade reduces the acyl-ghrelin/des-acyl-ghrelin ratio, des-acyl-ghrelin administration could be a promising therapeutic approach for metabolic dysfunction (Barnett et al., 2010). It is possible that the increased acyl-ghrelin/des-acyl-ghrelin ratio in individuals with obesity may promote insulin resistance and hyperinsulinemia (Barazzoni et al., 2007b). Insulin resistance and hyperinsulinism may increase the prevalence of AD by increasing Aβ-related metabolism and inflammation in the brain (Craft, 2007). Additionally, insulin transport to the brain is reduced, causing insulin deficiency (Baura et al., 1996). Furthermore, neurofibrillary tangles containing phosphorylated tau were observed in the hippocampus of insulin receptor substrate 2 knockout mice, indicating that insulinlike growth factor-1 and insulin are associated with tau phosphorylation (Schubert et al., 2003). These results suggest that metabolic abnormalities such as hyperinsulinemia and insulin resistance promote AD development (DiStefano et al., 2007). Both *in vitro* and *in vivo* studies have reported that an optimal concentration of insulin reduced Aβ production through increasing the levels of α-secretase ADAM10, sAPPα, and C83 and decreasing the levels of β-secretase BACE1, sAPPβ, and C99 (Vandal et al., 2014; Wang et al., 2014). Furthermore, antidiabetic drugs such as metformin and peroxisome proliferator-activated receptor-γ agonists may have beneficial effects on preventing or improving cognitive dysfunction and pathogenesis of AD (Crisby et al., 2002; Cong et al., 2010; Akter et al., 2011). Therefore, given that ghrelin plays major roles in metabolism, it may be a noteworthy therapeutic target for AD (Gahete et al., 2011; Eslami et al., 2018). Nonetheless, considering the fact that the area under the curve value of ghrelin was increased by glucose loading only in male patients with AD, not in female patients (Theodoropoulou et al., 2012), and the higher basal ghrelin levels in female healthy and opposite-sex twin pair subjects than men (Makovey et al., 2007; Song et al., 2017), difference in effects of ghrelin for AD-related metabolic syndrome according to gender should be examined in the future.

The Role of Ghrelin in AD-Related Depression

Depression is the most common secondary symptom in patients with AD and is associated with accelerated cognitive impairment (Bassuk et al., 1998; Modrego, 2010). In particular, late-onset depression is considered to be a risk factor for AD development and is more strongly associated with cognitive decline than early-onset depression (Devanand et al., 1996; van Reekum et al., 1999; Wilson et al., 2002). An increase in glucocorticoid production is characteristic of early AD (Rasmuson et al., 2001), and hypothalamic–pituitary–adrenal (HPA) axis dysfunction caused by excessive glucocorticoid secretion and reactivity promotes the development of depression (Zunszain et al., 2011). In addition, the limbic lobe, hippocampus, amygdala, and anterior and posterior cingulate cortices are involved in the pathophysiology of depression; a decrease in the density/structural plasticity of these areas has been identified in patients with depression (Rajkowska, 2000; Nestler et al., 2002; Ries et al., 2009) and in patients with early AD (Braak et al., 1993; Minoshima et al., 1997; Gastard et al., 2003; Poulin et al., 2011). Moreover, dysfunction of the monoaminergic system, in particular the serotonergic and noradrenergic systems, has been shown to occur in both depression and AD (Ressler and Nemeroff, 2000; Versijpt et al., 2003; Kepe et al., 2006; Chalermpalanupap et al., 2013).

Chronic stress–induced glucocorticoid upregulation promotes neuronal damage, induces structural changes,

and decreases the expression of brain-derived neurotrophin-3 and neurotrophic factor (BDNF) mRNA in the hippocampus (Smith et al., 1995; Nestler et al., 2002). Ghrelin, which has a protective effect on metabolic disturbances induced by chronic stress, has been reported to also have protective effects against depressive-like responses in experimental animals (Lutter et al., 2008; Labarthe et al., 2014). In addition, the rat model of diabetes exhibits lower hippocampal BDNF mRNA levels compared to control rats, while administration of ghrelin significantly upregulates BDNF mRNA levels in a rat model of diabetes (Ma et al., 2011). Olfactory bulbectomy induced depressive-like behavior in mice, and this deficit was reversed by ghrelin administration, indicating that ghrelin exhibits an antidepressant-like effect (Carlini et al., 2012). Moreover, olfactory bulbectomy decreased noradrenaline levels and serotonin turnover and increased the levels of proinflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) (Hellweg et al., 2007; Song et al., 2009; Yang et al., 2014; Chang et al., 2016). However, exogenous ghrelin inhibited the release of proinflammatory cytokines and increased noradrenaline levels and serotonin turnover, further demonstrating the antidepressant-like effect of ghrelin (Date et al., 2006; Kawakami et al., 2008; Waseem et al., 2008; Hansson et al., 2014). Moreover, increased ghrelin levels induced by calorie restriction led to anti–depressant-like effects. By contrast, the calorie restriction–induced anti–depressive-like effects were not observed in growth hormone secretagogue receptor (GHS-R) null mice, and these animals exhibited increased social avoidance compared to their wild-type littermates (Lutter et al., 2008). Notably, GHS-R1 is known to be involved in various psychological conditions, including depression (Guo et al., 2019). Thus, ghrelin may alleviate depressive-like responses by acting on GHS-R1–expressing neurons (Abizaid et al., 2006; Diano et al., 2006; Lutter et al., 2008).

Mechanisms related to the pathogenesis of depression include HPA axis dysfunction, monoaminergic system deficiency, inflammation, and neurodegeneration (Zunszain et al., 2011). Therefore, ghrelin may alleviate depressive symptoms by upregulating BDNF mRNA, decreasing glucocorticoid levels, rebalancing the monoaminergic system, stimulating GHS-R1–expressing neurons to modulate mood and synapse formation, and regulating the release of proinflammatory cytokines such as IL-1β and TNF-α. Unfortunately, few studies have investigated the role of ghrelin in AD-related depression. However, given the antidepressant-like effect of ghrelin observed in previous animal studies, we hypothesize that ghrelin may have a therapeutic effect on depression in AD patients.

Other neuropeptides, including neurotensin and neuropeptide Y (NPY), have been shown to be involved in the pathogenesis of depression. Interestingly, the effects of neurotensin were opposite to those of ghrelin on food intake (Cooke et al., 2009). In addition, neurotensin neurons are known to play important roles in regulation of energy balance controlled by ghrelin and leptin (Brown et al., 2017). Notably, mRNA levels of ghrelin and expression of its G protein–coupled receptors (neurotensin receptors 1 and 2) are decreased, whereas levels of neurotensin tend to decrease in the temporal lobe of patients with AD (Gahete et al., 2010). In another study, density of amyloid plaque in the occipital cortex was negatively correlated with density of neurotensin neurons in postmortem suprachiasmatic nucleus (SCN) (Hu et al., 2013). Moreover, neurotensin receptor 1 knockout mice showed increased depressive-like behaviors in the tail suspension test (Fitzpatrick et al., 2012). Despite the conflicting results from clinical studies examining the roles of NPY in depression, evidence strongly supports the involvement of NPY in pathogenesis of depression (Morales-Medina et al., 2010). In addition, levels of NPY vary by the locations of sampling and models of AD (Duarte-Neves et al., 2016). Considering, ghrelin cross talks with NPY neurons in the arcuate nucleus (ARC) in rats (Kohno et al., 2003) and the evidence that ghrelin increases gene expression of NPY in the ARC in hypothalamic cultures of rats (Goto et al., 2006), the regulatory effect of ghrelin on NPY in AD-related depression should be examined in the future. Although the interacting mechanisms among ghrelin, neurotensin, and NPY in AD-related depression remain to be examined, neurotensin and NPY, at least, seem to be mediating some AD-related depression-like behaviors by interacting with ghrelin.

The Role of Ghrelin in AD-Related Sleep–Wake Disturbances

Maintaining a normal circadian rhythm is essential in order to optimize quality of life and preserve health. Sleep–wake disturbances are common secondary symptoms of AD that have been observed in studies on patients with AD (Uddin et al., 2020) and on the 3×Tg and 5×FAD mouse models of AD (Sterniczuk et al., 2010; Sethi et al., 2015). Moreover, the pineal gland, which adjusts sleep patterns by producing melatonin, and the SCN, which is involved in the regulation and production of biological rhythms, are vulnerable regions in AD (Buijs and Kalsbeek, 2001; Wu and Swaab, 2005; Roy et al., 2019). A recent study using magnetic resonance imaging of the brain showed that the pineal volume was decreased in mild cognitive impairment (MCI) patients who converted to AD than in MCI patients who did not convert to AD (Matsuoka et al., 2020). Furthermore, sleep–wake cycle disturbances showed to increase Aβ plaques in the brain of AD mouse models (Kang et al., 2009; Rothman et al., 2013). The level of $A\beta_{42}$ protein in cerebrospinal fluid of healthy middle-aged individuals was increased in the sleep deprivation group compared to that in the unrestricted sleep group (Ooms et al., 2014). In particular, sundowning, a common symptom of AD with circadian rhythm disruption, occurs in the afternoon and evening and is accompanied by seven destructive actions: combativeness, agitation or purposeless movement, wandering, prolonged incoherent vocalization, hallucinations, confusion, and disorientation (Gallagher-Thompson et al., 1992; Volicer et al., 2001). Regulations of sleep and brain functions are related to regulatory pathways including hippocampal signaling pathway and common neurotransmitter systems such as orexinergic and GABAergic systems (Prince and Abel, 2013). However, dysfunction of sleep function destabilizes physiology, disturbs sleep–wake timing, and promotes other pathological symptoms such as cognitive and metabolic deficits (Wulff et al., 2010). Surprisingly, the orexigenic peptide ghrelin regulates circadian rhythm (Yannielli et al., 2007; LeSauter et al., 2009;

Steiger et al., 2011). Studies have been shown that administration of ghrelin decreased REM sleep and increased slow wave sleep in elderly men (Kluge et al., 2010) and promoted non-REM sleep in male mice (Obal et al., 2003). Several studies have been reported that GHS-R1 mRNA is highly expressed in the SCN (Zigman et al., 2006) and ARC (Jeon et al., 2019). It is well known that neurons in the SCN are projected to the dorsal parvocellular paraventricular nucleus (PVHd), and neurons in the PVHd are projected to sympathetic preganglionic neurons, which in turn regulate melatonin secretion by the pineal gland (Saper et al., 2005). Therefore, ghrelin could alleviate sleep–wake disturbances through increasing melatonin secretion by binding to GHS-R1 in the SCN and enhancing the regulatory pathways that stimulate the pineal gland. Additionally, the ARC neurons innervate to the ventrolateral preoptic nucleus (VLPO) and lateral hypothalamus (LH) via the dorsomedial hypothalamus. The VLPO is involved in sleep, and the LH is associated with wakefulness by regulating melanin-concentrating hormone (Saper et al., 2005). Thus, ghrelin could enhance sleep–wake cycle by stimulating the VLPO and LH through binding to GHS-R1 in the ARC. Moreover, ghrelin affects circadian locomotor output cycles kaput (CLOCK)–dependent functions (Garaulet et al., 2011). Taken together, these data indicate that ghrelin may alleviate sleep-wake disturbances by stimulating the SCN and ARC and ultimately regulate the function of CLOCK-related activity.

TABLE 1 | The role of ghrelin in metabolic syndrome and secondary symptoms of Alzheimer's disease.

	Subjects or experimental models	Major findings	References
Metabolic syndrome	Growth hormone–deficient patients	Combined treatment with acyl-ghrelin and des-acyl-ghrelin enhanced insulin sensitivity, while administration of acyl-ghrelin alone reduced insulin sensitivity	Gauna et al., 2004
	Patients with metabolic syndrome	Patients with metabolic syndrome exhibited lower total ghrelin levels and a higher acyl-ghrelin/des-acyl-ghrelin ratio than non-obese individuals with metabolic syndrome	Barazzoni et al., 2007b
	Obese children with metabolic syndrome	Obese children with metabolic syndrome exhibited decreased levels of des-acyl-ghrelin and an increased acyl-ghrelin/des-acyl-ghrelin ratio compared to obese children without metabolic syndrome	Pacifico et al., 2009
	Obese patients with normoglycemia and type 2 diabetes mellitus	Obese individuals with normoglycemia and type 2 diabetes mellitus exhibited increased plasma levels of acyl-ghrelin and decreased levels of des-acyl-ghrelin compared to lean individuals	Rodriguez et al., 2009
	Patients with moderate Alzheimer's disease	Patients with Alzheimer's disease exhibited a lower area under the curve value for ghrelin compared to control patients	Theodoropoulou et al., 2012
	Healthy young male subjects	Acyl-ghrelin reduced insulin levels and increased glucose levels, whereas des-acyl-ghrelin antagonized these effects	Broglio et al., 2004
	Healthy young subjects	Administration of des-acyl-ghrelin reduced the area under the curve for glucose and free fatty acid. In addition, des-acyl-ghrelin time-dependently increased the area under the curve of insulin	Benso et al., 2012
	Isolated rat adipocytes	Acyl-ghrelin inhibited lipolysis	Muccioli et al., 2004
	Isolated mice pancreatic islets	Acyl-ghrelin decreased spontaneous pancreatic polypeptide release, and des-acyl-ghrelin counteracted this	Kumar et al., 2010
	ddY mice	Administration of des-acyl-ghrelin decreased food intake and gastric emptying and increased the gene expression of hypothalamic neuropeptides such as cocaine- and amphetamine-regulated transcript and urocortin	Asakawa et al., 2005
	Transgenic mice overexpressing des-acyl-ghrelin	Mice overexpressing des-acyl-ghrelin exhibited a decrease in gastric emptying rate, body weight, food intake, fat pad mass, and plasma triglyceride levels	
	Transgenic mice overexpressing des-acyl-ghrelin	Overexpression of des-acyl-ghrelin inhibited adipose tissue development and improved glucose tolerance and insulin sensitivity	Zhang et al., 2008
	C57BL/6 mice	Inhibition of ghrelin-O-acyltransferase reduced body weight and fat mass	Barnett et al., 2010
Depression	Mice subjected to bilateral olfactory bulbectomy	Intracerebroventricular administration of ghrelin reversed the depressive-like phenotype induced by olfactory bulbectomy	Carlini et al., 2012
	Calorie-restricted mice growth hormone secretagogue receptor null mice	Increased ghrelin levels induced by calorie restriction promoted antidepressant-like responses, whereas these effects were abolished in growth hormone secretagogue receptor null mice	Lutter et al., 2008
Sleep–wake disturbances	Sprague–Dawley rats	Microinjection of ghrelin into the lateral hypothalamus stimulated wakefulness and food consumption	Szentirmai et al., 2007
	C57BL/6J mice mPeriod2^{Luciferase} mice	After food deprivation, intraperitoneal injection of ghrelin or growth hormone–releasing peptide-6 altered circadian rhythm by directly acting on the suprachiasmatic nucleus	Yannielli et al., 2007
	Overweight/obese patients	Ghrelin affected a circadian locomotor output cycle kaput-dependent mechanism	Garaulet et al., 2011
Abnormal eating behaviors	Healthy volunteers	Ghrelin increased appetite and food intake	Wren et al., 2001
	Male Wistar rats	Intracerebroventricular injection of ghrelin increased food intake	Wren et al., 2000
	Neuropeptide Y knockout mice	Intracerebroventricular injection of ghrelin increased food intake and body weight	Tschop et al., 2000

Areas such as the SCN, ARC, and pineal gland that influence the regulation and production of biological rhythms are damaged in patients and mouse models of AD, and these damaged regions cause sleep–wake disturbances (Do et al., 2018; Roy et al., 2019; Matsuoka et al., 2020). In addition, sleep–wake disturbances increase level of Aβ protein and plaques in healthy individuals and mouse models of AD (Kang et al., 2009; Rothman et al., 2013). Accumulating evidence has demonstrated that ghrelin not only has beneficial effects on sleep–wake cycle, but also stimulates areas involved in biological rhythms (Yannielli et al., 2007; LeSauter et al., 2009; Steiger et al., 2011). Because there is almost no study on the effects of ghrelin in AD-related sleep–wake disorders, further well-controlled clinical trials regarding the positive effects of ghrelin on disruption in the circadian rhythm and quality of life in patients with AD are needed.

The Role of Ghrelin in AD-Related Abnormal Eating Behaviors

In a previous clinical study, patients with AD exhibited weight loss (Barrett-Connor et al., 1998). Aging causes changes in appetite and growth hormone secretion (Creyghton et al., 2004), and these changes are referred to as "anorexia of aging." Anorexia of aging causes several sequelae such as undernutrition, frailty, and sarcopenia (Cox et al., 2019). In addition, aging increases insulin resistance and reduces glucose metabolism (Shou et al., 2020). Insulin levels increase with age, and insulin may promote the development of anorexia. Moreover, aging-related leptin and ghrelin resistance may be related to anorexia of aging (Chapman et al., 2002; Chapman, 2004, 2007; Di Francesco et al., 2007).

Ghrelin is known to increase food intake (Tschop et al., 2000; Wren et al., 2000; Wren et al., 2001) and promote gastric emptying (Inui et al., 2004; Overduin et al., 2012). In particular, ghrelin regulates fatty acid metabolism in the ventromedial nuclei of the hypothalamus (VMH) to regulate food intake. The orexigenic effect of ghrelin is mediated via the phosphorylation of hypothalamic AMPK, which decreases malonyl-CoA levels and increases carnitine palmitoyltransferase-1 activity (Lopez et al., 2008). In the hypothalamic ARC, agouti-related protein and NPY are expressed in orexigenic neurons, and proopiomelanocortin (POMC) and amphetamine- and cocaine-regulated transcript are expressed in anorexigenic neurons (Zheng et al., 2003; Chen et al., 2004). In a previous study, ghrelin suppressed the activity of POMC-expressing neurons in the ARC by activating NPY-expressing neurons, which promoted the release of GABA (Cowley et al., 2003). Moreover, ghrelin reduced malonyl-CoA levels by suppressing the expression of fatty acid synthase in the VMH (Lopez et al., 2008). Indeed, intracerebroventricular infusion of ghrelin stimulated food intake via a mechanism involving the dopamine D_1 receptor in rats (Overduin et al., 2012). In addition, ghrelin administration stimulated cerebral responses to food in the amygdala, anterior insula, orbitofrontal cortex, and striatum of healthy subjects (Malik et al., 2008). Interestingly, rivastigmine administration increased appetite by increasing acyl-ghrelin/des-acyl-ghrelin ratio in AD patients

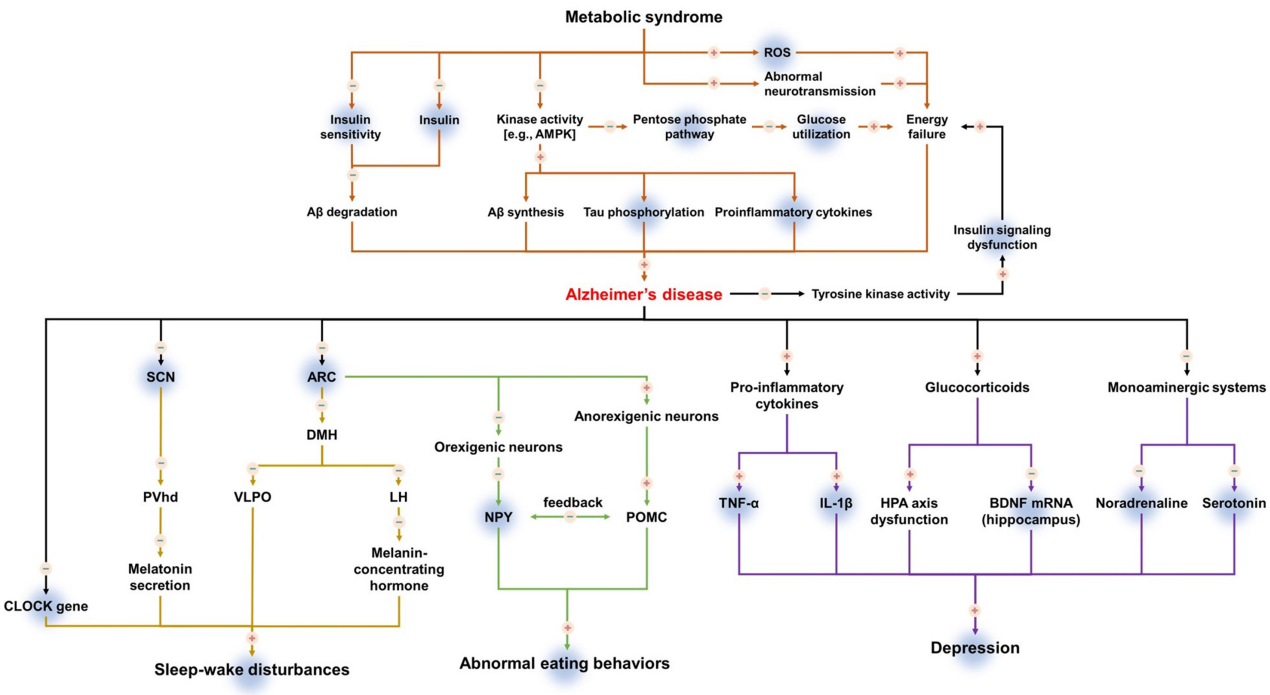

FIGURE 1 | The mechanism of action of ghrelin in metabolic syndrome and secondary symptoms of Alzheimer's disease. Upregulation is indicated by plus (+), downregulation is indicated by minus (–). Shadowed background color indicates the beneficial effects of ghrelin on metabolic syndrome and secondary symptoms of Alzheimer's disease.

(Furiya et al., 2018) implying AD-related cachexia could potentially be alleviated by promoting appetite through ghrelin administration. Thus, the orexigenic effect of ghrelin may prevent the loss of body weight and lean mass in AD patients.

CONCLUSION

Taken together, there has been a lack of evidence demonstrating that ghrelin can alleviate metabolic syndrome and secondary symptoms associated with AD. However, it has been suggested that ghrelin may affect the progression of AD by alleviating metabolic syndrome. Moreover, it is thought that ghrelin may control secondary symptoms of AD such as depression, sleep–wake disturbances, and abnormal eating behaviors (**Table 1** and **Figure 1**). Given the evidence for the involvement of ghrelin at various stages of AD progression, it is necessary to further examine the role of ghrelin in metabolic syndrome and in the secondary symptoms of AD.

AUTHOR CONTRIBUTIONS

SK, YN, SJS, YHP, SGJ, J-iK, and MM wrote this review article. SK, YN, SJS, YHP, SGJ, J-iK, M-JK, and MM revised this review article. All authors approved the submitted version.

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ORIGINAL RESEARCH
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Effects of Cerebrolysin on Hippocampal Neuronal Death After Pilocarpine-Induced Seizure

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Epilepsy is one of the most common and severe brain diseases. The exact cause of epilepsy is unclear. Epilepsy often occurs following brain damage, such as traumatic brain injury (TBI) and ischemia. Cerebrolysin is a porcine brain peptide that is a unique neurotropic and neuroprotective agent. Cerebrolysin has been reported to increase neuroprotective effects after TBI, ischemia, and other CNS diseases. However, the effects of cerebrolysin on seizures are not known. Therefore, this study aimed to investigate the effects of neuropeptide cerebrolysin on neuronal death in the hippocampus after a seizure. To confirm the effects of cerebrolysin, we used a pilocarpine-induced seizure animal model. Cerebrolysin (2.5 ml/kg, i.p., once per day for 7 days) was immediately injected after a seizure induction. After 1 week, we obtained brain tissues and performed staining to histologically evaluate the potentially protective effects of cerebrolysin on seizure-induced neuronal death in the hippocampus. We found that cerebrolysin decreased hippocampal neuronal death after a seizure. In addition, an increase in brain-derived neurotrophic factor (BDNF) was confirmed through Western blot analysis to further support our hypothesis. Therefore, the present study suggests that the administration of cerebrolysin can be a useful therapeutic tool for preventing neuronal death after a seizure.

Keywords: epileptic seizure, pilocarpine, cerebrolysin, neurotropic, neuroprotective, neuropeptide, brain-derived neurotrophic factor

INTRODUCTION

Epilepsy is one of the most common brain diseases, affecting about 70 million people worldwide (Thijs et al., 2019). Temporal lobe epilepsy (TLE) is the most common type of partial epilepsy, accounting for at least 20% of all patients with epilepsy (Babb, 1999). Although epilepsy has diverse subtypes and several co-occurring symptoms, the main causes of re-occurring epilepsy are the chronic downregulation of inhibitory neurotransmission or the overactivation of excitatory synaptic neurotransmission (Goldberg and Coulter, 2013). In addition, epilepsy often occurs after other brain diseases, such as traumatic brain injury and ischemia (Goldberg and Coulter, 2013). Thus, if the hippocampus is damaged by seizure, damage to the neuronal cells and changes in metabolic processes can result in the hippocampus failing to function normally (Holmes, 1991;

Najm et al., 1998; Pitkanen and Sutula, 2002; Haut et al., 2004; Lukoyanov et al., 2004; Vingerhoets, 2006). Cognitive impairment and neuronal injury in epileptic patients remain important medical problems (Jeong et al., 2017; Lee S. H. et al., 2018). Various animal models have been developed to help identify therapeutic interventions to prevent these negative epileptic outcomes (Xie et al., 1995; Smolders et al., 2002; Remy et al., 2003). Thus, pilocarpine-induced seizure causes severe and extensive neuronal damage in the cerebral cortex and hippocampus (Turski et al., 1983; Leite et al., 1990; Lee M. et al., 2018).

The use of pilocarpine-induced seizures in rodents is an animal model that is commonly applied to the study of epilepsy (Levesque and Avoli, 2013; Lee et al., 2019; Wang et al., 2019). Pilocarpine acts on the M1 muscarinic receptor and serves as an agonist for the muscarinic acetylcholine receptor. When M1 is activated, phospholipase C is also activated, producing inositol triphosphate (IP3) and diacylglycerol (DG), which change the currents of K(and Ca^2(and increase brain excitability. Moreover, increased glutamate activates AMPA/KA receptors into the cell. As a result, this phenomenon removes Mg^2(, which inhibits NMDA receptors, leading to an increase in Ca^2(permeability into the postsynaptic neurons, excitotoxicity, and neuronal cell death (Segal, 1988; Scorza et al., 2009).

Cerebrolysin is a small molecule peptide extracted from the porcine brain (Khalili et al., 2017) and has been previously used as a nootropic drug (Plosker and Gauthier, 2009). In another study, cerebrolysin was confirmed to reduce neuronal cell death and increase neurogenesis and brain functions in diverse brain diseases, such as mouse closed head injury (mCHI) and stroke (Zhang L. et al., 2013; Zhang Y. et al., 2013). In addition, it has been shown in many previous studies that cerebrolysin promotes neuroprotection and neurogenesis by increasing the expression of factors such as NGF and brain-derived neurotrophic factor (BDNF) (Rockenstein et al., 2015; Shishkova et al., 2015; Alvarez et al., 2016). In this way, cerebrolysin has shown positive effects in several brain diseases. However, the effects of cerebrolysin on epilepsy are still unknown.

BDNF, as a neurotrophin, promotes nerve differentiation, survival, and neurogenesis (Noble et al., 2011). BDNF simulates the growth and differentiation of new neurons (Alderson et al., 1990; Knusel and Hefti, 1991) and promotes neuronal survival (Hofer and Barde, 1988) and long-term potentiation (LTP) (Korte et al., 1996). BDNF is abundantly expressed throughout the central nervous system (CNS) (Lommatzsch et al., 1999).

In our lab, we hypothesized that BDNF might be increased by cerebrolysin, which would lead to reduced neuronal death. Therefore, this study investigated the effects of cerebrolysin in epilepsy, with BDNF signaling as our primary candidate for promoting neuroprotection after injury.

MATERIALS AND METHODS

Ethics Statement

This study was exhaustively approved according to the rules of the Laboratory Animals Guide and Laboratory Animals published by the National Institutes of Health (NIH). Animal experiments were performed according to the criteria of the Committee on Animal Habitation (Protocol # Hallym 2018-73). We made every effort to minimize the pain of the animals, which were ultimately sacrificed by isoflurane anesthesia.

Experimental Animals

This experiment used Sprague–Dawley male rats (250–350 g, DBL Co., Korea) aged 8 weeks. The animal rooms were kept at a constant humidity (55 ± 5%) and room temperature (22 ± 2°C). The room's lighting was set to automatically switch on at 12 h intervals (on at 6:00 and off at 18:00). This guideline was designed based on the ARRIVE (Animal Research: Reporting *in Vivo* Experiments) guidelines.

Seizure Induction

To confirm the effect of cerebrolysin on neuronal death after pilocarpine-induced seizure, the rats were administered lithium chloride (127 mg/kg, i.p, Sigma-Aldrich Co., St. Louis, MO, United States) 19 h before the administration of pilocarpine. Scopolamine (2 mg/kg, i.p., Sigma-Aldrich Co., St. Louis, MO, United States) was administered 30 min before the administration of pilocarpine (Biagini et al., 2009). Thirty minutes after scopolamine administration, status epilepticus (SE) was induced by the intraperitoneal administration of pilocarpine (25 mg/kg, i.p., Sigma-Aldrich Co., St. Louis, MO, United States). SE is observed according to the presence of five symptoms (1. mouth and facial movement, 2. head nodding, 3. forelimb clonus, 4. rearing with forelimb clonus, and 5. rearing and falling with forelimb clonus) that occur progressively in Racine's method. The animals were placed in individual cages for ease of observation. SE usually occurred within 20–30 min after pilocarpine injection (Persinger et al., 1988). Diazepam (10 mg/kg, i.p., Valium, Hoffman la Roche, Neuilly sur-Seine, France) was injected intraperitoneally 2 h after the last Racine's stage occurred (Biagini et al., 2001). If the animals presented consistent recurrent seizures, additional diazepam was injected (2 mg/kg, i.p.) (Kim et al., 2013).

Cerebrolysin Administration

Experimental groups were classified into four groups: sham-vehicle, sham-cerebrolysin, seizure-vehicle, and seizure-cerebrolysin. To evaluate the effect of cerebrolysin on pilocarpine-induced seizures, cerebrolysin groups were injected with cerebrolysin (2.5 ml/kg, i.p., Ever Neuro Pharma, Unterach, Austria) intraperitoneally daily for 1 week, 2 h after a seizure induction, and vehicle groups were injected with 0.9% saline intraperitoneally in the same way. Also, to evaluate the anticonvulsant effect of cerebrolysin on pilocarpine-induced seizure, cerebrolysin groups were injected with cerebrolysin (2.5 ml/kg, i.p.) 10 min before pilocarpine injection. The present study used this cerebrolysin concentration since several works have demonstrated a significant neuroprotective effect after brain insult (Zhang Y. et al., 2013; Liu et al., 2017; Zhang et al., 2019).

Brain Sample Preparation

Animals were sacrificed at 1 week after a seizure. Animals were injected with urethane (1.5 g/kg, i.p.) as anesthesia. After completely entering an anesthetic state, the animals were perfused with 0.9% saline and then 4% paraformaldehyde. The brains were harvested quickly and accurately and were fixed with 4% paraformaldehyde for 1 h. After fixation, the brains were immersed in a 30% sucrose solution as a cryoprotectant for 2 days (Vinet et al., 2016). Two days later, when the brains had sunk, the brains were frozen with a cryostat. The brains were then cut to a thickness of 30 μm on the cryostat, and the tissue was stored in a stock solution until histological evaluation was performed.

Microscope Equipment

Microscopy images were obtained with an Olympus IX70 microscope (Olympus, Shinjuku-ku, Tokyo) equipped with a U-HGLGPS (Olympus, Shinjuku-ku, Tokyo) and an INFINITY3 digital camera (Olympus, Shinjuku-ku, Tokyo). We obtained the image using the INFINITY ANALYZE software.

