NOVEL THERAPEUTIC POTENTIAL FOR PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP), VASOACTIVE INTESTINAL PEPTIDE (VIP) AND RELATED PEPTIDES IN COGNITION DEFICITS

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EDITORIAL
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Editorial: Novel Therapeutic Potential for Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP), Vasoactive Intestinal Peptide (VIP) and Related Peptides in Cognition Deficits

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Keywords: PACAP, VIP, synaptic plasticity, learning, cognitive deficit

Editorial on the Research Topic

Novel Therapeutic Potential for Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP), Vasoactive Intestinal Peptide (VIP) and Related Peptides in Cognition Deficits

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It is well-known that Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP), Vasoactive Intestinal Peptide (VIP) and related peptides act as neurotrophic and neuroprotective factors in the central nervous system (CNS). The present Research Topic highlights that these peptides also regulate learning and memory. In particular, several of the manuscripts (Ciranna and Costa; Cunha-Reis and Caulino-Rocha; Gilmartin and Ferrara; Johnson et al.; Solés-Tarrés et al.) demonstrate that PACAP modulates synaptic transmission and plasticity in the hippocampus, a brain area crucially involved in learning. It appears that PACAP released by projections from hippocampal hilar mossy cells increases the excitability of dentate gyrus (DG) neurons and modifies contextually-mediated fear memory (Johnson et al.). The circuit involving DG neurons is responsible for fear-related discrimination between similar contexts (Liu et al., 2012), thus a malfunction may disrupt the ability to discriminate between fearful and harmless situations linked to a similar context, a typical symptom of stress-related pathologies such as post-traumatic stress disorder (PTSD). Johnson et al. demonstrates that PACAP plays a crucial role in this process, with important translational implications for PTSD therapy. Interestingly, Gilmartin and Ferrara underlines sex-related differences, with defects in PACAP expression particularly affecting females, which might account for the increased vulnerability of females to PTSD symptoms. The authors also raise open questions concerning the role of PACAP in fear-related learning. As an intriguing speculative hypothesis, PACAP released during stressful events, by enhancing neuronal excitability, might determine which neurons are recruited into a memory circuit.

Cunha-Reis and Caulino-Rocha reviews VIP effects on cognition, illustrating in details the complex hippocampal circuits involving VIP-mediated transmission. This highlights that endogenous VIP is released by a subtype of hippocampal interneurons controlling pyramidal neurons both directly and indirectly through GABAergic interneurons. It appears from their analysis, that a malfunction of VIP-mediated signaling would be responsible for altered excitability

of pyramidal neurons, leading to epilepsy. Therefore, PACAP/VIP receptors are promising targets to prevent hippocampal epileptogenesis and subsequent cognitive decline.

New data are emerging regarding endogenous molecules mediating PACAP/VIP effects in the CNS. In particular, PACAP and VIP stimulate the synthesis of Activity-Dependent Neuroprotective Protein (ADNP), an astroglial-secreted protein playing a crucial role in CNS development (Zusev and Gozes, 2004). Sragovich et al. shows that ADNP regulates cognition since ADNP-deficient mice displayed impaired memory in the Morris Water Maze task, which was rescued by intranasal administration of SKIP, a synthetic peptide derived from ADNP. This finding suggests that cognition might be improved by stimulating ADNP production through the PACAP and VIP system.

Several studies demonstrate that PACAP and VIP are involved in pathologies with cognition impairment, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) (Ciranna and Costa; Solés-Tarrés et al.). Using a transgenic mouse model of AD, Perényi et al. shows cellular damages correlated with deficits of the PACAPergic system on peripheral organs. For instance, Aβ accumulated in kidneys, with a parallel decrease in the expression level of PACAP receptors and of PACAP-activated signaling messengers PKA and CREB. In the same AD model, elevated physical activity protected the kidneys from Aβ accumulation and rescued PACAP receptor expression as well as PACAP-mediated signaling. These results indicate that the neuroprotective action of physical activity in AD might be, at least partly, mediated by PACAP.

Concerning PD, Mosley et al. shows that VPAC2 receptors exert neuroprotection acting through regulatory T cells (Tregs), a subtype of T cells exerting inhibitory control on the immune system. Systemic administration of LBT-3627, a novel selective VPAC2 receptor agonist, increased Treg activity; interestingly, LBT-3627 also reduced brain inflammatory microglia and increased survival of substantia nigra dopaminergic neurons in rat models of PD. Thus, activation of peripheral VPAC2 receptors, by improving Treg function, induced neuroprotection of central dopaminergic neurons. This interesting result opens new challenges to identify the biological cascade underlying peripheral-central crosstalk.

Solés-Tarrés et al. accurately describes the rescue processes of PACAP and VIP in AD, PD, and HD, and decipher the underlying molecular mechanisms, showing the role of each PACAP/VIP receptor subtype. Of note, in AD and HD murine models and PD cellular models, beneficial effects of PACAP are mediated at least in part by stimulation of BDNF production (Rat et al., 2011; Brown et al., 2013; Cabezas-Llobet et al., 2018).

PACAP and VIP deficiency may also contribute to amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) non-motor symptomatology. Administration of PACAP and VIP as a possible therapy of ALS and MS is under investigation with respect to neuroprotective effects; future studies would be important to explore the capacity of PACAP and VIP to fight cognitive decline in ALS and MS (Solés-Tarrés et al.).

Ciranna and Costa suggests that Fragile X Syndrome (FXS) patients might benefit from a treatment based on PACAP receptor activation. FXS is a genetic cause of intellectual disability associated with autism, epilepsy, and mood disorders; no specific cure is presently available. Studies from FXS animal models revealed malfunctions in many intracellular signaling pathways, among which a reduction in cyclic AMP (cAMP) levels and downstream pathways (Kelley et al., 2008). PACAP, a very potent stimulator of adenylate cyclase, rescued hippocampal synaptic plasticity in a FXS mouse model (Costa et al., 2018). Therefore, Ciranna and Costa discusses evidence of downregulation of cAMP signaling in FXS patients and FXS animal models, underlining a novel potential of PACAP for FXS therapy.

To come to the point, articles from the present Topic issue highlight that PACAP, VIP, and related peptides play a role in memory and cognition, thus are very promising candidates for novel therapies of cognitive impairment. In support of future clinical studies, this issue also reviews the most suitable administration routes for PACAP, VIP, and related peptides, as well as novel subtype-selective PACAP/VIP receptor agonists with improved systemic stability (Mosley et al.; Solés-Tarrés et al.; Sragovich et al.). Intranasal administration route of exogenous PACAP and VIP in association with excipients (cyclodextrins) improves bioavailability and favors preferential distribution in specific brain areas, increasing the efficiency of the neuropeptide for CNS treatments while limiting its side effects. Taken together, we believe that the reports from this Research Topic will promote development of clinical research based on PACAP, VIP, and related peptides as pharmacological tools for treatment of cognitive deficits.

AUTHOR CONTRIBUTIONS

LC wrote the first draft of the manuscript. DR, BC, and DV wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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ORIGINAL RESEARCH
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A Synthetic Agonist to Vasoactive Intestinal Peptide Receptor-2 Induces Regulatory T Cell Neuroprotective Activities in Models of Parkinson's Disease

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A paradigm shift has emerged in Parkinson's disease (PD) highlighting the prominent role of $CD4^+$ Tregs in pathogenesis and treatment. Bench to bedside research, conducted by others and our own laboratories, advanced a neuroprotective role for Tregs making pharmacologic transformation of immediate need. Herein, a vasoactive intestinal peptide receptor-2 (VIPR2) peptide agonist, LBT-3627, was developed as a neuroprotectant for PD-associated dopaminergic neurodegeneration. Employing both 6-hydroxydopamine (6-OHDA) and α-synuclein (α-Syn) overexpression models in rats, the sequential administration of LBT-3627 increased Treg activity without altering cell numbers both in naïve animals and during progressive nigrostriatal degeneration. LBT-3627 administration was linked to reductions of inflammatory microglia, increased survival of dopaminergic neurons, and improved striatal densities. While α-Syn overexpression resulted in reduced Treg activity, LBT-3627 rescued these functional deficits. This occurred in a dose-dependent manner closely mimicking neuroprotection. Taken together, these data provide the basis for the use of VIPR2 agonists as potent therapeutic immune modulating agents to restore Treg activity, attenuate neuroinflammation, and interdict dopaminergic neurodegeneration in PD. The data underscore an important role of immunity in PD pathogenesis.

Keywords: neurodegeneration, Parkinson's disease, vasoactive intestinal peptide, agonist, microglia, regulatory T cells, alpha-synuclein, 6-hydroxydopamine

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INTRODUCTION

Parkinson's disease (PD) is a progressive degenerative movement disorder characterized by loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and their striatal connections. No therapies are available to date that alter disease progression. While the etiology of PD remains enigmatic, misfolded and nitro-oxidation modified alpha synuclein (α-Syn) produce neurotoxic protofibrils and form Lewy bodies. After extraneuronal release of protofibrils, microglial activation

and inflammation ensue with consequent neuronal damage resulting in a cascade of misfolding self-proteins and chronic inflammation leading to progressive neurodegeneration.

In addition to the innate microglial neuroinflammation linked to neurodegeneration, peripheral adaptive immunity is recognized to influence neurodegeneration and, as such, opens new opportunities for therapy (Ha et al., 2012; Olson et al., 2015, 2016; Deng and Jin, 2017; Lee et al., 2017; Gelders et al., 2018). This is highlighted by the key roles that $CD4^+$ T cell subsets play in the pathobiology of neurodegenerative diseases. In support of the linkages between immunity and PD, genetic studies have recently identified links between MHC II and disease (Kannarkat et al., 2015). Indeed, the delicate balance between effector T cells (Teffs) and Tregs affect neurodestructive and neuroprotective outcomes for neurodegenerative activities (Reynolds et al., 2010; Mosley et al., 2012; Anderson et al., 2014; Gendelman and Mosley, 2015; Mosley and Gendelman, 2017). Thus, either a minimum frequency or an appropriate function of peripheral Tregs appear to be vital for central nervous system (CNS) homeostasis. The number of Tregs or their activity are diminished in PD, stroke, and amyotrophic lateral sclerosis (ALS), and as a consequence lead to changes in the diseased-brain microenvironment with concurrent oxidative stress, inflammation, and protein misfolding; all serve to augment or speed neurodegenerative processes (Beers et al., 2011; Rentzos et al., 2012; Saunders et al., 2012; Henkel et al., 2013; Hu et al., 2014; Chen et al., 2015; Gendelman et al., 2017; Duffy et al., 2018). As a result, therapeutic strategies have emerged employing classes of immunomodulatory agents to promote T cell differentiation or to increase their baseline activity that lead to attenuation in both inflammation and oxidative stress that emerge during disease (ClinicalTrials.gov Identifier: NCT03790670) (Romero-Ramos et al., 2014; Gendelman et al., 2017; Kustrimovic et al., 2018; Solleiro-Villavicencio and Rivas-Arancibia, 2018).

One Treg-promoting agent is vasoactive intestinal peptide (VIP), a 28-amino acid polypeptide with pleiotropic activities that also serves as a neurotransmitter for central and autonomic nervous systems (Said, 2007). Pleiotropic activities for VIP include effects on vasodilation, secretion, circadian rhythm, memory recall, and inflammation through activation of specific class B GPCRs, namely VIPR1 (VPAC1) and VIPR2 (VPAC2), as well as with the lower affinity pituitary adenylate cyclase activating polypeptide 1 receptor type 1 (ADCYAP1R1 or PAC1) (Said, 2007; Vosko et al., 2007; Gomariz et al., 2010; Waschek, 2013; Fan et al., 2015; Kamigaki and Dan, 2017). VIP profoundly affects innate and adaptive immunity (Delgado and Ganea, 2013), and in models of autoimmunity exerts immune modulatory activities. VIP treatment strategies for rheumatoid arthritis, type I diabetes, Sjögren's disease, inflammatory bowel disease, experimental autoimmune encephalomyelitis, and autoimmune uveitis are in development (Delgado et al., 2001; Juarranz et al., 2005; Fernandez-Martin et al., 2006; Deng et al., 2010; Jimeno et al., 2010; Ganea et al., 2015). VIP effects on inflammatory-mediated autoimmune disorders are elicited, in large measure, by induction of Tregs through tolerogenic DCs (Chorny et al., 2005; Maldonado and von Andrian, 2010). In PD, works from other and our laboratories have shown that VIP elicits significant dopaminergic neuroprotective responses in PD models (Delgado and Ganea, 2003; Reynolds et al., 2010; Olson et al., 2015). VIP is up-regulated in neuroimmune cells after injury (Kim et al., 2000; Sandgren et al., 2003; Nishimoto et al., 2011). VIP also affects inflammatory response cascades, including mononuclear phagocytes (MP; macrophage and microglia) and T cells by inhibiting production or release of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) (Gonzalez-Rey and Delgado, 2005, 2008; Delgado et al., 2008; Waschek, 2013; Higyno et al., 2015). Furthermore, a critical aspect of the underlying biology is that VIP has been shown to rebalance the polarization of T cell responses from type-1 T helper cell (Th1) and type-17 Th cell (Th17) toward a type-2 Th cell (Th2) phenotype (Jimeno et al., 2012; Tan et al., 2015; Villanueva-Romero et al., 2018). Additionally, studies have demonstrated that VIP elicits both natural and inducible subsets of Tregs (Delgado et al., 2005; Fernandez-Martin et al., 2006; Szema et al., 2011). The neuroprotective and immunomodulatory actions of VIP, and the related pituitary adenylate cyclase activating polypeptide (PACAP), support the idea for the development of VIP- and PACAP-activated receptors as therapeutic targets for neurodegenerative and neuroinflammatory diseases (Tan and Waschek, 2011; Waschek, 2013; Olson et al., 2015, 2016).

We synthesized two novel agonists that discriminate VIPR1 and VIPR2 and exhibit increased systemic stability with greater than 20-fold longer half-lives compared to native VIP (Olson et al., 2015). Treatment of naïve animals with the VIPR2 agonist, LBT-3627, increased Treg activity without expanding Treg numbers. We previously compared the neuroprotective capabilities of these selective peptides in an acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of dopaminergic neurodegeneration. Pre-treatment with the VIPR2 agonist, LBT-3627, provided robust and greater dopaminergic neuronal protection compared to pre-treatment with VIPR1 agonist or native VIP (Olson et al., 2015, 2016). Additionally, adoptive transfer of Treg-mediated activity from VIPR2 agonist- or VIP-treated animals decreased reactive microglia numbers and pro-inflammatory cytokine production by microglia (Reynolds et al., 2010; Olson et al., 2015). Moreover, that co-transfer of VIP-induced Tregs with nitrated-α-Syn-specific Th17 Teffs to MPTP intoxicated recipient mice provided greater levels of neuroprotection than non-induced Tregs, and that adoptive transfer of LBT-3627-induced Tregs afforded greater neuroprotection than VIP-induced Tregs, highlight the potential importance of VIPR2 agonists as a putative treatment for neurodegenerative disorders.