Detection of Live Neurons

Live neurons were evaluated by staining for neuronal nuclei (NeuN) to confirm the effect of cerebrolysin on pilocarpine-induced seizure. Following the brain cryostat section, we stained the cut tissue. After precleaning to eliminate the remaining blood cells in the tissues, we put the tissues in monoclonal mouse anti-NeuN antiserum (diluted 1:500, Billerica, Millipore Co., MA, United States) and kept them overnight for 16 h at 4°C. Sixteen hours later, the tissues were placed in anti-mouse IgG (diluted 1:250, Burlingame, Vector, CA, United States) for 2 h at room temperature and then placed in an ABC complex solution (Burlingame, Vector, CA, United States) for 2 h at room temperature. Then, the samples were transferred to slides after 3,3′-diaminobenzidine (DAB ager, Sigma-Aldrich Co., St. Louis, MO, United States) coloring for 1.5 min. Slides were then dried and mounted using Canada balsam. The tissues were observed through an Axioscope microscope. Live neurons were averaged by blind quantification. Live neurons were quantified in the stratum pyramidale (SP) of hippocampal cornu ammonis1 (CA1) and CA3 and expressed as the density (cell count/mm^2).

Detection of Microglial Cells

Microglial cells were evaluated by ionized calcium-binding adaptor molecule 1 (Iba1) to confirm the effect of cerebrolysin on pilocarpine-induced seizure. Following brain cryostat sectioning, we stained the cut tissue. After precleaning to eliminate the remaining blood cells in the tissues, we put the tissues in monoclonal goat anti-Iba1 antiserum (diluted 1:500, AbD Serotec, United Kingdom) and kept them overnight for 16 h at 4°C. Sixteen hours later, the tissues were placed in Alexa Fluor 594-conjugated donkey anti-goat IgG secondary antibody (diluted 1:250, Invitrogen, Grand Island, NY, United States) for 2 h at room temperature. Then, they were put on the slides. The slides were dried and mounted with DPX (Sigma-Aldrich Co., St. Louis, MO, United States), and the tissues were observed through an Axioscope microscope. Microglial cells were quantified in the stratum oriens (SO), stratum pyramidale (SP), and stratum radiatum (SR) of the hippocampal CA1 and CA3. Microglial cells were expressed as their density (cell count/mm^2).

Detection of Astroglial Cells

Astroglial cells were evaluated by the glial fibrillary acidic protein (GFAP) to confirm the effect of cerebrolysin on pilocarpine-induced seizure. Following brain cryostat sectioning, we stained the cut tissue. After precleaning to eliminate the remaining blood cells in the tissues, we put the tissues in monoclonal rabbit anti-GFAP antiserum (diluted 1:1,000, AbD Serotec, United Kingdom) and kept them overnight for 16 h at 4°C. Sixteen hours later, the tissues were placed in Alexa Fluor 488-conjugated donkey anti-rabbit IgG secondary antibody (diluted 1:250, Invitrogen, Grand Island, NY, United States) for 2 h at room temperature. Then, the samples were placed on the slides. The slides were dried and mounted with DPX (Sigma-Aldrich Co., St. Louis, MO, United States), and the tissues were observed through an Axioscope microscope. Astroglial cells were then quantified in the stratum oriens (SO), stratum pyramidale (SP), and stratum radiatum (SR) of hippocampal CA1 and CA3. Astroglial cells were expressed as their density (cell count/mm^2).

Detection of Apoptotic Cells

Apoptotic cells were evaluated by cleaved caspase-3 staining to confirm the effect of cerebrolysin on pilocarpine-induced seizure. Following brain cryostat sectioning, we stained the cut tissue. After precleaning to eliminate the remaining blood cells in the tissues, we put the tissues in polyclonal rabbit anticleaved caspase-3 antiserum (diluted 1:200, Cell signaling, Danvers, MA, United States) and kept them overnight for 16 h at 4°C. Sixteen hours later, the tissues were placed in Alexa Fluor 488 donkey anti-rabbit IgG secondary antibody (diluted 1:250, Invitrogen, Grand Island, NY, United States) for 2 h at room temperature. Then, the samples were placed on slides. The slides were dried and mounted with DPX (Sigma-Aldrich Co., St. Louis, MO, United States). The tissues were then observed through an Axioscope microscope. Apoptotic cells were counted by blind quantification in the hippocampal CA1 and CA3 regions.

Western Blotting

To verify the protein level of BDNF in the vehicle and cerebrolysin groups, we performed a Western blotting analysis. The bilateral hippocampus was obtained and homogenized in a RIPA buffer consisting of 10 mM Tris-HCl (pH 7.4), 1% Non-idet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, and 0.1% SDS. The homogenized hippocampus was centrifuged at 14,000 × g for 20 min at 4°C, and the supernatant was harvested. The harvested supernatant was incubated at 100°C for 10 min and stored at −80°C in an ultra-low freezer until use. The protein composition within the hippocampus was measured by a Bradford protein assay. Hippocampal proteins were diluted in an SDS electrophoresis sample buffer, separated on a 14% SDS-polyacrylamide gel, and then transferred to a PVDF (polyvinylidene difluoride) membrane. Non-specific binding was prevented by using 5% skim milk and 5% BSA (TNF-α) in TBST (50 mM Tris-HCl, pH 7.5, 0.1% Tween

20 and 150 mM NaCl) for over 1 h at room temperature. Protein-transferred membranes were incubated on primary antibodies (BDNF, ab108317, diluted 1:1,000, Abcam, TrkB, #4603, diluted 1:2,000, Cell signaling, phospho-TrkB (p-TrkB), ABN1381, diluted 1:1,000, Millipore, phospho-CREB (p-CREB), #9198, diluted 1:1,000, Cell signaling, TNF-α, ab6671, diluted 1:500, Abcam) overnight at 4°C in an incubator. After primary antibody incubation, the membranes were washed three times for 5 min in TBST. Afterward, the primary anti-BDNF, anti-TrkB, anti-phospho-TrkB, anti-phospho-CREB, and anti-TNF-α-reacted membranes were incubated for 1 h in anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (HRP, LF-SA8002, diluted 1:5,000, Ab Frontier). Last, to visualize the protein concentration, we used an ECL (enhanced chemiluminescence) solution (Cat.P90720, Millipore) before observation. The ECL solution-mounted membrane was made to react using a chemiluminescence imaging system device (Amersham imager 680 machine, GE healthcare). All data were analyzed by Image J.

Statistical Analysis

We used non-parametric tests to determine the statistical significance between the experimental groups. The data of four groups were analyzed by a Kruskal–Wallis test and Bonferroni *post hoc* analysis, and the data from the two groups were analyzed by a Mann–Whitney U test. The data are expressed as the standard error of the mean (SEM) and were regarded as significant when the difference was $p < 0.05$.

RESULTS

Experimental Procedure and Seizure Grade at Cerebrolysin Treatment

We confirmed the neuroprotective effects of cerebrolysin treatment on seizure-induced neuronal death. Cerebrolysin was injected for 1 week after pilocarpine-induced seizure (**Figure 1A**). Both groups were confirmed to have equally induced seizures (**Figure 1B**). Only seizure-induced rats were used in the experimental groups. In both groups, there was no difference in weight change during the 1-week period after a seizure induction (**Figure 1C**). In addition, to test whether cerebrolysin treatment has an anticonvulsant effect, we injected cerebrolysin 10 min before pilocarpine injection. There was no difference in seizure grade between the vehicle- and cerebrolysin-treated groups (**Supplementary Figure S1**).

Cerebrolysin Increases the Density of Live Neurons After Pilocarpine-Induced Seizure

We conducted neuronal nuclei (NeuN) staining to confirm the effects of cerebrolysin on neuronal survival after a seizure. We sacrificed the animals at 1 week after inducing seizure and quantified the density of their live neurons in the hippocampus. As a result, after comparing the density of the live neurons of the seizure-vehicle and seizure-cerebrolysin groups, we found that the density of live neurons was increased in the group treated with cerebrolysin in the hippocampal CA1 and CA3 regions, rather than the seizure-vehicle group (**Figure 2**). The data are the mean ± SEM ($n = 5$) for each sham group, and $n = 5$–7 for each seizure group {$^{*}p < 0.05$ vs. vehicle-treated group; $^{\#}P < 0.05$ vs. sham-operated group [Kruskal–Wallis test with *post hoc* test: (CA1) Chi square = 17.722, $df = 3$, $p = 0.001$ (CA3), Chi square = 17.024, $df = 3$, $p = 0.001$]}.

Cerebrolysin Decreases the Density of Glial Cells After Pilocarpine-Induced Seizure

Glial activation is increased not only during seizure but also after other diseases (Hong et al., 2018; Lee S. H. et al., 2018; Kho et al., 2019). Inflammation was triggered by activated glial cells after a seizure. To determine the effect of cerebrolysin on the density of glial cells, we performed ionized calcium-binding adaptor molecule 1 (Iba-1) and GFAP staining, which are immunofluorescent stains used to confirm microglia and astroglial cells, respectively. There was little staining of glial cells in the sham-treated groups. On the other hand, we found that the group treated with cerebrolysin showed a decreased density of glial cells in the hippocampal CA1 and CA3 regions compared to the seizure-vehicle group (**Figure 3**). The data are the mean ± SEM, $n = 5$, for each sham group, and $n = 5$–7 for each seizure group {$^{*}p < 0.05$ vs. vehicle-treated group; $^{\#}p < 0.05$ vs. sham-operated group [Kruskal–Wallis test with *post hoc* test: (Iba-1, CA1) Chi square = 17.244, $df = 3$, $p = 0.001$, (GFAP, CA1) Chi square = 17.153, $df = 3$, p0.001 (Iba-1, CA3) Chi square = 17.456, $df = 3$, $p = 0.001$, (GFAP, CA3) Chi square = 18.292, $df = 3$, $p < 0.001$]}.

Cerebrolysin Decreases Levels of TNF-α After Pilocarpine-Induced Seizure

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine (Decourt et al., 2017). To confirm if suppressed glial activation via the administration of cerebrolysin reduces proinflammation, we confirmed the protein level of TNF-α by Western blot analysis after a seizure. In the present study, we found that the seizure group showed increased TNF-α expression in the hippocampus compared to the sham group. We also found that TNF-α expression was significantly reduced via the administration of cerebrolysin after a seizure (**Figure 4**). The data are the mean ± SEM, $n = 3$–4, for each seizure-experienced group [$^{*}p < 0.05$ vs. vehicle-treated group; $^{\#}p < 0.05$ vs. sham-operated group (Kruskal–Wallis test with *post hoc* test: Chi square = 17.153, $df = 3$, $p = 0.001$)].

Cerebrolysin Decreases the Number of Apoptotic Cells After Pilocarpine-Induced Seizure

Cleaved caspase-3 staining is an immunofluorescent staining method used to confirm apoptosis. We performed cleaved caspase-3 staining to verify the effect of cerebrolysin on apoptosis after a seizure. There was no caspase-3 activation observed in the sham-operated groups. The seizure-experienced group showed

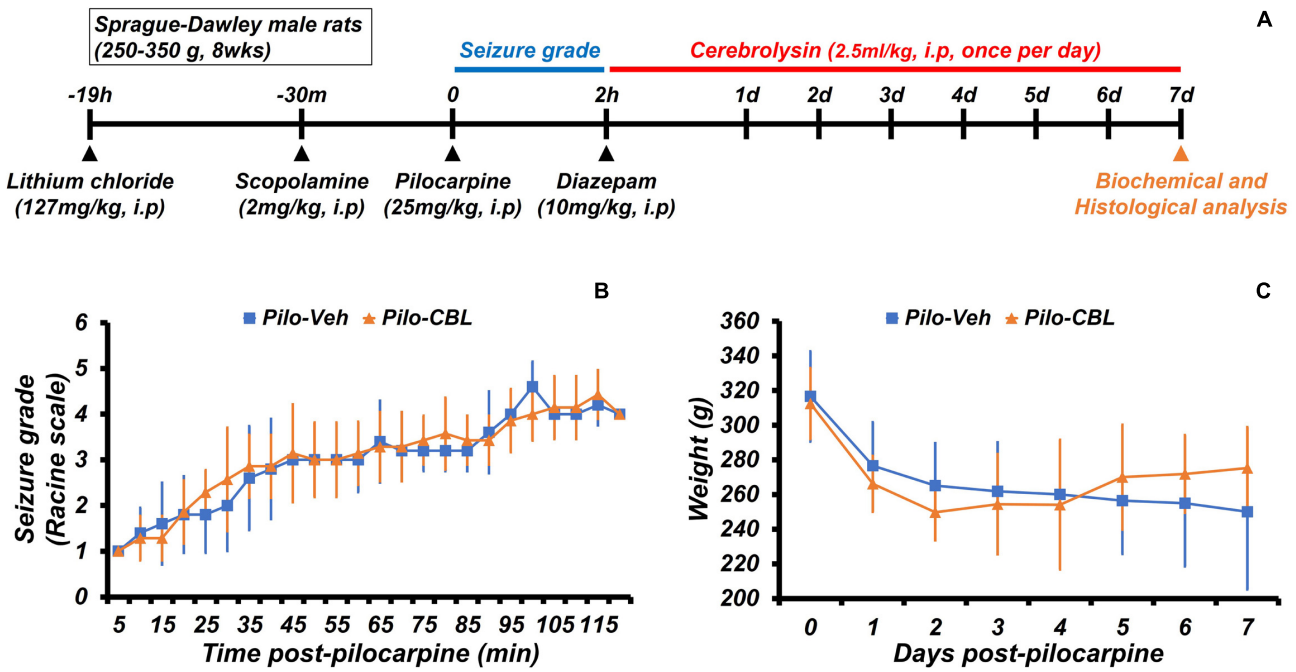

FIGURE 1 | Experimental procedure and seizure grade during cerebrolysin treatment. The experimental paradigm of this study and the seizure grade according to the Racine stage. **(A)** The experimental paradigm of this study. After a seizure was induced for 2 h by pilocarpine, cerebrolysin was administered once a day at a concentration of 2.5 ml/kg for 1 week. **(B)** A graph confirming the average value of the seizure grade based on the Racine stage after pilocarpine administration. **(C)** A graph of the average body weight for 1 week after pilocarpine-induced seizure; n = 5–7 for each seizure group.

an increased level of caspase-3 activation in the hippocampal CA1 and CA3 regions. However, the administration of cerebrolysin showed a decreased number of caspase-3-positive cells in the hippocampal CA1 and CA3 regions compared to the seizure-vehicle group (**Figure 5**). The data are the mean ± SEM, n = 5–7, for each seizure group {$^{*}p < 0.05$ [Mann–Whitney U test: (CA1) $z = 2.842$, $p = 0.03$, (CA3) $z = 2.517$, $p = 0.01$]}.

Cerebrolysin Increases Levels of BDNF After Pilocarpine-Induced Seizure

BDNF is regarded as a potent neural modulator, which is beneficial to neuronal functions and promotes neuroprotection (Chen et al., 2013). To confirm the increase in BDNF and determine whether the increase was mediated by cerebrolysin, we performed a Western blot analysis to confirm the level of the brain-derived neurotropic factor (BDNF) after a seizure. By comparing the BDNF expressions of the sham and seizure groups, we confirmed that the seizure group experienced increased BDNF expression in the hippocampus compared to the sham group. Moreover, comparing the BDNF expression of the seizure-vehicle and seizure-cerebrolysin groups, we demonstrated that the administration of cerebrolysin increased BDNF expression in the hippocampus is greater than in the seizure-vehicle group (**Figures 6A,B**). The data are the mean ± SEM, n = 3–4, for each seizure group [$^{*}p < 0.05$ vs. vehicle-treated group; $^{\#}p < 0.05$ vs. sham-operated group. (Kruskal–Wallis test with *post hoc* test: Chi square = 9.705, $df = 3$, $p = 0.021$)].

Cerebrolysin Increases Levels of p-TrkB/Tyrosine Kinase Receptor B After Pilocarpine-Induced Seizure

Tyrosine kinase receptor B (TrkB) is known as a receptor for BDNF (Budni et al., 2015). To evaluate BDNF receptor activation, we analyzed the levels of p-TrkB/TrkB by Western blot after a seizure. In the present study, we found that the seizure-experienced group showed increased levels of p-TrkB/TrkB expression in the hippocampus compared to the sham group. We found that cerebrolysin further increased the level of p-TrkB/TrkB expression in the hippocampus compared to the vehicle-treated group after seizure (**Figures 6C,D**). The data are the mean ± SEM, n = 3–4 for each seizure group [$^{*}p < 0.05$ vs. vehicle-treated group; $^{\#}p < 0.05$ vs. sham-operated group (Kruskal–Wallis test with *post hoc test*: Chi square = 9.029, $df = 3$, $p = 0.029$)].

Cerebrolysin Increases Levels of p-CREB After Pilocarpine-Induced Seizure

Phospho-cAMP-response-element-binding (p-CREB) is a transcription factor present downstream of BDNF that promotes neuronal protection and cell survival (Walton and Dragunow, 2000; Li et al., 2018). We evaluated the levels of p-CREB by Western blot analysis after seizure. We found that the seizure-experienced group showed increased levels of p-CREB expression in the hippocampus compared to the sham-operated group. Moreover, as seen in the p-TrkB/TrkB expression, the administration of cerebrolysin further increased the p-CREB expression in the seizure-experienced group (**Figures 6E,F**).

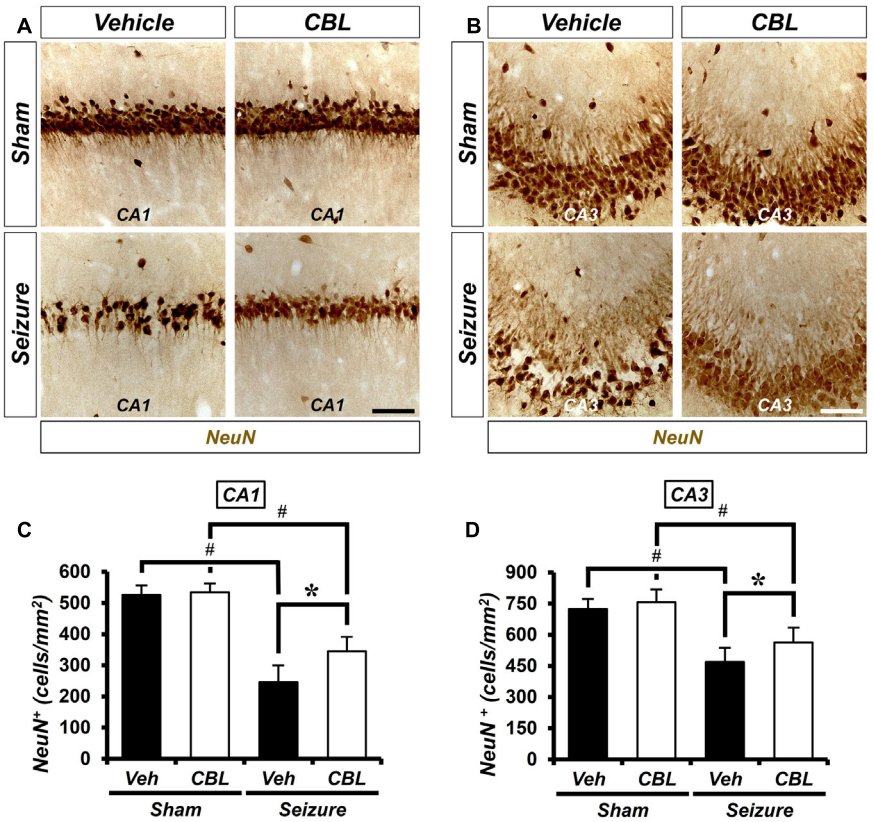

FIGURE 2 | Cerebrolysin increases the density of live neurons after pilocarpine-induced seizure. The administration of cerebrolysin decreases neuronal death after pilocarpine-induced seizure. **(A,C)** NeuN (+) neurons in the hippocampal CA1 and CA3 regions. After a seizure, the administration of cerebrolysin for 1 week increased the density of live neurons in the hippocampal CA1 and CA3 regions compared with the seizure-vehicle groups. Scale bar = 100 μm. **(B,D)** Graphs that show the density of live neurons. The data are the mean ± SEM, n = 5 from each sham group. N = 5–7 for each seizure group. $^{*}p < 0.05$ vs. vehicle-treated group; $^{\#}p < 0.05$ vs. sham-operated group [Kruskal–Wallis test with *post hoc* test: **(C)** Chi square = 17.722, df = 3, p = 0.001, **(D)** Chi square = 17.024, df = 3, p = 0.001].

The data are the mean ± SEM, n = 3–4 for each seizure group [$^{*}p < 0.05$ vs. vehicle-treated group; $^{\#}p < 0.05$ vs. sham-operated group (Kruskal–Wallis test with *post hoc* test: Chi square = 11.895, df = 3, p = 0.008)].

DISCUSSION

In the present study, we verified that cerebrolysin exerts powerful neuroprotective effects after pilocarpine-induced seizure. Seizure is one of the most common neurological diseases, but methods for preventing the cell death mechanisms that occur post-seizure and repairing this injury after a seizure remain uncertain. Seizures cause serious damage to the hippocampus, and neuronal death is ultimately caused by a series of cell death cascades involving excessive inflammation, glial activation, apoptosis, oxidative stress, and zinc accumulation (Kim et al., 2012; Jeong et al., 2017). Here, we focused on the rescue of seizure-induced neurological damage with cerebrolysin.

Cerebrolysin is neuropeptide extracted from porcine brains and has been used as a nootropic drug (Plosker and Gauthier, 2009). It is known that cerebrolysin can pass intact across the blood–brain barrier (BBB) (Muresanu et al., 2015; Bornstein et al., 2018). When cerebrolysin is administered, it is not known how much cerebrolysin reaches the brain. However, with status epilepticus, there is a known loss of integrity at the BBB with the entry of proteins such as albumin (Kim et al., 2015; Lee et al., 2017; Mendes et al., 2019). Disruption of the BBB, therefore, enables the entry of cerebrolysin into the brain. Furthermore, several studies have shown that the neuropeptide cerebrolysin can pass intact across the BBB (Muresanu et al., 2015; Bornstein et al., 2018). Cerebrolysin demonstrated significant neuroprotective effects and increased neurogenesis after brain insults when injected intraperitoneally (Zhang L. et al., 2013; Liu et al., 2017). Several previous studies have demonstrated that under head trauma and ischemic conditions, the administration of cerebrolysin attenuates brain damage (Zhang L. et al., 2013; Zhang Y. et al., 2013). In addition, previous studies have shown that cerebrolysin increases the level of the BDNF by inhibiting the activity of glycogen synthase kinase-3 beta (GSK-3β) (Alvarez et al., 2016). It has also been shown that enhancing BDNF expression decreases neuronal damage and inflammation and increases neurogenesis (Wada et al., 2003; Chen et al., 2013; Rockenstein et al., 2015).

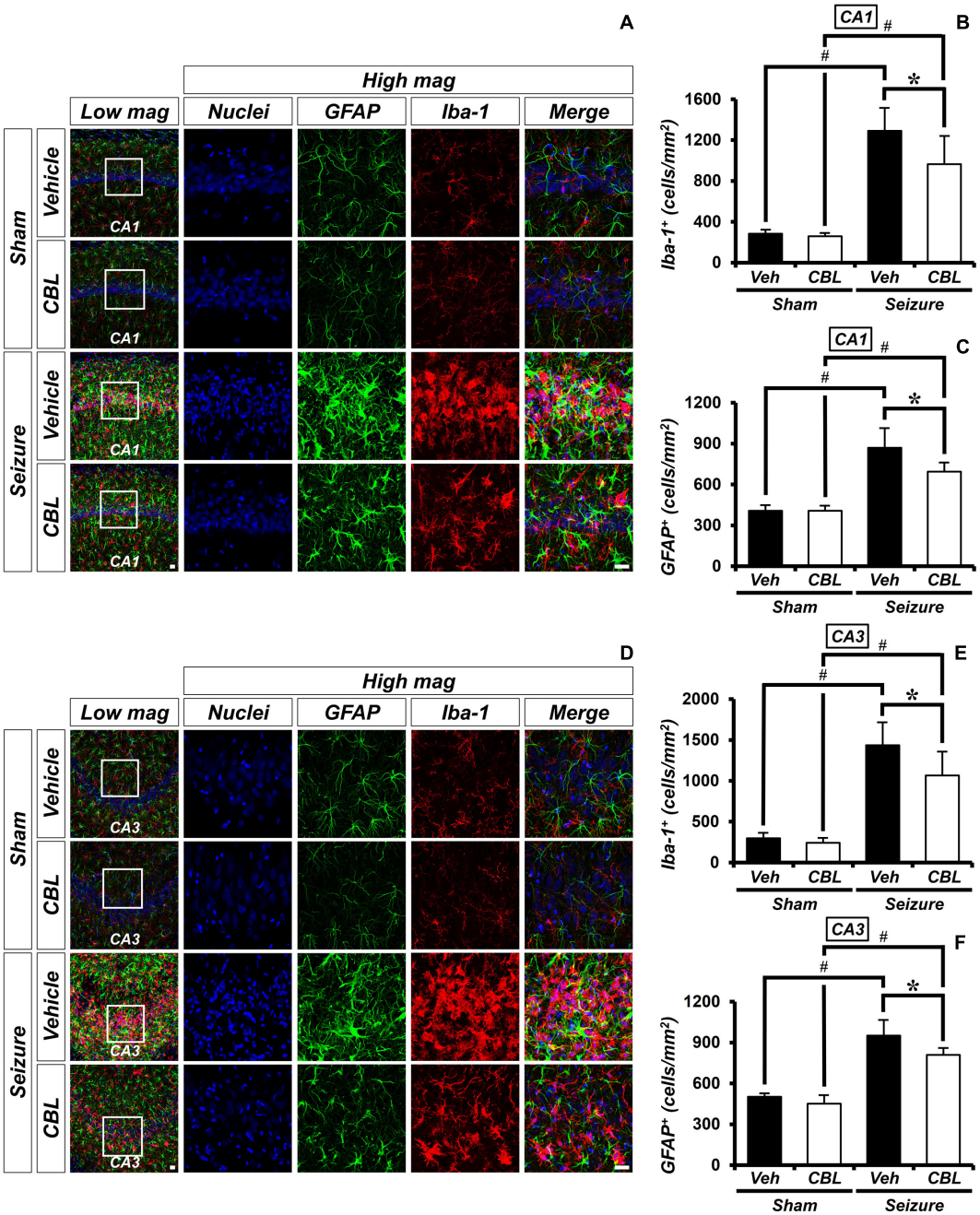

FIGURE 3 | Cerebrolysin decreases the density of glial cells after pilocarpine-induced seizure. The administration of cerebrolysin decreased the density of glial cells after pilocarpine-induced seizure. **(A,D)** Iba-1 (red), GFAP (green), and DAPI (blue) in the hippocampal CA1 **(A)** and CA3 **(D)** regions. The administration of cerebrolysin after a seizure decreased the density of glial cells in the hippocampal CA1 and CA3 regions compared to the seizure-vehicle groups. Scale bar = 20 μm. **(B,C,E,F)** A graph of the density of glial cells according to the standard. The data are the mean ± SEM, $n = 5$, from each sham group; $n = 5$–7 for each seizure group. $^{*}p < 0.05$ vs. vehicle-treated group; $^{\#}p < 0.05$ vs. sham-operated group [Kruskal–Wallis test with *post hoc* test: **(B)** Chi square = 17.244, $df = 3$, $p = 0.001$, **(C)** Chi square = 17.153, $df = 3$, $p < 0.001$, **(E)** Chi square = 17.456, $df = 3$, $p = 0.001$, **(F)** Chi square = 18.292, $df = 3$, $p < 0.001$].

However, the effects of cerebrolysin on seizure-induced neuronal death are not well known. Based on these previous findings, we hypothesized that the administration of cerebrolysin after pilocarpine-induced seizure would attenuate neuronal death by increasing levels of BDNF.

To evaluate the neuroprotective effects of cerebrolysin, we first injected cerebrolysin once a day for 1 week after a seizure and then confirmed the neuroprotective effects. Then, we performed several histological evaluations after pilocarpine-induced seizure. We next performed staining for NeuN, a specific

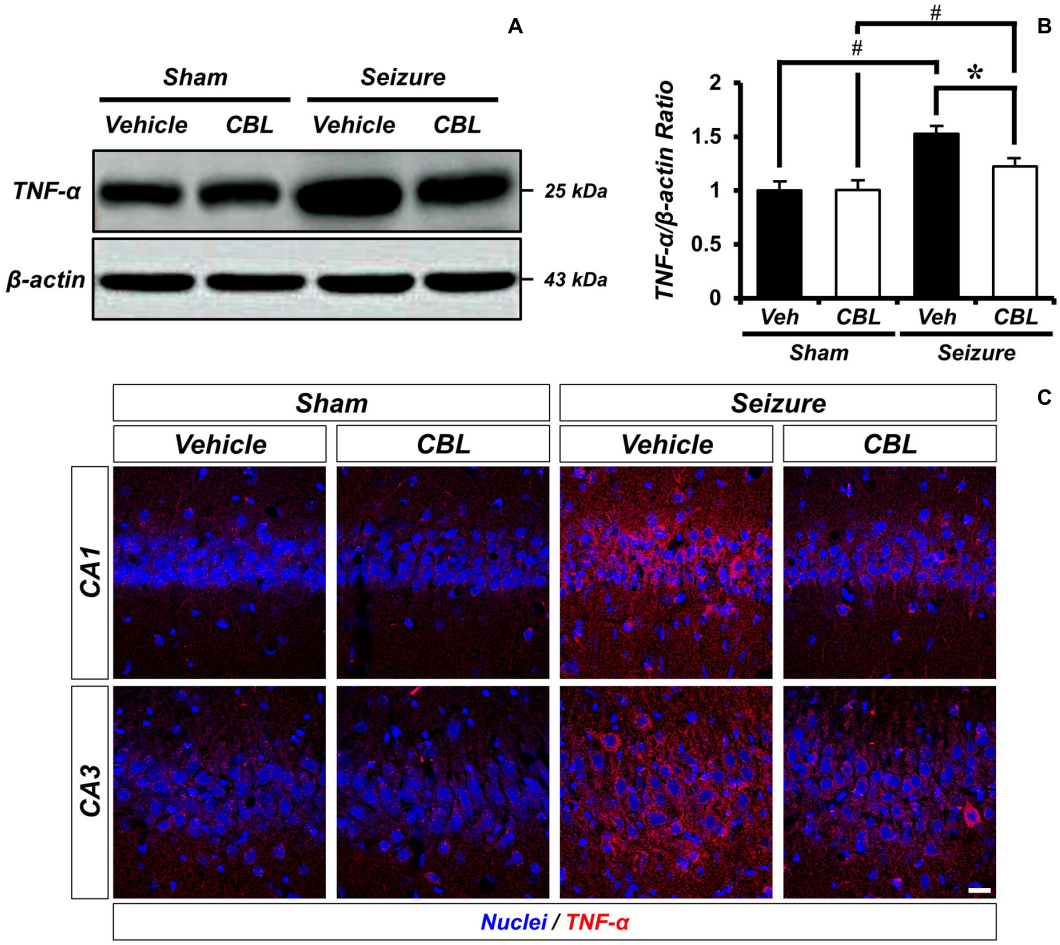

FIGURE 4 | Cerebrolysin decreases level of TNF-α after pilocarpine-induced seizure. The administration of cerebrolysin decreased TNF-α after pilocarpine induced seizure. **(A)** indicates the level of TNF-α in the hippocampus. After seizure, the administration of cerebrolysin decreased TNF-α expression in the hippocampus compared to vehicle groups. **(B)** A graph of the TNF-α. **(C)** TNF-α (red) and DAPI (blue) in the hippocampal CA1 and CA3 regions. The data are mean ± SEM, n = 3–4 from each seizure group. $^{*}p < 0.05$ vs. vehicle-treated group; $^{\#}p < 0.05$ vs. sham-operated group (Kruskal–Wallis test with *post hoc* test: Chi square = 17.153, df = 3, p = 0.001).

marker of live neurons, and cleaved caspase-3, a specific marker for apoptotic cells, to confirm the effects of cerebrolysin on neuronal death after pilocarpine-induced seizure. It is already well known that the number of live neurons decreases after a seizure (Kim et al., 2012; Jeong et al., 2017; Lee S. H. et al., 2018). Also, it is already well established that the number of apoptotic cells increases after a seizure (Lee J. M. et al., 2018; Li et al., 2019). To confirm the effects of cerebrolysin on neuronal death in a damaged hippocampus following a seizure, the density of live neurons was recorded to determine whether cerebrolysin treatment could rescue neuronal damage after a seizure. In the results, the sham-operated groups showed no differences in the density of their NeuN positive neurons between the vehicle and cerebrolysin groups. However, in the seizure-operated groups, the cerebrolysin-administered group showed a significant increase in the density of NeuN-positive neurons in hippocampal CA1 and CA3 compared to the vehicle group.

In addition to directly damaging neurons, other cells in the region are also affected by seizure. Epileptic seizure-induced neuroinflammation is triggered by activated glial cells, which include microglia and astroglia (Scorza et al., 2009). The damage caused by seizures is a trigger for glial activation (Scorza et al., 2009). If excessive inflammation persists, it promotes subsequent deleterious effects, such as cellular damage and neurotoxicity (Peng et al., 2015; Stein et al., 2017). We proceeded to stain for Iba-1, a specific marker for microglia, and GFAP, a specific marker for astroglia, to assess the effect of cerebrolysin on the density of glial cells after a seizure. We confirmed the presence of glial cells in the hippocampus after a seizure. In the sham-operated groups, there was no observable difference in the density of microglial cells between the vehicle and cerebrolysin groups. However, in the seizure-operated groups, the cerebrolysin-administered group showed a significant decrease in the density of microglial cells in the hippocampal CA1 and CA3 regions compared to the vehicle group. The density

of astroglial cells in the sham-operated groups showed no difference between the vehicle and cerebrolysin groups. However, in the seizure-operated groups, the cerebrolysin-administered group showed a significantly attenuated density of astroglial cells in the hippocampal CA1 and CA3 regions compared to the vehicle group.

Pilocarpine-induced seizure triggers glial activation and promotes the production of various inflammatory mediators, thus initiating a cascade of inflammatory processes in the hippocampus (Shapiro et al., 2008; Vezzani et al., 2011). The release of pro-inflammatory molecules can aggravate neuronal excitability and disturb the normal physiological functions of the glia, which perturbate glial–neuronal communications. In the pilocarpine-induced seizure model, the density of microglial cells thus contributes to decreasing the seizure threshold and compromising neuronal survival (Vezzani et al., 2008; Riazi et al., 2010). In the present study, we found that cerebrolysin administration decreased the density of microglial cells after a seizure. In addition, we evaluated the protein levels of TNF-α, a proinflammatory cytokine, by Western blot analysis to determine whether the cerebrolysin-induced reduction of microglial activation is associated with pro-inflammatory cytokine. Here, we found that the protein level of TNF-α in the cerebrolysin-administered group was decreased compared to that in the seizure-vehicle group.

Next, we confirmed that the observed reduction in neuronal death was associated with an increase in apoptosis induction through cleaved caspase-3 staining, which detects apoptotic cells. In the seizure-operated groups, the cerebrolysin-administered group showed a significant decrease in the number of apoptotic cells in the hippocampal CA1 and CA3 regions compared to the vehicle group. Cerebrolysin attenuates hippocampal

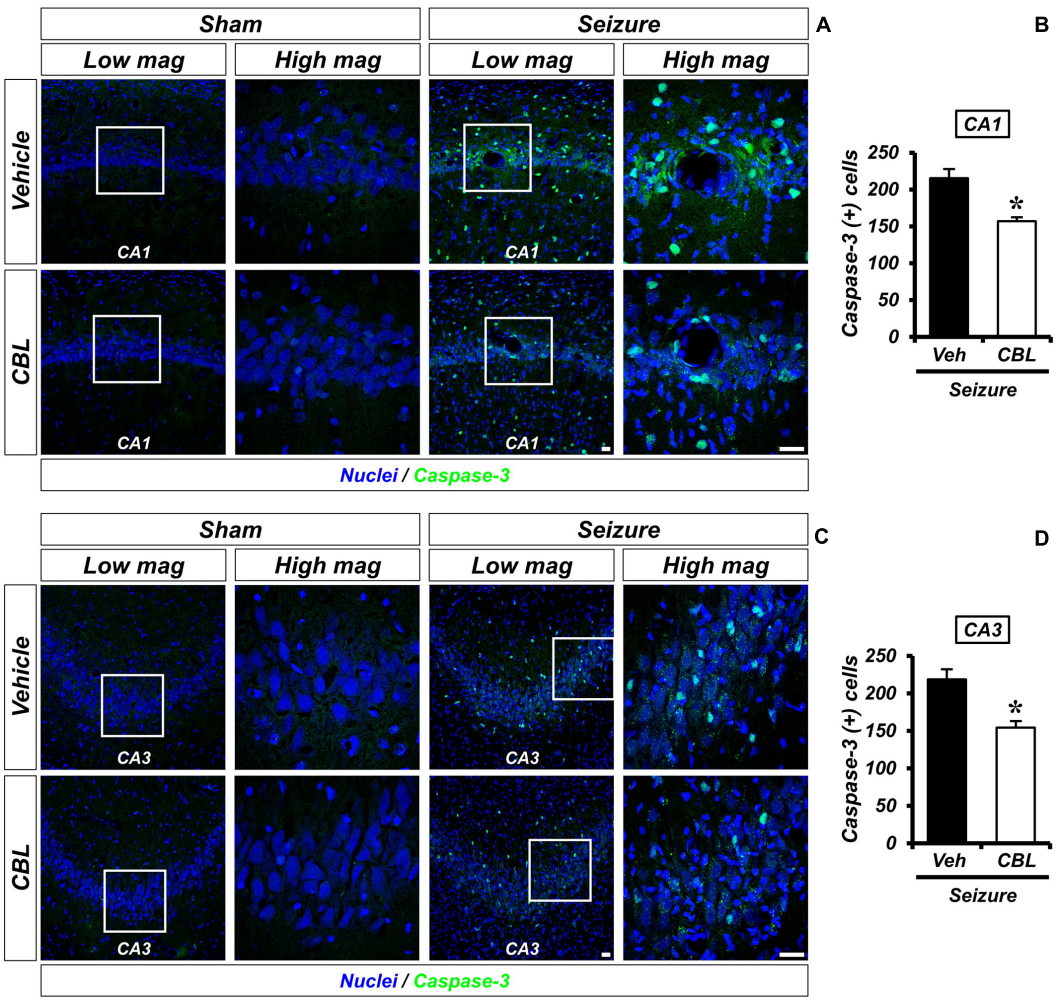

FIGURE 5 | Cerebrolysin decreases the number of apoptotic cells after pilocarpine-induced seizure. The administration of cerebrolysin decreases apoptosis after pilocarpine-induced seizure. **(A,C)** Cleaved caspase-3 (green) in the hippocampal CA1 and CA3 regions. After a seizure, the administration of cerebrolysin for 1 week decreased the number of apoptotic cells in the hippocampal CA1 and CA3 regions compared to the seizure-vehicle groups. Scale bar = 20 μm. **(B,D)** A graph that shows the number of apoptotic cells. The data are the mean ± SEM, n = 5–7 for each seizure group. *$p < 0.05$ [Mann–Whitney U-test: **(B)** $z = 2.842$, $p = 0.03$, **(D)** $z = 2.517$, $p = 0.01$].

neuronal death and apoptotic cell damage by increasing the concentration of the protease "furin," which upregulates BDNF levels (Rockenstein et al., 2015). Increased BDNF elevates TrkB activity, which is associated with antiapoptosis signaling and inhibits neuronal apoptosis (Chen et al., 2013). Following the above logic, we assumed that this process caused the cerebrolysin group to decrease neuronal apoptosis and increase the density of live neurons compared to the vehicle group (Rockenstein et al., 2005).

Several studies have demonstrated that neurotrophic factors, including NGF and BDNF, are elevated after status epilepticus, which promotes the survival of neurons post-seizure

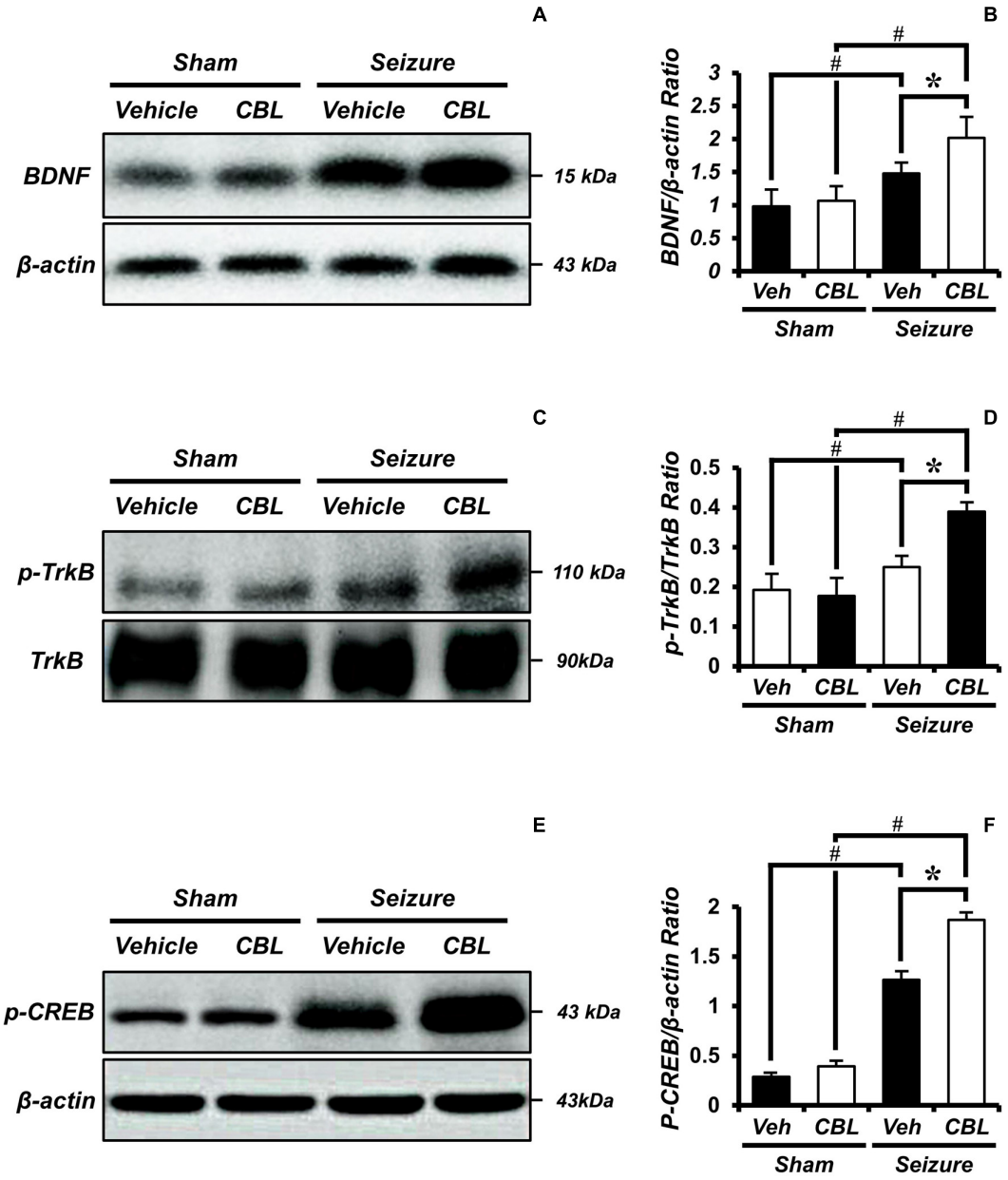

FIGURE 6 | Cerebrolysin increases the levels of brain-derived neurotrophic factor (BDNF), phospho-tyrosine kinase receptor B (p-TrkB), and phospho-cAMP-response-element-binding (p-CREB) after pilocarpine-induced seizure. The administration of cerebrolysin increased BDNF after pilocarpine-induced seizure. **(A)** The level of BDNF in the hippocampus. After a seizure, the administration of cerebrolysin increased BDNF expression in the hippocampus compared to the seizure-vehicle groups. **(B)** A graph of the BDNF. **(C)** The level of p-TrkB in the hippocampus. After a seizure, the administration of cerebrolysin increased p-TrkB expression in the hippocampus compared to the seizure-vehicle groups. **(D)** A graph of the p-TrkB. **(E)** The level of p-CREB in the hippocampus. After a seizure, the administration of cerebrolysin increased p-CREB expression in the hippocampus compared to the vehicle groups. **(F)** A graph of the p-CREB. The data are the mean ± SEM, $n = 3$–4, for each seizure group. $^{*}p < 0.05$ vs. vehicle-treated group; $^{\#}p < 0.05$ vs. sham-operated group [Kruskal–Wallis test with *post hoc* test: **(B)** Chi square = 9.705, $df = 3$, $p = 0.021$, **(D)** Chi square = 9.029, $df = 3$, $p = 0.029$, **(F)** Chi square = 11.895, $df = 3$, $p = 0.008$].

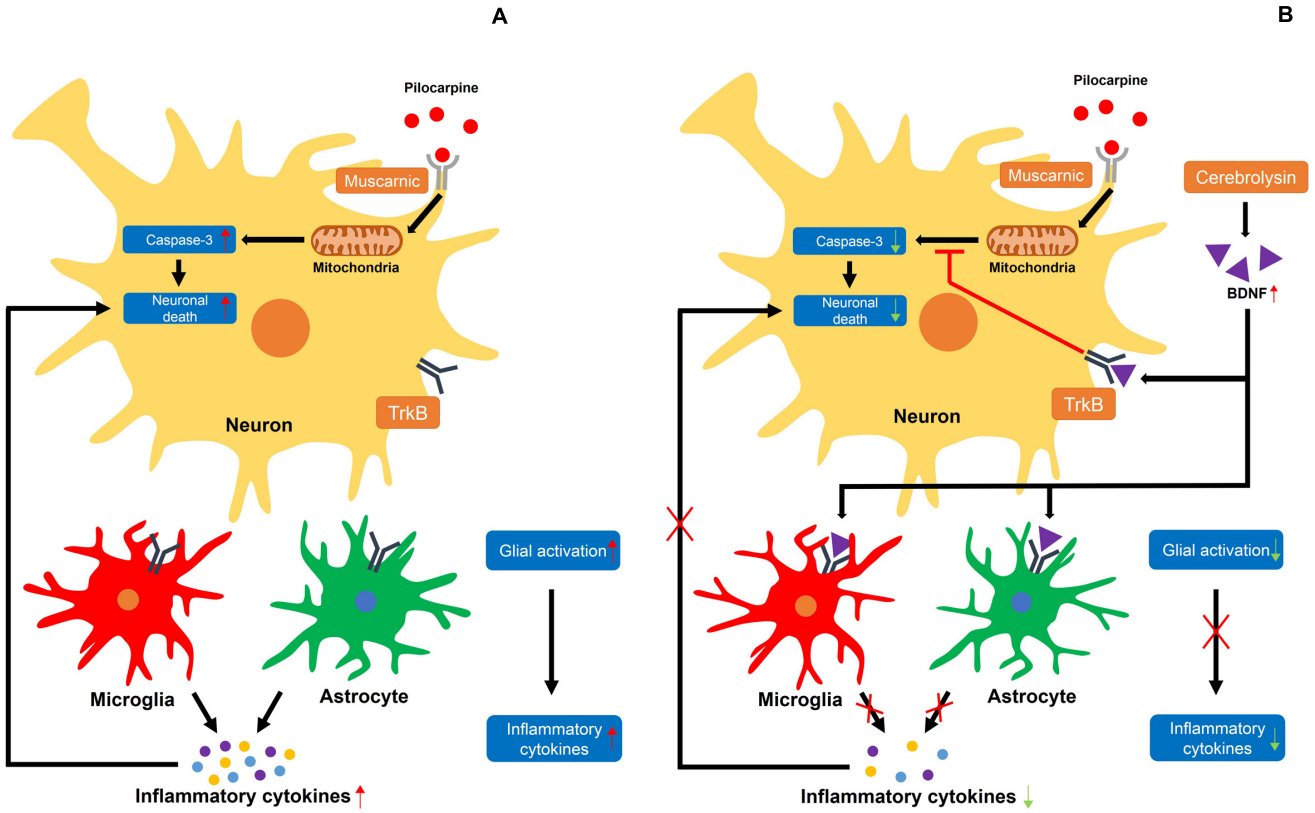

FIGURE 7 | Proposed mechanism for the effects of cerebrolysin on seizure-induced neuronal death. **(A)** A schematic drawing that proposes the possible cellular pathways through which a seizure may induce neuronal death. **(A)** The damage caused by excessive neuronal excitation when pilocarpine is bound by a muscarinic receptor increases caspase-3-dependent neuronal death. In addition, activated glial cells secrete inflammatory cytokines, increasing neuronal death. **(B)** Increased BDNF levels by cerebrolysin can result in the inhibition of caspase-3 and glial activation, which are thought to occur following pilocarpine-induced seizure. A red X indicates inhibition.

(VonDran et al., 2014; Lima et al., 2015; Sanna et al., 2017). Thus, we evaluated the protein levels of BDNF by using a Western blot analysis to test whether an increase in neuroprotection after cerebrolysin administration is correlated with increases in BDNF. Here, we found that BDNF expression was increased in the seizure group compared with the sham operated group, as also shown by another group. Previous studies have already shown that BDNF is increased after pilocarpine-induced seizure (da Penha Berzaghi et al., 1993; Metsis et al., 1993; Schmidt-Kastner et al., 1996). This is considered to be a defense mechanism in the brain to protect against neuronal degeneration. However, this mechanism is insufficient for neuroprotection or the rescue of damaged neurons (Schmidt-Kastner et al., 1996). The present study found that cerebrolysin administration after pilocarpine-induced seizure further increased BDNF concentrations compared to the vehicle-treatment. In addition, cerebrolysin-administration increased the phosphorylation of TrkB, as well as the protein level of p-CREB downstream of the TrkB signal. This result supports our hypothesis that the increased BDNF concentrations by cerebrolysin contribute to neuroprotection in the hippocampus after status epilepticus (**Figure 7**).

It is well-established that neuronal death occurs in the brain after a seizure (Kim et al., 2012; Jeong et al., 2017; Lee S. H. et al., 2018), but the mechanisms through which this injury occurs remain elusive. When neuronal death occurs, an inflammatory response is initiated to restore tissue homeostasis and remove dead cells (Chapkin et al., 2009; Karin and Clevers, 2016). However, if the inflammatory response that occurs is excessive due to widespread neuronal death, the tissues are not repaired but can actually sustain further damage (Fontana, 2009; Park et al., 2011). Here, we identified an excessive inflammatory response after a seizure. The inflammatory cytokines released by an excessive inflammatory response increase glial activation (Watkins et al., 2014; Wu and Watabe, 2017). It was previously shown that BDNF has anti-inflammatory effects that counteract various inflammatory cytokines (Chen et al., 2013; Papathanassoglou et al., 2015; Liang et al., 2019). In this study, we used cerebrolysin, which increases BDNF, to reduce excessive inflammatory responses and confirmed that cerebrolysin administration after a seizure reduces inflammation and promotes neuronal survival.

Despite BDNF showing potential neuroprotective effects in stroke, traumatic brain injury, and Alzheimer's disease, the

therapeutic delivery of BDNF has many obstacles related to its short *in vivo* half-life and uncertain BBB permeability. BDNF is relatively unstable, and only a small fraction can cross the BBB after administration. If the level of administered BDNF is too small due to its short half-life and limited permeability, it may not show observable neurotrophic effects (Geral et al., 2013; Tanila, 2017; Wurzelmann et al., 2017). The commercial product, cerebrolysin® (Ever Neuro Pharma, Unterach, Austria), is a mixture involving fragments of different neurotrophic factors, including BDNF. It has been demonstrated that BBB permeability promotes long-lasting BDNF supply to the brain (Sharma et al., 2012; Geral et al., 2013). Therefore, in the present study, we injected the neuropeptide cerebrolysin to increase BDNF levels in the brain.

CONCLUSION

The present study found that the administration of cerebrolysin decreased seizure-induced neuronal death and glial activation by increasing BDNF levels. Although the precise mechanism through which cerebrolysin promotes increased BDNF production and downregulates microglial activation after a seizure remains unclear, the present study suggests that the administration of cerebrolysin can be a useful therapeutic agent to prevent neuronal death in this setting. However, substantial further research is needed to determine the mechanism by which cerebrolysin increases BDNF and promotes other neuroprotective outcomes.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on Animal Habitation.

AUTHOR CONTRIBUTIONS

DK researched the data and reviewed and edited the manuscript. BC reviewed and edited the manuscript. SL, AK, JJ, DH, BK, MP, HS, and HC researched the data. M-SL and SS contributed to the discussion and wrote, reviewed, and edited the manuscript. HS, HC, M-SL, and SS take full responsibility for the manuscript and its originality. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2020.568813/full#supplementary-material

Supplementary Figure 1 | Pre-treatment of cerebrolysin has no anti-conversant effect on pilocarpine-induced seizure.

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REVIEW
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Cytoprotective and Neurotrophic Effects of Octadecaneuropeptide (ODN) in *in vitro* and *in vivo* Models of Neurodegenerative Diseases

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Octadecaneuropeptide (ODN) and its precursor diazepam-binding inhibitor (DBI) are peptides belonging to the family of endozepines. Endozepines are exclusively produced by astroglial cells in the central nervous system of mammals, and their release is regulated by stress signals and neuroactive compounds. There is now compelling evidence that the gliopeptide ODN protects cultured neurons and astrocytes from apoptotic cell death induced by various neurotoxic agents. *In vivo*, ODN causes a very strong neuroprotective action against neuronal degeneration in a mouse model of Parkinson's disease. The neuroprotective activity of ODN is based on its capacity to reduce inflammation, apoptosis, and oxidative stress. The protective effects of ODN are mediated through its metabotropic receptor. This receptor activates a transduction cascade of second messengers to stimulate protein kinase A (PKA), protein kinase C (PKC), and mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) signaling pathways, which in turn inhibits the expression of proapoptotic factor Bax and the mitochondrial apoptotic pathway. In N2a cells, ODN also promotes survival and stimulates neurite outgrowth. During the ODN-induced neuronal differentiation process, numerous mitochondria and peroxisomes are identified in the neurites and an increase in the amount of cholesterol and fatty acids is observed. The antiapoptotic and neurotrophic properties of ODN, including its antioxidant, antiapoptotic, and pro-differentiating effects, suggest that this gliopeptide and some of its selective and stable derivatives may have therapeutic value for the treatment of some neurodegenerative diseases.

Keywords: gliopeptide ODN, neurodegeneration, cell protection, cell differentiation, oxidative stress

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INTRODUCTION

The existence of binding sites for benzodiazepines (BZs), the most widely prescribed and therapeutically used drugs for their anxiolytic, sedative, and muscle relaxant properties, has prompted several teams to search for endogenous ligands of the BZ receptors. Thus, the team of Erminio Costa has isolated from rat brain extracts an 11-kDa polypeptide able to competitively displace tritiated diazepam on synaptosomes, which has been called diazepam binding inhibitor (DBI) (1). Two major compounds, generated from DBI, have been identified in rat brains, the triakontatetraneuropeptide (TTN, DBI_{17-50}) and the octadecaneuropeptide (ODN, DBI_{33-50}). DBI and its processing products, collectively grouped under the generic term of endozepines (2), are currently considered as the endogenous ligands of BZ receptors. DBI is also known as an acyl-coenzyme A-binding protein (ACBP) due to its ability to bind acyl-coenzyme A esters and to stimulate fatty acid synthesis in different cell types (3).

In this review, which will only refer to endozepines as natural ligands of BZ receptors, the acronym DBI will be used. In addition, we will focus on ODN, which is the major form of endozepines produced in the brain and whose structure has been well-preserved during evolution, suggesting that it exerts important biological functions in the central nervous system (CNS). Supporting this hypothesis, high concentrations of DBI mRNA and DBI-derived peptides are detected in the rat brain during ontogenesis. Furthermore, it has been reported that in the cerebellar cortex, ODN is expressed by Bergmann glia, which controls cerebellar granule neuron migration (4), indicating that ODN may act as a neurotrophic factor during brain development.

ORGANIZATION AND REGULATION OF THE OCTADECANEUROPEPTIDE GENE PRECURSOR (DIAZEPAM BINDING INHIBITOR) EXPRESSION

Cloning and characterization of cDNAs encoding DBI were first performed from a rat brain cDNA library (5). Since then, DBI has been cloned from other tissues (adrenal, liver, and testis) and from various animal species, including human (6), cattle (7), frog (8), and carp (9). The cDNA encoding DBI has also been cloned in invertebrate species such as the fruit fly *Drosophila melanogaster* (10), the butterfly *Bombyx mori* (11), and the tobacco maggot *Manduca sexta* (12), different plant species such as *Arabidopsis thaliana* (13), and unicellular organisms such as the yeast *Saccharomyces cerevisiae* (14) and the bacteria *Escherichia coli* (15, 16).

Southern blot analysis of genomic DNA from human (6), rat (5), and mouse (17) revealed the existence of several genes encoding DBI, but only one of which is functional. In human, the active gene is located on the q12-21 region of the long arm of chromosome 2 (18) and the pseudogenes are located on chromosomes 5, 6, 11, and 14 (19). The human and murine genes are organized into four exons (E1–E4), coding for regions 1–2, 3–41, 42–62, and 63–86 of the DBI, respectively (20, 21), while in *Drosophila*, there are only three exons (10). In human and murine adipocytes, a novel exon, named E1c, has been identified in the DBI gene, which codes with the three other exons for a longer transcript. Genomic DNA analysis shows that the E1c exon is located in the intron between exons E1 and E2, and that its expression would be under the control of a second promoter located in intron 1 (22, 23). These data reinforce the work of Helledie et al. (24), which showed that the DBI gene, cloned from human and murine adipocytes, contains a functional consensus sequence on intron 1, the peroxisome proliferator-activated receptor (PPAR)-response element (PPRE), capable of binding the transcription factors PPARγ and retinoid X receptor α. All these data indicate that the expression of the DBI gene is under the control of two promoter regions (P1 and P2) located upstream and downstream of the E1 exon initially described in human and rat (**Figure 1**).

The absence of TATA and CCAAT boxes, the presence of several element initiators of transcription, and the high content of nucleotide acids C and G in a promoter are often considered as the hallmarks of a domestic gene. Nevertheless, the expression level of DBI gene is regulated in different cell types (25). In addition, the P1 and P2 regions of the DBI gene have several consensus sequences for transcription factors including a glucocorticoid-response element (GRE), a CAAT-binding transcription factor/nuclear factor 1 (CTF/NF1), a hepatocyte nuclear factor (HNF), a sterol regulatory element-like sequence (SRE), and a PPAR sequence. Moreover, it has been reported that glucose and hormones, such as insulin or androgens, regulate the expression of the DBI gene (21, 26–28). Several research groups have also shown a modification of the levels of DBI gene expression in the brain in various experimental conditions. For instance, a decrease in DBI mRNA levels is observed after starvation (29) or castration (30) in rat, while an increase of DBI expression is shown after high-frequency vestibular stimulation in rabbits (31). Drug addiction (alcohol, morphine, and nicotine) also leads to an increase in DBI gene expression in the rodent brain (32, 33), and abrupt interruption of the consumption of these substances further increases DBI mRNA levels (33). *In vitro* studies on cultured astrocytes have shown that DBI gene expression is stimulated by different cell stress inducers such as peptide β-amyloid (34) and hydrogen peroxide (H_2O_2) (35). All these observations reveal that the DBI gene cannot be considered as a "housekeeping gene."

DISTRIBUTION OF OCTADECANEUROPEPTIDE FAMILY PEPTIDES IN THE NERVOUS SYSTEM

At Tissue Level

In all vertebrate species studied, endozepines are present both in the CNS and in many peripheral organs and tissues (25, 36). Although DBI and its derivatives are found in the gonads (126 pM), duodenum (100 pM), kidneys (73 pM), heart (30 pM), liver (22 pM), skeletal muscle (18 pM), adrenals (15 pM), lungs (13 pM), and spleen (11 pM) (25), the highest concentrations are found in rat brain (10–50 μM). Peptides of the ODN family

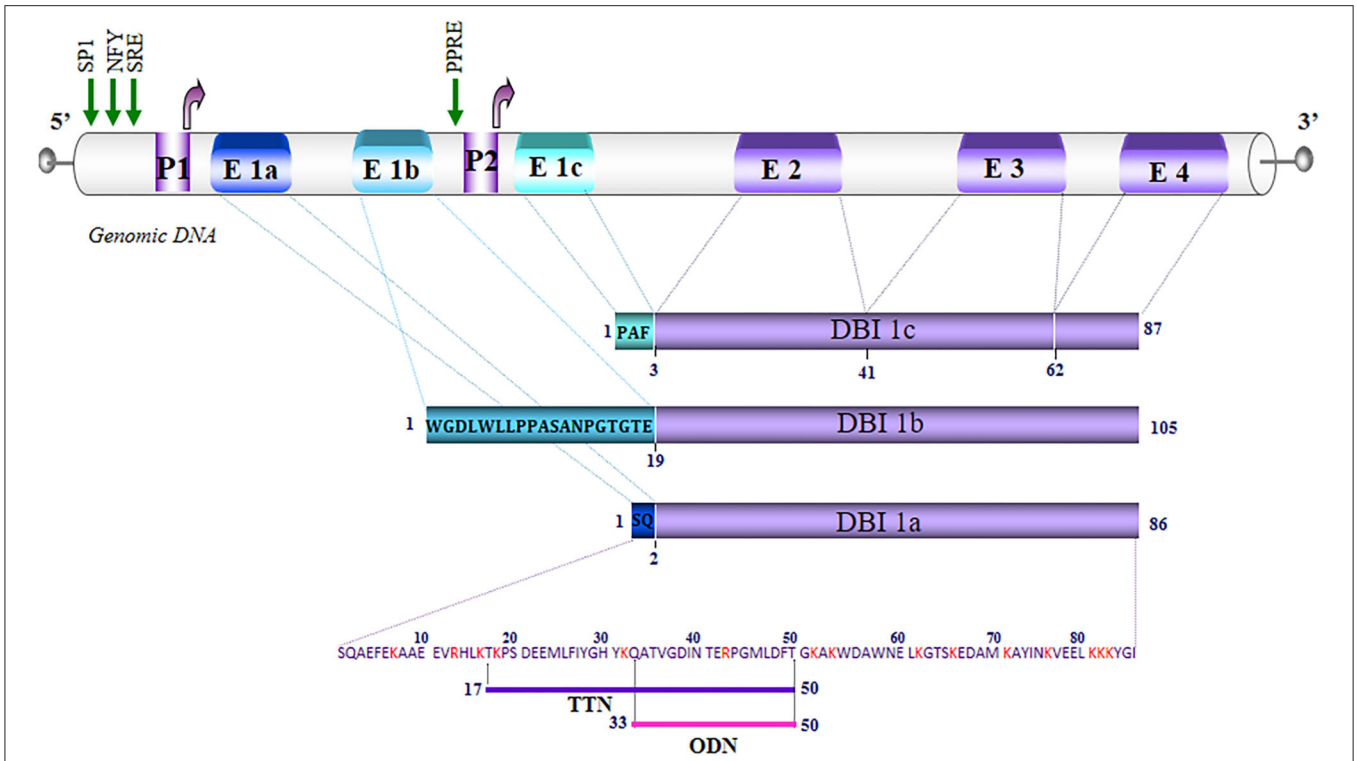

FIGURE 1 | Schematic representation of the organization of the diazepam-binding inhibitor (DBI) gene in humans. The exons (E) are numbered from 1 to 4. Alternative splicing of exon 1, which is composed of three sub-exons E1a, E1b, E1c, generates DBI 1a (86 amino acids), 1b (105 amino acids), and 1c (87 amino acids). The letters in the blue rectangles correspond to the amino acid sequence of the regions translated by E1a, E1b, and E1c. The P1 promoter region controls the expression of DBI 1a and 1b. The P1 and P2 promoters control the expression of DBI 1c. The green arrows correspond to the sites of transcriptional initiation from promoters P1 and P2. The regions that are common to the three DBI isoforms are represented by rectangles in purple. The basic residues are noted in red, and the underlined amino acids correspond to the sequences of triakontatetraneuropeptide (TTN) (DBI_{17-50}) and octadecaneuropeptide (ODN) (DBI_{33-50}). NFY, nuclear transcription factor Y; SP1, transcription factor specificity protein 1; SRE, sterol response element; PPRE, peroxisome proliferator-response element.