Although LBT-3627 induced Treg-mediated neuroprotection in MPTP intoxicated mice, whether VIPR2 agonist induction of Tregs is conserved and protective in diverse models of PD is not known. To these ends, the VIPR2 agonist, LBT-3627 was tested for its abilities to augment Treg numbers and function, and to elicit neuroprotective activities in two models of nigrostriatal neurodegeneration. Stereotactically delivered 6-hydroxydopamine (6-OHDA) was used as a neurotoxic alternative to MPTP as no known immune toxic effects have been reported, while stereotactic overexpression of human α-Syn

from adeno-association human α-Syn (AAV-α-Syn) constructs was also used as the slower progression is considered a better model to capture the pathobiology of PD (St Martin et al., 2007; Theodore et al., 2008; Torres et al., 2011; Decressac et al., 2012). Additionally, the 6-OHDA model was included as considerable evidence showed VIP provided protective effects as demonstrated by diminished oxidative stress and apoptosis, and improvements in measures of dopaminergic neuron survival, spine densities for medium spiny neurons, neurotransmitter levels, synaptic plasticity, rotational behavior, and striatal astrocyte activity (Tuncel et al., 2005, 2012; Korkmaz et al., 2010, 2012; Yelkenli et al., 2016; Korkmaz and Tuncel, 2018). In these studies, both immune function and neuroprotection by LBT-3627 were evaluated in both models at multiple dose levels to determine parallel model effects. The results indicated that LBT-3627 diminished the number of reactive microglia at all doses tested. Moreover, we demonstrated that this VIPR2 agonist enhances Treg function peripherally, which leads to attenuation of microglial inflammatory responses and increased dopaminergic neuroprotection at dose levels that can be translated for human use. Lastly, due to the historical nature of VIP and the role of VIP2R in vasodilation (Koga et al., 2014), initial pre-clinical testing of LBT-3627 was performed for heart rate, pulse, and systolic blood pressure. All readings recorded were within average values. Taken together, we provide strong translational merit toward moving forward a unique pharmaceutical agent for PD neuroprotection.

MATERIALS AND METHODS

Animals

Male 7-week old Sprague Dawley and Lewis rats were ordered from SASCO and Charles River Laboratories, respectively. Typical weights were 180–200 g at the time of surgery or regulatory T cell (Treg) functional studies. All rodent procedures were performed with approval of the UNMC Institutional Animal Care and Use Committee and in accordance with NIH Guide for the Care and Use of Laboratory Animals.

LBT-3627 Treatment and Stereotactic Injections of 6-OHDA and AAV Vectors

Rats were treated initially with 5 daily s.c. doses of LBT-3627 at 0.06, 0.2, 0.6, 2.0, or 6.0 mg/kg/100 μl dose. Animals treated with an equal volume of PBS vehicle served as negative controls. Immediately after delivery of AAV vectors or 6-OHDA, rats began a regimen of LBT-3627 or vehicle for 5 days and every other day thereafter. For stereotaxic delivery, rats were anesthetized with 2% isoflurane in oxygen and placed in a stereotaxic device (Leica Biosystems Inc., Buffalo Grove, IL, United States). For each rat, the scalp was retracted to expose the skull and a 1–2 mm hole was drilled in the skull. Injections were accomplished using sterile Hamilton syringes (model 8100) affixed with 26-gauge needles and delivered by with syringe pump (LEGATO 210, cat 78-8212, KD Scientific, Holliston, MA, United States). For α-Syn overexpression studies, AAV2/1-α-SYN-IRES-eGFP-WPRE (AAV-α-Syn) (cat Standaert-5713) or control AAV2/1-IRES-eGFP-WPRE (AAV-GFP) (cat Standaert-5712) were obtained from the University of Iowa Vector Core (Iowa City, IA, United States) with kind permission from Dr. David G. Standaert (Department of Neurology, University of Alabama-Birmingham, Birmingham, AL, United States) (St Martin et al., 2007; Theodore et al., 2008). For these studies, 3×10^9 genomic copies of AAV-vectors were delivered in 3 μl of PBS to the left hemisphere above the substantia nigra at coordinates relative to the bregma, AP, −5.3 mm; ML, −2.0 mm; and −7.5 mm according to the stereotaxic atlas (Paxinos and Watson, 1986; Decressac et al., 2012). For studies using 6-OHDA, rats received 10 μg of 6-OHDA in 5 μl PBS delivered to the right hemisphere above the medial forebrain bundle at coordinates AP, −4.5 mm; ML, 1.5 mm; DV, −7.3 mm, and relative to the bregma (Paxinos and Watson, 1986; Torres et al., 2011).

Immunohistochemistry and Stereological Analysis

Following administration of terminal pentobarbital anesthesia, rats were transcardially perfused with DPBS followed by 4% paraformaldehyde in DPBS. The cohort that received 6-OHDA injections were euthanized on day 14, while the cohort that received AAV-only injections were euthanized on day 28. Brains were obtained and cryosectioned through the ventral midbrain and striatum. Frozen midbrain sections (30 μm) were collected in PBS as free-floating sections, and every 8th section was immunostained for 48 h as free-floating sections for tyrosine hydroxylase (TH) (anti-TH, 1:2000, EMD Millipore, Burlington, MA, United States) (Benner et al., 2008). Sections were washed and reacted with biotinylated secondary antibody for 1 h and washed. To visualize antibody-labeled tissues, sections were incubated in streptavidin-HRP solution (ABC Elite Vector Kit, Vector Laboratories, Burlingame, CA, United States) and color was developed using a glucose/glucose oxidase/H_2O_2 generation system and diaminobenzidine (DAB) chromogen (Sigma-Aldrich). Sections were mounted to microscope slides, counterstained for Nissl substance, dried, coated with mounting medium (Cytoseal 60, Thermo Fisher Scientific), and covered with a coverslip. To determine the microglia reactivity, midbrain sections were immunostained as free-floating sections for ionized calcium binding adaptor molecule 1 (Iba1) (anti-Iba1, 1:1000, Wako, Richmond, VA, United States) and biotinylated secondary antibody, and were visualized as previously described. The slides were coded by an investigator not familiar with treatment regimens and were assessed by blinded investigators. Total numbers of reactive $Iba1^+$ microglia/mm^2, TH^+Nissl^+ (dopaminergic neurons), and TH^-Nissl^+ (non-dopaminergic) neurons in the substantia nigra were determined by stereological analyses using the Fractionator probe from StereoInvestigator software (MBF Bioscience, Williston, VT, United States) interfaced with an Eclipse 90i microscope (Nikon, Melville, NY, United States). To sample through the substantia nigra using every 8th section required 10 sections/rat for enumeration of total nigral neurons and microglia density. The densities of

TH^+ expression for striatal termini were determined by digital densitometry using image J software (National Institutes of Health, Bethesda, MD, United States) (Benner et al., 2008). Briefly for each rat, optical densities from 6 striatal sections stained for expression of TH were obtained from bit map probes of standardized areas, and background densities were determined from non-striatal areas. Striatal TH densities were determined for each section by subtracting the densities of background from TH^+ areas and the mean of the 6 sections served as the striatal TH density/animal.

Treg Function Assessment

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from rat spleens using $CD4^+$ T Cell isolation kit (cat 19642) and PE selective kit (cat 18557) (StemCell, Vancouver, BC, Canada). Briefly, isolated spleen cells were collected and placed in a petri dish with 70 μm mesh cell strainer in 5 ml HBSS. A syringe plunger was used to gently mash tissue to obtain single cells. Red blood cells were removed with ACK lysis buffer (3 mL per spleen). Spleen cells were stained to obtain $CD4^+$ cells according to the manufacturer's protocol. $CD4^+$ cells were stained with anti-CD25 PE (cat 55486, BD Biosciences, San Jose, CA, United States) for 20 min at a concentration of 0.75 μg/ml per 15×10^6 cells and anti-PE-magnetic beads were used for positive selection of $CD4^+CD25^+$ T cells. These cells were greater than 90% Tregs as determined by expression of forkhead box P3 (Foxp3) by flow cytometric analysis (**Figure 1B**). The $CD4^+CD25^-$ T cell fraction was collected in the flow through and served as conventional responding T cells (Tresps) to be used in proliferation assays. Tresps were labeled with carboxyfluorescein succinimidyl ester (CFSE) (cat C34554, Thermo Fisher Scientific). Tregs were serially diluted by twofold dilutions in a 96 well U-bottom microtiter plate to contain 100, 50, 25, 12.5, and 6.25×10^3 Tregs in 100 μl of media. To each well was added 100 μl of 50×10^3 CFSE-stained Tresps to yield Treg:Tresp ratios of 2, 1, 0.5, 0.25, and 0.125:1, and Tresps in media served as controls. To each well was added 25×10^3 rat T cell activating CD3/CD28 beads (1 bead: 1 Tresp) and cultures were incubated at 37°C in 5% CO_2 for 72 h. The cells were fixed with 1% formaldehyde in PBS and analyzed by flow cytometric analysis. T cell activating CD3/CD28 beads were prepared with Dynabeads M-450 epoxy (cat 14011, Thermo Fisher Scientific) conjugated with anti-rat CD3 (cat 5012338, Thermo Fisher Scientific) and anti-rat CD28 (cat 5014270, Thermo Fisher Scientific) according to manufacturer's protocol. For conjugation, the CD3:CD28 ratio was 1:1 and bead to antibody ratio was 1000 beads with 200 μg antibody (100 μg CD3 and 100 μg CD28). Coupled beads were stored at 2–8°C at a concentration of 4×10^7 beads/ml in PBS, pH 7.4 with 0.1% bovine serum albumin (BSA) and were utilized over a 1 year period.

Flow Cytometric Analysis

Blood was collected from the rat left atrium to determine Treg number. Blood (50 μl) was stained with a mixture of BV-421-anti-CD3 (2.5 μl, cat 563948, BD Bioscience), PerCP-eFluor 710-anti-CD4 (2 μl, cat 46-0040-82, eBioscience, Thermo Fisher Scientific) and PE-anti-CD25 (2.5 μl, cat 554866, BD Bioscience) and incubated on ice for 30 min. Cells were washed with DPBS with 2% BSA and fixed. To probe for intracellular transcription factors, cells were permeabilized according to the manufacturer's protocol (Permeabilization Kit, cat 005523-00, eBioscience) and were reacted with APC-anti-Foxp3 (5 μl, cat 77-5775-40, eBioscience) for 1 h. Samples were analyzed using a BD LSRII flow cytometer and FACSDiva Software (BD Biosciences) at the UNMC Center Flow Cytometry Research Facility.

Statistics

Data are presented as means ± SEM. Data were assessed for normal distribution by probability plots against the theoretical cumulative normal distribution function. Homoscedasticity of data was assessed by Cochran C, Hartley, Bartlett test, and Levene's test. Normally distributed, homoscedastic data were evaluated by parametric one-way ANOVA followed by Newman-Keuls *post hoc* tests (Statistica, v13, TIBCO Software, Inc., Palo Alto, CA, United States). Measurements of Treg function were assessed by linear regression analyses as a function of Treg:Tresp ratios. Differences in multiple linear Treg functions were determined as significant differences in slope or intercepts (Prism, v7, GraphPad Software, Inc., San Diego, CA, United States).

Cardiovascular Safety Study in Dogs

The *in vivo* studies pertaining to this portion of the project were performed at Charles River Laboratories, formerly WIL Research Laboratories. All animals were housed individually in clean stainless steel cages in an environmentally controlled room. The cages were elevated above stainless steel flush pans, which were cleaned daily. Animals were individually housed as to prevent cross contamination as part of the Latin-square cross over design. Dogs were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). The facilities at WIL Research were fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). Environmental controls were set to maintain a temperature of 71°F ± 5°F (22°C ± 3°C) and relative humidity of 50% ± 20%. Temperature and relative humidity were monitored continuously. Fluorescent lighting was set to provide illumination for a 12-h light/12-h dark photoperiod. Reverse osmosis-purified municipal water was available *ad libitum*.

Male Beagle dogs, 8–12 months of age and weighing 9.5 ± 038 kg, were previously implanted with radiotelemetry transmitters and used in a Latin square cross-over design for administration of LBT-3627 or vehicle (10 mM Tris + 267 mM glucose, pH 8.5). Radiotelemetry implantation was performed by Charles River Laboratories in naïve colonies of dogs following standard operating procedures. The telemetrized dogs were then maintained as a non-naïve colony for non-GLP CV safety monitoring with an adequate washout period between administrations of each test article. At the time of implantation, dogs were surgically fitted with telemetry transmitters [TL11M3-D70-PCTR, Data Sciences International (DSI), Minnesota,

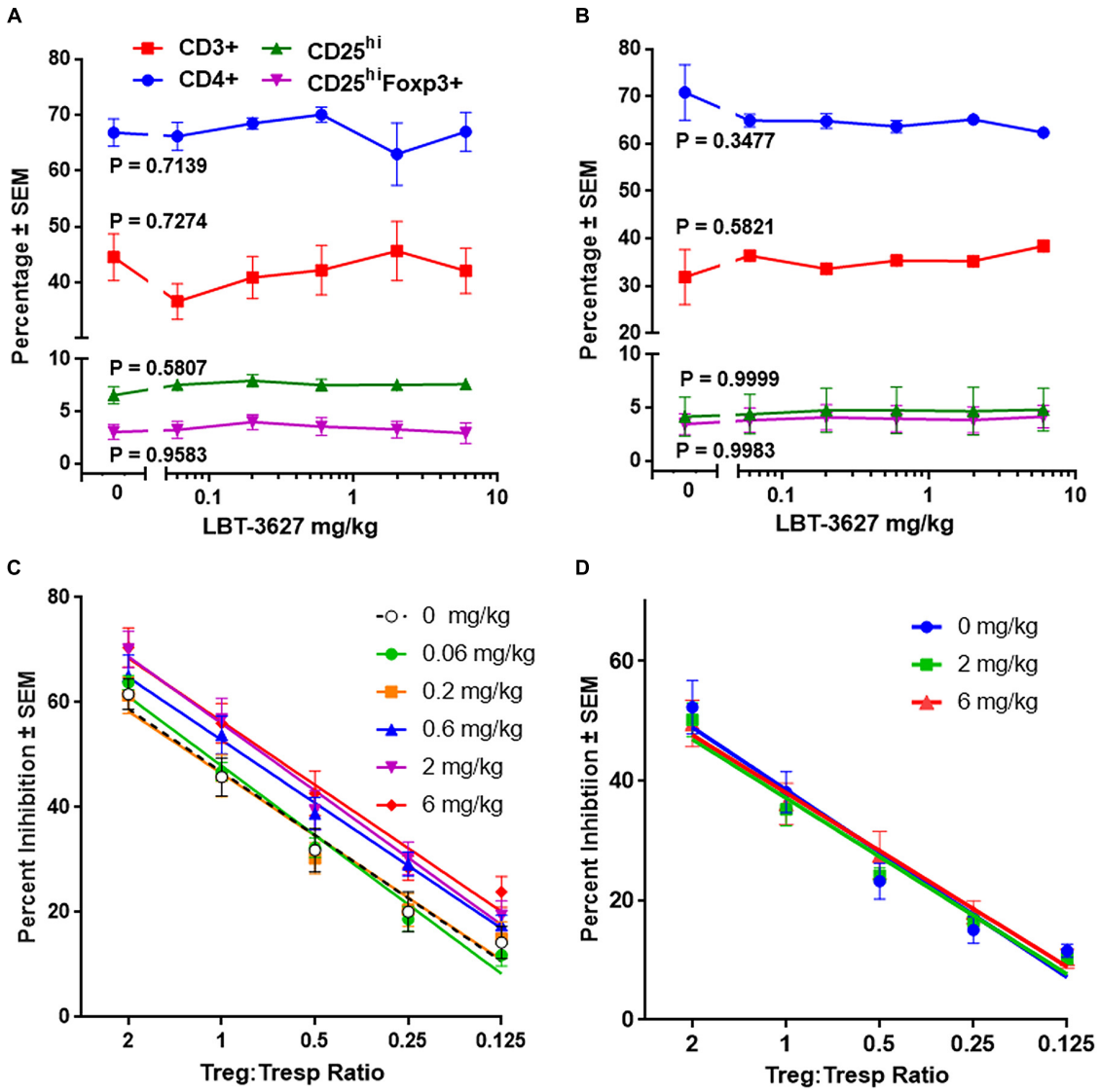

FIGURE 1 | Multiple doses of VIPR2 agonist augments Treg activity. Naïve Lewis rats were administered 5 daily s.c. injections of PBS vehicle (designated 0) or LBT-3627 at doses of 0.06, 0.2, 0.6, 2.0, or 6.0 mg/kg (n = 5 rats/group). One day after last injection, cells from peripheral blood **(A)** and spleens **(B)** were assessed for T cell and Treg frequencies. Percentages of cell populations included $CD3^+$ T cells among lymphocytes (red circles), $CD4^+$ T cells among $CD3^+$ cells (blue squares), $CD25^{hi}$ T cells among $CD3^+CD4^+$ cells (green triangles), and $CD25^{hi}Foxp3^+$ among $CD3^+CD4^+$ cells (purple inverted triangles). Mean percentages and SEMs were determined from 5 rats/group and significant differences between dosages were assessed for each population by one-way ANOVA. P values for each T cell population are denoted next to the concentration curve. **(C)** Spleen cells obtained from the above multiple-dosed animals **(B)** or from rats receiving a single s.c. dose of LBT-3627 at 2.0 or 6.0 mg/kg (n = 5 rats/group) **(D)** were enriched for $CD3^+CD4^+CD25^{hi}$ Tregs and assessed for Treg function. Percent Treg-mediated suppression was determined by flow cytometric analysis. Significant differences were determined from linear regression analysis with 5 rats/group and triplicate assays for each rat. Regression analysis for all lines indicated that $R^2 > 0.96$ with P values < 0.0054.