TABLE 1 | Octadecaneuropeptide-like immunoreactivity (ODN-LI) content in different rat brain structures.

Brain region	ODN-LI level (ng/region)
Olfactory bulb	57.2
Cerebral cortex	427.8
Hippocampus	55.7
Striatum	28.5
Cerebellum	258.6
Hypothalamus	73.6

are widely distributed in the brain, with the highest levels measured in the olfactory bulb, hypothalamus, hippocampus, cerebellum, striatum, cerebral cortex, and circumventricular organs (**Table 1**).

At the Cellular Level

A limited number of immunocytochemical studies using antibodies against DBI show a localization of endozepines in neurons (37, 38). However, the majority of the data in the literature indicate that endozepines are primarily expressed in glial cells (25). For example, ODN- and/or DBI-like immunoreactivities are detected in astrocytes in many brain areas including the cerebral cortex, hippocampus, amygdala, and olfactory bulb (39, 40), in the ependymocytes bordering the cerebral ventricles (39, 41), in the tanycytes of the median eminence (39, 42), in Bergmann cells from the cerebellum (40), and in Gomori-positive astrocytes from the arcuate nucleus (43). Similarly, in the peripheral nervous system, immunostaining for ODN and/or DBI is associated with Schwann cells (44). Finally, in the retina, endozepines are exclusively expressed by specialized glial cells, the Müller cells (45).

REGULATION OF OCTADECANEUROPEPTIDE RELEASE

Ultrastructural studies revealed that DBI immunoreactivity is diffused throughout the cytoplasm (39), excluding its packaging in secretory vesicles. This is consistent with the absence of signal peptide in the primary sequence of DBI that could direct the protein into the endoplasmic reticulum/Golgi system. Nevertheless, mass spectrometry analysis of rat astroglial cell secretome revealed the presence of DBI in incubation media

(46). The endozepine secretion is insensitive to brefeldin A, an inhibitor of Golgi vesicular transport (46), and can be stimulated by induction of autophagy by rapamycin (47). These data indicate that DBI and its derived peptides may be released by a mechanism independent of the conventional vesicular exocytosis pathway. The translocation of endozepines to the extracellular space could be provided by carriers called ATP-binding cassettes (ABCs), as demonstrated for the release of interleukin (IL)-1β by astrocytes (48) and annexin 1 by follicle-stellar cells (49), two proteins whose precursors are devoid of a signal peptide sequence. Consistent with this hypothesis, a site-directed mutagenesis study conducted with the amoeba *Dictyostelium discoideum* has shown the existence of a direct interaction between DBI and ABC-serine protease transporter (TagA) (50).

Although the mechanism of secretion of ODN and other DBI-derived peptides is not yet clearly elucidated, the secretion of endozepines is modulated in different pathophysiological situations and regulated by many factors. An assay using antibodies against ODN shows that the level of ODN-like immunoreactivity (ODN-LI) is increased in the plasma of septic shock patients (51). *In vitro* studies reveal that the release of ODN-LI is positively regulated by numerous neuroactive compounds, including neuropeptides [pituitary adenylate cyclase-activating polypeptide (PACAP) (52), urotensin-II (UII), UII-related peptide (53), and peptide β-amyloid (54)], steroid hormones [cortisol, pregnenolone, or progesterone (55)], cytokines [tumor necrosis factor-α and IL-1β (56)], or elevated extracellular potassium concentrations (57), and conversely repressed by the neuropeptide somatostatin (58) and the neurotransmitter gamma aminobutyric acid (GABA) (59). Pharmacological studies indicate that the release of ODN-LI from astroglial and retinal cells is mediated by ABC transporter activity through a mechanism that is dependent on the phosphorylation by protein kinases A and C (PKA and PKC) (52, 54, 60) (**Figure 2**), and blockage of the phosphorylation of ABC transporter inhibits the basal release of ODN-LI by about 50%. A phosphorylated form of ODN is also released by astrocytes but appears less active than the non-phosphorylated peptide, suggesting the existence of a posttranslational regulation mechanism (61).

In culture media from astrocytes exposed to moderate oxidative stress, the quantity of authentic ODN is significantly higher than that detected in culture media from control astrocytes (35). Clinical studies have shown that the levels of endozepines, ODN, and DBI, increase in the plasma or cerebrospinal fluid (CSF) of patients suffering from pathological disorders related to oxidative stress, such as systemic inflammation, hepatic encephalopathy, and neurodegenerative diseases (25). Such an increase in ODN release could be responsive for the stimulation of antioxidative defenses (induction of Mn-superoxide dismutases, catalase, glutathione peroxidase-1, sulfiredoxin-1 gene transcription, and glutathione biosynthesis) in glia and neurons (62, 63) in agreement with the emerging concept indicating that ODN acts as a potential cytoprotective actor preventing the deleterious action of oxidative insults.

BIOLOGICAL ACTIVITIES OF OCTADECANEUROPEPTIDE IN THE CENTRAL NERVOUS SYSTEM

As endogenous ligands of BZ receptors, ODN interacts with the central-type BZ receptors, which is an integrated component of the GABA-A receptor (GABA-A-R)/Cl^- channel complex (64, 65). Like DBI, ODN has been shown to displace diazepam and modulate GABAergic transmission *via* an allosteric reaction (1). Electrophysiology and cell functional studies indicate that ODN may act as an agonist, i.e., a positive allosteric modulator (PAM) promoting the action of GABA, or as an inverse agonist, i.e., a negative allosteric modulator (NAM) reducing the action of GABA, depending on the subunit composition of the GABA-A-R (57, 66). Studies conducted on cultured astrocytes have demonstrated that ODN is also the endogenous ligand of a G protein-coupled receptor, which is functionally and pharmacologically different from the conventional BZ receptors (67, 68). In particular, ODN stimulates the metabolism of polyphosphoinositides (PIPs) in rat astrocytes and cerebellar granule neurons *via* a phospholipase C (PLC) coupled to a G protein sensitive to pertussis toxin (63, 69). In addition, in cultured astrocytes, ODN increases the intracellular concentration of calcium from intracellular pools (68, 70). In the same cell type, ODN stimulates the formation of cAMP through activation of adenylyl cyclase (AC) activity (61, 71). The ability of ODN to stimulate phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK 1 and 2) is also found in both astrocytes and cerebellar granule neurons (63, 71). These data collectively indicate that ODN is the natural and specific ligand of a $G_{i/0}$ or G_S protein-coupled receptor, which activates complementary transduction mechanisms depending on the cell type.

The structure–activity studies of ODN in relation to its ability to activate the metabotropic receptor expressed in rat astrocytes show that the C-terminal octapeptide of ODN (OP, ODN_{11-18}) is the shortest biologically active fragment of the peptide (72). OP is able to mimic the effects of ODN on calcium mobilization and cAMP level increase observed in astrocytes (71, 72). Conversely, OP has no effect on the binding of [^{3}H]flumazenil in cerebellar granular cells or of [^{3}H]PK11195 in rat astrocytes (73). The effects of ODN on the activation of the cAMP/PKA, PIPs/calcium/PKC, and MAPK-ERK transduction pathways in astrocytes and neurons are completely suppressed by a specific antagonist of this receptor, the $cyclo_{(1-8)}$[$DLeu^5$]OP (63, 68, 72).

Effect of Octadecaneuropeptide on Cell Proliferation

ODN, *via* activation of the central-type BZ receptors, stimulates the incorporation of [^{3}H]thymidine into cultured rat astrocytes (74). Consistent with this proliferative effect of ODN, *in vivo* studies have demonstrated that ODN promotes the proliferation of neuronal progenitor stem cells from the germinative sub-ventricular zone in adult rat (75). Conversely, inhibition of DBI gene transcription by shRNA transfection *in vivo* reduces the number of proliferating cells and the number of neurons newly

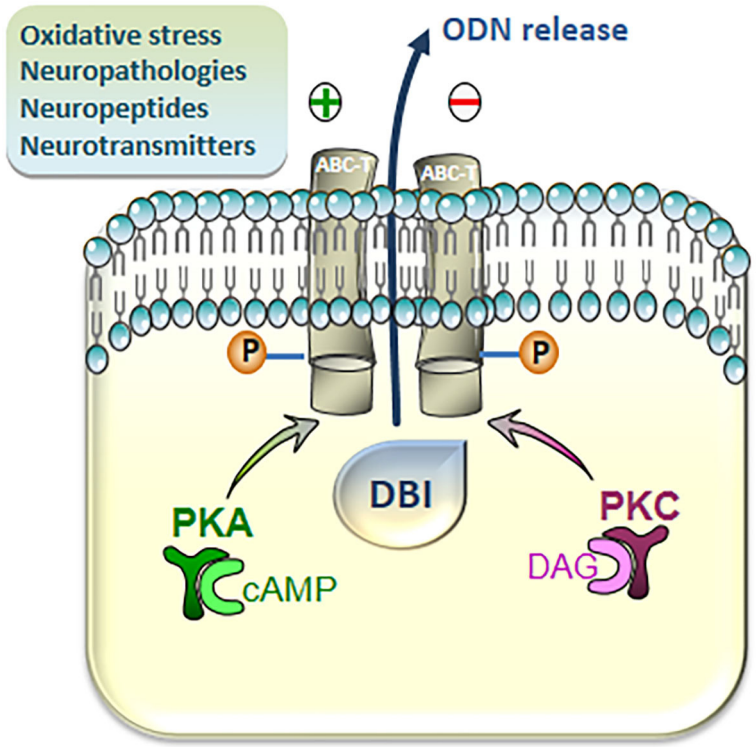

FIGURE 2 | Intracellular pathways involved in the regulation of the release of diazepam-binding inhibitor (DBI)-related peptides from cultured astroglial cells. Activation of cAMP/protein kinase A (PKA) and Diacylglycerol (DAG)/protein kinase C (PKC) cascades stimulates the release of DBI-related peptides through phosphorylation, at consensus sites, of transmembrane ATP-binding cassette transporters (ABC-Ts). The production of the octadecaneuropeptide (ODN), which occurs extracellularly, is finely regulated (positively or negatively) by various neuronal mediators, including classical neurotransmitters and neuropeptides, and Ⓟ under cerebral pathologies and oxidative injuries.

formed at the level of olfactory bulbs but does not affect stem cell survival (75). Furthermore, the fact that ODN can inhibit neuronal cell death and stimulate neurogenesis suggests that it could also play a key role during brain development. In support of this hypothesis, it has been shown that, in the cerebellar cortex, endozepines are exclusively expressed by Bergmann glia (2, 4), which control cerebellar granule neuron migration. Taken together, these data indicate that ODN may exert a neurotrophic effect and reinforce the notion that ODN may promote cell proliferation, survival, and/or differentiation.

Protective Effects of Octadecaneuropeptide on Neuronal Cells

ODN has been shown to rescue neurons and glial cells from neurotoxicity induced by several substances such as H_2O_2 (35, 71, 76, 77), 6-hydroxydopamine (6-OHDA) (62, 63), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (35). Indeed, exposure of cultured granule neurons and N2a neuronal cell line to very low concentrations (in the subpicomolar range) of ODN totally abolishes the deleterious effects of 6-OHDA (63) and H_2O_2 (76–78). ODN also exerts a strong protective effect against oxidative stress-induced apoptosis on cultured astrocytes (62, 78, 79). ODN acts by preventing (i) the accumulation and overproduction of intracellular reactive oxygen species (ROS), (ii) the depletion of glutathione (GSH) levels, and (iii) the decrease of the expression and activity of the antioxidant enzymes provoked by oxidative stress (79). Furthermore, ODN prevents apoptotic cell death by inhibiting (i) the overexpression of the proapoptotic protein Bax as well as the repression of the antiapoptotic protein Bcl-2 and (ii) the drop of the mitochondrial membrane potential responsible for the stimulation of caspase-3 activity. Treatment of neuroblastoma cell lines and cerebellar granule neurons with astrocyte-conditioned medium significantly promotes neuron survival under oxidative injury induced by 6-OHDA (80) and H_2O_2 (76, 81). Treatment of cerebellar granule neurons with ODN metabotropic receptor antagonist greatly attenuated the protective action of astrocyte-conditioned medium. Quantitative measurement of ODN by mass spectrometry indicates that the amount of ODN present in glial conditioned medium is in the same range of concentration as the one necessary for the neuroprotective action of the peptide on granular neurons against apoptotic cell death induced by oxidative damage, suggesting a neuroprotective effect of the endogenous gliopeptide (76).

Some behavioral studies have shown that in the same way as ODN, intracerebroventricular administration of TTN induces proconflict- and anxiety-related behavior. At the cellular level, TTN also promotes proliferation and intracellular calcium increase in glial cells (70, 74). However, the potential

neuroprotective and/or neurotrophic activity of TTN has never been reported.

The neuroprotective activity of ODN has also been observed *in vivo*, in an MPTP mouse model of Parkinson's disease (35). A single intracerebroventricular injection of 10 ng ODN, 1 h after the last administration of MPTP, is sufficient to prevent the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) induced by the toxin and to block the degeneration of nerve fibers in the striatum (35). ODN-mediated neuroprotection is associated with a reduction of the number of glial fibrillary acidic protein-positive reactive astrocytes and a strong inhibition of the expression of pro-inflammatory genes induced by MPTP in the SNpc. Moreover, ODN blocks the inhibition of the antiapoptotic gene Bcl-2 and the stimulation of the proapoptotic genes Bax and caspase-3 (35). ODN also prevents the accumulation of ROS and lipid oxidation products both in the SNpc and the striatum. Furthermore, $DBI^{-/-}$ mice exhibit more vulnerability to MPTP injection than wild-type animals ($DBI^{+/+}$). Thus, ODN-knockout (KO) mice are more sensitive to MPTP-induced inflammatory and oxidative brain damages, suggesting that the endogenous ODN may also be neuroprotective (35). These data indicate that the induction of ODN production in pathological conditions, similar to the one observed at the early stages of neurodegenerative processes, may correspond to a compensatory mechanism, initiated by reactive astrocytes, to reduce their sensitivity to oxidative aggression and to limit the progression of brain damages.

Altogether, these results demonstrate that, based on its antioxidative (77, 82), anti-inflammatory (35), and antiapoptotic effects (63, 71), the gliopeptide ODN, which acts as a potent neuroprotective agent, could lead to the development of effective therapeutic agents for the treatment of cerebral injuries involving oxidative neurodegeneration. It should nevertheless be pointed out that in the acute phase of stroke, ODN, through its allosteric modulation of GABA A receptor, boosts the excitability of cortical neurons and as a consequence increases neuronal damages (83). Consistent with this observation, $DBI^{-/-}$ mice exhibit increased infarct volume compared to wild-type animals. However, when administered in the subacute period after stroke, ODN improves functional recovery, showing that if provided at the right time, ODN can also be of interest for the treatment of stroke patients.

Differentiating Activity of Octadecaneuropeptide on N2a Cells

There is evidence indicating that neuropeptides, such as PACAP (84, 85), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) (86), promote both neuronal survival and differentiation, suggesting that the gliopeptide ODN, which protects neurons and astrocytes from apoptosis (25, 80), could also have a differentiating activity. We have thus tested whether ODN can have a neurotrophic property on murine N2a cells and stimulate neurite (axons and dendrites) outgrowth, in indded, at very low concentrations (in the fentomolar range, 10^{-14} M), ODN exerts a protective action on H_2O_2-induced N2a cell apoptosis and induces cell differentiation in the presence or absence of fetal bovine serum (87) (**Figure 3**). The cytoprotective effects of ODN on N2a cells (capacity to prevent damages induced by oxidative stress) are observed at similar concentrations to those inducing neuronal differentiation (35, 63), which leads us to propose that ODN can be considered as a potent neurotrophic factor. It is noteworthy that no cytotoxic effect (inhibition of cell growth and cell adhesion, loss of transmembrane mitochondria potential, ROS overproduction, loss of lysosomal integrity, or cell death induction) is observed with ODN in the concentration range inducing neurite outgrowth (87). During neuronal differentiation, cells also acquire excitability and express genes with functional identity (88).

To define the signaling pathway associated with the differentiating effect of ODN on N2a cells, inhibitors that are linked to the pathways involved in the cytoprotective action of ODN, such as the PKA inhibitor H89, the PLC inhibitor U73122, the PKC inhibitor chelerythrine, and the MEK inhibitor U0126, were used. All these molecules were able to block ODN differentiating activity (79, 87), indicating that ODN's cytoprotective and differentiating activities use common signaling pathways. The MEK–ERK pathway may be activated by both PKA and PKC (63, 71). Some other pathways such as the phosphoinositide 3-kinase (PI3K)/Akt involved in cell differentiation and activated by other neurotrophic factors could also contribute to ODN-induced N2a cell differentiation.

Since the growth of neurites (dendrites and/or neurons) requires significant energy and lipid synthesis, the effect of ODN on mitochondria and peroxisomes was investigated (89–91). In the presence of ODN, topographical changes, especially regarding mitochondria distribution in the N2a cells, are observed not only in the soma but also in neurites (dendrites and/or axons) (**Figure 4**). It is well-established that mitochondria are capable of producing fatty acids and cholesterol, which are essential for neurite outgrowth, and that peroxisome also contributes to cholesterol biogenesis *via* the production of acetyl-CoA (92, 93). Noteworthy, higher levels of cholesterol and its precursors (lanosterol, desmosterol, and lathosterol) are detected in cells treated with ODN (10^{-14} M) (87). It should be noted that there is a preference for the Bloch pathway for the cholesterol biogenesis as shown by the increased levels of lathosterol and desmosterol after treatment with ODN (87). Altogether, these data provide some of the mechanisms involved in the differentiation of the neuronal cell line N2a by ODN.

TRANSDUCTION PATHWAYS INVOLVED IN THE NEUROPROTECTIVE EFFECTS OF OCTADECANEUROPEPTIDE

Pharmacological studies have shown that central BZ receptor agonists are unable to mimic the protective effects of ODN (25). Similarly, central-type BZ receptors and translocator protein (TSPO) antagonists fail to suppress ODN-induced protection of neurons and glial cells from injuries, indicating that "classical" BZ receptors are not involved in the protective activity of ODN. However, OP and $cyclo_{(1-8)}$OP, two agonists of the

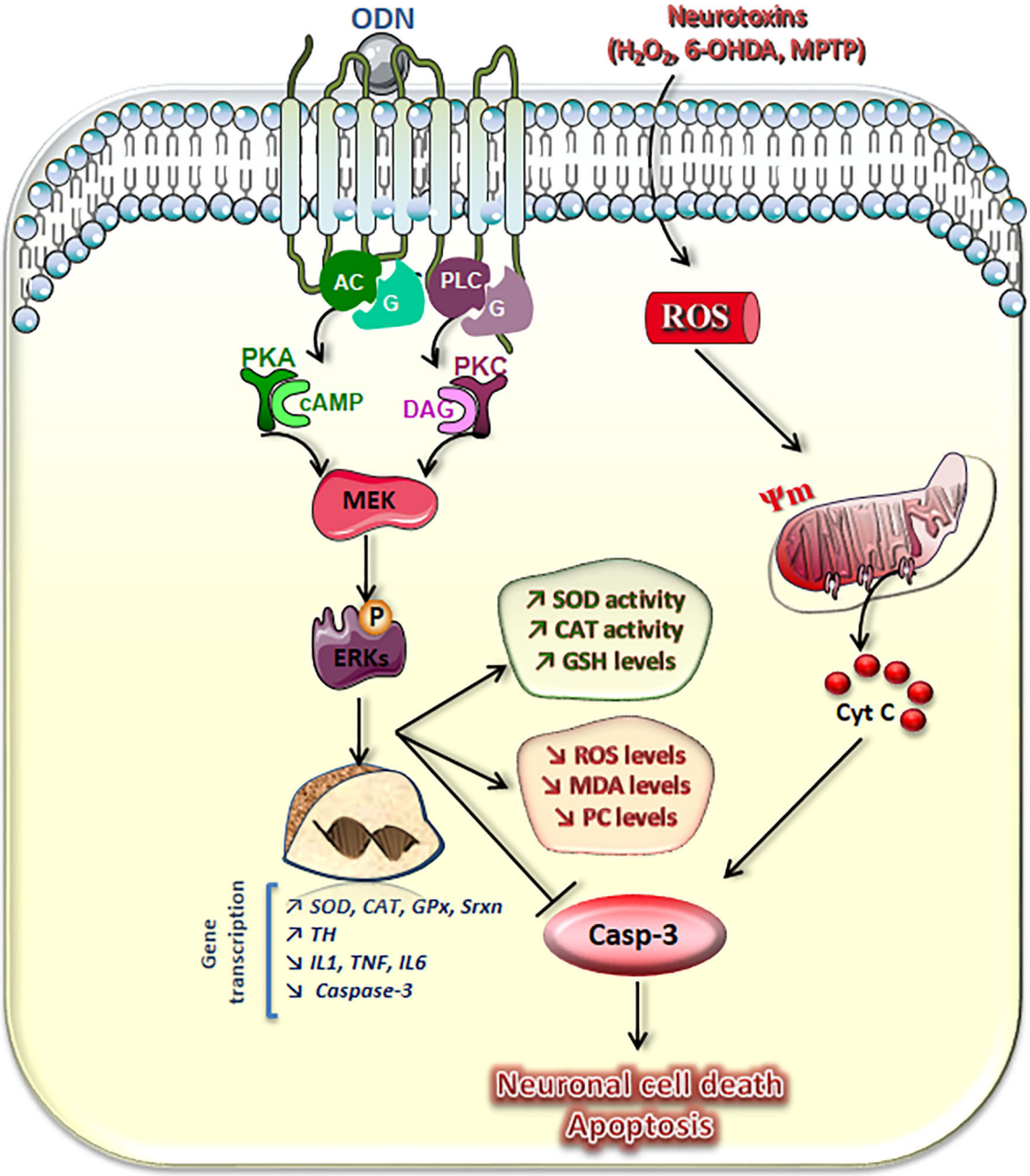

FIGURE 3 | Schematic representation of transduction pathways involved in the neuroprotective effects of octadecaneuropeptide (ODN). ODN, through activation of its metabotropic receptor and the AC–protein kinase A (PKA), phospholipase C (PLC)–protein kinase C (PKC), and mitogen-activated protein (MAP)-kinases/extracellular signal-regulated kinase (ERK) signaling pathways, stimulates superoxide dismutase (SOD), and catalase (CAT) activities and glutathione (GSH) cellular contents, which prevent the drop of the mitochondrial membrane potential (Ψ) and activation of caspase-3 (Casp-3) induced by hydrogen peroxide (H_2O_2) or 6-hydroxydopamine (6-OHDA) in cultured neurons and astrocytes. ODN also abolished neurotoxin-induced overproduction of reactive oxygen species (ROS) and blocked oxidative damage of cell molecules, i.e., formation and accumulation of lipid oxidation products [malondialdehydes (MDAs)] and protein carbonyl compounds (PCs). Concomitantly, in cultured astrocytes, ODN stimulates *Mn-SOD*, *CAT*, *glutathione peroxidase-1* (GPx), and *sulfiredoxin-1* (Srxn) gene transcription and rescues 6-OHDA-associated reduced expression of endogenous antioxidant enzymes. In 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice, ODN prevents the degeneration of dopaminergic neurons in the *substantia nigra pars compacta*, abrogates the effect of toxin on inhibition of *tyrosine hydoxylase* (TH) gene transcription, and blocks the stimulation of the expression of pro-inflammatory genes, such as *interleukins (IL) 1* and *6, tumor necrosis factor-α (TNF-α)*, and the proapoptotic gene *caspase-3*. Taken together, these results show that the gliopeptide ODN exerts a potent neuroprotective effect through mechanisms inhibiting oxidative stress, neuroinflammation, and apoptosis.

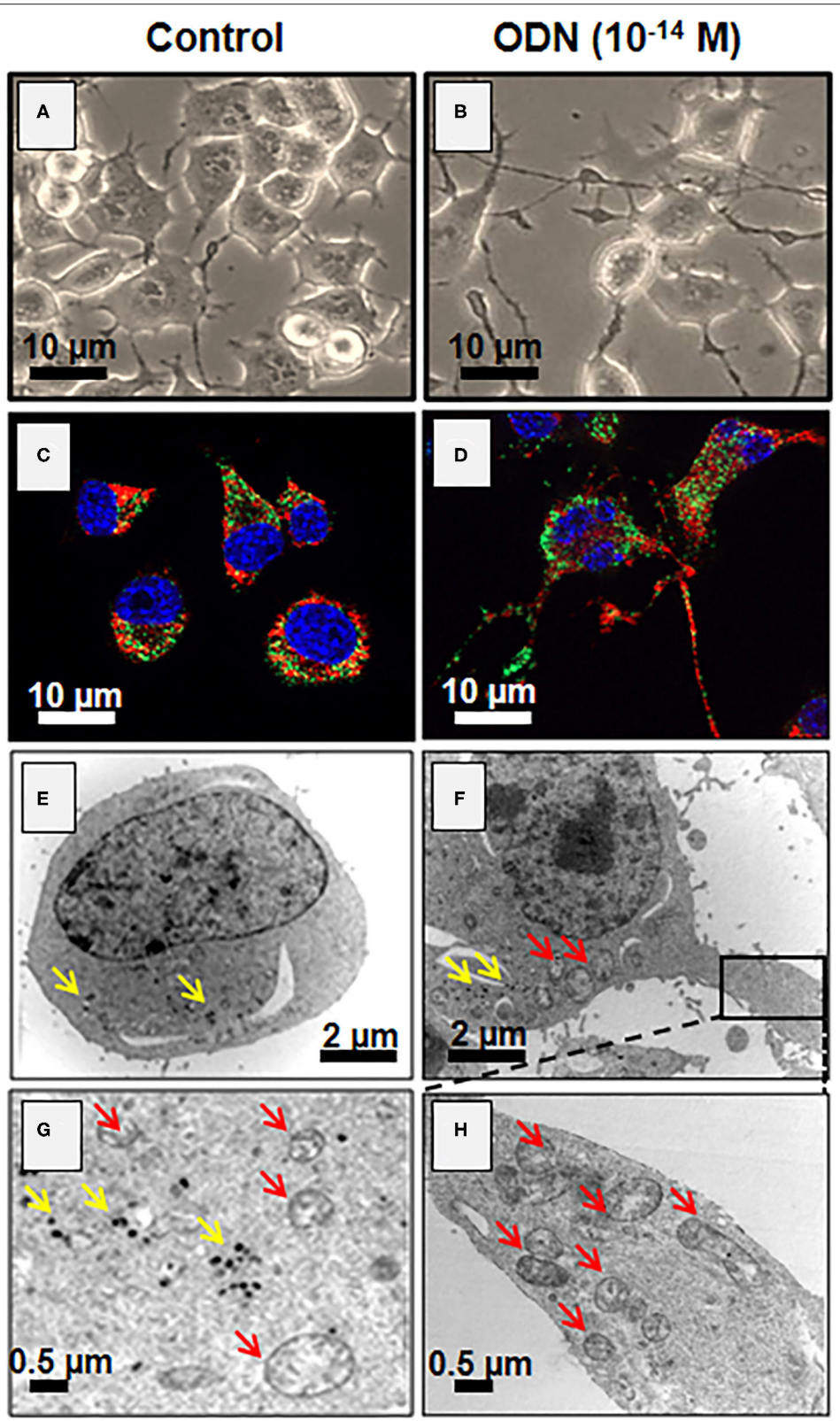

FIGURE 4 | Induction of neuronal differentiation in octadecaneuropeptide (ODN)-treated N2a cells. Murine neuronal N2a cells were treated with ODN (at 10^{-14} M for 48 h). **(A,B)** Undifferentiated [control, **(A)**] and differentiated cells (characterized by morphological aspects based on neurite outgrowth, **(B)** cells were observed by

(Continued)

FIGURE 4 | phase contrast microscopy. Due to the presence of numerous mitochondria and peroxisomes in neurites, these later can be considered functional. **(C,D)** The mitochondria were detected with Mitotracker Red (red fluorescence) and the peroxisomes by indirect immunofluorescence after staining with an antibody raised against the ABCD3 peroxisomal transporter (green fluorescence) in control **(C)** and ODN-treated cells **(D)**. The nuclei were counterstained with Hoechst 33342; the images were acquired under a fluorescent microscope coupled with an Apotome (Zeiss). The green spots point toward peroxisomes, and red spots point toward mitochondria**. (E–H)** Visualization by transmission electron microscopy of mitochondria (red arrows) and peroxisomes (yellow arrows) in control **(E,G)** and differentiated N2a cells [treated with ODN, **(F,H)**].

ODN metabotropic receptor, exhibit a protective activity when used in the same range of concentration as ODN. In addition, cyclo$_{(1-8)}$[DLeu^5]OP, a potent antagonist of this metabotropic receptor, blocks the protective effects of ODN on neurons and astrocytes (62, 63, 71, 82).

Downstream of the receptor, several transduction pathways can mediate the protective effect of ODN according to the cell type. In astrocytes, activation of ODN metabotropic receptor implies stimulation of the activity of the AC/cAMP/PKA pathway (71). The neuroprotective effect of ODN against 6-OHDA-induced cerebellar granule cell death involves activation of the PLC/IP3/PKC pathway (63). Downstream to PKA and PKC activations, ODN stimulates the phosphorylation of the ERK1 and ERK2 proteins in astrocytes and neurons (79). The phosphorylation of ERK1 and ERK2 in turn stimulates the expression of the Bcl-2 protein and concomitantly inhibits the expression of Bax, thereby preventing mitochondrial dysfunction and blocking cell death *via* the intrinsic apoptotic pathway (84, 94). The stimulating effect of ODN on the expression of antioxidant enzyme genes and their enzymatic activities is dependent on the activity of the cAMP/PKA transduction pathway as well as that of MAPK–ERKs in astrocytes (62, 77, 82). ODN promotes cerebellar granule cell survival from apoptosis through the PKC pathway (63), while another neuropeptide, PACAP, protects the same neurons from apoptosis through the PKA pathway (95). It would thus now be of interest to investigate if combining the two molecules leads to an enhanced and/or prolonged antiapoptotic effect.

OCTADECANEUROPEPTIDE, AN ENDOGENOUS NEUROPROTECTIVE AGENT

Clinical studies have revealed that endozepine levels are significantly increased in the CSF of patients suffering from neurological disorders such as epilepsy, hepatic encephalopathy (96, 97), or Alzheimer's and Parkinson's diseases (98). Mass spectrometry, radioimmunoassay (RIA), and q-RT PCR analyses reveal that moderate oxidative stress induces stimulation of the endogenous production of ODN, as well as the expression of the ODN precursor gene DBI from cultured astrocytes (76). Furthermore, induction of endogenous ODN production is responsible for (i) the stimulation of the expression and activity of antioxidant enzymes and thus rapid resorption of ROS, (ii) the protection of bio-macromolecules from oxidative damages, and (iii) the prevention of astrocytes and neuron cell death induced by oxidative stress. In contrast, inhibition of ODN release by the PKA inhibitor H89 or blockage of the effects of ODN with an antagonist of its metabotropic receptor, the cyclo$_{(1-8)}$[DLeu^5]OP, exacerbates damages induced by oxidative stress on astroglial cell viability (76). Consistently, knockdown of ODN precursor expression, by DBI siRNA, induces morphological alterations with loss of membrane integrity and the formation of apoptotic bodies and increases the vulnerability of oxidative stress inducing cell death (76). It has also been shown, by using DBI-KO animals, that ODN precursor deficiency increases brain sensitivity to MPTP toxicity, highlighting the neuroprotective role of endogenous ODN against neuronal degeneration (35). Taken as a whole, these data indicate that the induction of ODN production in pathological conditions reproducing early stages of neurodegenerative processes may represent a compensatory mechanism, initiated by reactive astrocytes, to reduce their sensitivity, as well as that of their surrounding neurons, to oxidative aggression and to limit the progression of the neurodegeneration process in some neurological disorders.