United States] into the abdominal cavity under anesthesia. The transmitters have a fluid-filled catheter (coated with an antithrombotic film to inhibit thrombus formation) with the tip filled with a patented gel for blood pressure collection, and 2 ECG leads surgically implanted emulating a Lead II configuration. All dogs were allowed to recover for at least 2 weeks from the implantation of the telemetry device before administration of the test articles. LBT-3627 was provided to WIL Research by Longevity Biotech, Inc. A total of 4 dogs were on study with each of the 4 dogs receiving each dose with at least a 3-day washout period in between any single dose. The test compound, LBT-3627, was administered via s.c. injection at 3 dose levels and a vehicle alone (0, 0.14, 0.6, and 1.4 mg/kg). These dose levels were allometrically scaled from the dose ranges that were observed to be effective to prevent neurodegeneration in a mouse model of PD (Olson et al., 2015). When scaled from dog, these doses would equate to approximately 0.5, 2, and 5 mg/kg in rats, which is comparable

to the three doses used in the current neurodegenerative models (0.6, 2, and 6 mg/kg).

Following a single injection of the test compound or vehicle to the dog, a host of parameters were tracked by radiotelemetry. Clinical observations included detailed daily physical examinations (before and after dosing) and continual observation for the first 4 h after dosing. Dogs were also weighed before, after, and throughout the study. Baseline arterial blood pressure (systolic, diastolic, and mean), pulse pressure, heart rate, electrocardiographic (ECG) waveforms, and body temperature were collected continuously for 1 h prior to administration of vehicle or test compound. Following administration of vehicle or test compound, the same parameters were collected continuously for at least 24 h. Cardiovascular parameters and body temperature data were averaged for 4 dogs per group to appropriate time intervals for statistical analysis. Two way ANOVA was used to determine statistically significant differences between groups over time for each of the parameters that were tracked.

RESULTS

Multiple Doses of VIPR2 Agonist Increase Treg Activity Without Expanding $CD4^+$ T Cells or Tregs

To assess the effects of VIPR2 agonist on Treg numbers and function, we treated rats with 5 daily s.c. doses of LBT-3627 at 0.06, 0.2, 0.6, 2.0, or 6.0 mg/kg/dose or with PBS vehicle (0 mg/kg). The day after the last injection, T cell and Treg frequencies from peripheral blood and spleens were assessed. Compared to vehicle-treated controls, no significant differences were detected in percentages of (a) $CD3^+$ T cells among lymphocytes, (b) $CD4^+$ T cells among $CD3^+$ T cells, $CD25^{hi}$ T cells among $CD3^+CD4^+$ T cells, or $CD25^{hi}Foxp3^+$ Tregs among $CD3^+CD4^+$ T cells in either peripheral blood (P value ranges = 0.5807 – 0.9583) (**Figure 1A**) or spleen (P value ranges = 0.3477 – 0.9999) (**Figure 1B**). Spleen cells from individual animals were enriched for $CD3^+CD4^+CD25^{hi}$ Tregs and assessed for suppressive activity toward Tresp. Flow cytometric analysis indicated those $CD4^+CD25^+$ T cells exhibited a Treg phenotype with greater than 90% expressing Foxp3 (**Figure 1B**). Compared to animals treated with vehicle, Treg activity was significantly increased in animals treated with LBT-3627 at 0.6. 2.0, and 6.0 mg/kg once daily for 5 days, but no effect was detected in rats treated with lower concentrations at 0.06 and 0.2 mg/kg (**Figure 1C**). We also assessed whether a single dose of VIPR2 agonist was sufficient to upregulate Treg activity. We found that one dose of LBT-3627 at levels required to upregulate Treg activity with multiple doses did not alter Treg activity compared to control (P_{slope} and $P_{intercept} > 0.8702$) (**Figure 1D**). These data together demonstrated that LBT-3627 elevates Treg function without increasing frequencies and that multiple doses of LBT-3627 are necessary to increase Treg activity. Those effective dosing paradigms were chosen for evaluation in the two neurodegenerative models.

Dopaminergic Neurodegeneration and Reactive Microgliosis Are Induced by Human Wild Type α-Syn Overexpression

We utilized the overexpression of human α-Syn in rat ventral midbrain as a model of dopaminergic neurodegeneration to assess putative neuroprotective modalities. Initially, to delineate the kinetics of neurodegeneration and inflammation in that model, Sprague-Dawley rats were stereotactically injected in the left hemisphere with AAV-α-Syn, AAV-GFP, or were sham-treated by insertion of the needle into the dura without infusion (SHAM). At 21, 28, and 35 days post-injection, animals were sacrificed, brains collected, processed, sectioned through the ventral midbrain and striatum, and stained for expression of TH for dopaminergic neurons and termini. TH staining was similar in sections isolated from rats treated with either sham (**Figure 2A**) or AAV-GFP (**Figure 2B**) in ipsilateral and contralateral hemispheres of the ventral midbrain and the striatum regardless of time after viral treatment indicating that viral delivery itself resulted in little if any neurodegenerative effect. In contrast, TH staining from animals treated with AAV-α-Syn showed reductions of TH staining within the substantia nigra and striatum of the ipsilateral hemisphere compared to the non-injected contralateral hemisphere (**Figure 2C**). These reductions are particularly evident 28 and 35 days after infection. To validate those observations, we counted TH^+ and TH^- neuronal numbers in both the ipsilateral and contralateral hemispheres by stereological analysis and compared ratios of ipsilateral and contralateral neuronal counts. Significant loss of TH^+Nissl^+ neurons were not detected after treatment with AAV-GFP vector as ipsilateral/contralateral ratios at any time were greater than 0.91 and $P > 0.3225$ (**Figure 2D**). However, treatment with AAV-α-Syn resulted in 24, 36, and 55% loss of TH^+Nissl^+ neurons correlating to diminution of ipsilateral/contralateral ratios of 0.77, 0.53, and 0.41 on days 21, 28, and 35, respectively. No significant effects of treatment and/or day were detectable for non-dopaminergic (TH^-Nissl^+) neurons within the substantia nigra ($P > 0.4603$). While densities of striatal TH^+ termini were slightly diminished 21 days after treatment with AAV-α-Syn, significant diminution was realized only after 28 and 35 days compared to treatment with AAV-GFP (**Figure 2E**).

To assess the effects of α-Syn overexpression on neuroinflammation, sections of ventral midbrain were stained for Iba1 expression. $Iba1^+$ microglia from sham (**Figure 3A**) and AAV-GFP (**Figure 3B**) treated rats showed mostly ramified morphologies with densities that were unaffected regardless of hemisphere, treatment regimen, or time after treatment. In contrast, by day 21 and through day 35 after AAV-α-Syn treatment, staining of amoeboid $Iba1^+$ microglia were increased in the ipsilateral hemisphere compared to the contralateral hemisphere (**Figure 3C**). Stereological analysis confirmed these observations showing that ipsilateral/contralateral ratios of microglial densities (1.0 – 1.3) for AAV-GFP treated animals were not significantly different; however, AAV-α-Syn treatment increased those ratios to 4.2-fold by days 21 and 28 post-injection (**Figure 3D**). By day 35, the ratio diminished to twofold, which

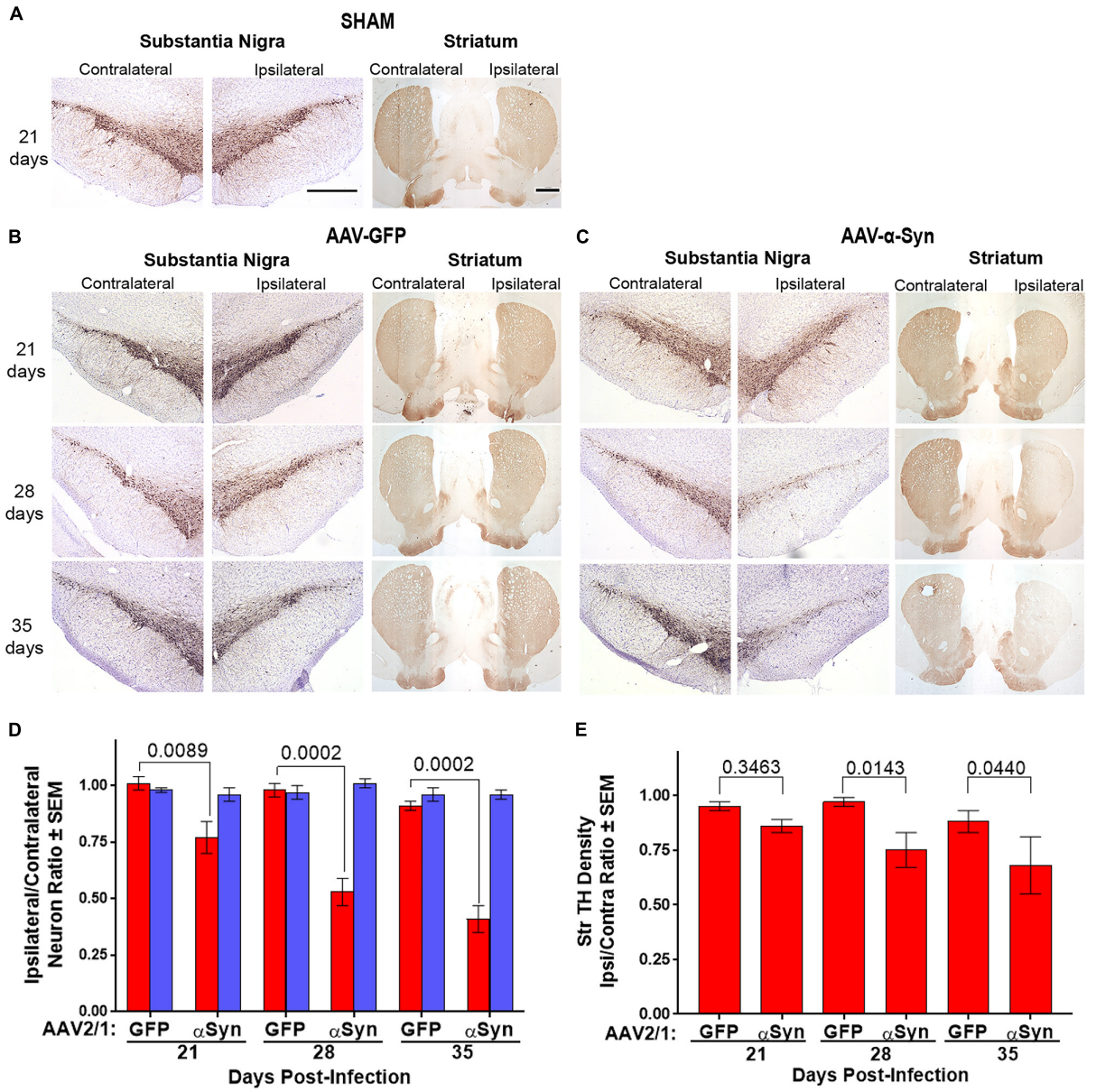

FIGURE 2 | Dopaminergic neurodegeneration by overexpression of human α-Syn. Sprague-Dawley rats were stereotactically sham injected without infusion (n = 3/day) **(A)** or injected with either AAV-GFP vector (n = 5/day) **(B)** or AAV-α-Syn vector (n = 4–5/day) **(C)**. Brains were removed on days 21, 28, and 35 post injection. Sections of midbrain and striatum were stained to visualize tyrosine hydroxylase (TH) expression to reveal dopaminergic neurons in the substantia nigra and efferent termini in the striata. **(D)** Ipsilateral/contralateral ratios of TH^+Nissl^+ (red bars) and TH^+Nissl^- (blue bars) neuron numbers as determined from stereological analysis. **(E)** Ipsilateral/contralateral ratios of TH^+ striatal densities as determined from digital image analysis. Means and SEM were determined from 4 to 5 rats/group. Significant differences were assessed by one-way ANOVA and Newman-Keuls *post hoc* tests. *P*-values are denoted above connecting comparisons. Scale bars, 1000 μm.

was significantly lower compared to those on day 21 and 28 ($P < 0.0003$), but not significantly different than AAV-GFP treated animals. These data suggested that by day 35, reactive microglia responses were beginning to be resolved, even in the context of progressive dopaminergic neuronal loss. Thus, we selected day 28 after AAV-α-Syn injection as an optimal time to assess the neuroprotective and anti-inflammatory capacities of VIPR2 agonist.

VIPR2 Agonist Protects From Dopaminergic Neurodegeneration and Attenuates Neuroinflammation Induced by α-Syn Overexpression

To assess the effects of a VIPR2 agonist on neurodegeneration by α-Syn overexpression, we stereotactically injected rats with AAV-α-Syn and administered LBT-3627 in 5 sequential daily s.c.

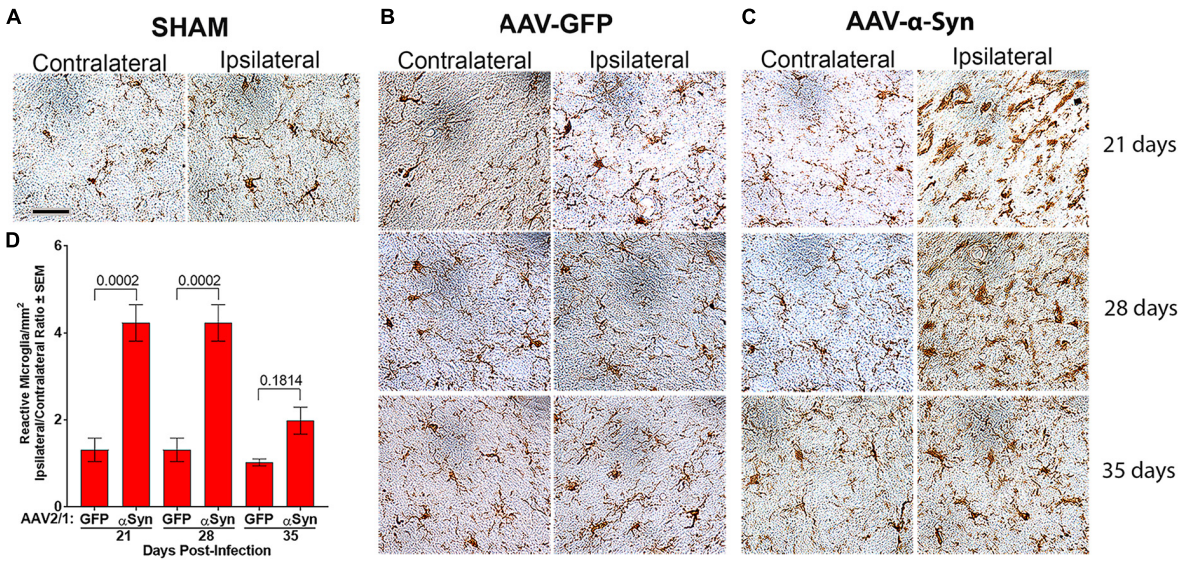

FIGURE 3 | Human α-Syn overexpression increases reactive microglia. Sprague-Dawley rats (**Figure 2**) were stereotactically injected without infusion (SHAM) (n = 3/day) **(A)** or injected with either AAV-GFP vector (n = 5/day) **(B)** or AAV-α-Syn vector (n = 4–5/day) **(C)**. Brains were removed on days 21, 28, and 35 post injection. Sections of midbrain were stained to visualize Iba1 expression to reveal reactive microglia in the substantia nigra. **(D)** Numbers of Iba1$^+$ amoeboid microglia were estimated by stereological analysis. Means and SEM were determined from 4 to 5 rats/group. Significant differences were assessed by one-way ANOVA and Newman-Keuls *post hoc* tests. *P*-values are denoted above connecting comparisons. Scale bar, 40 μm.

doses, followed by one dose every other day thereafter. Drug concentrations of 0, 0.6, 2.0, or 6.0 mg/kg/dose were delivered to each group. Control groups were treated with AAV-GFP or LBT-3627 vehicles. Assessment of brain sections from 28 days after AAV injections revealed that AAV-α-Syn led to reduced TH expression in the substantia nigra and striata compared to sham and AAV-GFP controls (**Figure 4A**). In contrast, intensities of TH expression within the ipsilateral hemispheres were increased in ipsilateral sections of the substantia nigra and striatum from AAV-α-Syn rats treated with LBT-3627 compared to those sections from vehicle-treated AAV-α-Syn rats. Stereological analysis validated findings within the nigra which demonstrated differences between the ipsilateral and contralateral hemispheres taken as ratios of TH$^+$ neurons (**Figure 4B**). TH$^+$ neuron ratios were significantly diminished in AAV-α-Syn treated animals compared to sham- and GFP-treated rats. While treatment with LBT-3627 after AAV-α-Syn injection increased TH$^+$ neuron ratios, only 2.0 mg/kg significantly increased the ipsilateral/contralateral ratio by 43% compared to rats treated with AAV-α-Syn and vehicle. The increase ratio was due to increased numbers of dopaminergic neurons in the ipsilateral hemisphere compared to that of the AAV-α-Syn controls. Additionally, 2.0 mg/kg of LBT-3627 significantly spared the density of striatal TH$^+$ termini by 24% compared to AAV-α-Syn controls (**Figure 4C**).