AUTHOR CONTRIBUTIONS

OM-K, AN, GL, JL, M-CT, and DV provided valuable editorial comments and relevant bibliographic references. YH, SB, IG, and DV provided valuable editorial comments. OM-K wrote the manuscript with the contribution of AN, GL, and DV. TG, JC, and BL contributed to the review and editing revised version. All authors contributed to the article and approved the submitted version.

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REVIEW
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Cancer Treatment by Caryophyllaceae-Type Cyclopeptides

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Cancer is one of the leading diseases, which, in the most cases, ends with death and, thus, continues to be a major concern in human beings worldwide. The conventional anticancer agents used in the clinic often face resistance among many cancer diseases. Moreover, heavy financial costs preclude patients from continuing treatment. Bioactive peptides, active in several diverse areas against man's health problems, such as infection, pain, hypertension, and so on, show the potential to be effective in cancer treatment and may offer promise as better candidates for combating cancer. Cyclopeptides, of natural or synthetic origin, have several advantages over other drug molecules with low toxicity and low immunogenicity, and they are easily amenable to several changes in their sequences. Given their many demanded homologues, they have created new hope of discovering better compounds with desired properties in the field of challenging cancer diseases. Caryophyllaceae-type cyclopeptides show several biological activities, including cancer cytotoxicity. These cyclopeptides have been discovered in several plant families but mainly are from the Caryophyllaceae family. In this review, a summary of biological activities found for these cyclopeptides is given; the focus is on the anticancer findings of these peptides. Among these cyclopeptides, information about Dianthins (including Longicalycinin A), isolated from different species of Caryophyllaceae, as well as their synthetic analogues is detailed. Finally, by comparing their structures and cytotoxic activities, finding the common figures of these kinds of cyclopeptides as well as their possible future place in the clinic for cancer treatment is put forward.

Keywords: anticancer activity, Caryophyllaceae-type cyclopeptides, dianthins, longicalycinin A, plant family, synthetic analogues

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INTRODUCTION

Cancer cells, often present as malignant tumors, are described as compiled abnormal cells with fast growth and division in an uncontrolled manner (1). They may migrate and invade every part of the body, a phenomenon that is named metastasis (1). Cancer cells do not die, and so apoptosis, a programmed cell death that occurs in normal cells, does not happen in cancerous cells, and as a result, any old, damaged, and defective cells survive and come together with newly born unwanted cells (2). The progression of cancer and its metastasis ultimately ends with patient death. On the

basis of a WHO report, it is estimated that 1 in 6 deaths globally is due to cancer (3). As time passes, the incidence of cancer cases increases year by year such that it will involve 29.5 million people in 2040 (4).

Cancer treatment by traditional chemotherapeutics may have several drawbacks, including the danger of disease recurrence due to emerging resistance to these agents and the appearance of drug side effects during long-term usage (1). Therefore, cancer treatment requres changing of anticancer drug protocols many times, which obviously imposes high costs and a major economic burden on the patients and their relatives (5). The problem is compounded if there are social and emotional pressures on the patients (5).

The successful use of peptides for treating various diseases (6, 7) has attracted researchers to apply these agents to combating cancer (8, 9). That is because peptides have several advantages over other chemotherapeutics. These noticeable characteristics of peptides may be enumerated as good efficacy, high potency, and low immunogenicity (1). Peptides are amenable to many changes in their sequences in a way of showing selective action in targeted cells while causing low toxicity to normal cells (1). In addition, peptides cause low incidence of resistance in malignant cells (10). However, peptides contain some properties that are considered to be disadvantages (1, 9). Among these unfavorable characteristics, low intolerability against lytic enzymes is the most important feature (1). To improve low stability, some strategies have been suggested by researchers (11). These are reordering peptide sequences (12), choosing D-amino acid instead of L-amino acid (13, 14), and converting linear peptide into a cyclized form (15). Cyclization can also cause peptide conformation to become more suitable for binding to a peptide biologically active site (16).

Cyclic peptides (also called cyclopeptides) have many potential therapeutic properties (17) and are suitable to be used as drugs in the clinic (18). The plant cyclopeptides show many attractive biological activities. They are divided into eight types (19). Among them, Caryophyllaceae-type cyclopeptides composed of cyclo di-, penta-, hexa-, hepta-, octa-, nona-, deca-, undeca-, and dodeca-amino acid residues are mounted by more than 200 kinds (20). These cyclopeptides are extracted from several plant families, mainly from Caryophyllaceae. This is a large family of flowering plants that contains approximately 81 genera and 2625 species (21). Discovery of cyclopeptides from Caryophyllaceae dates back to 1959, when the first cyclopeptide, named Cyclolinopeptide A, was extracted from the seeds of *Linum usitatissimum* (22). It is a potent immunosuppressive agent (23). Since then, Caryophyllaceae-type cyclopeptides, defined as homomonocyclopeptides, have been discovered from higher plants. These peptides show several biological activities, such as antimalarial, antiplatelet, immunomodulating, immunosuppressive, cyclooxygenase inhibitory, tyrosinase and melanogenesis inhibitory, Ca^{2+} antagonism, and estrogen-like and cytotoxic activities (19, 24). Demonstrating various activities, including an anticancer effect, by Caryophyllaceae-type cyclopeptides encouraged us to review and summarize the latest biological findings on these peptides. In this review, the focus is on discussing the cytotoxic action of Caryophyllaceae-type cyclopeptides, especially dianthins and their synthetic analogues. Moreover by this review, attempt will be made to obtain information about the relations between structure and anticancer activity (SAR) of the cyclic peptides, which is useful for finding good candidates among cyclopeptides as anticancer agents with optimum structures for application in the clinic.

SEGETALINS

Discovery of segetalin cyclopeptides from the seeds of *Vaccaria segetalis* (Caryophyllaceae) started in 1994, when the first cyclopeptide, called segetalin A, was isolated, and its structure was proved by instrumental analyses, i.e., two-dimensional nuclear magnetic resonance (2D NMR) and electrospray ionization mass spectrometry (ESI-MS)/MS as well as by chemical and enzymatic hydrolysis methods. The sequence of its structure was found to be cyclo(Ala-Gly-Val-Pro-Val-Trp-). It was shown that this cyclopeptide produces an estrogenic activity on the uterine weight of ovariectomized rats (25). Soon after, the isolation of other segetalins, such as segetalins B, C, and D, was reported from the same plant (26). The estrogenic activity of segetalin B was higher than segetalin A, whereas segetalins C and D did not show such activity noticeably (26). The resemblance of the part of the peptide sequence between segetalin A and segetalin B (Gly-Val and Trp-Ala) was suggested to be the reason for such similar activity. (The peptide sequences are given in **Table 1**.) Discovery of the natural segetalins E, F, G, and H from *Vaccaria segetalis* is documented in several reports (27–30). Further examining the biological activity, segetalins G and H in addition to A and B displayed estrogenic

TABLE 1 | Segetalin cyclopeptides with their sequences and biological activities.

Peptide names	Peptide sequence	Cytotoxic activity	Pharmacological activities	Anti-infective activities
Segetalin A	Cyclo(-Gly-Val-Pro-Val-Trp-Ala-)	—	Estrogenic	
Segetalin B	Cyclo(-Gly-Val-Ala-Trp-Ala-)	—	Estrogenic, Contractile	
Segetalin C	Cyclo(-Gly-Leu-His-Phe-Ala-Phe-Pro-)	—		
Segetalin D	Cyclo(-Gly-Leu-Ser-Phe-Ala-Phe-Pro-)	—		
Segetalin E	Cyclo(-Gly-Tyr-Val-Pro-Leu-Trp-Pro-)	On P-388 cells, DLA cells, EAC cells		Antihelimintic
Segetalin F	Cyclo(-Tyr-Ser-Ser-Lys-Pro-Ser-Ala-Ser)		Vasorelaxant	
Segetalin G	Cyclo(-Gly-Val-Lys-Tyr-Ala)		Estrogenic, Vasorelaxant	
Segetalin H	Cyclo(-Gly-Tyr-Arg-Phe-Ser)		Estrogenic, Vasorelaxant	
Segetalin J	Cyclo(-Phe-Gly-Thr-His-Gly-Leu-Pro-Ala-Pro-)	—		
Segetalin K	Cyclo(-Gly-Arg-Val-Lys-Ala-)	—		

activity in ovariectomized rats (27, 31). It was assumed that this property was due to the kind of sequence as well as conformation of the peptides (31). The sequences of Trp-Ala-Gly-Val or Tyr-Ala-Gly-Val, β-turn occurring, one in segetalin B (between Trp^4 and Ala^5) and two in segetalin A (between Trp^5 and Ala^6 and between Val^2 and Pro^3) as well as cyclic rather than acyclic shapes of the peptides are important factors in demonstrating estrogenic activity (31). When the biological activity of segetalin E (**Figure 1**) was examined on cancer cells using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, it was found that segetalin E had moderate inhibitory activity against the growth of lymphocytic leukemia P-388 cells (IC_{50} 40 μg/mL^{-1} ≈ 49.2 μM) (32) and higher inhibitory actions against Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines (with IC_{50} values of 3.71 and 9.11 μM, respectively) (29) (**Chart 1**).

Segetalin E also showed antihelmintic activity against two earthworms *M. konkanensis* and *P. corethruses* by a dose of 2 mg/mL. In another study, the structure as well as absolute stereochemistry of segetalin F were determined by analytical and chemical means (30). In an experiment designed to determine the vasorelaxant activity of segetalins against rat aorta contraction induced by norepinephrine, some segetalines, i.e., segetalins F, G, and H, isolated from *Vaccaria segetalis*, showed relatively strong relaxant activity, and segetalin B exhibited contractile activity. This interesting finding was interpreted as a result of the basic Lys and Arg residues being present in segetalins F, G, and H, whereas in segetalin B, there is no basic residue; instead, a Trp residue is present (27, 30).

As cyclopeptides may be produced biosynthetically by ribosomal- or nonribosomal-dependent peptide synthases, some authors investigated the biosynthesis of Caryophyllaceae-like cyclopeptides in the developing seeds of *Saponaria vaccaria*. They found that there are genes to encode the precursors of cyclic peptides, which ultimately are cyclized and result in the final products. This finding was further confirmed by expressing a cloned DNA (cDNA) in the roots of transformed *S. vaccaria* to encode a synthetic precursor of segetalin A. During the investigation, the authors predicted the presence of two more segetalins, i.e., segetalins J and K in *S. vaccaria* seeds. The prediction by sequence analysis also revealed the existence of genes for encoding cyclic peptide precursors in *Dianthus caryophyllus* and *Citrus* species (33). The sequences of all segetalins are shown in **Table 1**.

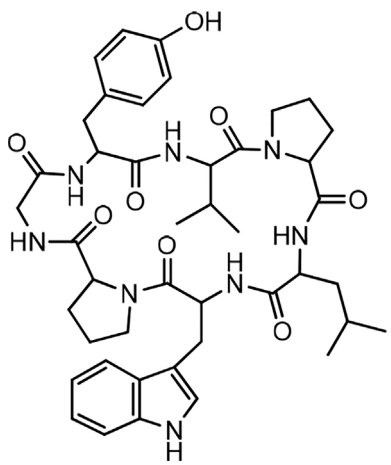

FIGURE 1 | Structure of segetalin E.

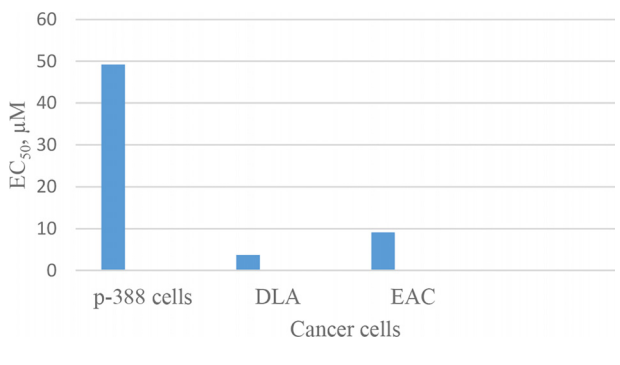

CHART 1 | Inhibitory action of segetalin E against cancer cells.

YUNNANINS

In 1994, the isolation of two cyclic peptides, yunnanin A and B, was reported from the root of *Stellaria yunnanensis* (Caryophllaceae) (34). The peptide structures were confirmed by spectroscopic analysis and chemical methods (34, 35). Yunnanin A showed cytotoxic activity against p-338 cells (36). Yunnanin C, isolated from the root of the same plant (36), as well as yunnanin A, indicated *in vitro* antiproliferative activities against three cell lines—J774.A1 (murine monocyte/macrophage cell line), WEHI-164 (murine fibrosarcoma cell line), and HEK-293 (human epithelial kidney cell line)—after three days of incubation (IC_{50} s ranging from 2.1 to 7.5 μg/mL) (37). Interestingly, the synthetic counterparts of these cyclopeptides did not show the same level of cytotoxicity against the above cell lines (IC_{50} s less than 100 μg/mL). This discrepancy was discussed as it might be as a result of subtle conformational changes of proline units during the synthesis process, which ended by diverse arrangements of proline residues in the synthetic cyclopeptides (37). In another experiment, the synthesized yunnanin A showed weak antimicrobial, anti-inflammatory, and anthelmintic activities (38). Recently, a cyclic analogue of yunnanin A was synthesized by eliminating tyrosine residue and introducing a phthalimide structure instead, inside the ring structure, through photo inducing a single electron transfer reaction (SET) (**Figure 2**). The hydroxyl group attached to the isoindolinone part of the phthalimide structure could have a role similar to the hydroxyl group of the eliminated tyrosine residue. It is shown that this analogue exhibits strong toxicity against HepG-2 and Hela cell lines (IC_{50} s 29.25 μg/mL and 65.01 μg/mL, respectively) (**Chart 2**). This cytotoxicity had almost no toxic activity against normal cells, L929 cell lines (IC_{50} 203.25 μg/mL) (**Chart 2**). It is suggested that special intramolecular hydrogen binding and γ, β–turn secondary structures of this cyclic peptide analogue could be possible reasons for producing such high activity (39).

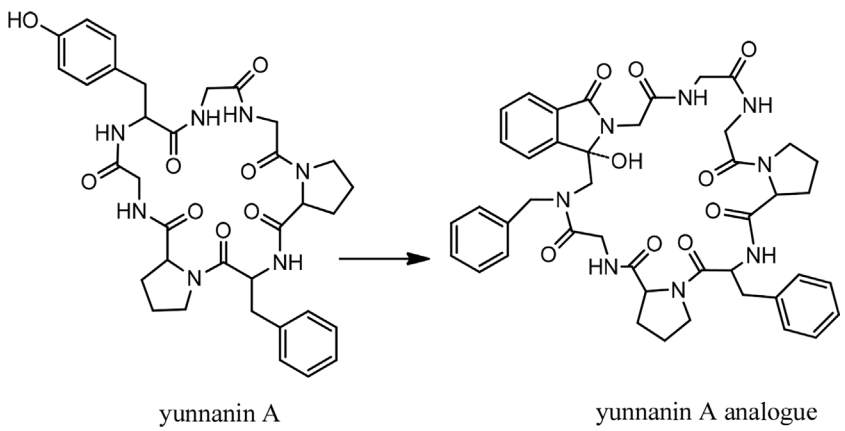

FIGURE 2 | The structure of yunnanin A and its designed analogue.

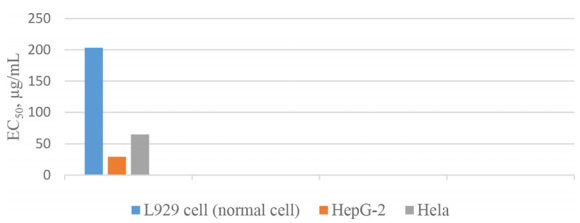

CHART 2 | Inhibitory action of the yunnanin A analogue against cell lines.

On the other hand, the synthesis of yunnanin C was also reconsidered along with the preparation of 9 related mutated analogues by the method of serine/threonine ligation (STL)–mediated cyclization (40). Three other yunnanins, named yunnanins D, E, and F, were isolated from *Stellaria* yunnanensis, and their structures were confirmed by spectroscopic and chemical analysis (41). Later, in 2005, the total synthesis of yunnanin F was reported using a disconnection approach (42). By screening for antimicrobial and pharmacological activities, yunnanin F showed moderate-to-good inhibitory activity against the growth of bacterial cells and weak activity against fungal cells (42). Meanwhile, this cyclopeptide demonstrated a good anthelmintic activity against earthworms (42). From the point of pharmacological activity, yunnanin F produced moderate anti-inflammatory activity (42). The structures of yunnanin B, C, and D are shown in **Figure 3**. The peptide sequences and information on biological activities of all the yunnanin cyclopeptides are given in **Table 2**.

DICHOTOMINS

Dichotomins A to E were isolated from the roots of *Stellaria dichotoma* L. vat. *lanceolata* Bge, and their structures were defined by 2D NMR spectroscopy, X-ray, and chemical analysis (43, 44). In addition, the biological activities of these cyclopeptides were also reported by the same authors. Dichotomins A, B, C, and E demonstrated inhibitory action against the growth of p-388 lymphocytic leukemia cells (IC_{50} s were 2.5, 3.5, 5.0, 2.0 μg/mL, respectively) (**Chart 3**). Interestingly, dichotomin D did not show such activity. It is argued that dichotomins A, B, and C, as hexacyclopeptides, have identical sequences except at the sixth residues. To show similarity between dichotomin E, a pentacyclopeptide, with the three dichotomins A, B, and C, the

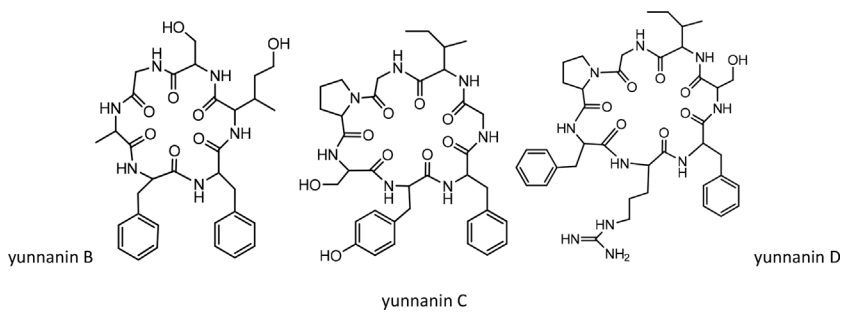

FIGURE 3 | The structures of yunnanin B, C, D.

TABLE 2 | Yunnanin cyclopeptides with their sequences and biological activities.

Peptide names	Peptide sequence	Cytotoxic activity	Pharmacological activities	Anti-infective activities
Yunnanin A	Cyclo(-Gly-Tyr-Gly-Gly-Pro-Phe-Pro)	+	weak anti-inflammatory	weak antimicrobial, anthelmintic
Yunnanin B	Cyclo(-Gly-Ser-δ-HO Ile-Phe-Phe-Ala)	+		
Yunnanin C	Cyclo(-Gly-Ile-Gly-Phe-Tyr-Ser-Pro)	+		
Yunnanin D	Cyclo(-Gly-Ile-Ser-Phe-Arg-Phe-Pro)	+		
Yunnanin E	Cyclo(-Gly-Ser-δ-HO Ile-Phe-Phe-Ser)			
Yunnanin F	Cyclo(-Gly-Val-Thr-Trp-Tyr-Pro-Ser-Ser)		anti-inflammatory	Antimicrobial, anthelmintic

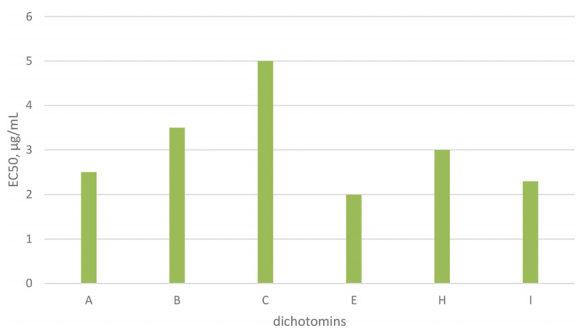

CHART 3 | Anticancer activity of dichotomins on p-388 cells.

sequence of Tyr-Ala-Phe in dichotomin E was compared with the sequence of Phe-Leu-Tyr in these hexacyclopeptides. By this comparison, a comment was made that a common feature exists as one aliphatic residue is present within two aromatic residues among A, B, C, and E cyclopeptides. Dichotomin D, as a hexacyclopeptide, had neither an identical sequence with the sequence of dichomins A, B, and C nor an aliphatic residue separating two aromatic residues. In fact, the two aromatic residues of dichotomin D were close together. Instead of cytotoxic activity, dichotomin D showed a strong cyclooxygenase inhibitory action (about 73% inhibition at 100 μM concentration compared with the control). Dichotomin A did not show activity against cyclooxygenase (44). Later on, dichotomins F and G were isolated from the same plant source, and their structures were confirmed by instrumental and chemical methods (45). Both dichotomin F and G demonstrated inhibitory action on cyclooxygenase. In another report, dichotomins H and I were found from the same plant, and their biological activities were studied (46). Using an MTT assay, dicotomins H and I were shown to have inhibitory activity against the growth of p-388 cells (IC_{50} s 3.0 and 2.3 μg/mL, respectively) (**Chart 3**). In a further study, dichotomin J, naturally originated from the roots of *Stellaria dichotoma*, was synthesized and screened for antibacterial, antifungal, and anthelmintic activities and compared with the appropriate related standard drugs, such as ciprofloxacin, griseofulvin, and albendazole, respectively. It is shown that this cyclopeptide contains good inhibitory action against bacteria (*S. aureus*, *B. substilis*) and moderate activity against fungi (*C. albicans*, *A. niger*) in 50 μg/mL concentration and high killing activity against earthworm (27.65 min, mean death time comparable with 22.78 min mean death time found for albendazole) (47). The structures of anticancer dichotomins are shown in **Figure 4**. The peptide sequences and information on biological activities of all the dichotomin cyclopeptides are given in **Table 3**.

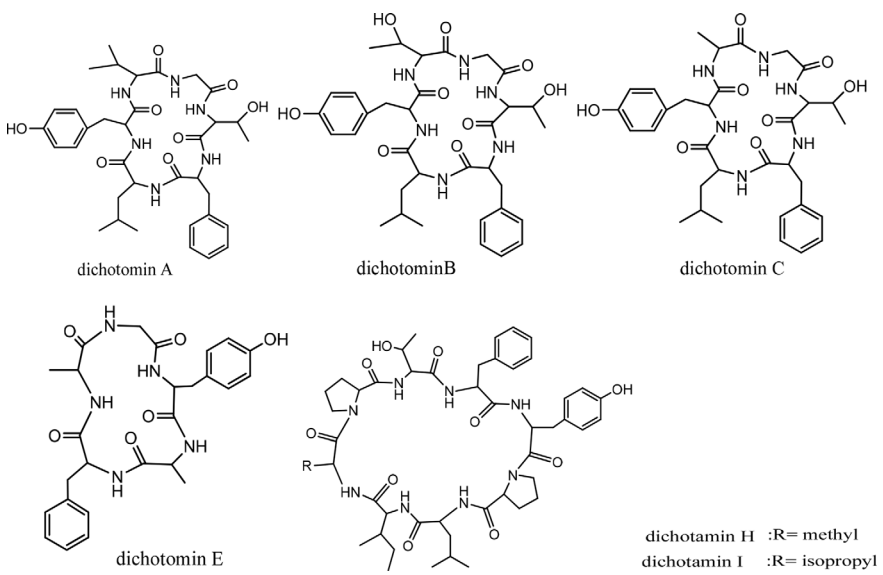

FIGURE 4 | The structures of dichotamins.

TABLE 3 | Dichotomin cyclopeptides with their sequences and biological activities.

Peptide names	Peptide sequence	Cytotoxic activity	Pharmacological activities	Anti-infective activities
Dichotomin A	Cyclo(-Gly-Thr-Phe-Leu-Tyr-Val)	+		
Dichotomin B	Cyclo(-Gly-Thr-Phe-Leu-Tyr-Thr)	+		
Dichotomin C	Cyclo(-Gly-Thr-Phe-Leu-Tyr-Ala)	+		
Dichotomin D	Cyclo(-Gly-Val-Gly-Phe-Tyr-Ile)		Cyclooxygenase inhibitor	
Dichotomin E	Cyclo(-Gly-Tyr-Ala-Phe-Ala)	+		
Dichotomin F	Cyclo(-Pro-Tyr-Phe-Val-Leu-Pro-Ser-Val-Tyr)		Cyclooxygenase inhibitor	
Dichotomin G	Cyclo(-Pro-Leu-Pro-Ile-Pro-Pro-Phe-Tyr-Ser)		Cyclooxygenase inhibitor	
Dichotomin H	Cyclo(Ala-Pro-Thr-Phe-Tyr-Pro-Leu-Ile)	+		
Dichotomin I	Cyclo(-Val-Pro-Thr-Phe-Tyr-Pro-Leu-Ile)	+		
Dichotomin J	Cyclo(-Gly-Ile-Phe-Leu-Tyr-Ala)			antibacterial, antifungal, anthelmintic

CYCLOLEONURIPEPTIDES

Cycloleonuripeptides as proline-rich cyclopeptides were discovered from the fruits of *Leonurus heterophyllus* (Labiatae). Cycloleonuripeptides A, B, and C are nonacyclopeptides (**Figure 5**). Among them, cycloleonuripeptide B is epimer with cycloleonuripeptide C. The stuctures of these cyclopeptides were elucidated by 2D NMR and chemical analysis (48). The presence of proline residues in the peptide backbone could end with several possible conformations as a result of cis-trans isomerization of amide bonds involving proline. Therefore, conformational analysis of the cyclopeptides were carried out by distance geometry calculation and restrained energy minimization using NMR data. Although five proline residues are present in these peptide sequences, a single stable conformer was observed for them. In addition, it was found that the skeleta of cycloleonuripeptides A, B, and C contain two β-turns (49). Cycloleonuripeptides B and C exhibit inhibitory action on the growth of p-338 lymphocytic leukemia cells (IC_{50} s 6.0 and 3.7 μg/mL, respectively) (**Chart 4**) (48). Further experiments on the fruit extract of *Leonurus heterophyllus* resulted in the discovery of cycloleonuripeptides D (50), E, and F (51). Cycloleonuripeptide D shows inhibitory action against a cyclooxygenase enzyme, and cycloleonuripeptides E and F demonstrate moderate activities as vasorelaxant agents in rat aorta (50, 51). The peptide sequences and information on biological activities of all the cycloleonuripeptides are given in **Table 4**.

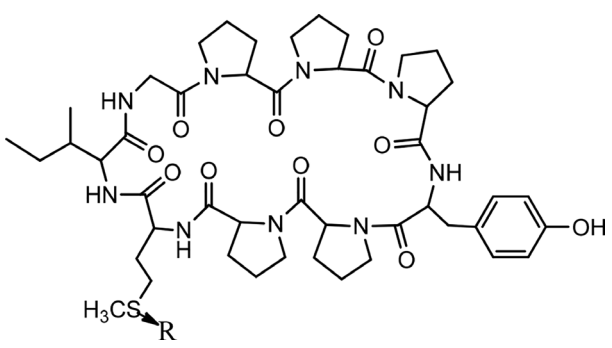

FIGURE 5 | The structures of cycloleonuripeptides A, (B,C) (R=O). B and C are isomers.

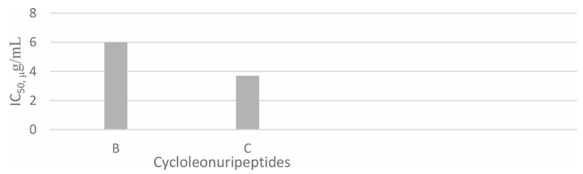

CHART 4 | Inhibitory activity of cycloleonuripeptides on p-338 lymphocytic leukemia cells.

CYCLOLINOPEPTIDES

Cyclolinopeptide A (CLA), as a first natural cyclopeptide, was isolated from the seeds of *Linum usitatissimun* (linseed oil) in 1959 (52). Conformational analysis by NMR shows that cyclolinopeptide A occurs as at least four types of conformers in solution state, and none of them contains hydrogen binding intramolecularly. Therefore, high flexibility of the peptide molecule can result in solution (53). This peptide is active as a potent immunosuppressive compared with cyclosporine A (23). The search for more cyclolinopeptides was followed by the discovery of cyclolinopeptides B–E, and their structures were elucidated by 2D NMR and chemical analysis (54). Studying the biological activities of these cyclopeptides showed that cyclolinopeptide B possesses inhibitory activity against concanavalin A-induced mitogenic response of human peripheral blood lymphocytes (IC_{50} 44 ng/mL), comparable with cyclosporine A (55). Cyclolinopeptides A, B, and E also show moderate inhibition on the proliferation of mouse lymphocyte cells induced by concanavalin A (IC_{50} s 2.5, 39, and 43 μg/mL, respectively) (**Chart 5**) (54). In contrast, cyclolinopeptides C and D do not give such a level of activity (IC_{50} >100 μg/mL). In another study, four cyclolinopeptides F–I were found in the seeds of *Linum usitatissimun*. The structures of isolated cyclopeptides were determined by instrumental and chemical methods. In addition, the immunosuppressive activity of these compounds was evaluated against mouse splenocytes (56). Cyclolinopeptides F–I also do not show immunosuppressive activity (IC_{50} >100 μg/mL). For these different results between cyclolinopeptides A, B, and C on one hand and cyclolinopeptides C, D, and F–I on the other, it is inferred that the biological activity is very dependent on the sequence as well as conformation of the peptides. To confirm this hypothesis, the three-

TABLE 4 | The sequences of Cycloleonuripeptide cyclopeptides and related biological activities.

Peptide names	Peptide sequence	Cytotoxic activity	Pharmacological activities
Cycloleonuripeptide A	Cyclo(-Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Met-Ile)		
Cycloleonuripeptide B	Cyclo(-Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Met(O)-Ile)	+	
Cycloleonuripeptide C	Cyclo(-Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Met(O)-Ile)	+	
Cycloleonuripeptide D	Cyclo(-Ser-Pro-Pro-Pro-Tyr-Phe-Gln-Thr-Pro-Ile)		Cyclooxygenase inhibitor
Cycloleonuripeptide E	Cyclo(-Ala-Pro-Ile-Val-Ala-Ala-Phe-Thr-Pro)		Vasorelaxant
Cycloleonuripeptide F	Cyclo(-Gly-Tyr-Pro-Leu-Pro-Phe-Tyr-Pro-Pro)		Vasorelaxant

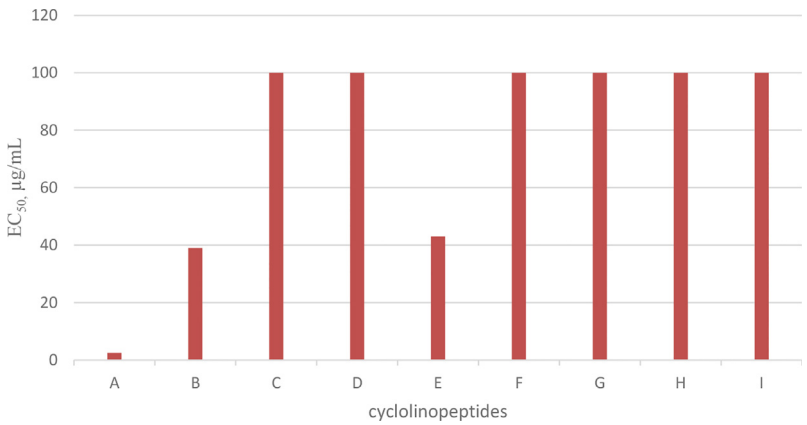

CHART 5 | Antiproliferative activity of cyclolinopeptides on mouse lymphocyte cells.

dimensional structure of cycylolinopeptide A, studied by X-ray, and its distance geometry calculations were compared with that of cyclolinopeptide B. It was found that, in the solid state, conformation of cyclolinopeptide A was similar to that of the both cyclopeptides A and B in the solution state (57). The structures of cytotoxic cyclolinopeptides are shown in **Figure 6**. The peptide sequences and information on biological activities of all the cyclolinopeptides are given in **Table 5**.

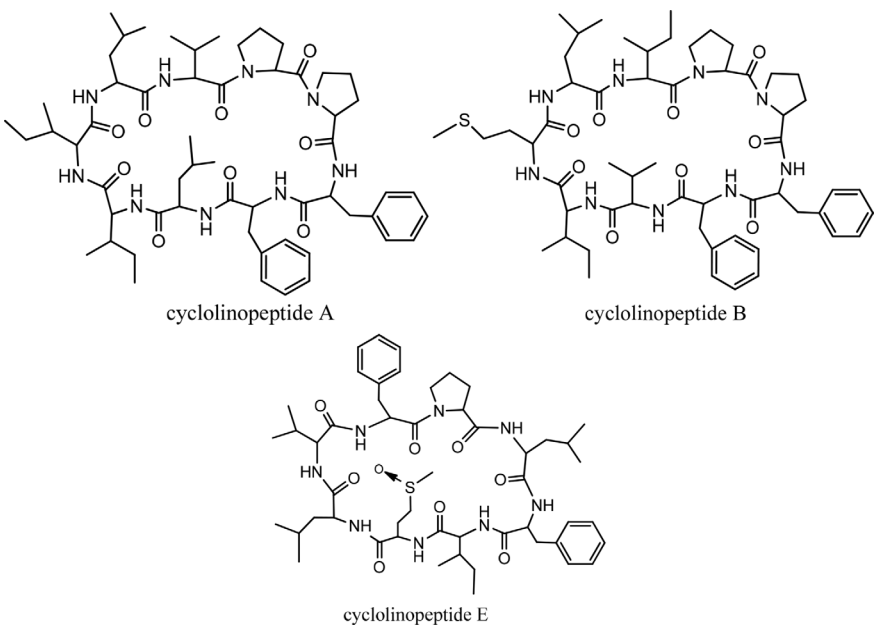

FIGURE 6 | The structures of cyclolinopeptides.

TABLE 5 | The sequences of cyclolinopeptides and their biological activities.

Peptide names	Peptide sequence	Cytotoxic activity	Pharmacological activities
Cyclolinopeptide A	Cyclo(-Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val)	+	immunosuppressive
Cyclolinopeptide B	Cyclo(-Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-Ile)	+	immunosuppressive
Cyclolinopeptide C	Cyclo (-Pro-Pro-Phe-Phe-Val-Ile-Met(O)-Leu-Ile)		
Cyclolinopeptide D	Cyclo(-Pro-Phe-Phe-Trp-Ile-Met(O)-Leu-Leu)		
Cyclolinopeptide E	Cyclo(-Pro-Leu-Phe-Ile-Met(O)-Leu-Val-Phe)	+	immunosuppressive
Cyclolinopeptide F	Cyclo(-Pro-Phe-Phe-Trp-Val-Met(O)-Leu-Met(O)		
Cyclolinopeptide G	Cyclo(-Pro-Phe-Phe-Trp-Ile-Met(O)-Leu-OMet)		
Cyclolinopeptide H	Cyclo(-Pro-Phe-Phe-Trp-Ile-Met(O)-Leu-Met)		
Cyclolinopeptide I	Cyclo(-Pro-Phe-Phe-Trp-Val-Met-Leu-Met(O))		

CHERIMOLACYCLOPEPTIDES

Cherimolacyclopeptides A and B were isolated from the seeds of *Annona cherimola* Miller. Tandem mass spectrometry and 2D NMR spectroscopy were used to determine the peptide sequences (**Figure 7**). The solution state structure of cherimolacyclopeptide A was also studied. It is shown that this cyclopeptide contains two β-turns and one new type of β-bulge compared with other cyclopeptides. A cytotoxicity study showed that cherimolacyclopeptide A is a potent cytotoxic agent (IC_{50} 0.6 μM), but cherimolacyclopeptide B is a weak cytotoxic agent against KB tumor cells (IC_{50} 45 μM) (**Chart 6**) (58). The peptide sequences and information on biological activities of cherimolacyclopeptides are given in **Table 6**.

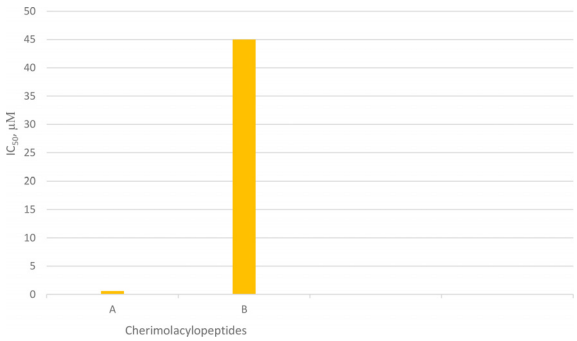

CHART 6 | Cytotoxic activity of cherimolacylopeptides on KB tumor cells.

DIANTHINS

Dianthus is a genus of *Caryophyllaceous* that includes 300 species (59). From some *Dianthus* species, three classes of compounds have been isolated and studied; 1) triterpenoid saponins, called dianosides A and B (60), C–F (61), and G–I (62); 2) dianthin proteins dianthin-30 and dianthin-32 (63) and dianthin-29 (64); and 3) dianthin cyclic peptides A and B (65), C–F (59), G and H (66), and I (67, 68). It should be noted that, because proteins and cyclopeptides of *Dianthus* have the common name of dianthin, these two different classes must not be confused. In this review, isolation, synthesis, and biological activities of dianthin cyclopeptides are discussed. Discussion of the biological activities is focused on the anticancer activity of these cyclopeptides.

Dianthin Isolation and Synthesis

Isolation

Historically, the plant *Dianthus superbus* L. has been used in China as a traditional medicine for its several biological activities, including diuretic, anti-inflammatory, urinary anti-infective, and

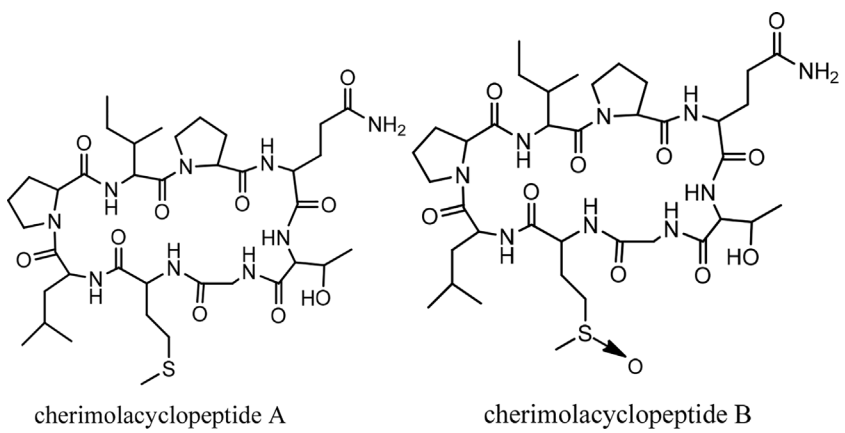

FIGURE 7 | The structures of cherimolacyclopeptides.

TABLE 6 | The cherimolacyclopeptides, their sequences, and biological activities.

Peptide names	Peptide sequence	Cytotoxic activity
Cherimolacyclopeptide A	Cyclo(-Pro-Gln-Thr-Gly-Met-Leu-Pro-Ile)	+++ (high potency)
Cherimolacyclopeptide B	Cyclo(-Pro-Gln-Thr-Gly-OMt-Leu-Pro-Ile)	+ (low potency)

anticancer effects (69). In an initial study, two cyclopeptides were isolated from this plant, and their structures were determined as cyclo(-Ala-Tyr-Asn-Phe-Gly-Leu) (dianthin A) and cyclo(-Ile-Phe-Phe-Pro-Gly-Pro) (dianthin B), using instrumental analysis (65). In the following study, four cyclopeptides, dianthins C, D, E, and F, are isolated from the extract of the same plant, and their structures are documented by mass spectrometry, 2D NMR analysis, and some chemical methods (59). In this study cytotoxicity of dianthin E is also reported. In another study, two other cyclopeptides are identified from the extract of *Dianthus superbus* by employing ESI tandem mass fragmentation, 2D NMR analysis, and X-ray diffraction (28). The proliferative activities of dianthins G and H are also evaluated in this study. A few years ago, the isolation of a new cyclopeptide named dianthin I was reported from *Dianthus chinensis*, and its structure was determined (67). From *Dianthus superbus* var. *longicalysinus*, along with six other known compounds, a cyclopeptide called longicalycinin A was isolated and its structure identified by instrumental analysis and reported as cyclo(-Gly-Phe-Tyr-Pro-Phe) (**Figure 1**). The biological evaluation of this compound shows its cytotoxicity against the HepG2 cancer cell line (70). So far, there are no other reports on the discovery of new longicalycinin although total synthesis of this and some other cyclopeptides from *Dianthus superbus* has been documented in several studies (71–75). The structures of dianthins and longicalycinin A are presented in **Figures 8** and **9**. The peptide

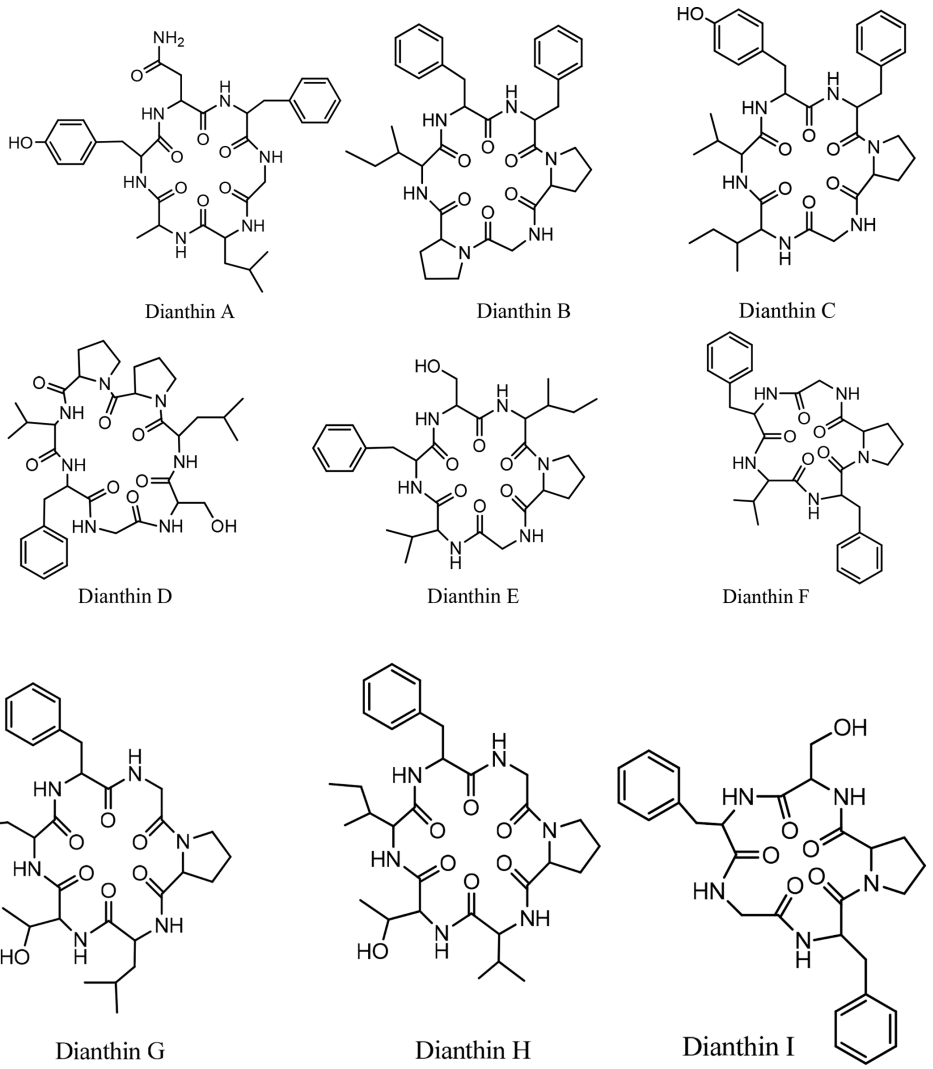

FIGURE 8 | The structures of Dianthins.

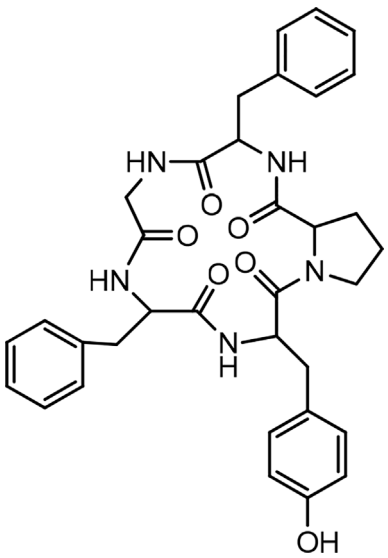

FIGURE 9 | Structure of Longicalycinin A.

sequences and information on biological activities of dianthins and longicalycinin A are given in **Table 7**.

Synthesis of Dianthins

Dianthin A, also called cyclopolypeptide (XIII), was synthesized through several chemical steps using a solution phase peptide synthesis strategy (71). The synthesis of dianthin I by the solid phase method was reported by a group of scientists (68). The synthesis of longicalycinin A was also reported through solution phase (72) as well as solid phase methods (73, 74). In addition, several analogues of longicalycinin A were synthesized in order to achieve information about relationships between structure and activity of this cyclopeptide (74). For the synthesis of longicalycinin A analogues, a two-step synthesis strategy was chosen. To explain more, linear peptides at first were synthesized on 2-chlorotrityl (2-CTC) resin and then detached from the resin as protected peptides in the partial cleavage step. In the second step, final deprotection was applied in the solution phase in order to achieve fully unprotected linear peptides. For preparing cyclic peptide analogues, after cleaving protected linear peptides from the resin, conditions for peptide cyclization were employed in the solution phase followed by final deprotection. The reaction product was then solidified in cold diethyl ether. In the other effort, linear and cyclic heptapeptide analogues of longicalycinin A were also synthesized by employing two cysteine molecules (75). The cysteine molecules were added as one at the C-terminal and the other at the N-terminal position of the longicalycinin A linear peptide analogues. In this experiment, the peptide cyclization was performed in an oxidation condition in order to make a disulfide bond between two SH groups of cysteine residues of the linear peptides.

Biological Activities of Dianthin Cyclopeptides

Various biological activities have been reported in the literature concerning dianthins A–I and longicalycinin A. The studies were made on the evaluation of antiprotozoal, antifungal, antiduretic, anti-inflammatory, and anticancer activities of these cyclopeptides. The survey of these studies is presented as follows.

Anticancer Activity of Dianthins

As previously mentioned, *Dianthus superbus*, a genus of plant family *Caryophyllaceae*, has been used for anticancer activity (69, 70). In one study, the antioxidant activity (free radical scavenging ability) and cytotoxic effect of several fractions from an ethanol extract of *Dianthus superbus* on three human cancerous cell lines, HepG2, HeLa, and Bel-7402, were reported (76). It was demonstrated that, among these fractions, the ethyl acetate part containing a high content of phenolic compounds with high reducing ability showed the most antioxidant activity. Using the MTT assay, the ethyl acetate part also showed considerable cytotoxicity (IC_{50} 20–36 μg/mL) against the three cell lines. In the other study, by employing the ethyl acetate fraction, apoptotic activation was observed in the HepG2 cell line (77). Treating with 80 μg/mL of the fraction over 24 h caused a considerable increase in the percentage of cells in the sub-G1 phase in which a high amount of apoptotic nuclear fragment bodies was seen. In a further experiment exposing HepG2 cells to the ethyl acetate fraction for 48 h, it was shown that the expressions of Bcl-2 and NF-κB were suppressed, and the amount of cytochrome c was increased in cytosol due to the release from mitochondria. Caspases-9 and -3 were also activated. All these data infer that the ethyl acetate fraction of the ethanol extract of *Dianthus superbus* induces apoptotic phenomena in Hep-G2 cells by a

TABLE 7 | Dianthins, their sequences, and biological activities.

Peptide names	Sequence	Anticancer activity	Pharmacological activities	Anti-infective activities
Dianthin A	cyclo-(Ala-Tyr-Asn-Phe-Gly-Leu)	+		Anthelmintic Antifungal
Dianthin B	cyclo-(Ile- Phe- Phe-Pro- Gly-Pro)			
Dianthin C	cyclo-(Gly-Pro-Phe-Tyr-Val-Ile)	+		
Dianthin D	cyclo-(Gly-Ser-Leu-Pro-Pro-Ile-Phe)	+		
Dianthin E	cyclo-(Gly-Pro-Ile-Ser-Phe-Val)	+	Proliferation stimulant	
Dianthin F	cyclo-(Gly-Pro-Phe-Val-Phe)	+		
Dianthin G	cyclo-(Gly-Pro-Leu-Thr-Leu-Phe)		Proliferation stimulant	
Dianthin H	cyclo-(Gly-Pro-Val-Thr-Ile-Phe)	+	Proliferation stimulant	
Dianthin I	cyclo-(Gly–Phe–Pro–Ser–Phe)			
Longicalycinin A	cyclo-(Gly-Phe-Tyr-Pro-Phe).	+		anthelmintic

mitochondrial intrinsic pathway (77). Also, there are several reports considering the anticancer activities of dianthin cyclopeptides originally isolated from *Dianthus superbus.* In one study, the biological activities of the synthesized dianthin A, previously isolated from the whole plant of *Dianthus superbus*, are reported (71). Dianthin A shows a noticeable cytotoxic activity against two cancer cell lines, DLA and EAC cells (cytotoxic concentration inhibitory of 50% growth as CTC_{50}, 15.1 and 18.6 μM, respectively) (**Chart 7**). 5-Fluorouracil (5-FU), as a standard drug, shows CTC_{50}, 37.36 and 90.55 μM against the two cell lines, respectively (71). In another study, MTT as a cytotoxicity assay was used to examine the anticancer activity of all the compounds isolated from the methanol extract of *Dianthus superbus* (59). By employing different cancerous cell lines, Hep G2, Hep 3B, A-549, MCF-7, and MDA-MB-231, it was shown that, among dianthins C–F, only cyclopeptide E was effective against HepG2 with IC_{50}, 2.37 μg/mL (**Chart 8**). Doxorubicin was chosen as the control drug in this experiment (IC_{50}, 0.19 μg/mL) (59). Interestingly, by the MTT assay, it was shown that dianthins G, H, and E increased cell division (proliferation) of rat osteoblast cells, MC3T3-E1 *in vitro* (66). The highest cell stimulation activity for all three compounds was achieved at 1×10^{-5} mM. It was, therefore, suggested that these cyclic peptides may be potential candidates for osteoporosis therapy. In one study, by isolating different compounds from *Dianthin superbus* var. *longicalysinus*, only longicalycinin A, as a cyclopeptide, showed cell toxicity against HepG2 with IC_{50} value 13.52 μg/mL by MTT assay (**Chart 8**) although several cell lines were employed, including human hepatocellular carcinoma Hep G2, Hep 3B, human breast carcinoma MCF-7, MDA-MB-231, and human lung carcinoma A-549 (70). The cytotoxic activity of longicalycinin A was also examined against DLA (NCRC 101) and EAC (NCRC 69) cell lines. 5-FU was used as the reference drug. Cytotoxic concentration of longicalycinin A for 50% growth inhibition (CTC_{50}), determined by a graphical extrapolation method, was calculated as 2.62 and 6.37 μM for DLA and EAC cell lines, respectively (**Chart 7**) (72). The standard drug reported was also 5-FU with the previous CTC_{50} reports (71). In a recent publication, longicalycinin A and its several analogues were synthesized, and their cytotoxic activities were examined against HepG2 and HT-29 using different experiments, including MTT, flow cytometry analysis, and Lysosomal membrane integrity assays. The results show that the two cyclopeptide analogues of longicalycinin A, cyclo-(Thr-Val-Pro-Phe-Ala), and cyclo-(Phe-Ser-Pro-Phe-Ala) were effective cytotoxic agents that were even

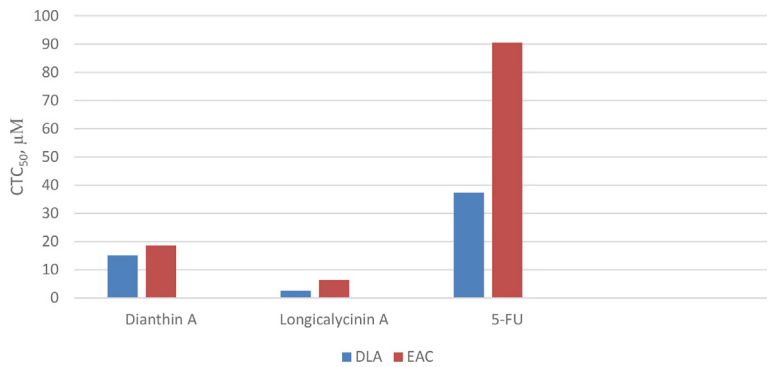

CHART 7 | Cytotoxicity of longicalycinin A, compared with dianthin A and 5- Fluorouracil (5-FU) as standard drug on cancer cells, Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC).

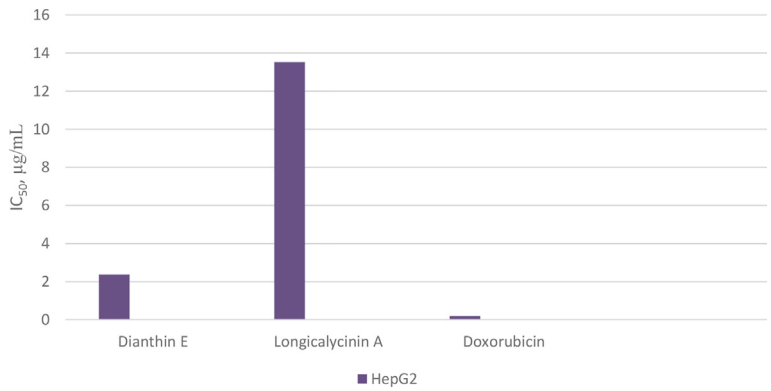

CHART 8 | Cytotoxicity of longicalycinin A on cancer cells, HepG2, compared with dianthin E and doxorubicin (as standard drug).

better than longicalycinin A (74). Cytotoxic activity of linear and cyclic heptapeptide analogues of longicalycinin A containing two cysteine residues were also examined on the two cell lines HepG2 and HT-29, using MTT assay and flow cytometry analysis. Skin fibroblast cells were included in the experiment to evaluate if any toxicity of these peptides occurs on normal cells. As a cytotoxic reference, 5-FU was chosen (75). The result of the MTT assay show that the cyclic heptapeptide analogue was toxic against the cancer cell lines more than the linear heptapeptide congener. In addition, flow cytometry analysis demonstrates that apoptosis of the cancer cells can occur by the cyclic heptapeptide in higher percentages than by the linear heptapeptide analogue. In fact, the linear peptide showed no harmful effect on cancer cells as well as on skin fibroblast cells, whereas the cyclic heptapeptide could impose an apoptotic event (about 90%) on all the cancerous and fibroblast cells (75). As a conclusion from this experiment, further and better designed heptapeptide analogues of longicalycinin A containing a disulfide bond are needed to differentiate toxicity between cancer cells and normal cells. In the later study, various fractions of methanol extract of *Dianthus superbus*, i.e., ethyl acetate, butanol, and distilled water fractions as well as their bioactive compound content were evaluated *in vitro* from the points of antioxidant, anti-influenza, and cell toxicity activities (78). A cytotoxic activity assay against four cell lines, Hela (ovarian cancer cell), SKOV (ovarian cancer cell), Caski (cervical cancer cell), and NCL-H1299 (human lung cancer cell) showed that the ethyl acetate fraction was the most potent part of the methanol extract (with IC_{50} s 9.5, 9.6, 13.8, and 69.9 μg/mL against the four cell lines, respectively). Because SKOV cells, as the most resistant ovarian cells, do not respond to the usual anticancer drugs such as Adriamycin and cis-platin, these results could be interesting. The authors show that the cyclic peptide content of the ethyl acetate

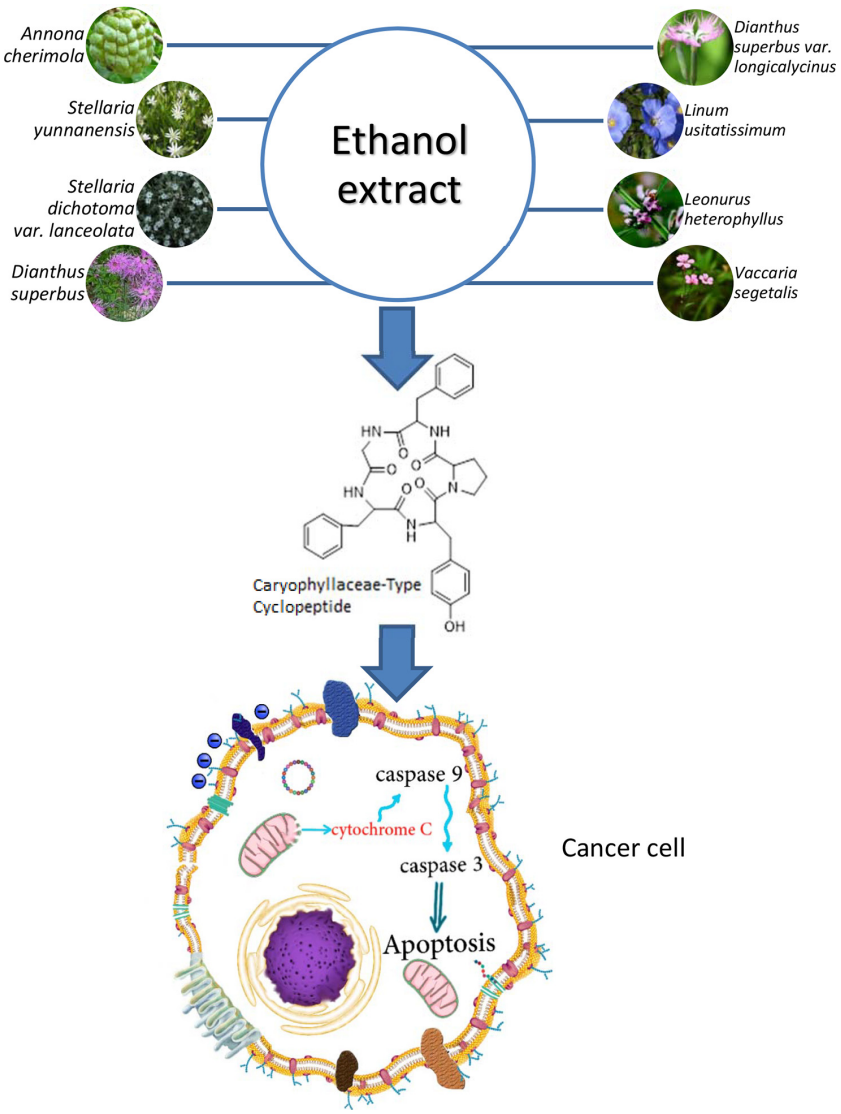

FIGURE 10 | Caryophyllaceae-type cyclopeptides, isolated from the ethanol extract of various higher plants of Caryophyllaceae type and their cancer cell cytotoxicity.

extract was more than that of the other extracts. The butanol extract is shown to be of secondary importance with fewer phenolic compounds and, thus, with less cyclic peptide content. Moreover, dianthines C, D, H, and F, identified in the extracts by LC-MS/MS and measured by the correlation study, are shown to have cytotoxicity effects in the range of 75.7% to 99.7% (78).

Other Biological Activities of Dianthins

Various species of *Dianthus* have been used in China, Korea, Iran, and Mongolia (78) for medicinal purposes other than carcinoma (65, 79). In one report, *Dianthus superbus* is shown to enhance cognition and improve memory in memory impaired mice under scopolamine induction. Thus, it is suggested that the plant could be useful in preventing Alzheimer disease (80).

Dianthus superbus has been also considered as a source of antioxidants for scavenging reactive oxygen radical species (ROS) (76, 81). That is because it contains phenolic compounds with high reducing power. These phenolic compounds mainly correlate with cyclic peptides' content of the plant. In fact, antimicrobial and cytotoxic activities of *Dianthus superbus* may be somehow in parallel with the kinds of dianthin cyclopeptides present in the plant (76, 78, 81). On the other hand, antiviral activity of *Dianthus superbus* has been also reported against influenza viruses, but this activity corresponded to the presence of flavonol glycosides detected in the butanol fraction of the methanolic extract of the plant (78).

There are reports concerning the utilization of dianthins as antiparasitic agents (71, 72). Dianthin A is shown to have a strong antifungal activity against *Candida albicans* (MIC, 6 μg/mL) compared with griseofulvin. This cyclopeptide also demonstrated a moderate anthelmintic activity on earthworms in 2 mg/mL concentration, using membendazole/piperazine citrate as the standard drugs (71). A good anthelmintic activity of longicalycinin A was also reported in the literature (72). In addition, longicalycinin A showed moderate activity against dermatophytes (72).

CONCLUDING REMARKS

Caryophyllaceae-type cyclopeptides, as natural peptides isolated from the ethanol extract of various higher plants of Caryophyllaceae type (**Figure 10**), have shown anticancer activity against several cancerous cell lines. The common feature in these peptides, apart from the cyclic structure, is the hydrophobic characteristic of their whole molecules due to the high amount of nonpolar amino acid residues present in their structures (≈80%). As can be roughly calculated from **Table 8**, proline, phenylalanine, and glycine, in order, make up the higher percentage of residue content in the cyclopeptide scaffolds compared with other residues. Polar amino acids make less contribution (around 20%) in the cyclic structures of peptides. Therefore, from the point of structure–activity relationship (SAR) studies, it may be estimated that these naturally occurring cyclopeptides insert their toxicity on cancer cells by a hydrophobic/hydrophilic characteristic balanced equal to a 4/1 ratio. This estimate is in accordance with a study that reports higher hydrophobic peptides infuse better into the cancer cells through the nonpolar part of the cell membrane, which results in cell disruption and necrosis (82, 83). Moreover, the presence of proline as well as glycine in the peptides is important for interaction with the cancer cell membrane (84). Phenylalanine residue can also

TABLE 8 | Caryophyllaceae-type cyclopeptides as cytotoxic agents, comparing the properties of amino acid (AA) content in their sequences. Each (+) stands for one AA. AAs with colored names indicate their importance in cytotoxic activity of the cyclopeptides.