During the same study, we also assessed the effects of VIPR2 agonist on microglia-mediated neuroinflammatory responses. Sections of ventral midbrain from the above studies were probed for Iba1 expression and the densities of Iba1$^+$ reactive microglia were measured. Sections showed that microglial morphologies and Iba1 intensities in ipsilateral and contralateral hemispheres were virtually identical 28 days after sham or AAV-GFP treatment (**Figure 5**). In contrast, α-Syn overexpression increased the intensity of staining for Iba1$^+$ amoeboid microglia within the ipsilateral ventral midbrain compared to the contralateral hemisphere. LBT-3627 treatment diminished the intensity of Iba-1 staining in tissues from rats overexpressing α-Syn. To validate those observations, we enumerated reactive microglial by stereological analyses. AAV-α-Syn overexpression increased the numbers of ipsilateral Iba1$^+$ reactive microglia by 2.5-fold compared to the contralateral hemisphere. Treatment with LBT-3627 at doses of 0.6, 2.0, and 6.0 mg/kg reduced reactive microglia by 26, 27, and 36%, respectively. Interestingly, doses of 6.0 mg/kg returned microglia numbers to levels not significantly different from either sham or AAV-GFP controls, resulting in nearly complete ablation of the microglia response.

VIPR2 Agonist Rescues Treg Activity Diminished by α-Syn Overexpression

As Treg function has been shown to be deficient in both PD patients and in mice immunized with α-Syn, and since LBT-3627 augments Treg activity with subsequent neuroprotection (Reynolds et al., 2010; Saunders et al., 2012; Olson et al., 2015), we assessed the effects of VIPR2 activation on Treg numbers and function in the context of α-Syn overexpression. As described above, rats were stereotactically injected with AAV-α-Syn and treated with varying doses of LBT-36327. After 28 days, splenic cells were enriched for Tresp and Treg populations and tested for proliferative or suppressive functions, respectively. Tresps were stained with CFSE, stimulated with CD3/CD28 beads in the absence of Tregs, and assessed by flow cytometric analysis for

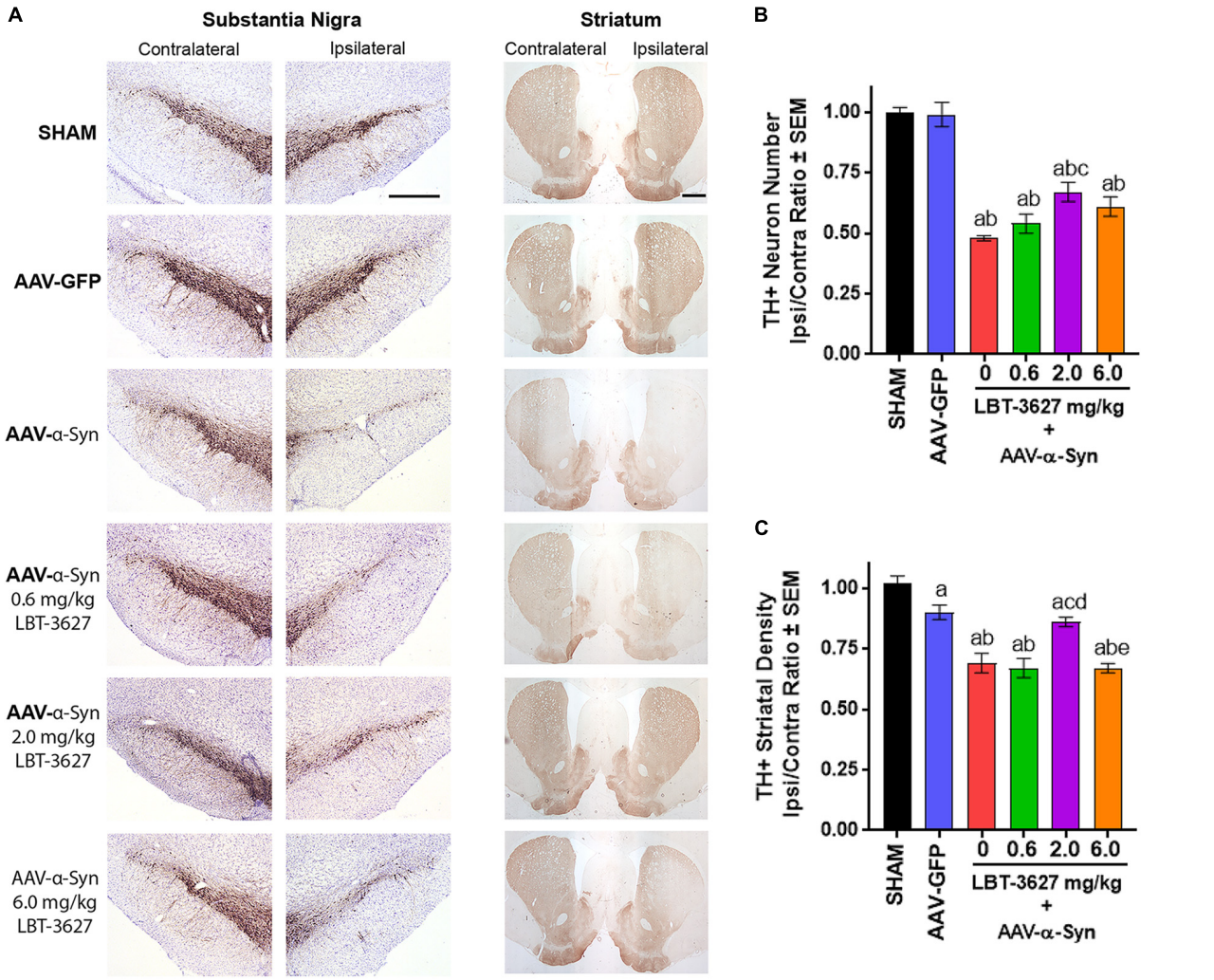

FIGURE 4 | VIPR2 agonist protects dopaminergic neurons from α-Syn-mediated neurodegeneration. Sprague-Dawley rats were stereotactically injected, but not infused (SHAM) (n = 5) or infused with AAV-GFP (n = 6) or AAV-α-Syn vector (n = 6–7/group). AAV-α-Syn-treated rats began a regimen of PBS (0) (n = 7) or LBT-3627 at doses of 0.6, 2.0, or 6.0 mg/kg (n = 6/group) administered as daily s.c. injections for 5 days and every other day thereafter. On day 28 after infection, brains were obtained and sections of midbrain and striatum were stained for expression of TH **(A)**. **(B)** Numbers of TH^+ neurons were assessed in the substantia nigra by stereological analysis and ipsilateral/contralateral ratios calculated. **(C)** Ipsilateral/contralateral ratios of TH^+ densities were determined from digital image analysis of striatal TH expression. Means and SEM were determined from 5 to 7 rats/group. Significant differences were assessed by one-way ANOVA and Newman-Keuls *post hoc* tests. $P \leq 0.05$ compared to treatment as [a]sham controls, [b]AAV-GFP, or animals treated with AAV-α-Syn and [c]0 (vehicle), [d]0.6, or [e]2.0 mg/kg LBT-3627. Scale bars, 1000 μm.

proliferative capacity after 3 days of incubation. No significant differences in the percentages of proliferating Tresps were discernable between treatment groups (P = 0.2028) (**Figure 6A**). In contrast, Treg function from AAV-α-Syn-treated rats (red line) was significantly diminished compared to Treg function from AAV-GFP controls (blue line) (P = 0.0115) (**Figure 6B**). Treatment with 0.6 mg/kg of LBT-3627 (green line) increased Treg function compared to that from AAV-α-Syn alone treated rats (P = 0.0065), but was still lower than that from AAV-GFP controls (blue line) (P = 0.0168). Only doses of 2.0 (purple) and 6.0 (orange) mg/kg of LBT-3627 were sufficient to increase Treg activity above that of GFP controls (P < 0.007). While no significant differences in Treg function were detected between 2.0 and 6.0 mg/kg doses, 2.0 mg/kg doses of LBT-3627 tended to show better efficacy affecting Treg function and TH^+ neuron survival.

Higher Doses of VIPR2 Agonist Are Required to Protect Dopaminergic Neurons in the 6-OHDA Model

We sought to validate the neuroprotective capacity of VIPR2 agonist in a second model of dopaminergic neurodegeneration. For these studies, 6-OHDA was delivered to the medial forebrain bundle by stereotactic injection into the left hemisphere while the contralateral hemisphere served as a control. Following a similar

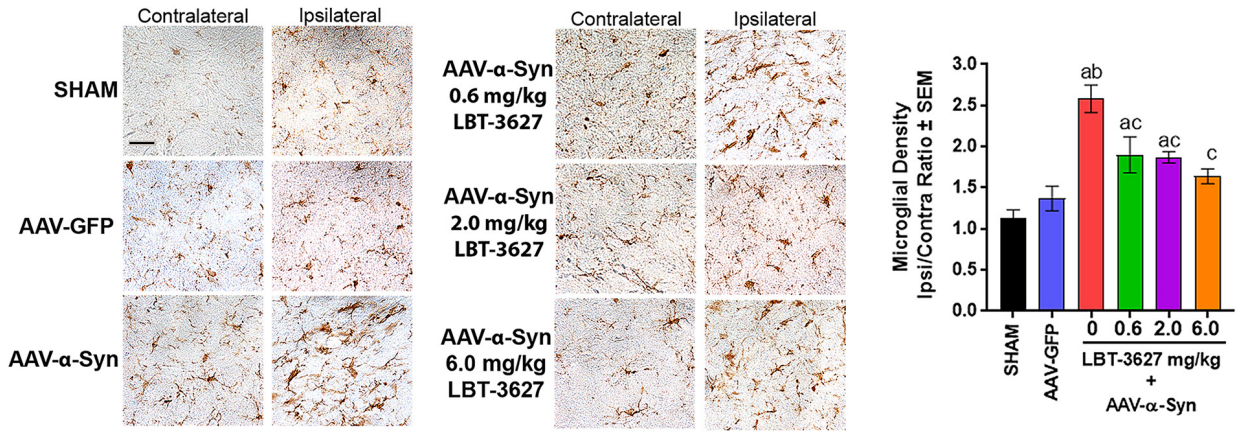

FIGURE 5 | VIPR2 agonist diminishes microglial response to α-Syn overexpression. Sprague-Dawley rats were treated as sham control (n = 5) or stereotactically injected with AAV-GFP (N = 6) or AAV-α-Syn vector (n = 6–7/group). AAV-α-Syn-treated rats started the same regimen as in **Figure 4** of vehicle (0) (n = 7) or LBT-3627 administered at 0.6, 2.0, or 6.0 mg/kg (n = 6/group). On day 28 after infection, brains were obtained and sections of midbrain were stained for expression of Iba1 by microglia. Numbers of Iba1$^+$ amoeboid microglia were determined by stereological analysis. Means and SEM were determined from 5 to 7 rats/group. Significant differences were assessed by one-way ANOVA and Newman-Keuls *post hoc* tests. $P \leq 0.05$ compared to treatments as [a]sham controls, [b]AAV-GFP, or AAV-α-Syn and [c]PBS. Scale bar, 40 μm.

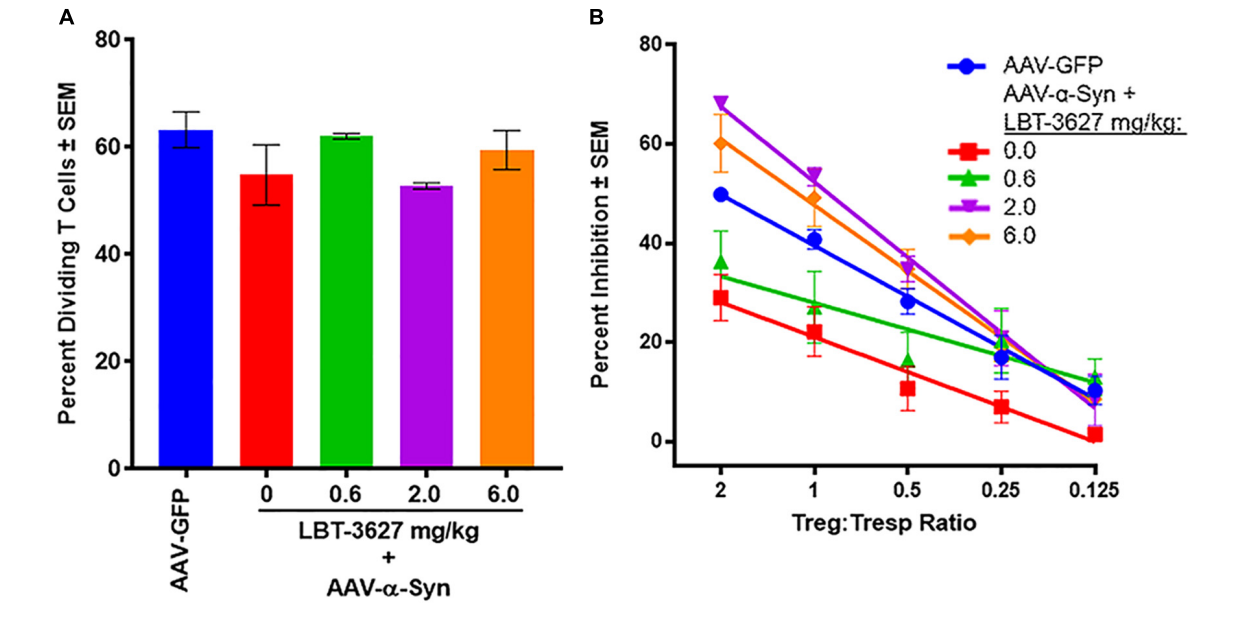

FIGURE 6 | VIPR2 augments Treg activity in rats overexpressing human α-Syn. Sprague-Dawley rats were treated by stereotaxic injection with AAV-GFP (n = 3) or AAV-α-Syn vector. Rats treated with AAV-α-Syn-began a regimen of LBT-3627 administrated at 0.6, 2.0, or 6.0 mg/kg or vehicle (0 mg/kg) (n = 3/group) by daily administration for 5 days and every other day thereafter. On day 28 after infection, spleen cells were enriched for conventional CD4$^+$ T cells and assessed for percentage of proliferating T cells **(A)**, or enriched for Tregs and assessed for percentage inhibition of proliferating T cells **(B)**. Note, treatment regimens for **(A,B)** are color-matched. **(A)** Means and SEM were determined for 3 rats/group. No significant differences were detected by one way ANOVA, P = 0.2028. **(B)** Significant differences of Treg activities between treatment groups were determined from linear regression analysis of 3 rats/group. Regression analysis for all treatments indicated that each regression exhibited $R^2 > 0.83$ with P values < 0.032.