Peptide name	Sequence	AA, acidic	AA, basic	AA, polar	AA, nonpolar
Segetalin E	Cyclo(-Gly-Tyr-Val-Pro-Leu-Trp-Pro-)			++	+++++
Yunnanin A	Cyclo(-Gly-Tyr-Gly-Gly-Pro-Phe-Pro)			++++	+++
Yunnanin B	Cyclo(-Gly-Ser-δ-HO Ile-Phe-Phe-Ala)			+++	+++
Yunnanin C	Cyclo(-Gly-Ile-Gly-Phe-Tyr-Ser-Pro)			++++	+++
Yunnanin D	Cyclo(-Gly-Ile-Ser-Phe-Arg-Phe-Pro)		+	++	++++
Dichotomin A	Cyclo(-Gly-Thr-Phe-Leu-Tyr-Val)			+++	+++
Dichotomin B	Cyclo(-Gly-Thr-Phe-Leu-Tyr-Thr)			++++	++
Dichotomin C	Cyclo(-Gly-Thr-Phe-Leu-Tyr-Ala)			+++	+++
Dichotomin E	Cyclo(-Gly-Tyr-Ala-Phe-Ala)			++	+++
Dichotomin H	Cyclo(Ala-Pro-Thr-Phe-Tyr-Pro-Leu-Ile)			++	++++++
Dichotomin I	Cyclo(-Val-Pro-Thr-Phe-Tyr-Pro-Leu-Ile)			++	++++++
Cycloleonuripeptide B	Cyclo(-Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Met(O)-Ile)			+++	++++++
Cycloleonuripeptide C	Cyclo(-Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Met(O)-Ile)			+++	++++++
Cherimolacyclopeptide A	Cyclo(-Pro-Gln-Thr-Gly-Met-Leu-Pro-Ile)			**+++**	**+++++**
Cherimolacyclopeptide B	Cyclo(-Pro-Gln-Thr-Gly-OMt-Leu-Pro-Ile)			**++++**	**++++**
Dianthin A	cyclo-(Ala-Tyr-Asn-Phe-Gly-Leu)			**++**	**++++**
Dianthin C	cyclo-(Gly-Pro-Phe-Tyr-Val-Ile)			**++**	**++++**
Dianthin D	cyclo-(Gly-Ser-Leu-Pro-Pro-Ile-Phe)			**++**	**+++++**
Dianthin E	cyclo-(Gly-Pro-Ile-Ser-Phe-Val)			**++**	**++++**
Dianthin F	cyclo-(Gly-Pro-Phe-Val-Phe)			**+**	**++++**
Dianthin H	cyclo-(Gly-Pro-Val-Thr-Ile-Phe)			**++**	**++++**
Longicalycinin A	cyclo-(Gly-Phe-Tyr-Pro-Phe).			**++**	**+++**
Cyclolinopeptide A	Cyclo(-Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val)				**+++++++++**
Cyclolinopeptide B	Cyclo(-Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-Ile)				**+++++++++**
Cyclolinopeptide E	Cyclo(-Pro-Leu-Phe-Ile-OMet-Leu-Val-Phe)			**+**	**+++++++**

increase the affinity of the peptides for interaction with the membrane of cancer cells (85). On the other hand, tyrosine residue as a polar amino acid may raise the toxicity of the peptides toward cancer cells (83, 86). In addition, the cyclic form of the peptides with fixed conformational structure and less occupying space can cause peptides to face less of a physical barrier to enter the cells (87). However, modification of these cyclic peptides by chemical means, either in a hydrophobic direction (e.g., acylation) or in a hydrophilic way (e.g., phosphorylation to impose negative charge or replacing/adding lysine or arginine within the cyclic peptide structure to add positive charge), is needed (88). By such favorable modification, these cyclic peptides can meet most all the requirements of SAR data needed to make them good candidates for cancer treatment in the clinic. On the other hand, conjugation of these peptides through tyrosine residue with an anticancer drug may even increase their physicochemical properties. These peptides considered cell-penetrating/targeting peptides can improve specificity and reduce side effects of the present anticancer drugs.

Because medicinal plants containing cyclopeptides, including caryophyllaceae-type cyclopeptides, have been used traditionally for a long time, it is inferred that these cyclopeptides should not be toxic to healthy cells/tissues of human body, so this is another reason for considering these cyclopeptides as safer compounds for cancer therapy compared with the conventional chemotherapy agents. However, modified caryophyllaceae-type cyclopeptides, like any other newly introduced agent as drugs, must pass all the requirements necessary to ensure that these cyclopeptides are safe as well as biologically active enough to add to the treatment schedules of cancer therapy.

AUTHOR CONTRIBUTIONS

A part of this manuscript concerning Longicalycinin A synthesis and its analogues was experimentally involved by MG, AB, and BM. All authors contributed to the article and approved the submitted version.

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Intranasal Administration of PACAP Is an Efficient Delivery Route to Reduce Infarct Volume and Promote Functional Recovery After Transient and Permanent Middle Cerebral Artery Occlusion

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Intranasal (IN) administration appears to be a suitable route for clinical use as it allows direct delivery of bioactive molecules to the central nervous system, reducing systemic exposure and sides effects. Nevertheless, only some molecules can be transported to the brain from the nasal cavity. This led us to compare the efficiency of an IN, intravenous (IV), and intraperitoneal (IP) administration of pituitary adenylate cyclase-activating polypeptide (PACAP) after transient or permanent middle cerebral artery occlusion (MCAO) in C57BL/6 mice. The results show that the neuroprotective effect of PACAP is much more efficient after IN administration than IV injection while IP injection had no effect. IN administration of PACAP reduced the infarct volume when injected within 6 h after the reperfusion and improved functional recovery up to at least 1 week after the ischemia.

Keywords: pituitary adenylate cyclase-activating polypeptide, intranasal administration, cerebral ischemia, infarct volume, functional recovery

INTRODUCTION

Stroke can either be hemorrhagic, when a cerebral blood vessel bursts, or ischemic, when an artery gets obstructed by a clot (1). In 85% of the cases, stroke is of ischemic origin due to a permanent or temporary occlusion of a cerebral artery, which in one third of the patients concerns the middle cerebral artery (MCA) (2). Stroke induced blood flow disruption, impairs oxygen and nutrients supply, inducing irreversible neuronal loss responsible for cognitive disorders, fatigue, paralysis, and even death of the patient. Actually, stroke is the second leading cause of death worldwide and the

third leading cause of disability in adults (3). Stroke affects almost 17 million people worldwide each year, i.e., one victim every 2 s (4). As a consequence, millions of stroke survivors must adapt to a life with restrictions in daily activities and depend on other's continuous support (5).

A prolonged and severe reduction of cerebral blood flow (CBF) causes neuronal necrosis responsible for irreversible neurological deficits. In contrast, a moderate and gradual decline in CBF at a distance from the occlusion induces a benign oligemia which can be tolerated and remains asymptomatic. In between the core where necrosis occurs and the peripheral benign oligemia, the ischemic penumbra is a territory where neurons are on the verge of death but are still salvageable by rapid blood flow restoration and/or proper pharmacological treatment (6).

The approved medical treatments for acute ischemic stroke are intravenous (IV) thrombolysis with recombinant tissue plasminogen activator (tPA) and/or mechanical thrombectomy, which promote the recanalization of the occluded artery and therefore the rapid restoration of a normal CBF. However, tPA can only be administered within the first 4.5 h after the manifestation of the symptoms since after this short therapeutic time window tPA administration becomes deleterious (7). Mechanical thrombectomy can be carried out in people with large vessel ischemic stroke within the first 6 h after the occlusion but needs competent practitioners for this act and access to an appropriate diagnostic imaging unit (8). Application of these very limited therapeutical options is also restricted by cost, time and age of patients, so that less than 15% of stroke victims benefit from thrombolysis. Furthermore, functional recovery after thrombolysis is only partial but so far, all other therapeutic approaches targeting the acute phase of stroke have failed (9, 10). Thus, there is an urgent need for additional, more widely applicable, treatment options.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide of the VIP/secretin/glucagon/growth hormone-releasing hormone family, naturally produced by the organism and widely distributed in the brain and peripheral tissues, notably in the endocrine pancreas, gonads, respiratory and urogenital tracts (11). PACAP acts as a neurohormone, neurotrophic factor and neuroprotective agent, through three receptors, i.e., PAC1, VPAC1, and VPAC2 (12). PACAP has high affinity for the PAC1, VPAC1, and VPAC2 receptors, whereas VIP has ~1,000 times greater affinity for VPAC receptors than for the PAC1. The 3 receptors are largely distributed in peripheral organs (13), but PAC1 is more expressed than VPAC1 and VPAC2 in the central nervous system (14). In relationship with their large distribution, activation of those three G-protein coupled receptors initiates multiple signaling pathways involved in the regulation of important biological functions such as stress response, cardiovascular effects, food intake, circadian rythm, and reproduction (15, 16).

Besides those effects on the control of physiological functions, numerous studies have highlighted the neuroprotective potential of PACAP in various neurological diseases involving neuronal cell death (17–20). For instance, PACAP protects dopaminergic neurons in a model of Parkinson's disease (21, 22), reduces amyloidopathy in a model of Alzheimer's disease (23–25), suppresses cortical damages in mice after traumatic brain injury (26), and improves memory performance in a model of Huntington's disease (27).

PACAP also decreases the infarct volume and improves functional recovery after stroke. One particularity of PACAP is that it counteracts many of the deleterious processes activated by stroke through its anti-excitotoxicity, anti-apoptotic, anti-inflammatory, antioxidant, and immuno-modulatory activities (28–31). Furthermore, PACAP may act beyond the acute phase of stroke by promoting neurogenesis, plasticity and angiogenesis (32, 33).

However, to consider possible clinical applications, several parameters remain to be clarified, such as the optimal administration route, the lower efficient dose and the therapeutic window of PACAP. The present study aimed to investigate some of these issues in 2 stroke animal models.

Abbreviations: ACVR1, Activin A receptor 1; AQP4, Aquaporin 4; ATF3, Activating transcription factor 3; BBB, Blood brain barrier; BCL10, B cell lymphoma/leukemia 10; BDNF, Brain derived neurotrophic factor; CASP9, Caspase 9; CAT, Catalase; CBF, Cerebral blood flow; CXCR1, Chemokine receptor type 1; CXCR4, Chemokine receptor type 4; DBI, Diazepam binding inhibitor; DDIT3, DNA damage inducible transcript 3; DUSP6, Dual specificity phosphatase 6; FAS, Fas cell surface death receptor; GAD1, Glutamate decarboxylase 1; GADD45A, Growth arrest and DNA damage inducible alpha; GFAP, Glial fibrillary acidic protein; GJB6, Gap junction beta 6; GJD2, Gap Junction Protein Delta 2; GPX1, Glutathione peroxidase 1; HMOX1, Heme oxygenase 1; HOMER1, Homer scaffold protein 1; HSPA1B, Heat shock protein A (Hsp70) 1B; HSPB1, Heat shock protein beta 1; HSPD1, Heat shock protein D (Hsp60) 1; IFNAR1, Interferon alpha and beta receptor subunit 1; IL12A, Interleukin-12 subunit alpha; IL6, Interleukin-6; IN, Intranasal Injection; IP, Intraperitoneal injection; IRF1, Interferon regulatory factor 1; IRF5A, Interferon regulatory factor 5a; IV, Intravenous injection; JUN, Jun proto-oncogene; MCAO, Middle cerebral artery occlusion; NEUROD1, Neurogenic differentiation 1; NOS1, Nitric oxide synthase 1; PAC1, Pituitary adenylate cyclase-activating polypeptide type I receptor; PACAP, Pituitary adenylate cyclase-activating polypeptide; PIK3R1, Phosphatidylinositol 3-kinase regulatory subunit alpha; pMCAO, permanent middle cerebral artery occlusion; SLC16A7, Solute Carrier Family 16 Member 7 (Monocarboxylate Transporter 2) TGFB1, Transforming growth factor-beta 2; TGFB2, Transforming growth factor-beta 2; TGFBR1, Transforming growth factor-beta receptor type 1; TIMP1, TIMP metallopeptidase inhibitor 1; TLR4, Toll-like receptor 4; tMCAO, Transient middle cerebral artery occlusion; tPA, Tissue plasminogen activator; VCAM1, Vascular cell adhesion molecule 1; VEGFA, Vascular endothelial growth factor A; VIP, Vasoactive intestinal polypeptide; VPAC1, Vasoactive intestinal polypeptide receptor 1; VPAC2, Vasoactive intestinal polypeptide receptor 1.

MATERIAL AND METHODS

Animals

Two-month-old C57BL/6 (27.3 ± 2.3 g; Janvier Labs) male mice, kept in a humidity- and temperature-controlled environment under an established photoperiod with free access to food and water, were used in this study. Experiments were approved by the regional committee of ethics for animal experimentation (CENOMEXA; approval number #7619-2016101417048165) and conducted in an accredited animal facility (C7645104),

according to the recommendations of the European Union under the supervision of authorized investigators.

Reagents

PACAP38 was synthesized using solid phase strategy combined with the Fmoc chemistry methodology as previously described (34). For administration, PACAP was dissolved in a 0.9% NaCl solution.

tMCAO and pMCAO Chirurgical Procedures

Occlusion was performed on the MCA, because approximately 70% of the human ischemic strokes affect this artery (10).

Transient middle cerebral artery occlusion (tMCAO) was induced under general anesthesia (isoflurane 1.5% to 2% infused by air) by occlusion of the right MCA by the mean of the intraluminal filament technique (35). Briefly, a nylon thread (0.1 mm in diameter) with a distal cylinder (1 mm in length and 0.18 mm in diameter) was inserted into the lumen of the internal carotid artery and advanced to the origin of the MCA. The nylon thread was removed 45 min later to allow reperfusion. To monitor the occlusion, a laser-Doppler flowmeter probe (0.7 mm in diameter, FloLab Moor Instruments) was positioned and glued on the right parietal bone, after skin and muscle incision, and then CBF was measured continuously before the occlusion and up to 10 min after reperfusion in order to monitor the efficiency of the occlusion and success of the reperfusion. Based on those laser-Doppler flowmeter recordings, 11% of the operated animals were excluded from further analysis. For sham-operated animals, the nylon thread was put in place but not pushed to the origin of the MCA. Each treated mouse received 1 h after waking up and 24 h later, 500 µl of NaCl 0.9% by subcutaneous injection to limit dehydration.

Permanent middle cerebral artery occlusion (pMCAO) was induced under general anesthesia (isoflurane 1.5% to 2% infused by air) using bipolar-forceps connected to a high frequency coagulation generator (KLS Martin ME 102). Briefly, skin was incised between the ear and the eye, the temporal muscle was detached from the skull without totally removing it and the MCA was localized below the transparent skull. The bone was carefully withdrawn above the artery. Once the MCA exposed, the artery was electrocoagulated with bipolar forceps. After checking the absence of recanalization, the temporal muscle was replaced and wound sutured. No electrocoagulation was performed on sham operated mice.

Following MCAO, mice were randomized in 3 groups, i.e., NaCl group (composed of ischemic mice treated with 0.9% NaCl), a Sham group (composed of mice having undergone all the surgical procedure but without occlusion of the MCA and treated with 0.9% NaCl) and a PACAP group (composed of ischemic mice treated with various concentrations of PACAP according to the different protocols).

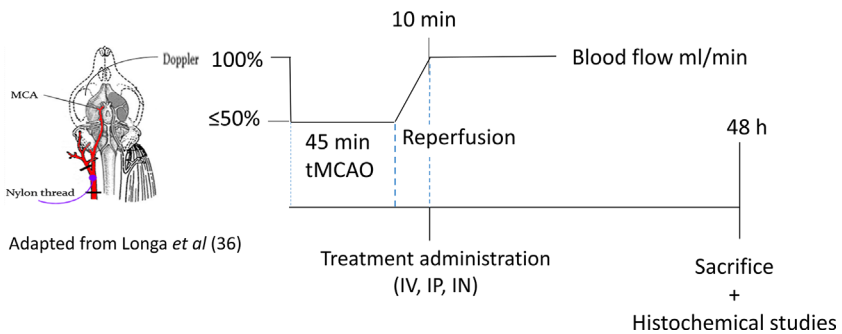
Behavioral tests were performed 2 and 8 days after stroke. Brains were collected at different time points in order to perform histological experiments to measure infarct volumes and/or to quantify gene expression.

PACAP Administration Routes

After tMCAO, mice were randomized into four groups corresponding to the different types of treatment: NaCl group (control), IV group, IP group, and IN group. Ten minutes after reperfusion, mice from the control group (Ctrl, n = 8) received 200 µl of NaCl intravenously; those from the IV group (IV, n = 7) received 200 µl of PACAP at the concentration of 0.02 µg/kg with 50% of the dose given as a bolus while the rest was provided as a 30-min infusion; those from the intraperitoneal (IP) group (IP, n = 8) received 200 µl of PACAP as a bolus at a concentration of 0.02 µg/kg; and finally those from the intranasal (IN) group (IN, n = 7) received 10 µl of PACAP solution at a concentration of 1 µg/µl. Forty-eight hours after reperfusion brains were collected for morphological analysis and gene expression studies (**Scheme 1**). The choice of these doses was based on the most efficient dose reported by Dejda et al. for IV injection (28) and the one used by Rat et al. for IN administration (23).

PACAP Therapeutic Window

After tMCAO, mice were randomized into five groups corresponding to the different times of treatment after stroke: a NaCl group who received 10 µl of NaCl intranasally 10 min after reperfusion and 4 groups who received 10 µl of PACAP at a concentration of 1 µg/µl intranasally 10 min, 1 h, 6 h, or 15 h



SCHEME 1 | The schema of PACAP administration routes.

after reperfusion. Forty-eight hours after reperfusion brains were collected for further analysis (**Scheme 2**).

PACAP Dose Response Efficiency

Animals were divided into NaCl, Sham and PACAP groups. PACAP animals were treated with 4 different concentrations of peptide ranging from 1 µg/µl to 1 fg/µl. Excepted sham animals, all other mice were subjected to permanent occlusion of the middle cerebral artery (pMCAO). One hour after cerebral ischemia animals received a single IN administration of 10 µl of PACAP or Nacl. Behavioral studies were conducted 48 h after treatment, just before animals were sacrificed and brains collected (**Scheme 3**).

PACAP Delayed IN Daily Administration Efficiency

To determine whether delayed IN delivery of PACAP durably improves functional recovery after cerebral ischemia, mice received, 6 h after pMCAO, an IN injection of a 0.9% NaCl solution (control, sham) or of a PACAP solution (1 µg/µl or 1 ng/µl). Such IN administration was repeated daily until day 6 (D6).

Behavioral studies were performed 48 h (on day 2; D2) and 192 h (on day 8; D8) after pMCAO. The animals were sacrificed on day 8 and brains collected (**Scheme 4**).

Functional Recovery Assessment

The **walking fault test** was used to detect deficits in motor skills and balance (36). In this test, the mouse had to walk across an elevated wooden beam (1 m long and 9 mm of diameter) to reach a safe platform (home cage). The time to cross the beam and the number of walking faults were scored. The **adhesive removal test** was used to assess sensorimotor deficits (37). Performance on this test was quantified by measuring the time taken by the mouse to detect the presence of the adhesive (first contact) and the time taken by the mouse to remove it from both paws.

Infarct Volume Measurement

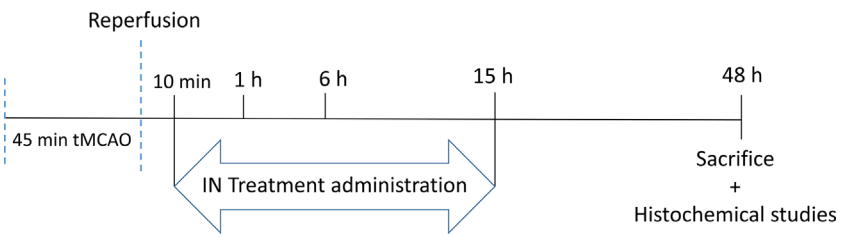
After sacrifice, brains were collected and frozen in cooled isopentane for cryosectionning. Twenty-micron thick sections were mounted on slides and stained with cresyl violet according to the Kapelsohn's method. The slides were scanned and the measurement of infarct volume was performed by using ImageJ program. Infarct volumes were then calculated as follows:

$$v_c = v_i \left[v_i - \left(\frac{v_{ih}}{v_{ih}} - v_{\text{ch}} \right) \right]$$

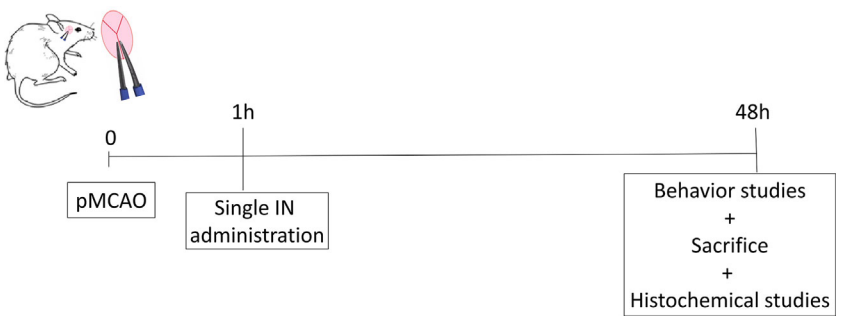
v_c, corrected volume; v_i, infarct volume; v_{ih}, ipsilateral hemisphere volume; and v_{ch}, controlateral hemisphere volume.

Animal Weight Monitoring and Food Intake

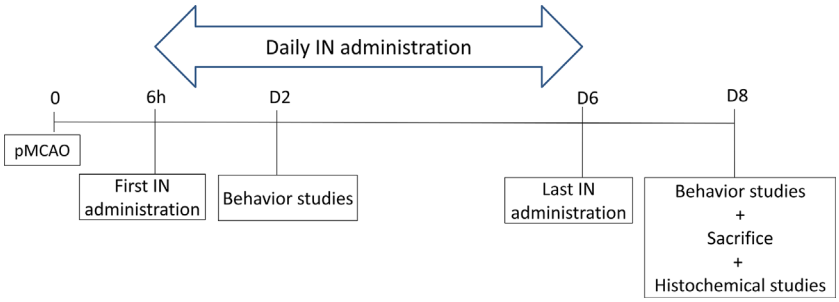
Animals body weight was assessed before MCAO surgery (day 0) and then daily until sacrifice. For the food intake experiments, animals have been fasted overnight. After NaCl or PACAP (10 µg/µl) IN administration, pre-weighted pellets were provided and then food consumption was recorded every 30 min for 3 h using metabolic cages.



SCHEME 2 | The schema of PACAP therpauetic window.



SCHEME 3 | The schema of PACAP dose response efficiency.



SCHEME 4 | The schema of PACAP delayed IN daily administration efficiency.

Gene Expression Analysis (qPCR)

Some brain tissue from ipsilateral hemispheres was collected from a series of crysotat's brain sections (20-µm thick) in TRI-reagent (Sigma) and RNAs were extracted according to the manufacturer's protocol. RNAs were further purified with the NucleoSpin® RNA II kit (Macherey-Nagel) and total RNAs were reverse transcribed into cDNA with the Promega® ImProm-II Reverse Transcription System kit. Real-time polymerase chain reaction was performed on a QuantStudio 12K Flex (Life Technologies) to quantify the expression of genes potentially regulated by ischemia.

Statistical Analysis

Results are expressed as mean ± SEM ($n \geq 6$ animals per group). We used ANOVA tests followed by *post hoc* tests for statistical analysis thanks to GraphPad Prism 6. A value of $P^* < 0.05$ was considered as significant.

RESULTS

PACAP Administration Route

In the present study, the efficiency of IV administration, which has already been used in previous stroke studies (28–39), was compared with IP and IN administration routes. The results show that PACAP IN administration 10 min after reperfusion induced a three-fold reduction of the infarct volume (**Figure 1A**). This neuroprotective action of a PACAP IN delivery was much more efficient than the IV treatment, while the IP administration had no effect (**Figure 1B**).

PACAP Therapeutic Window

In a second step, the neuroprotective effect of a delayed single IN PACAP administration 10 min, 1 h, 6 h and 15 h after reperfusion was investigated in a tMCAO mice model. Those experiments were conducted using a single IN administration of 10 µl of PACAP at a concentration of 1 µg/µl, which was, as indicated in **Figure 1B** the most efficient. The results show that 10 µl of PACAP at a concentration of 1 µg/µl induced 48 h after the occlusion a decrease of the lesioned volume of 72.8%, 38.7%, and 22.1% compared to NaCl treated animals when administered 10 min, 1 h, or 6 h after reperfusion, respectively (**Figure 1C**). In contrast, when IN administration of PACAP was conducted 15 h after reperfusion, the peptide had no more effect.

Gene Expression (qPCR)

Stroke is a complex multifunctional physiopathology and MCAO activates a diversity of cellular and molecular mechanisms related to excitotoxicity, oxidative stress, post-ischemic inflammation and apoptosis (40, 41). To analyse and compare the different mechanisms potentially involved in the effects of PACAP IN administration 10 min, 1 h, 6 h, and 15 h after reperfusion in a tMCAO mice model, a transcriptomic analysis was performed by measuring the expression of 88 genes known to be regulated after stroke. Among them, 42 were indeed regulated 48 h after tMCAO in one of the experimental conditions (**Table 1**). The results indicate that 64% of these 42 genes were regulated by stroke with only 4 genes repressed, all the others being increased. Among the 27 genes regulated by stroke, 89% were also regulated by PACAP after stroke in an opposite manner, showing that the peptide exerts a strong anti-excitotoxicity, antioxidant, anti-inflammatory, and anti-apoptotic effect as evidenced by its ability to inhibit the expression of genes such as GJB6, NOS-1, IL6, BCL10, and caspase-9 (CASP9) which were induced by stroke. On the opposite, PACAP could counteract the decrease of genes such as Vegfa involved in vascular remodelling after stroke. PACAP also upregulated the brain expression of 15 genes not affected by stroke such as NEUROD1 or BDNF but known to promote synaptic plasticity and which contribute to improve functional recovery. Among the 42 genes of our list, only 4 were not regulated by PACAP, 24 being regulated when PACAP was injected 10 min after reperfusion, 18 when PACAP was injected 1 h after reperfusion, 19 when PACAP was injected 6 h after reperfusion and 17 when PACAP was injected 15 h after reperfusion. This highlights that most genes were regulated at different time points of PACAP treatment even if an early injection of PACAP after stroke affects more genes. Some genes such as CASP9, Tpa, or IL6 are regulated by PACAP regardless when the peptide is administered. Few genes such as Homer 1, not affected by PACAP at early times of treatment can be regulated when the peptide is administered in a delayed

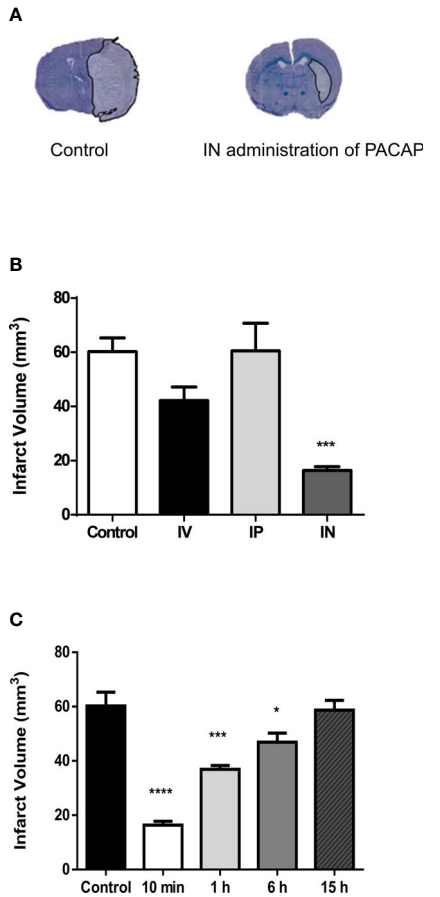


FIGURE 1 | Effect on cerebral infarct volume of PACAP administration 2 days after tMCAO. **(A)** Illustration of the brain infarct area after NaCl (Control) or PACAP (IN administration of PACAP) intranasal administration, 48 h after tMCAO. **(B)** Quantification of the brain infarct volume of mice treated 10 min after the reperfusion by intranasal administration of NaCl (0.9%; Ctrl; n = 7), intravenous administration of PACAP (200 µl at a concentration of 0.02 µg/kg with 100 µl in the form of a bolus and then 100 µl by infusion over a 30-min period; IV; n = 7), intraperiotoneal administration of PACAP (200 µl in the form of a bolus at a concentration of 0.02 µg/kg; IP; n = 8) or intranasal administration of PACAP (10 µg in 10 µl; IN; n = 7), 48 h after a 45-min tMCAO. **(C)** Quantification of the brain infarct volume of mice treated 10 min (n = 8), 1 h (n = 8), 6 h (n = 8) or 15 h (n = 7) after reperfusion by an intranasal administration of PACAP (10 µg in 10 µl; IN). Control animals (Ctrl; n = 7) received 10 µl NaCl 0.9% IN administration. Volume was expressed in mm^3. ANOVA followed by the Bonferroni's test. *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$ vs. Ctrl.

manner and vice versa, few genes such as Hmox1 or Gjd2 are only regulated by PACAP when it is administered to the animals just after the reperfusion.

PACAP Dose Response Efficiency

After putting in evidence the efficiency of an IN administration of PACAP for stroke treatment and the existence of a therapeutic window of a least 6 h in the tMCAO mice model, we looked for the most efficient dose of the peptide. For those experiments, the neuroprotective effect of a single IN administration of different concentrations of PACAP ranging from 1 µg/µl to 1 fg/µl, 1 h after permanent middle cerebral artery occlusion (pMCAO) was tested. The choice of using a pMCAO mice model was both to evaluate the efficiency of the treatment in another commonly used stroke model as recommended by the STAIR guidelines and to reduce the number of animals used, given that reproducibility and survival rate in the pMCAO is higher than in the tMCAO. Effectively we recorded that the mortality rate in tMCAO model (20%) was approximately twice higher than in the pMCAO one. The other advantage of the pMCAO model is that it produces a smaller infarct area more in line with what is observed in human stroke (42). Results highlight that a single IN administration of very low concentrations of PACAP decreased infarct volume (**Figures 2A, B**) when administered 1 h after stroke in a pMCAO mice model. Effectively, 48 h after the occlusion, an 86.3% reduction of the infarct volume compared to controls was observed when animals received 1 µg/µl IN PACAP treatment (**Figures 2A, B**). This protection diminished when decreasing the doses of PACAP, with a reduction of the infarct volume of only 24.6% for animals receiving 1 fg/µl of PACAP (**Figure 2B**). In addition, to these histological effects, PACAP also improved functional recovery (**Figures 2C, D**). In particular, PACAP administered 1 h after pMCAO decreased the number of slips from 16.3 ± 0.6 for control mice to 6.6 ± 0.3 for animals treated with the highest concentration of PACAP (**Figure 2C**). PACAP also reduced the time spent to cross the beam from 29.6 ± 0.9 s for control mice to 18 ± 1.6 s for animals treated with 1 µg/µl PACAP. Finally, PACAP treatment enhanced performances in the adhesive removal test compared to control animals after pMCAO with asymmetrical symptoms (**Figure 2D**). It is interesting to note that several of those tests revealed functional improvement even with the lowest doses of PACAP (**Figures 2C, D**).

PACAP Delayed IN Daily Administration Efficiency

The efficacy of an IN administration of PACAP at very low doses in a pMCAO mice model being demonstrated, we tested whether the treatment is still efficient and persistent when started 6 h after ischemia in the pMCAO mice model. For those experiments, animals received 1 µg/µl or 1 ng/µl daily administration of PACAP. The results show that at day 8, the infarct volume of 3.8 ± 0.5 mm^3 for mice from the control group was reduced to 2.6 ± 0.6 and 1.5 ± 0.2 mm^3 for animals treated intranasally with 1 µg/µl and 1 ng/µl of PACAP, respectively (**Figures 3A, B**). Such treatments also improved the sensorimotor performances by reducing on the beam test, the number of slips and the time spent to cross the beam both 2 and 8 days after the ischemia (**Figures 3C, D**) and by reducing for the adhesive removal test, the time spent to feel the presence of the adhesive and to withdraw it from both forepaws (**Figures 3E, F**). In most pMCAO mice, an asymmetrical symptom was observed with firstly withdrawal of the adhesive from the right paw, which is

TABLE 1 | Effect of acute and delayed IN PACAP administration after reperfusion (tMCAO protocol) on gene expression measured 48 h after the occlusion.

Events	Gene	ctrl vs sham			10 min vs ctrl			1 h vs ctrl			6 h vs ctrl			15 h vs ctrl		
			SEM	stat		SEM	stat		SEM	stat		SEM	stat		SEM	stat
Excitotoxicity	GJD2	↘ **0.22**	**0.09**	***	↗**7.88**	**0.19**	****	↗1.83	0.1		↗1.01	0.05		↗1.66	0.12	
	GJB6	↗**1.29**	**0.06**	**	↘**0.34**	**0.07**	****	↘**0.72**	**0.12**	***	↗1.45	0.34		↗1.26	0.14	
	GAD1	↘0.88	0.19		↘0.378	0.44		↗**2.49**	**0.21**	**	↗**2.13**	**0.14**	*	↗**2.52**	**0.28**	**
Stress Response	CAT	↗1.56	0.38		↘**0.80**	**0.03**	***	↘**0.30**	**0.13**	**	↗1.04	0.14		↘**0.40**	**0.09**	**
	GPX1	↗**1.78**	**0.34**	*	↘0.58	0.4		↘0.10	0.32		↗1.157	0.17		↘0.61	0.07	
	NOS1	↗**2.26**	**0.18**	**	↘**0.46**	**0.21**	**	↘**0.50**	**0.14**	**	↘**0.54**	**0.22**	**	↘**0.58**	**0.19**	*
	HMOX1	↗**19.55**	**1.25**	***	↘**0.47**	**0.49**	**	↘0.84	3.57		↘0.87	1.52		↗1.02	2.97	
	JUN	↗**1.53**	**0.26**	*	↘0.83	0.19		↘0.30	0.14		↘0.87	0.31		↘**0.53**	**0.06**	**
	HSPA1B	↗**3.20**	**0.43**	*	↘0.40	0.53		↘0.42	0.1		↘0.92	0.58		↘0.61	0.38	
	HSPB1	↗**4.42**	**0.64**	**	↘0.77	0.93		↗1.04	0.45		↗1.09	0.44		↘0.72	0.58	
	HSPD1	↗**2.04**	**0.06**	*	↘**0.73**	**0.14**	*	↘0.59	0.16		↘0.81	0.31		↘0.87	0.27	
Apoptosis	DDIT3	↘0.78	0.23		↗1.05	0.23		↗1.11	0.16		↗**2.18**	**0.09**	**	↘0.844	0.25	
	DUSP6	↘0.75	0.26		↗1.73	0.41		↗**2.56**	**0.36**	*	↗**2.72**	**0.34**	*	↗1.66	0.2	
	BCL10	↗**1.90**	**0.05**	*	↘0.65	0.31		↘0.993	0.12		↘**0.17**	**0.03**	***	↘0.81	0.22	
	FAS	↗1.31	0.18		↗**2.89**	**0.21**	*	↗**3.39**	**0.65**	**	↗2.34	0.26		↗1.80	0.27	
	CASP9	↗**2.21**	**0.08**	****	↘**0.34**	**0.02**	****	↘**0.50**	**0.09**	***	↘**0.10**	**0.04**	****	↘**0.49**	**0.2**	***
	GADD45A	↘0.63	0.42		↗**2.34**	**0.19**	**	↗**2.90**	**0.24**	***	↘0.90	0.04		↗**1.97**	**0.15**	*
	ATF3	↗**37.34**	**2.24**	**	↘**0.16**	**0.07**	*	↗**2.64**	**4.2**	***	↗1.04	8.65		↗1.16	2.82	
	PIK3R1	↗1.05	0.11		↘0.78	0.27		↘**0.36**	**0.07**	**	↗1.10	0.13		↘0.65	0.09	
Inflammation	IL12A	↗2.20	0.64		↘0.49	0.55		↘0.92	0.41		↘**0.24**	**0.14**	*	↘0.84	0.2	
	IL6	↗**4.68**	**0.1**	****	↘**0.23**	**0.05**	****	↘**0.445**	**0.24**	****	↘**0.16**	**0.15**	****	↘**0.33**	**0.52**	****
	IFNAR1	↗**3.87**	**0.34**	*	↘**0.12**	**0.06**	*	↘0.63	0.32		↘**0.27**	**0.13**	*	↘0.48	0.85	
	IRF1	↗**2.74**	**0.52**	**	↘**0.49**	**0.17**	*	↘**0.47**	**0.18**	**	↗1.11	0.26		↘**0.43**	**0.21**	**
	IRF5A	↗**3.66**	**0.94**	*	↘**0.51**	**0.07**	*	↘0.73	0.98		↗1.48	0.58		↘0.44	0.27	
	CXCR4	↗**4.47**	**0.47**	***	↘**0.44**	**0.50**	*	↘**0.458**	**0.37**	*	↗1.09	0.57		↘**0.46**	**0.53**	*
	CXCR1	↗1.03	0.08		↘0.95	0.02		↗1.59	0.24		↗**1.99**	**0.44**	*	↗1.47	0.14	
	VCAM1	↘0.60	0.57		↗1.71	0.48		↗**1.47**	**0.2**	*	↗1.51	0.08		↗**1.96**	**0.13**	**
	TLR4	↗**4.06**	**0.33**	****	↘**0.58**	**0.28**	**	↘**0.78**	**0.03**	*	↘0.85	0.39		↘**0.52**	**0.23**	****
Synaptic/neurogenesis/Angiogenesis/ cellular protection and activity	NEUROD1	↘0.48	0.55		↗**2.07**	**0.32**	**	↗1.08	0.1		↗**2.28**	**0.1**	*	↗1.32	0.04	
	BDNF	↘0.52	0.39		↗1.33	0.12		↗6.07	0.95		↗**3.31**	**0.21**	**	↗1.35	0.18	
	SLC16A7	↘0.74	0.18		↘0.20	0.03		↘0.60	0.16		↘0.99	0.21		↗**1.91**	**0.18**	*
	DBI	↘**0.37**	**0.06**	**	↗**2.83**	**0.46**	*	↘0.99	0.1		↗1.26	0.09		↗1.53	0.14	
	VEGFA	↘**0.47**	**0.15**	**	↗**1.75**	**0.27**	**	↗1.19	0.1		↗**2.32**	**0.08**	***	↘0.93	0.07	
	TGFB2	↗1.02	0.15		↗1.55	0.26		↗1.17	0.08		↗1.27	0.11		↗**1.76**	**0.33**	*
	TGFB1	↗**6.46**	**0.66**	****	↘**0.40**	**0.06**	****	↘**0.49**	**0.95**	**	↘**0.54**	**0.47**	***	↘**0.16**	**0.32**	****
	TGFBR1	↗1.25	0.17		↗1.73	0.52		↗1.44	0.08		↗1.30	0.18		↘0.83	0.24	
	GFAP	↗**21.61**	**3.71**	***	↘**0.42**	**1.03**	**	↘0.80	3.13		↘0.86	1.88		↘**0.52**	**3.46**	*
	HOMER1	↘**0.27**	**0.53**	*	↘0.17	0.09		↗1.72	0.12		↗**3.59**	**0.25**	*	↗1.62	0.16	
	TIMP1	↗**46.90**	**8.63**	***	↘0.75	0.59		↘0.74	2.56		↘**0.36**	**1.85**	**	↘**0.456**	**4.55**	*
	TPA	↗**7.95**	**0.57**	****	↘**0.17**	**0.03**	****	↘**0.52**	**1.32**	**	↘**0.17**	**0.21**	***	↘**0.334**	**1.04**	***
	AQP4	↗**2.86**	**0.22**	**	↘**0.61**	**0.23**	*	↘0.59	0.19		↘**0.43**	**0.27**	*	↘0.95	0.61	
	ACVR1	↗**2.33**	**0.22**	**	↘**0.34**	**0.08**	**	↘**0.36**	**0.23**	**	↘**0.28**	**0.17**	**	↘0.36	0.33	

*PACAP was administered 10 min, 1 h, 6 h, or 15 h after reperfusion. Ctrl corresponds to animal subjected to tMCAO and sham corresponds to operated animals who were not subjected to tMCAO. Results are expressed in relation to the ones of sham ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).*
The arrow also indicate a up and down gene regulation.

controlled by the ipsilateral hemisphere, before elimination in a second time of the adhesive from the left paw, which is controlled by the contralateral hemisphere.

Food Intake and Body Weight Variation

The anorexigenic effects of PACAP have mainly been studied in various species such as chickens (43), fishes (44, 45), and rodents (46, 47), using different routes of administration from systemic ones such as IV or IP to direct brain administrations such as ICV or amygdala central nucleus injections. In the present study the effect of IN administration of PACAP was evaluated on body weight variation and food intake. The results showed no significant differences between control and PACAP treated groups in terms of food intake (**Figure 4A**). Interestingly, weight loss was usually slightly less important after PACAP IN acute or delayed administration, 2 and 4 days after pMCAO (**Figure 4B**). A significant decrease in the weight loss was even observed in the pMCAO model after 8 days of 1 ng/µl PACAP daily IN administration. Those experiments also highlight that the weight loss is significantly more important for the mice subjected to a

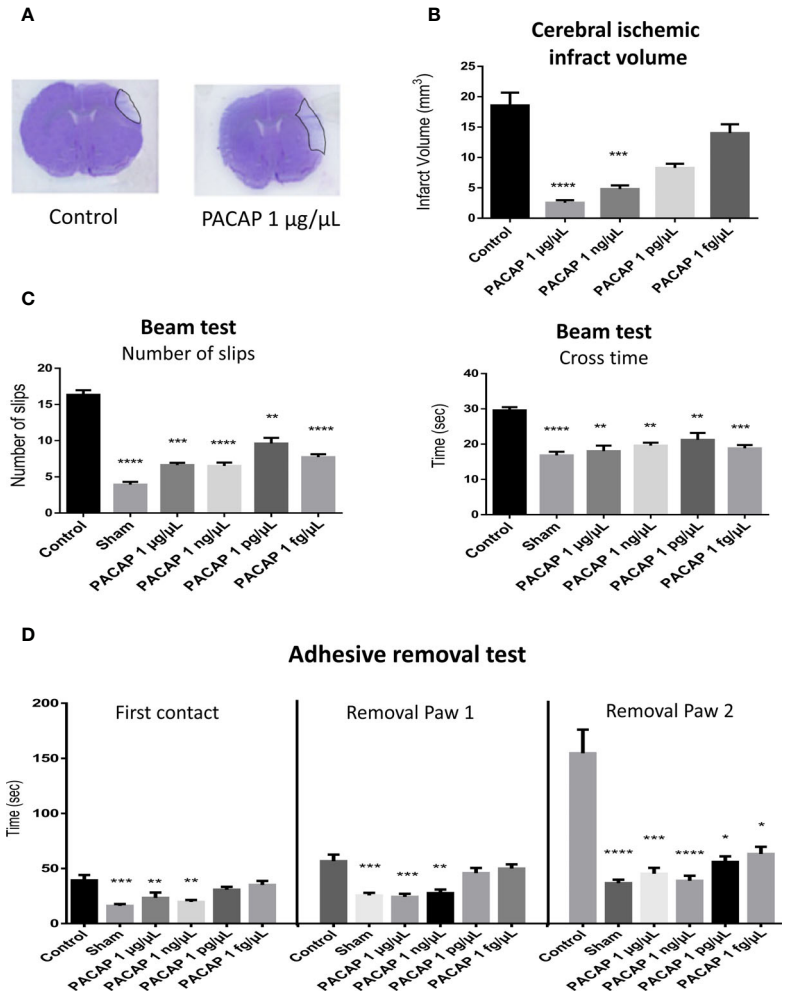


FIGURE 2 | Effects on the infarct volume and functional recovery, 2 days after stroke, of a single intranasal (IN) administration of decreasing concentrations of PACAP provided 1 h after pMCAO. Male C57BL6 mice were treated with a saline solution (NaCl 0.9% for control and sham) or with 10 µl of different PACAP concentrations (1 µg/µl, 1 ng/µl, 1 pg/µl, and 1 fg/µl dissolved in NaCl 0.9%). **(A)** Illustration of the brain infarct zone after NaCl (Control) or PACAP 1 µg/µl IN administration, 48 h after pMCAO. **(C)** Cerebral ischemic infarct volume (mm^3) 48 h after pMCAO. **(C)** Beam test indicating cross times and number of slips of mice on 9-mm beam. **(D)** Adhesive removal test with the time taken for first contact and adhesive removal from mice paws 2 days after stroke. PACAP treatment promoted functional recovery and reduced cerebral ischemic lesions in stroke mice. Variations of n ≥ 6 animals per group are reported as S.E.M. (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).

tMCAO than for the animals included in the pMCAO protocol, which is probably due to the heavier surgery that represents the intraluminal filament technique, comforting the necessity to use various stroke models which have different outcomes that may interfere differently with the subsequent treatment.

DISCUSSION

Stroke is the second leading cause of death worldwide and the leading cause of disability in adults. The complex processes involved in stroke induced brain damages explain the difficulties to develop effective pharmacological agents, making it a major health problem. Thus so far, reperfusion is the only approach really recommended but its use is restricted because of cost, short therapeutic window or risks of causing a hemorrhage, so that it can only benefit to a small proportion of stroke victims. This therefore led over the last couple of years to intense research targeting some of the numerous mechanisms activated after brain artery occlusion such as excitotoxicity, oxidative stress, inflammation and apoptosis in order to block neuronal cell death and/or stimulate neurogenesis, synaptogenesis and angiogenesis in order to promote functional recovery. However, most trials so far have failed, probably because molecules could only target part of stroke induced deleterious pathways. In this context, PACAP may be a promising molecule as it could counteract most if not all the deleterious processes induced by stroke and improve functional

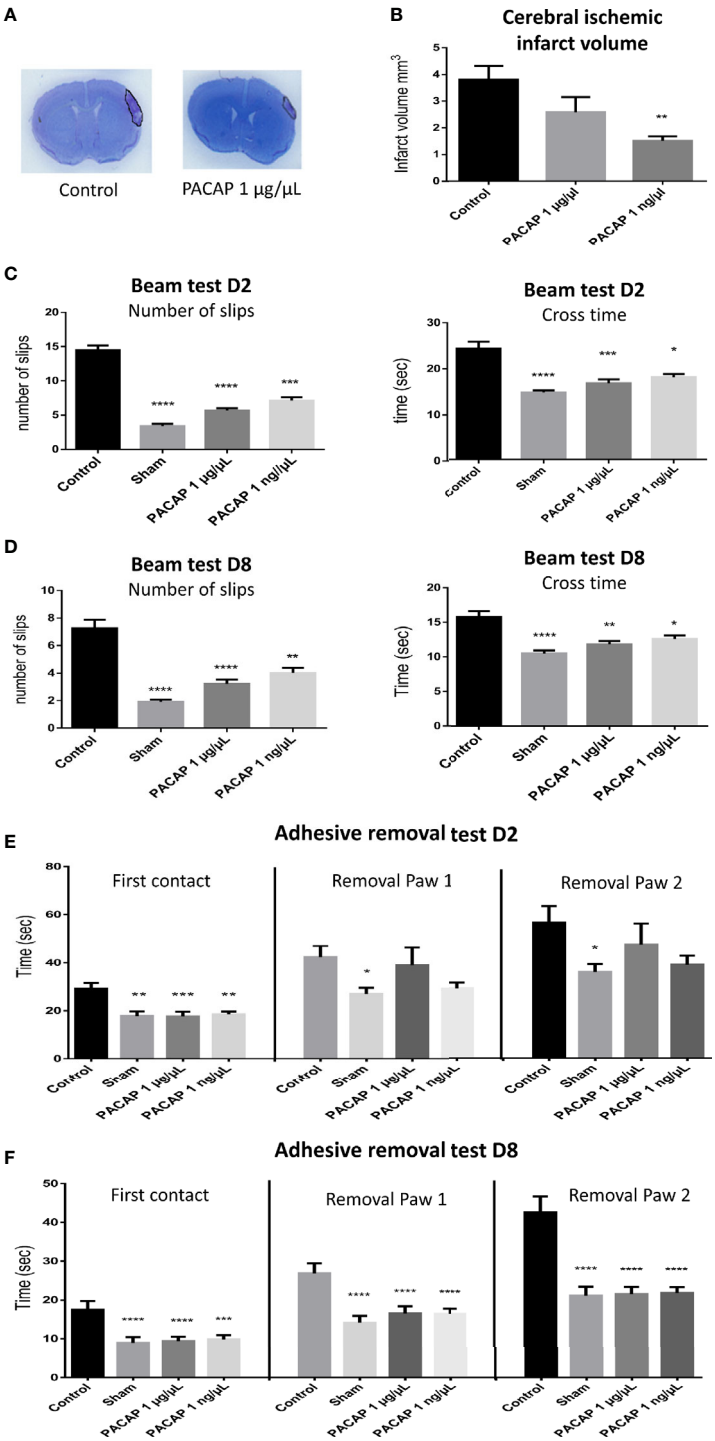


FIGURE 3 | Effects on the infarct volume and functional recovery of delayed (6 h post pMCAO) PACAP daily administration, 2 and 8 days after brain ischemia. Male C57BL6 mice were treated with a saline solution (control, sham) or with different concentrations of PACAP. **(A)** Illustration of the brain infarct zone after NaCl (control) or PACAP 1 µg/µl intranasal administration, 8 days after pMCAO. **(B)** Cerebral ischemic infarct volume (mm^3) 8 days after pMCAO. **(C, D)** Beam test indicating cross times and number of slips of mice (sham and stroked animals) on 9 mm beam 2 and 8 days after stroke. **(E, F)** Adhesive removal test with the time taken for first contact and adhesive removal from mice paws 2 and 8 days after stroke. PACAP treatment promoted functional recovery and reduced cerebral ischemic lesions in stroke mice. Errors are reported as Mean ± S.E.M and n ≥ 8 animals per group. (*P < 0.05; **P < 0.01; ***P < 0.001, ****P < 0.0001).

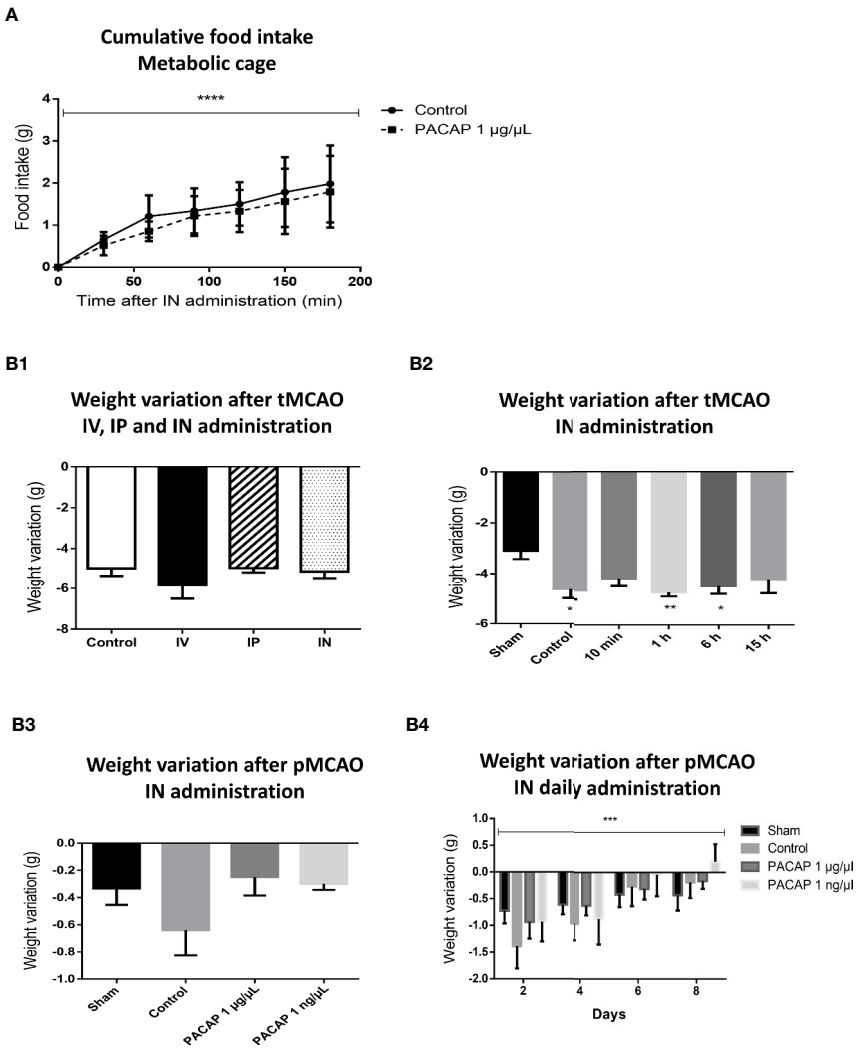


FIGURE 4 | Effects of stroke and/or PACAP treatment on cumulative food intake and body weight variation. **(A)** Cumulative food intake after PACAP IN delivery over 3 h of time in non-operated mice. **(B)** Variation of body weight after PACAP administration following different stroke protocols, i.e., PACAP administration routes protocol (B1.), PACAP therapeutic window protocol (B2.), PACAP dose response efficiency protocol (B3.) and PACAP delayed IN daily administration efficiency protocol (B4.). Errors are reported as Mean ± S.E.M. (*P < 0.05; **P < 0.01; ***P < 0.001). Two-way ANOVA was performed for figure A and B4 showing significant means only for time factor (minutes and days), while the effects of treatment and the interaction are not significant.

recovery in various MCAO models (39–48). However, to consider the use of PACAP in clinic, several points remain to be evaluated and/or improved. Indeed, PACAP, acting *via* 3 different receptors (PAC1, VPAC1, and VPAC2) widely distributed in the brain and peripheral organs, regulates numerous biological functions, including hormone release or systemic decrease of blood pressure. It seems to be the VPAC2 receptor, highly expressed in the cardiovascular system and endocrine glands that is at the origin of most of the deleterious side effects of PACAP. Conversely, VPAC2 expression is very low in the brain (14).

In addition, although PACAP remains active after IV administration by passing the blood-brain barrier (BBB), it is very quickly degraded in blood with a half-life of less than 5 min due to a high sensibility to dipeptidyl-peptidase-IV (DPP-IV), endopeptidases, and carboxypeptidases. In particular, PACAP is hydrolyzed into PACAP3-38 acid amins and PACAP5-38 to create a shorter peptide with antagonist activity which may limit the effect of the treatment (34, 49, 50).

PACAP Administration Routes

Various administration routes of PACAP have already been reported from IP to intravitreal administration (16–51), leading either to systemic or topical delivery of the peptide. This diversity of PACAP administration routes led us to compare the efficiency of a single intranal (IN), IV, and IP administration of the peptide after tMCAO in C57Bl/6 mice. The results

revealed that the IN PACAP delivery led to the stronger neuroprotection, which could be explained by the fact that the quantity of PACAP able to reach the brain is variable, depending on i) the amount provided and ii) the route of administration. It is also likely that IN administration induces a direct delivery of PACAP to the brain through a rapid absorption *via* the nasal mucosa with highest uptake of the peptide in the occipital cortex and striatum (52). At the same time, IN delivery reduces systemic exposure, decreasing by the way peripheral side effects, in particular on the cardiovascular system.

Food Intake and Weight Variation

It is well established that PACAP regulates food intake and energy homeostasis through both central and peripheral action. In particular, PACAP injected centrally into the hypothalamic ventromedial nucleus or into the posterior region of the stria terminalis bed nucleus induced a rat weight loss 24 h post injection (53, 54). Also, an IP administration of PACAP to PAC1-deficient and wild-type mice shows that PACAP suppresses appetite *via* its PAC1 receptor by inhibiting ghrelin and increasing GLP-1 and leptin (55). The anorexigenic effect was also observed after IV and ICV administration of high concentrations of PACAP (56, 57). Although our results seem to be in contradiction with these previous reports, the discrepancy might be due to differences in the amount of PACAP which reaches the various brain regions after IN administration. It is estimated that less than 4% of the 10 μg of PACAP administered to the mice actually reach the brain (52). Furthermore, the main brain area playing a role in appetite regulation and energy homeostasis are hypothalamus and amygdala (58, 59) while the IN administration tend to promote peptide uptake toward the occipital cortex and striatum (52). In addition to those experiments on food intake, further thorough tests must be done to confirm the innocuity of this efficient, non-invasive and easy to set up IN administration of PACAP (60, 61). Moreover, this approach has started to be used with PACAP for the treatment, in animal models, of some neurodegenerative diseases such as Alzheimer or Huntington (23, 27, 62).

PACAP Therapeutic Window

The present results demonstrate that the therapeutic window of PACAP in mice could exceed 6 h. This observation is very promising when considering that rtPA, the approved medical treatment for acute ischemic stroke, has a therapeutic window of only 2 h in mice (63). Considering that in clinic rtPA therapeutic window reaches 4.5 h, we can extrapolate that in human, PACAP could still block development of the infarct area when injected 12 h after the ischemic event.

In mice, 15 h after reperfusion the infarct area is largely consolidated (64), so it is not surprising that PACAP has no more ability to reduce the lesion volume. Nevertheless, the gene expression data suggest that a delayed administration of PACAP after stroke could still shift the inflammatory response and stimulate synaptogenesis. These data correlate with the observation that injection of PACAP-producing stem cells, 3 days after permanent focal ischemia, do not reduce the ischemic lesion volume but shift the inflammatory response from a M1 to M2 phenotype and promote functional recovery (31).

PACAP Dose Response Efficiency

In accordance with the tMCAO protocol observations, a single IN administration of a very low dose of PACAP in a pMCAO also decreased infarct volume and enhanced sensorimotor performances, in accordance with what was previously reported through other routes of administration [(65, 66) and others referenced higher]. The fact that low doses of PACAP are sufficient to protect the brain from stroke, together with publications showing that PACAP-deficient mice exhibit increased lesions and enhanced neurological deficits (39, 67–69) suggests that any molecule, such as linagliptin, susceptible to increase endogenous PACAP levels could be of interest for the treatment of stroke.

PACAP Delayed IN Daily Administration Efficiency

Administration of PACAP started 6 h after pMCAO and repeated daily for 6 days led to a significant improvement in neurological function recovery and a reduction of the infarct volume. Surprisingly, when PACAP was administered 6 h after ischemia, the most important infarct volume reduction was observed in mice treated with the lowest concentration of PACAP (1 ng/μl/day). These results suggest that a daily reiteration of PACAP administration could at high doses induce desensitization of PAC1 receptors through sequestration away from the membrane (70). Nevertheless, the PACAP treatment remained efficient and this result highlights the potential of low doses of PACAP, which will certainly decrease the risk of side effects.

Gene Expression

Ischemia is known to cause an early wave of glutamatergic excitotoxicity on depolarizing or dying neurons, resulting in breakdown of the homeostatic and water balances. Interestingly, several genes such as GAD1, GJB6, GJD2, and AQP4 known to be induced or repressed by those early events of stroke (71–74) are regulated in an opposite manner by PACAP which contributes to explain how PACAP protects the brain after stroke. For instance, PACAP by inhibiting AQP4, reduces brain oedema, and promotes cell survival after MCAO (75, 76). Likewise, PACAP injections 10 min or 1 h after reperfusion probably inhibit the deleterious action of glutamate by down-regulating GJB6 and up-regulating GJD2 (77). The observed repression of Tpa mRNA expression should also reduce stroke induced NMDA receptor-mediated signaling (78). Conversely, the fact that GAD1 expression is increased when PACAP is injected 1, 6, and 15 h after reperfusion suggests that PACAP also contributes to restore altered GABA levels after stroke (79), which must in turn reduce neuronal hyperexcitability.

Homeostatic disruption and the massive entry of ions inside the cell provoke a reactive oxygen/nitrogen species overproduction such as nNOS and iNOS (80, 81) causing severe intracellular damages to macromolecules and mitochondrial impairment, leading to apoptosis. It is thus interesting to note that PACAP represses the ischemic induction of NOS1 (a gene encoding nNOS)

mRNA expression, which attenuates free radical production and in turn reduces oxidative stress. This may explain that 48 h after reperfusion, the expression of genes from the antioxidant system such as CAT, GPX1 and HMOX1 is reduced in PACAP treated animals. In addition, PACAP induction of GADD45A and GADD153/DDIT3 mRNA expression contributes to repair oxydative stress induced DNA damage (82–84), which reduces the number of cells entering apoptosis. The ability of PACAP to repress the expression of pro-apoptotic genes such as Caspase 9, PIK3R1, ATF3, FAS, or BCL10 and to promote the expression of anti-apoptotic genes such as Dusp6 provides further information on how PACAP prevents neuronal cell death. Of course, other molecular players are also involved in this neuroprotection mechanism. For example the blockage by PACAP of stroke induced c-Jun mRNA expression, supports the idea that it blocks apoptosis through an inhibition of the expression of proteins such as bax and a concomitant stimulation of BCL2 or BCL-XL as already shown in other experimental models (29, 85, 86). Besides its neuroprotective effect, PACAP also reduces reactive astrogliosis as shown by its ability to inhibit stroke induced GFAP and VIM expression. Such effect of PACAP on GFAP and VIM expression decreases post-stroke astrocytic hypertrophy and contributes to brain protection (87–89).

Within minutes to hours after ischemia, an inflammatory response is initiated (41–90) whose intensity is strongly correlated with stroke severity (91). As already shown PACAP can skew the inflammatory response from a deleterious phenotype to a protective one (28, 31, 67). The present results obtained with TLR4, IL6, or IRF1 confirm that PACAP can inhibit most proinflammatory mediators both when given rapidly or in a delayed manner after ischemia.

The ability of PACAP to stimulate the expression of genes such as BDNF, SLC16A7, NEUROD1, VEGFA, HOMER 1, or DBI/ACBP when administered either just after the occlusion or at later time points, highlights the ability of PACAP to not only block stroke induced apoptosis but also to promote subsequent synaptic plasticity and brain regeneration.

Taken together, the present gene expression results highlight the many protective pathways activated by PACAP after stroke both when administered within minutes after the reperfusion but still when provided 15 h after the stroke onset. One of the strengths of PACAP is probably its ability to reduce both neuronal cell death in the acute phase of stroke, to sustainably skew the inflammatory response toward a protective phenotype and to promote during the chronic phase of stroke the release of neurotrophic factors responsible for subsequent neuronal plasticity.

CONCLUSION AND FUTURE PERSPECTIVES

Neuronal cell death triggered by MCAO is initiated by several cellular events including excitotoxicity, free radical damages and inflammation which often activate apoptosis. Current research is focusing on candidate-drugs that may slow or prevent brain ischemic injury. There have been many unsuccessful therapeutic trials in stroke due to a single angle of attack. PACAP by modulating simultaneously different deleterious pathways activated by stroke could be a promising therapeutic molecule for the treatment of brain ischemia. However, the rapid degradation of the peptide and its numerous effects on the body require a method of delivery which targets specifically the brain. In this perspective, the present results showing the high efficiency of an IN PACAP administration are very promising. Future development should focus on the use of PACAP analogs and vectorization methods to increase the half-life of the peptide and its selectivity for PAC1 and VPAC1 receptors to further enhance its neuroprotective action while reducing possible side effects. It will also be interesting to study a potential combination therapy with PACAP and traditional stroke reperfusion methods (rtPA or endovascular thrombectomy) in order to see if PACAP can act as a freezing molecule of the penumbra to enhance the therapeutic window of the already approved reperfusion methods (6).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Regional committee of ethics for animal experimentation (CENOMEXA; approval number #7619-2016101417048165) and conducted in an accredited animal facility (C7645104), according to the recommendations of the European Union under the supervision of authorized investigators.

AUTHOR CONTRIBUTIONS

AC and JM performed the experiments. BL and JL synthesized PACAP. Supervision and conceptualization were performed by DV. AC and DV wrote the manuscript. AC, JM, BL, JL, and DV revised the manuscripts. Final editing was performed by AC and DV. All authors contributed to the article and approved the submitted version.

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