dosing paradigm as described above, immediately after 6-OHDA delivery, LBT-3627 at doses of 0.6, 2.0, or 6.0 mg/kg was initiated with 5 daily s.c. injections followed every other day thereafter until day 21 post-injection. A control group included rats that received 6-OHDA and were treated with vehicle alone. Sections of midbrain and striatum immunostained for TH expression showed substantive loss of TH expression within the substantia nigra of the ipsilateral hemisphere compared to the contralateral side (**Figure 7A**). Meanwhile, examination of sections from animals treated with LBT-3627 suggested that ipsilateral expression of TH in the substantia nigra was increased in a dose-dependent fashion compared to 6-OHDA controls. TH$^+$

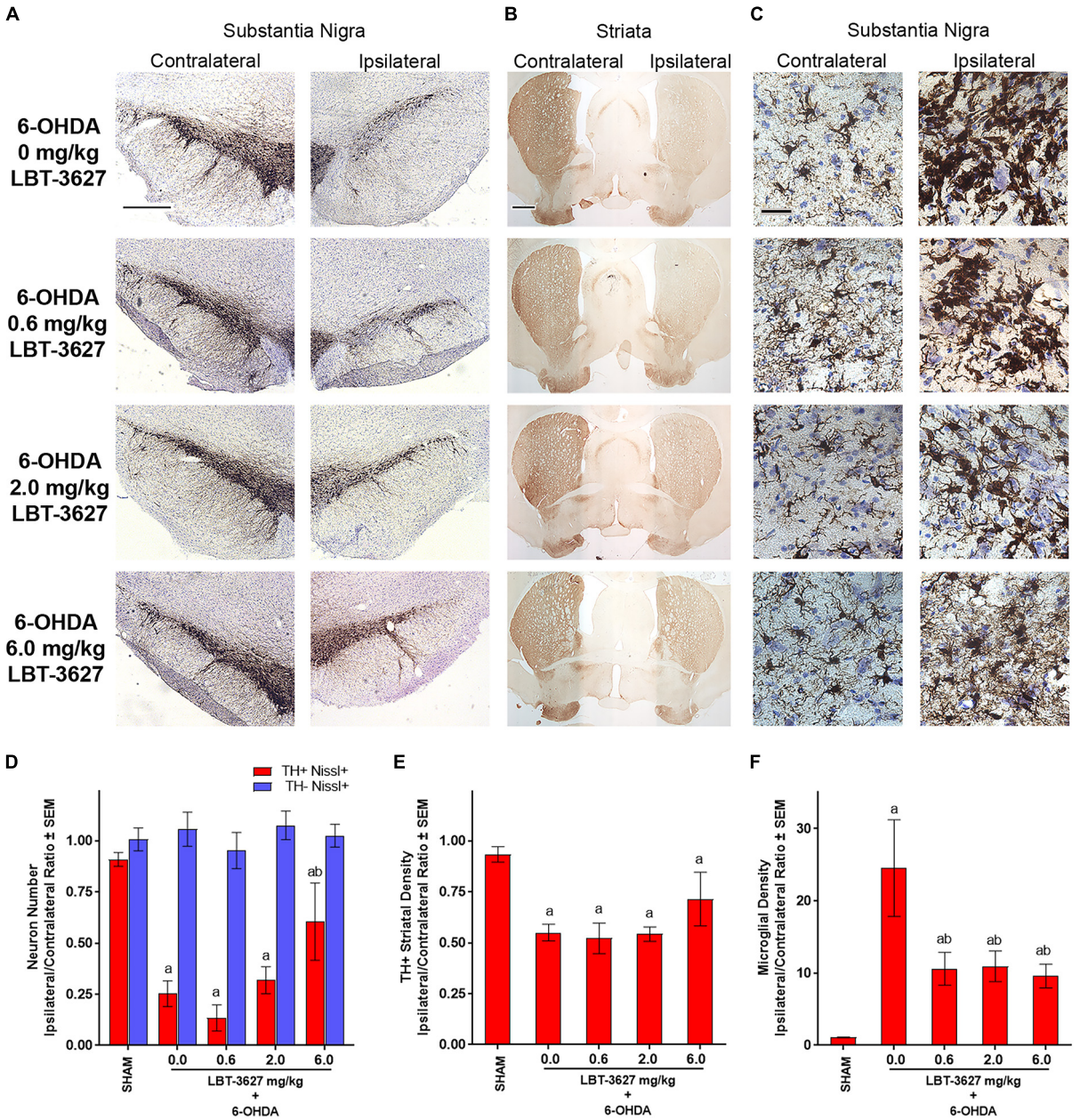

FIGURE 7 | VIPR2 agonist protects dopaminergic neurons and diminishes microglial response after 6-OHDA. Lewis rats were administered 10 μg of 6-OHDA in 5 μl of PBS by stereotaxic injection or were injected without infusion (SHAM controls) (n = 6). Immediately after injection, rats began a regimen of either vehicle (0.0 mg/kg) (n = 5) or 0.6 (n = 6), 2.0 (n = 6), or 6.0 (n = 5) mg/kg of LBT-3627 administered as daily injections for 5 days and every other day thereafter. After 14 days, brains were obtained, processed and stained for expression of TH in the substantia nigra **(A)** and striatum **(B)**, and for Iba1 in the substantia nigra **(C)**. **(D)** TH^+Nissl^+ (red bars) and TH^-Nissl^+ (blue bars) neurons within the substantia nigra were counted by stereological analysis and the ipsilateral/contralateral ratios of those numbers were determined. No significant differences in TH^-Nissl^+ neurons were discernible between treatment groups, P = 0.7671. **(E)** Densities of TH^+ termini were determined by digital image analysis and ipsilateral/contralateral ratios of those densities calculated. **(F)** Densities of amoeboid $Iba1^+$ microglia were assessed by stereological analysis and ipsilateral/contralateral ratios of those densities were determined. **(D–F)** Means and SEM were calculated for 5–6 rats/group. Significant differences were assessed by one-way ANOVA followed by Newman-Keuls *post hoc* tests. $P \leq 0.05$ compared to animals treated as [a]sham controls or with [b]6-OHDA + vehicle (0 mg/kg). Scale bars, **(A,B)** 1000 μm and **(C)** 40 μm.

stained termini in the striatum indicated that 6-OHDA resulted in a profound loss of TH expression in the ipsilateral hemisphere compared to contralateral hemisphere (**Figure 7B**). Additionally, sections stained for $Iba1^+$ reactive amoeboid microglia in the ventral midbrain, revealed that 6-OHDA treatment induced an intense activated microglial response (**Figure 7C**). Treatment with LBT-3627 at all doses substantially decreased neuroinflammatory responses as exhibited by diminished

Iba-1 expression intensity. Stereological analysis of TH^+Nissl^+ neurons within the ipsilateral substantia nigra revealed a 73% loss of dopaminergic neurons by 6-OHDA compared to sham control (**Figure 7D**). Treatment with 6.0 mg/kg of LBT-3627 spared 53% of the dopaminergic neurons, whereas doses of 0.6 and 2.0 mg/kg showed no significant effect compared to 6-OHDA control. No effect on TH^-Nissl^+ non-dopaminergic neurons was detected ($P = 0.7671$). Digital image analysis of TH^+ striatal termini showed a 41% loss of dopaminergic termini compared to sham controls, and regardless of LBT-3627 dose, losses of striatal termini were not significantly different from 6-OHDA controls ($P > 0.1226$) (**Figure 7E**). Meanwhile, to measure the inflammatory response of 6-OHDA, the density of $Iba1^+$ reactive amoeboid microglia were determined by stereological analysis. 6-OHDA induced a 22.6-fold increase in the density of reactive microglia in the ipsilateral hemisphere compared to that of the sham control (**Figure 7F**). Treatment with LBT-3627 decreased reactive microglia densities in the SN by 57–61%, regardless of dose. Taken together these data indicate that LBT-3627 is neuroprotective at the 6 mg/kg dose in 6-OHDA-induced dopaminergic neurodegeneration, while attenuation of microglial-mediated neuroinflammatory responses was possible at all dose levels evaluated.

LBT-3627 Tests on Cardiovascular Metrics

Despite promising immunomodulatory and related neuroprotective effects of VIPR2 activation observed in both rat models investigated in this report, a major gap between preclinical and human efficacy existed. To advance this VIPR2 strategy toward clinical evaluations warranted safety evaluations of LBT-3627. Thus, we administered LBT-3627 by subcutaneous (s.c.) injection to dogs at doses of 0.14, 0.6, and 1.4 mg/kg, which were allometrically scaled to mirror dose concentrations required for Treg induction. After the single injection of LBT-3627, dogs were monitored telemetrically for heart rate, pulse pressure, systolic blood pressure, diastolic blood pressure, and corrected Van de Water's QT (QTcV) interval (**Figure 8**). At the lowest dose level, none of the monitored parameters were changed over placebo during a 24-h observation period. At the two higher doses, transient, but limited, increases in heart rate, decreases in pulse pressure, and reductions in systolic pressure were observed. All changes were within the historical range of baselines as indicated by the dotted horizontal boundaries in all plots (**Figures 8A–E**).

DISCUSSION

The study provides further evidence that modulation of adaptive immunity through the production of Tregs leads to significant neuroprotection in PD animal models. As a peptide hormone and neurotransmitter, VIP's pleotropic effects modulate innate and adaptive immunity (Delgado et al., 2004; Pozo and Delgado, 2004; Reynolds et al., 2010; Ganea et al., 2015; Olson et al., 2015) that include the inhibition of macrophage and microglial release of inflammatory mediators (Gonzalez-Rey and Delgado, 2005, 2008; Delgado et al., 2008; Waschek, 2013; Higyno et al., 2015), regulation of lymphocytic Th1/Th2/Th17 differentiation (Voice et al., 2004; Jimeno et al., 2012; Tan et al., 2015; Villanueva-Romero et al., 2018), and alterations of immunoglobulin production by B lymphocytes (Samarasinghe et al., 2011; Xu et al., 2014). Our prior works also show that VIP and selective VIPR1 and VIPR2 receptor analogs affect Treg activity (Reynolds et al., 2010; Olson et al., 2015, 2016). In the setting of neurodegenerative diseases, VIP and VIP-based analogs downregulate microglia pro-inflammatory activities and spare dopaminergic neuronal damage in the nigra and striatum. These activities appear to be Treg-mediated as adoptive transfer from VIP-treated donors with α-Syn-specific Th17 effectors resulted in dopaminergic neuroprotection (Reynolds et al., 2010). Parallel experiments wherein Tregs were transferred from naïve donors were not as protective. In each of these cases, Treg-mediated activities induced by VIPR2 agonist performed better than those induced by VIPR1 agonist or native VIP (Olson et al., 2015). All together, these data show the importance of the VIPR2 receptor, not only as an immune modulator, but also as a potentiator of Treg-mediated neuroprotection (Dejda et al., 2005; Rangon et al., 2005; Delgado et al., 2008; Reynolds et al., 2010; Shioda and Gozes, 2011; Olson et al., 2015, 2016; Deng and Jin, 2017).

In parallel, VIP is associated with adverse effects in the gastrointestinal and the CNS (Bloom et al., 1973; Said and Faloona, 1975; Bloom, 1978; Kane et al., 1983; Guelrud et al., 1992; Masel et al., 2000; Cernuda-Morollon et al., 2015; Riesco et al., 2017). VIP's effects are due in large part by engaged signaling pathways associated with binding of specific receptors preferentially expressed by specific cell types. For instance, VIPR1 is preferentially expressed by gastrointestinal tissues, while VIPR2 is relegated largely to smooth muscle, lung, and myocardial organs (Schulz et al., 2015; Jayawardena et al., 2017). In the immune system, early reports suggested that modulatory effects were associated with the constitutively expressed VIPR1, however, recent data suggest that induced VIPR2 may play a larger role than previously suggested, especially for Treg induction or potentiation of suppressive function (Goetzl et al., 2001; Martinez et al., 2002; Samarasinghe et al., 2011; Olson et al., 2015, 2016). We recently synthesized specific agonists specific for VIPR1 or VIPR2 that do not interact with ADCYAP1R1 (PAC1), and display increased metabolic stability compared to VIP (Olson et al., 2015). We demonstrated that pretreatment with the VIPR2-specific agonist, LBT-3627, but not a VIPR1 agonist (LBT-3393), potentiated Treg neuroprotective activity in a dose-dependent manner in MPTP mice. These results supported Treg induction experiments in VIPR knockout animals which concluded that VIPR2, rather than VIPR1 plays a greater role in VIP-mediated augmentation of Tregs (Yadav et al., 2011; Tan et al., 2015). Moreover, adoptive transfer studies demonstrated that cells from LBT-3627-treated animals provide significantly greater protection for dopaminergic neurons in MPTP mice than that provided by VIPR1 agonist (Olson et al., 2015, 2016). However, whether VIPR2 agonist functions in different species and affords neuroprotection in more progressive models of dopaminergic neurodegeneration awaits further investigation.

In establishing the neuroprotective profile of VIPR2, adoptive transfer techniques were necessary to overcome

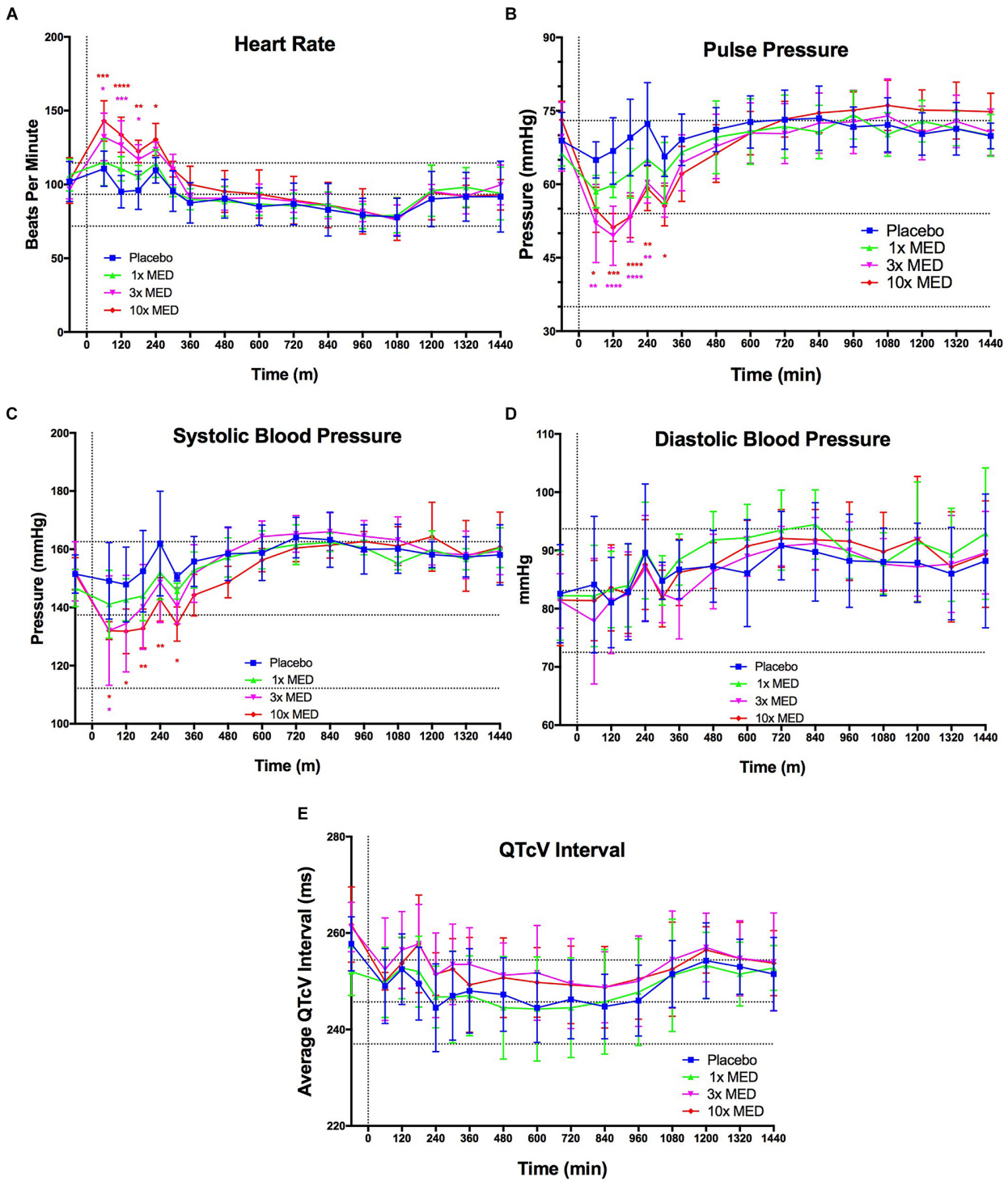

FIGURE 8 | VIPR2 agonist does not induce any overtly concerning effects on CV parameters. LBT-3627 was administered subcutaneously to beagle dogs at three dose levels (1× minimum effective dose (MED) = 0.14 mg/kg, 3× MED = 0.6 mg/kg, and 10× MED = 1.4 mg/kg), plus a vehicle only control (placebo), that allometrically scale to approximately match the three dose levels used in the neuroprotection portion of this study (0.6, 2.0, and 6.0 mg/kg) (n = 4/group). CV parameters were tracked for 24 h after the single s.c. injection. Additionally, horizontal dotted lines indicate the historic baseline averages, plus and minus one standard deviation, for the dogs within the testing colony. For the lowest dose level (1× MED), none of the CV parameters were significantly modulated compared to placebo **(A–E)**. Heart rate was significantly increased for both 3× and 10× MED for the first 3–4 h after administration and returned to placebo levels after 4 h **(A)**. Pulse pressure mirrors this response and is increased for the 3× and 10× MED groups for the first 4 h **(B)**. This increase in pulse pressure can be attributed to the increase in systolic pressure **(C)**, since the diastolic pressure was not modulated for any dose level compared to placebo **(D)**. QTcV interval was unaltered for any

(Continued)

FIGURE 8 | Continued
dose level compared to placebo as well **(E)**. Means and standard deviations were calculated for 4 dogs/group and significant differences were assessed by two-way ANOVA with Dunnett's *post hoc* tests. Compared to placebo treated animals, designated *P* values were *≤0.05, **≤0.01, ***≤0.001, or ****≤0.0001.

immunotoxicities intrinsic to the MPTP model. Herein, we used two different animal models that are not inherently immunotoxic and, therefore, preclude the need for adoptive transfer to evade toxicities and elicit neuroprotection. The α-Syn overexpression model results from this study are particularly interesting as they establish a clear link between human PD pathology (accumulation of α-Syn in the brain) and immune system dysregulation of innate and Treg functions (**Figures 3**, **6**). Of particular interest is that each dose level of LBT-3627 tested reduced microglial activation regardless of dose. Most interestingly, the middle dose of LBT-3627 (2.0 mg/kg), which trended to provide the highest suppressive activity, paralleled neuroprotection. Similar observations were recorded in the 6-OHDA rat model, wherein LBT-3627 reduced microglial activation across all three dose levels, but only the highest dose (6.0 mg/kg) was sufficient to afford significant neuronal survival. While the exact biological cascade fully describing how VIPR2 activation in the periphery leads to central neuroprotection remains incomplete, it is clear that peripheral T cell function plays a critical role. Several groups, ours included, are investigating specific genomic pathways outlining the crosstalk between CNS and immune compartments (Reynolds et al., 2010; Ransohoff and Brown, 2012; Kosloski et al., 2013; Anderson et al., 2014; Olson et al., 2015; Chan et al., 2016; Gendelman et al., 2017; Schutt et al., 2018; Limanaqi et al., 2019). Other groups have reported that the parenchyma and/or choroid plexus contribute to the crosstalk, which becomes more pronounced in diseased states, while others have sidestepped the issue and allude to passive diffusion pathways (Xu et al., 2010; Nunan et al., 2014; Balusu et al., 2016; Jayawardena et al., 2017; Marques et al., 2017; Arcuri et al., 2019).

However, should subsequent work determine that VIPR2 agonist is indeed directly responsible for both peripheral and central observations noted herein, without additional messengers, the neurotrophic aspects of the VIPR2 receptor would come into play. Specifically, VIP interneurons play several key roles in cognition. Recent work has revealed that VIP interneurons are able to affect the function of downstream pyramidal neurons. In optogenetic animal models, activation of these VIP interneurons improved memory retention over negative controls (Kamigaki and Dan, 2017). Furthermore, activation or disinhibition of these neurons, releases a self-imposed suppression on interneuron neurotransmitter signaling (Lee et al., 2013; Pi et al., 2013; Fu et al., 2014; Stryker, 2014; Zhang et al., 2014). Perhaps an underlying cause for this biological cascade affects circadian rhythm aspects relevant to VIP biology (Ono et al., 2016).

Aside from the biology described above, VIP is also responsible for attenuating microgliosis (Kim et al., 2000; Dejda et al., 2005; Waschek, 2013). This orthogonal, yet direct, mechanism of action is also beneficial in the context of a neurodegenerative disorder. If the complex biological pathways and peripheral to central crosstalk thesis is ignored as "too complex," one must conclude that the VIPR2 agonist is a lone actor with central actions being all neuroprotective in the diseased state and ultimately resulting in improved memory recall. While much work remains to be done to understand the peripheral to central transduction pathway along with a multitude of downstream molecular actors involved, the results herein clearly indicate that something is transferring from the peripheral to central compartments resulting in robust neuroprotection with attenuation of microgliosis. Therefore, based on our collective work, we proposed that VIPR2 activation, though minimally on Tregs, but potentially on other cell types, initiates a cascade of biological events that include (1) improved peripheral Treg function, (2) reduced reactive microglia inside the central compartment, (3) shifted balance of multiple pro- and anti-inflammatory cytokines, and (4) robust, dose response-mediated neuroprotection of TH^+ dopaminergic neurons.

Interestingly, an optimal range in the VIPR2 agonist dose appears to be necessary for Treg functional improvement in terms of neuroprotection. Specifically, in our earlier work, high concentrations of VIPR2 agonists result in a decrease in cAMP production via cell-based assays (Olson et al., 2015). This has been attributed to GPCR activation-induced receptor internalization (Valdehita et al., 2010). The rescue of Treg function in the α-Syn model was evident at the same dose (2 mg/kg) that induced the greatest neuroprotection. On the other hand, neuroprotection in the 6-OHDA model was attained only at the highest dose level. The case may be made that an appropriate balance must be achieved under different disease processes and conditions, and that a standard dose level may not be the best for each different set of circumstances. Another explanation to the non-traditional dose-response outcomes for neuroprotection in the α-Syn model may lie within the complex GPCR biological cascades that act through cAMP, β-arrestin1 or β-arrestin2 as second messengers. Furthermore, these underlying circuits may be wired differently for different cell types, possibly explaining the nuanced results reported herein. Multi-omic approaches would shed additional light on the specific wiring of this therapeutic pathway.

Regardless of the disease model, all dosing regimens of LBT-3627 reduced numbers of inflammatory microglia. For the overexpression of α-Syn model, treatment with LBT-3627 caused a dose-dependent reduction in microglial activation with 6 mg/kg yielding microglia levels indiscernible from sham or GFP controls, albeit not yielding statistically different levels from the other two lower doses. Similarly, LBT-3627 in 6-OHDA treated animals reduced microglia equally, regardless of dose, but did not attain levels of sham controls. This dichotomy may be due, in part, to different intensities of microglial responses in each model. Numbers of inflammatory microglia in

6-OHDA-treated animals increased 24-fold compared to sham controls, whereas overexpression of α-Syn increased microglial numbers by only 2.6-fold compared to sham controls. Thus, microglial responses in 6-OHDA treated rats intensities were 10-fold greater than those in α-Syn overexpression animals. The intensity of the microglial response may also affect the adaptive immune responses and exacerbate neuroinflammation and neurodegeneration as previously reported and reviewed (Benner et al., 2008; Gendelman and Mosley, 2015; Mosley and Gendelman, 2017). Indeed, 6-OHDA treated mice also have been reported to exhibit an extensive microglial response associated with IgG deposition, increased infiltration of T and B cells, and exacerbated dopaminergic neurodegeneration (Theodore and Maragos, 2015). Moreover, α-Syn overexpression induced an average of 50% dopaminergic neuronal loss in the substantia nigra, whereas 6-OHDA induced a 75% loss. Together, these data suggest that 6-OHDA induces greater severity of neuroinflammation and neurodegeneration than α-Syn overexpression.

It is well-known that peripheral VIPR2 activation can be directly attributed to central neuroprotection along with a reduction in microgliosis. Using doses of 2 mg/kg LBT-3627 led to protection of dopaminergic neurons in the SNpc and their terminal processes in the striatum reflecting our own prior works in MPTP intoxicated animals (Olson et al., 2015). Notably, doses of 2 mg/kg nearly restored striatal density to levels of AAV-GFP controls (**Figure 4C**). Nonetheless, in 6-OHDA-treated rats, doses of 2 mg/kg LBT-3627 were insufficient to elicit neuroprotection along the nigrostriatal axis and required at least 6 mg/kg to protect nigral neurons. Alternatively, differences in the exhibited effective doses for neuroprotection between models may be influenced by the difference in the severity of the models. Thus, regarding translational evaluation, the therapeutic paradigm may require personalization by dose titration or a biomarker guided treatment strategy using T cell-related markers as pharmacodynamic surrogates. For example, Treg activity may be one such marker since our works confirmed that VIPR2 agonism enhanced Treg activity, which confirmed others' previous results as well that required multiple injections to augment Treg activity in healthy subjects (Delgado et al., 2005; Chen et al., 2008; Deng et al., 2010; Jimeno et al., 2010; Reynolds et al., 2010; Olson et al., 2015, 2016).

While VIP and VIPR2 agonism promote Treg induction and function, the mechanisms are not well-defined. VIPR2 expression is either absent or minimal on resting $CD4^+$ T cells and Tregs, but can be upregulated by activated $CD4^+$ T cells (Lara-Marquez et al., 2001). Additionally, Tregs from lymphoid tissues of VIPR2 deficient mice are found at lower levels and have a diminished capacity to expand after stimulation, suggesting a role for VIPR2 in VIP- or agonist-induced maintenance or potentiation of Treg numbers or function. Whether that role is limited to direct interactions by Tregs or can indirectly exert its effects through other cell types has yet to be definitively resolved. Indeed, we previously showed that the VIPR2 agonist, LBT-3627 induces a 45-fold increase in GM-CSF expression among $CD4^+$ T cells (Olson et al., 2015), and that GM-CSF-induced tolerogenic DCs to transform naïve $CD4^+$ T cells to Tregs by OX40L and Jagged1 induced signaling via OX40 and Notch1, respectively (Gopisetty et al., 2013; Alharshawi et al., 2017; Schutt et al., 2018). Underscoring the translational potential of VIPR2 agonism is suggested by the demonstration that those downstream signaling outcomes via GM-CSF have successfully been tested in clinical trials of PD wherein sargramostim-treated patients showed increased Treg numbers and function, augmented motor neuronal activity, and improved UPDRS III scores (Gendelman et al., 2017). A direct mechanism is suggested for T cells activated through CD3/CD28, whereby VIP induced cell cycle arrest, inhibited IL-2 transcription, and suppressed transcription factor-mediated signaling with eventual transformation of Teff phenotypes to Treg phenotypes with high levels of CD25, CTLA4, and FOXP3 expression as well as potent suppressive activities (Anderson and Gonzalez-Rey, 2010). Indeed, the first translational use of VIP for regulation of immune conditions was tested with inhaled VIP for inflammatory sarcoidosis which increased Treg numbers and function from T cells in bronchoalveolar lavages (Prasse et al., 2010). Together, these data provide strength for a therapeutic strategy of VIP and VIP agonists as immune modulatory agents in inflammatory- or immune-mediated conditions.

Finally, we and others have shown that specific T cell responses are associated with PD (Baba et al., 2005; Brochard et al., 2009; Saunders et al., 2012; Levite, 2016; Gendelman et al., 2017; Mosley and Gendelman, 2017; Sulzer et al., 2017; Kustrimovic et al., 2018). Previous clinical studies showed diminution of Treg numbers or activities in PD patients compared to non-PD controls whereby those diminutions correlated to disorder severity (Saunders et al., 2012; Gendelman et al., 2017; Kustrimovic et al., 2018). Moreover, exposure to modified α-Syn has been shown to diminish Treg activity and Treg numbers (Reynolds et al., 2008, 2009, 2010; Labrador-Garrido et al., 2014; Schutt et al., 2018). Herein, we also demonstrated that Treg activity was diminished in rats that overexpress α-Syn compared to GFP controls (**Figure 6**), but showed no untoward effect on numbers or proliferative function of conventional $CD4^+$ T cells that serve as Tresps. Treatment of α-Syn overexpressing rats with LBT-3627 at 0.6 mg/kg slightly increased Treg activity from the nadir attained in α-Syn expressing rats, but not to Treg levels of controls. Rescue of Treg activity above control levels required treatment with VIPR2 agonist concentrations of 2–6 mg/kg. These data confirmed our previous data which demonstrated that immunization with nitrated α-Syn diminished Treg activity to levels significantly below naïve controls even with concomitant increases in Treg numbers (Reynolds et al., 2010). More recently, we found that nitrated α-Syn added during *in vitro* differentiation of naïve T cells to Tregs by tolerogenic dendritic cells significantly diminished numbers of differentiated Tregs (Schutt et al., 2018). In previous clinical studies, we found that Treg function levels were significantly diminished in PD patients compared to age- and environment-matched controls (Saunders et al., 2012; Gendelman et al., 2017). Similar to results here, we also found that treatment with VIP at the time of immunization with α-Syn rescued deficient Treg activity to levels significantly above controls (Reynolds et al., 2010). More importantly, treatment of PD patients or MPTP-intoxicated mice

with another immune modulatory agent, GM-CSF, also rescued Treg function and improved clinical scores as well as motor function (Kosloski et al., 2013; Gendelman et al., 2017). Taken together, these data indicate that that modified α-Syn regardless of endogenous or exogenous derivation, interferes with Treg differentiation or activation and diminishes Treg capability to control neuroinflammation. Thus, VIP or VIPR2 agonist may serve as a putative therapeutic to rescue deficits of differentiation and activation and return Treg function to neuroprotective levels.

DATA AVAILABILITY

Data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the UNMC Institutional Animal Care and Use Committee and Charles River Laboratories.

AUTHOR CONTRIBUTIONS

RM, JS, SS, and HG designed the experiments, interpreted the results, and wrote the manuscript. JS and SS provided the LBT-3627. YL, KO, JM, WY, and KN performed the experiments. YL and KO acquired and analyzed the data. RM performed the statistical analysis. All authors read and approved the final manuscript.

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Pituitary Adenylate Cyclase-Activating Polypeptide Modulates Hippocampal Synaptic Transmission and Plasticity: New Therapeutic Suggestions for Fragile X Syndrome

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Ciranna L and Costa L (2019) Pituitary Adenylate Cyclase-Activating Polypeptide Modulates Hippocampal Synaptic Transmission and Plasticity: New Therapeutic Suggestions for Fragile X Syndrome. Front. Cell. Neurosci. 13:524. doi: 10.3389/fncel.2019.00524

Pituitary adenylate cyclase-activating polypeptide (PACAP) modulates glutamatergic synaptic transmission and plasticity in the hippocampus, a brain area with a key role in learning and memory. In agreement, several studies have demonstrated that PACAP modulates learning in physiological conditions. Recent publications show reduced PACAP levels and/or alterations in PACAP receptor expression in different conditions associated with cognitive disability. It is noteworthy that PACAP administration rescued impaired synaptic plasticity and learning in animal models of aging, Alzheimer's disease, Parkinson's disease, and Huntington's chorea. In this context, results from our laboratory demonstrate that PACAP rescued metabotropic glutamate receptor-mediated synaptic plasticity in the hippocampus of a mouse model of fragile X syndrome (FXS), a genetic form of intellectual disability. PACAP is actively transported through the blood–brain barrier and reaches the brain following intranasal or intravenous administration. Besides, new studies have identified synthetic PACAP analog peptides with improved selectivity and pharmacokinetic properties with respect to the native peptide. Our review supports the shared idea that pharmacological activation of PACAP receptors might be beneficial for brain pathologies with cognitive disability. In addition, we suggest that the effects of PACAP treatment might be further studied as a possible therapy in FXS.

Keywords: pituitary adenylate cyclase-activating polypeptide, fragile X syndrome, cyclic adenosine monophosphate, long-term depression induced by metabotropic glutamate receptor, hippocampus, learning

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) was initially discovered in ovine hypothalamus as an endocrine regulator (Miyata et al., 1989). PACAP is highly expressed in the brain and in peripheral tissues in two forms of 38 and 27 amino acid residues, PACAP-38 and PACAP-27 (Arimura et al., 1991; Koves et al., 1991). In the present review, "PACAP" refers to

PACAP-38, the most abundant form in the brain (Arimura et al., 1991; Vaudry et al., 2009). We will specifically indicate PACAP-38 and PACAP-27 to underline differences between the two forms.

Pituitary adenylate cyclase-activating polypeptide activates two main classes of G protein-coupled receptors: PAC1 and VPAC (Vaudry et al., 2009). PAC1 is PACAP specific, with a high nanomolar affinity for PACAP and a 1,000-fold lower affinity for the structurally related vasoactive intestinal peptide (VIP). VPAC receptors include VPAC1 and VPAC2 subtypes, with equally high nanomolar affinity for PACAP and VIP. PAC1 and VPAC receptors are expressed in peripheral tissues and in the central nervous system (CNS), where PAC1 is the most abundant (Jolivel et al., 2009). All PACAP/VIP receptors are positively coupled to adenylate cyclase; PAC1 receptors can also activate phospholipase C and Ca^{2+} release. The brain localization, pharmacological features, signal transduction mechanisms, and biological effects of PACAP/VIP receptors are described in details in excellent reviews (Dickson and Finlayson, 2009; Vaudry et al., 2009; Harmar et al., 2012; Hirabayashi et al., 2018).

In the CNS, PACAP is a neurotrophic and a neuroprotective factor regulating differentiation of neuronal precursors, promoting neuronal survival, and exerting neuroprotective effects after brain damage (Arimura et al., 1994; Shioda and Nakamachi, 2015; Reglodi et al., 2018c). Acting on the brain, PACAP also regulates important physiological functions, among which are feeding (Sekar et al., 2017), circadian rhythm (Holland et al., 2018), body temperature (Tan et al., 2016), learning, and memory (see below). We will initially describe the physiological role of PACAP on learning and then highlight recent findings showing an involvement of PACAP in cognitive disability. Finally, we will discuss the possibility to use PACAP as a pharmacological tool in conditions of learning impairment.

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE MODULATES HIPPOCAMPAL SYNAPTIC TRANSMISSION, SYNAPTIC PLASTICITY, AND LEARNING IN PHYSIOLOGICAL CONDITIONS

Pituitary adenylate cyclase-activating polypeptide receptors are highly expressed in rat hippocampus (Shioda et al., 1997; Jaworski and Proctor, 2000; Joo et al., 2004). On cultured hippocampal neurons, PACAP stimulated axon outgrowth (Ogata et al., 2015) and increased the size and density of dendritic spines (Hayata-Takano et al., 2019). Accordingly, PACAP-deficient mice displayed a reduced hippocampal spine density with respect to wild type (Hayata-Takano et al., 2019), indicating an important role of PACAP on synapse formation.

Pituitary adenylate cyclase-activating polypeptide increases the firing rate of hippocampal neurons (Di Mauro et al., 2003; Liu et al., 2003) and inhibits potassium currents responsible for membrane repolarization (Taylor et al., 2014; Gupte et al., 2015), which likely accounts for PACAP-induced increase in intrinsic excitability. Importantly, PACAP modulates hippocampal synaptic transmission and plasticity (Yang et al., 2010) and hippocampus-dependent learning (Borbely et al., 2013). In CA1 neurons, PACAP dose dependently modulates glutamate-mediated transmission (Kondo et al., 1997; Roberto et al., 2001; Ciranna and Cavallaro, 2003; Ster et al., 2009), exerting different effects on AMPA (Costa et al., 2009; Toda and Huganir, 2015) and NMDA receptor-mediated synaptic responses (Yaka et al., 2003; Macdonald et al., 2005). PACAP also stimulates acetylcholine release in the rodent hippocampus (Masuo et al., 1993), which in turn affects glutamate-mediated transmission (Roberto and Brunelli, 2000; Roberto et al., 2001; Pecoraro et al., 2017).

Pituitary adenylate cyclase-activating polypeptide-deficient mice show reduced long-term depression (LTP) in the dentate gyrus (Matsuyama et al., 2003) and impaired memory (Ago et al., 2013; Takuma et al., 2014). Impaired hippocampal LTP was also observed in PAC1 receptor-deficient mice (Otto et al., 2001; Matsuyama et al., 2003), together with a specific deficit in contextual fear conditioning, an index of associative memory, but not in Morris water maze test performance, indicative of spatial discrimination (Sauvage et al., 2000; Otto et al., 2001). In wild-type rats, intravenous injection of PACAP improved spatial memory (Ladjimi et al., 2019); direct infusion of PACAP in the hippocampus and amygdala improved learning in contextual fear conditioning (Schmidt et al., 2015); intracerebroventricular administration of PACAP exerted bi-directional effects (initial impairment and later improvement) on fear conditioning memory (Meloni et al., 2016, 2018) and, at low doses, improved learning in passive avoidance response test (Sacchetti et al., 2001).

DYSREGULATION OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE FUNCTIONS IN CONDITIONS OF COGNITIVE IMPAIRMENT

Recent studies show reduced brain levels of PACAP and/or altered PACAP receptor expression in cognitive deficit (**Table 1**). PACAP-KO mice display neuronal apoptosis, oxidative damage, and neuroinflammation similar to those observed at old age; thus, PACAP deficiency has been proposed as a model of premature aging (Reglodi et al., 2018b). Accordingly, age-related cognition impairment was associated with reduced levels of PACAP in rhesus macaque brain (Han et al., 2017) and in an invertebrate model of aging, in which memory loss was rescued by activation of PAC1 receptors (Pirger et al., 2014).

Defects in PACAP-mediated functions are well documented in Alzheimer's disease (AD), the most devastating neurodegenerative disease leading to memory loss and dementia. Reduced PACAP levels were observed in the brain of different transgenic AD mouse models (Wu et al., 2006; Han et al., 2014b, 2017) and in cortical brain samples from AD patients, where

TABLE 1 | Involvement of PACAP in conditions involving cognitive impairment.

Condition	Data from human patients	Data from animal models	References
Aging	–	Aged pond snail (*Lymnaea stagnalis*): low brain levels of PACAP. PACAP administration rescued learning impairment.	Pirger et al., 2014
		PACAP-KO mice: neuronal apoptosis, oxidative damage, and neuroinflammation.	Reglodi et al., 2018b
		Aged rhesus macaque: reduced brain levels of PACAP (striatum, parietal, and temporal lobes).	Han et al., 2017
Alzheimer's disease (AD)		Transgenic AD mouse models: reduced PACAP gene expression.	Wu et al., 2006
		Transgenic AD mouse model: *in vivo* administration of PACAP exerted neuroprotective effects and improved learning.	Rat et al., 2011
	AD patients: reduced brain levels of PACAP.		Han et al., 2014a,b, 2015
		Transgenic AD mouse model: reduced PACAP gene expression and brain levels. PACAP protected cultured neurons against Aβ toxicity.	Han et al., 2014b, 2017
Parkinson's disease (PD)	–	6-OHDA-treated rats: *in vivo* administration of PACAP exerted neuroprotective effects and reduced behavioral deficits.	Reglodi et al., 2004, 2006
		MPTP-treated mice: *in vivo* administration of PACAP exerted neuroprotective effects.	Wang et al., 2008; Lamine et al., 2016; Lamine-Ajili et al., 2016
		MPTP-treated mice: *in vivo* administration of PACAP improved learning.	Deguil et al., 2010
		Prostaglandin J2-treated mice: PACAP-27 exerted neuroprotective effects.	Shivers et al., 2014
		MPTP-treated macaque: altered PAC1 receptor expression in basal ganglia	Feher et al., 2018
Huntington's disease (HD)	HD patients: reduced PAC1 receptor expression in the hippocampus.	Transgenic HD mouse models: reduced expression of PAC1, VPAC1, and VPAC2 receptors in the hippocampus. *In vivo* administration of PACAP rescued synapse formation, PAC1 receptor levels, and learning.	Cabezas-Llobet et al., 2018
Schizophrenia	Schizophrenia patients: mutations of genes coding for PACAP and PAC1 receptors.	–	Hashimoto et al., 2007
Fragile X syndrome	–	*Fmr1* KO mouse hippocampal slices: PACAP rescued abnormal synaptic plasticity.	Costa et al., 2018

PACAP, pituitary adenylate cyclase-activating polypeptide; AD, Alzheimer's disease; 6-OHDA, 6-hydroxydopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

PACAP levels were inversely related to the amount of amyloid plaques and neurofibrillary tangle, as well as to dementia rating scores (Han et al., 2014a). Reduced PACAP levels and altered PAC1 receptor expression in the brain of AD patients were detected since early stages of the progressive neurodegeneration characterized by mild cognitive impairment (Han et al., 2015). Interestingly, besides exerting a neuroprotective role, PACAP stimulates a non-amyloidogenic processing pathway of amyloid precursor protein (APP) (Kojro et al., 2006), suggesting that PACAP might be used in AD therapy. Administration of PACAP has proven to be effective against Aβ-induced toxicity in different AD mouse models (Rat et al., 2011; Han et al., 2014b). Intranasal administration of PACAP to APP-transgenic mice increased brain expression of PACAP and PACAP receptors, stimulated the production of neurotrophic and antiapoptotic factors [brain-derived neurotrophic factor (BDNF) and Bcl-2], enhanced the expression of the Aβ-degrading enzyme neprilysin, and improved learning (Rat et al., 2011). Accordingly, VIP decreased amyloid plaques and prevented brain atrophy in the 5xFAD mouse model of AD (Korkmaz et al., 2018). The same authors suggest that VIP-mediated neuroprotective effects might also be used for therapy of Parkinson's disease (PD) (Korkmaz and Tuncel, 2018).

Parkinson's disease, a neurodegenerative disorder characterized by loss of dopaminergic neurons in the substantia nigra, primarily affects motor control and also involves cognition deficit (Aarsland et al., 2017). A new study shows a decreased PAC1 receptor expression in basal ganglia in a macaque model

of PD (Feher et al., 2018), suggesting that reduced PACAP function contributes to neurodegeneration and PACAP might become a promising tool for PD therapy (Reglodi et al., 2017). Administration of PACAP to rats treated with the neurotoxin 6-hydroxydopamine (6-OHDA), a model of PD, prevented degeneration of nigral dopaminergic neurons and rescued behavioral deficits (Reglodi et al., 2004, 2006). Likewise, in a different murine PD model [mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)], intravenous injection of PACAP-27 prevented neuronal loss in the substantia nigra (Wang et al., 2008) and rescued learning deficit (Deguil et al., 2010). Still in MPTP-treated mice, PACAP treatment exerted neuroprotective effects in the substantia nigra (Lamine et al., 2016) and reduced abnormal autophagy, a mechanism that might contribute to neuronal death (Lamine-Ajili et al., 2016). PACAP-27 also prevented dopaminergic neuronal loss and motor deficit in prostaglandin J2-treated mice, another proposed PD model (Shivers et al., 2014).

A reduced expression of all PACAP receptor subtypes was observed in two different mouse models of Huntington's disease (HD), an inherited degenerating motor and cognitive disease, and PAC1 receptors were downregulated in postmortem hippocampal samples from HD patients (Cabezas-Llobet et al., 2018). Remarkably, intranasal administration of PACAP to HD mice increased PAC1 receptor expression, stimulated BDNF production, reduced the formation of huntingtin aggregates, prevented the loss of hippocampal glutamatergic synapses, and improved memory (Cabezas-Llobet et al., 2018).

Finally, mutations of the genes coding for PACAP and for PAC1 receptors were found in schizophrenic patients together with reduced hippocampal volume and impaired memory (Hashimoto et al., 2007). Schizophrenia involves dysregulation of brain dopaminergic system (Weinstein et al., 2017). Interestingly, genetic ablation of D3 receptors increased the expression of PACAP and PACAP receptors in mouse hippocampus and enhanced memory (Marzagalli et al., 2016), suggesting a close interplay between PACAP and dopamine on learning and memory.

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE RESCUES SYNAPTIC PLASTICITY IN A MOUSE MODEL OF FRAGILE X SYNDROME

Fragile X syndrome (FXS) is a genetic form of intellectual disability affecting 1/4,000 males and 1/6,000 females. FXS patients show cognitive and language deficits; subgroups of patients also display autistic features, epilepsy, attention deficit and hyperactivity disorder (ADHD), and mood disorders (Bardoni et al., 2006; Maurin et al., 2014; Gross et al., 2015; Hagerman et al., 2017). FXS is caused by transcriptional silencing of the FMR1 gene coding for fragile X mental retardation protein (FMRP) (Verkerk et al., 1991), an mRNA-binding protein mostly functioning as a repressor (Garber et al., 2006) and in some cases as an enhancer (Bechara et al., 2009) of protein translation (Darnell and Klann, 2013). Hundreds of mRNAs have been identified as FMRP targets, particularly mRNAs coding for proteins involved in synapse development and function (Bassell and Warren, 2008; Bear et al., 2008). Cortical neurons from *Fmr1* knock out (*Fmr1* KO) animal models of FXS (Comery et al., 1997) and FXS patients (Irwin et al., 2000) display an increased density of dendritic spines, with a long and thin morphology reminiscent of immature filopodia. Abnormal dendritic spine morphology has crucial consequences on synaptic function. Many alterations of glutamate-mediated synaptic transmission and plasticity were found in the brain of *Fmr1* KO mice. Among the first discovered, hippocampal LTP induced by metabotropic glutamate receptors (mGluR-LTD) is abnormally enhanced (Huber et al., 2002). Exaggerated mGluR-LTD led to formulation of the "mGluR theory" of FXS, pointing out excessive signaling downstream activation of mGluRs (Bear et al., 2004). In *Fmr1* KO neurons, mGluRs also show altered cell-surface mobility, abnormal coupling to NMDA receptors, and impaired mGluR-LTD of NMDA-mediated synaptic currents (Aloisi et al., 2017). Other malfunctions of glutamatergic synapses in *Fmr1* KO mouse brain include a reduced coupling of mGluRs to Homer proteins (Giuffrida et al., 2005), a reduced NMDA/AMPA ratio (Yun and Trommer, 2011; Gocel and Larson, 2012; Aloisi et al., 2017), and altered NMDA-dependent plasticity (Uzunova et al., 2014; Bostrom et al., 2015). An increased expression of Ca^{2+}-permeable AMPA receptors was recently found in human neural precursors derived from FXS patients (Achuta et al., 2018). Inhibitory synapses are also affected in the brain of FXS animal models, with a deficit of GABAergic inhibition (Martin et al., 2014; Braat and Kooy, 2015) and abnormal functioning of $GABA_A$ receptors (He et al., 2014).

At a cellular level, FMRP absence is associated with dysregulation of many signaling pathways, among which upregulation of PI3K/Akt/mTOR pathway (Sharma et al., 2010; Huber et al., 2015), overactivation of GSK3 (Min et al., 2009), and altered MAPK/ERK signaling (Kim et al., 2008; Osterweil et al., 2010). The large amount of data now available on the molecular basis of FXS provides several cues for a possible therapy of FXS, currently under investigation (Santoro et al., 2012; Sethna et al., 2014; Gross et al., 2015; Castagnola et al., 2017). Each proposed strategy might be useful in subsets of FXS patients, owing to a large individual heterogeneity with respect to the type and severity of symptoms (Jacquemont et al., 2014).

Interestingly, early observations on FXS patients and latest findings on FXS animal models have pointed out a downregulation of the cyclic adenosine monophosphate (cAMP) pathway, originating a "cAMP theory" of FXS (Kelley et al., 2008). A recent study shows that the mRNA coding for phosphodiesterase 2A (PDE2A), a cAMP-degrading enzyme, is among the most prominent targets of FMRP (Maurin et al., 2018a). In the brain of *Fmr1* KO mice, PDE2A is overexpressed and overactive, causing reduced cAMP formation and dysregulation of cAMP downstream signaling (Maurin et al., 2018b). In line with this, synaptic plasticity, learning, and behavior in *Fmr1* KO mice are rescued by agonists of

serotonin 5-HT_7 receptors, positively coupled to adenylate cyclase (Costa et al., 2012, 2015, 2018; Ciranna and Catania, 2014), by PDE4 inhibitors (Choi et al., 2015, 2016), and by a selective PDE2A inhibitor (Maurin et al., 2018b). Of note, inhibition of PDE2A also corrected abnormal dendritic spine morphology of cortical neurons from *Fmr1* KO mice (Maurin et al., 2018b). All these data confirm a deficit in cAMP-mediated signaling in *Fmr1* KO neurons and demonstrate that pharmacological manipulations increasing cAMP levels can rescue synaptic morphology and function, learning, and behavior in animal models of FXS.

Pituitary adenylate cyclase activating polypeptide is a potent stimulator of adenylate cyclase activity (Miyata et al., 1989; Vaudry et al., 2009). Consistent with the cAMP hypothesis of FXS, we found that PACAP reversed mGluR-LTD in the CA3-CA1 hippocampal synapse in wild-type mice and reduced exaggerated mGluR-LTD in *Fmr1* KO, thus correcting a synaptic defect typically observed in FXS mouse models (Costa et al., 2018). This result offers novel suggestions for a possible therapy of FXS, for which no specific cure is presently available. In future studies, it would be interesting to test if PACAP can correct other abnormal features in *Fmr1* KO neurons (dendrite development, synapse formation and function, ion channel expression, membrane excitability, and intracellular signaling) and rescue learning and behavior when administered *in vivo* to *Fmr1* KO mice.

Dysregulation of cyclic nucleotide pathways was found at different levels (synthesis, functioning, and/or degradation by PDE) in aging and age-related cognitive decline (Kelly, 2018). Accordingly, inhibition of PDE activity improved memory in animal models of AD (Gulisano et al., 2018) and HD (Saavedra et al., 2013) and seems to prevent memory loss in elderly humans and in AD patients (Prickaerts et al., 2017). As indicated above, in FXS, the cAMP pathway is also dysregulated, and increasing cAMP rescues several phenotypes. Therefore, altered cAMP signaling might be a common feature in cognitive deficits of very different origin, in which administration of PACAP might be beneficial.

SYSTEMIC ADMINISTRATION ROUTES FOR EFFECTIVE BRAIN DELIVERY OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE

Pituitary adenylate cyclase activating polypeptide-38 is actively transported into the brain across the blood–brain barrier (BBB) by a saturable carrier, whereas PACAP-27 passes the BBB by transmembrane diffusion (Banks et al., 1993). After intravenous administration, the amount of PACAP-38 brain uptake was high enough to exert neuroprotective effects (Banks et al., 1996). The rate of PACAP-38 transport varies largely in different brain areas, with maximal uptake in the hypothalamus and in the hippocampus (Nonaka et al., 2002), and can be altered in pathological conditions (Banks et al., 1998; Nonaka et al., 2002; Rhea et al., 2018).

On the other side, PACAP-38 and PACAP-27 are transported out of the brain by a common efflux mechanism, reducing their brain levels (Banks et al., 1993). It is noteworthy that antisense inhibition of peptide transport system-6 (PTS-6) improved brain uptake and neuroprotective effects of PACAP-27 in murine models of AD and stroke (Dogrukol-Ak et al., 2009), suggesting that inhibition of PTS-6 efflux component might become a therapeutic strategy to enhance central effects of PACAP.

Some issues were raised about intravenous administration of PACAP to humans: PACAP-38 induced headache in healthy and migraine-suffering subjects (Schytz et al., 2009). Headache was also reported after intravenous infusion of PACAP-27 to healthy subjects (Ghanizada et al., 2019a) and migraine patients (Ghanizada et al., 2019b).

Other issues against intravenous administration of PACAP concern metabolic stability and selectivity. In fact, PACAP-38 showed a very short half-life (<5 min) in human plasma *in vitro*, being converted by the blood enzyme dipeptidyl peptidase IV into shorter peptides that behave as PACAP receptor antagonists, whereas PACAP-27 was relatively stable (Bourgault et al., 2008).

Besides, parenteral administration of PACAP can induce undesired peripheral actions, among which are cardiovascular (Runcie et al., 1995; Farnham et al., 2012) and hormonal effects (Tsutsumi et al., 2002). To overcome these limitations, new synthetic agonists of PACAP receptors have been developed with improved metabolic stability, higher brain uptake, selectivity for PAC1 receptors (the predominant PACAP receptors in the CNS), and reduced side effects (Bourgault et al., 2008; Dejda et al., 2011; Doan et al., 2011; Lamine et al., 2016).

Another strategy exploits conjugation of PACAP with a TAT peptide, improving passage through the BBB (Yu et al., 2012a,b). Carrier vesicles can also be used to protect peptides from blood-degrading enzymes (Dufes et al., 2004).

Administration routes for brain delivery of PACAP are illustrated in details in a recent review (Reglodi et al., 2018a). A promising non-invasive and easy route is intranasal application, by which PACAP reaches the brain fast and effectively in rodents, exerting neuroprotective effects in mouse models of AD (Rat et al., 2011; Nonaka et al., 2012) and HD (Cabezas-Llobet et al., 2018). Intranasal administration of PACAP was also tested on human volunteers, showing good safety and tolerability (Doberer et al., 2007; Reglodi et al., 2018a) with only mild local adverse reactions (Kinhult et al., 2003). Interestingly, headache was not reported after intranasal application of PACAP-38 (Doberer et al., 2007), suggesting this route as a valuable alternative to intravenous administration.

CONCLUDING REMARKS

Pituitary adenylate cyclase activating polypeptide plays an important role in learning and has a therapeutic potential in cognition deficits associated with disruption of cyclic nucleotide signaling. We suggest that the effects of PACAP might also be

studied for a possible therapy of FXS, in which a deficit in cAMP formation and downstream signaling were evidenced. For future translational applications, it would be interesting to test *in vivo* effects of PACAP on learning and behavioral deficits in FXS animal models.

Pituitary adenylate cyclase activating polypeptide is brain permeant and has already proved to be safe on healthy humans and thus might be tested in clinical trials. To improve PACAP brain uptake, it would be worth focusing on new selective PAC1 receptor agonists with enhanced metabolic stability, on delivery carriers, and/or on suitable administration routes.

AUTHOR CONTRIBUTIONS

LCi designed and directed the research projects and wrote the manuscript. LCo performed the experiments and data analysis, and contributed to the manuscript preparation.

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BRIEF RESEARCH REPORT
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VIP/PACAP-Based Drug Development: The ADNP/NAP-Derived Mirror Peptides SKIP and D-SKIP Exhibit Distinctive *in vivo* and *in silico* Effects

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Activity-dependent neuroprotective protein (ADNP) was discovered and first characterized in the laboratory of Prof. Illana Gozes to be regulated by vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase-activating peptide (PACAP) toward neuroprotection. Importantly, ADNP is a master regulator of >400 genes, essential for brain formation, while its haploinsufficiency causes cognitive impairments. Recently, *de novo* mutations in ADNP were identified as leading to the autism-like *ADNP* syndrome, mimicked by the *Adnp*-deficient mouse model. Furthermore, novel peptide derivatives of the neuroprotective ADNP-snippet NAP (NAPVSIPQ), developed in our laboratory, include SKIP and the mirroring all D-amino acid SKIP (D-SKIP). We now extended previous evidence suggesting potential antagonistic features for D-SKIP, compared with the neuroprotective peptide SKIP, as was observed by NMR analysis and social/olfactory functional testing. Here, an impact of the *Adnp* genotype was observed in the Morris Water Maze (MWM) test measuring cognition, coupled with improvement by SKIP, opposing the inert/exacerbating effect of D-SKIP. In the elevated plus-maze and open field tests measuring anxiety-related behaviors, contrasting effects of SKIP and D-SKIP were found, with SKIP improving/preserving the normal phenotype of the mouse, and D-SKIP causing alterations. Lastly, an *in silico* analysis suggested that SKIP and D-SKIP bind the microtubule end binding (EB) proteins EB1 and EB3 in different conformations, thereby indicating distinctive natures for the two peptides, potentially mediating differential *in vivo* effects. Altogether, our findings corroborate the notion of D-SKIP acting as an antagonist, thus distinguishing it from the neuroprotective SKIP.

Keywords: ADNP, NAP, SKIP, D-SKIP, VIP, PACAP, ASD

INTRODUCTION

Activity-dependent neuroprotective protein (ADNP) and activity-dependent neuroprotective factor (ADNF) were originally discovered and characterized in our laboratory (IG) in collaboration with the laboratory of Dr. Douglas E. Brenneman as astroglial secreted proteins, found to mediate the neurotrophic/neuroprotective activity of vasoactive intestinal peptide (VIP; Brenneman and Gozes, 1996; Bassan et al., 1999), as well as pituitary adenylate cyclase-activating peptide (PACAP; Zusev and Gozes, 2004). Importantly, ADNP is essential for mammalian brain formation (Bassan et al., 1999; Zamostiano et al., 2001; Pinhasov et al., 2003; Vulih-Shultzman et al., 2007), and function, as it was shown that complete knockout of the *Adnp* gene in mice results in embryonic lethality due to a failure in neural tube closure (Pinhasov et al., 2003). Nevertheless, *Adnp*-haploinsufficient mice ($Adnp^{+/-}$) survive but exhibit cognitive and social deficits, as well as microtubule-tau pathology and neurodegeneration (Vulih-Shultzman et al., 2007), even when being outbred (Malishkevich et al., 2015; Amram et al., 2016; Hacohen-Kleiman et al., 2018), thus confirming the impact of ADNP on neuronal function and *in vivo* behavior.

Furthermore, the Gozes laboratory identified a small active 8-amino-acid peptide (NAPVSIPQ = NAP, also called davunetide or CP201), derived from ADNP. NAP was shown to enhance cognitive function in $Adnp^{+/-}$ mice (Vulih-Shultzman et al., 2007; Hacohen-Kleiman et al., 2018; Sragovich et al., 2019a), by interacting with tubulin/microtubule end binding (EB) proteins through its SIP motif (NAPVSIPQ; Oz et al., 2014), eventually leading to microtubule stabilization and fortification (Gozes et al., 2005). Similarly, additional peptides developed in our lab were derived from ADNF, namely ADNF-14 (VLGGGSALLRSIPA) and ADNF-9 (SALLRSIPA = SAL), both sharing the SIP motif with NAP (Gozes et al., 2000b), and exhibiting neuroprotective activity (Brenneman and Gozes, 1996; Brenneman et al., 1998). Interestingly, both D- and L-amino acid forms of ADNF-9 (SAL) exhibited similar neuroprotective potency, with the D-amino acid form being protease stable and suitable for oral administration (Brenneman et al., 2004; Wilkemeyer et al., 2004; Parnell et al., 2007), further found to be neuroprotective *in vitro* and *in vivo* (Gozes et al., 2008; Shiryaev et al., 2011).

NAP safety and efficacy profiles were also translated to humans, showing favorable intranasal brain bioavailability, broad safety profile (Gozes et al., 2005), as well as cognitive and functional protection in clinical trials involving patients suffering from amnestic mild cognitive impairment and schizophrenia (Javitt et al., 2012; Jarskog et al., 2013; Morimoto et al., 2013a,b). Based on the SIP motif, serving as the NAP EB binding site, a NAP-derived 4-amino acid peptide, SKIP, was developed. This peptide contains only the EB-binding site, and was previously found to protect social memory, axonal transport, as well as correct hippocampal gene expression in the $Adnp^{+/-}$ mouse model (Amram et al., 2016). In parallel, a dextrorotatory analog peptide, D-SKIP, was also developed in the lab, and further suggested to have an antagonistic activity, as was observed by NMR analysis and social/olfactory functional testing (Amram et al., 2016).

Recently, ADNP was found to be mutated *de novo* in 0.17% of autism spectrum disorder (ASD) cases, thus causing the *ADNP* syndrome. A thorough characterization of this pathologic condition has revealed cognitive deficits (Helsmoortel et al., 2014), global developmental delays, intellectual disabilities (ID), speech impediments and motor dysfunctions (Gozes et al., 2017a; Van Dijck et al., 2019). In this regard, the human *ADNP* syndrome patient was shown to be mimicked by the *Adnp*-haploinsufficient mouse model, implying a strong impact of the *Adnp* genotype, which included neurodevelopmental delays, as well as cognitive, social, and motor deficits (Hacohen-Kleiman et al., 2018). At the synapse level, reduced dendritic spine density and altered synaptic gene expression were also observed in *Adnp*-deficient mice. In terms of therapeutics, systemic/nasal administration of the drug candidate, NAP, provided improvement at all investigated levels spanning from synapse to behavior in $Adnp^{+/-}$ mice (Hacohen-Kleiman et al., 2018). Therefore, these findings established the *Adnp*-haploinsufficient mouse as a valid model for the human *ADNP* syndrome patient and provided a solid basis for the further clinical development of NAP (CP201) in children suffering from the *ADNP* syndrome (Hacohen-Kleiman et al., 2018).

In the current article, we sought to characterize and provide additional evidence for the antagonistic nature of D-SKIP. For this purpose, the study involved male mice subjected to several *in vivo* behavioral tests including the Morris Water Maze (MWM) test measuring cognition, as well as elevated plus maze (EPM) and open field tests measuring anxiety-related behavior. Results showed cognitive impairments in $Adnp^{+/-}$ mice, observed in the MWM test, coupled with improvement by SKIP treatment. In contrast, D-SKIP exhibited a distinctive effect, with an aggravated phenotype displayed in $Adnp^{+/+}$ mice in the MWM, and with no amelioration observed in $Adnp^{+/-}$ mice. Anxiety-related behavior assessed in the EPM and open field tests were improved/normally preserved by SKIP treatment in $Adnp^{+/+}$ mice or remained unaffected in their $Adnp^{+/-}$ counterparts. As opposed, D-SKIP-treated mice of both genotypes presented an altered phenotype. Lastly, *in silico* bioinformatics analysis showed that SKIP and D-SKIP bind EB1 and EB3 proteins in different conformations. Therefore, our present results provide further characterization/substantiation of D-SKIP as a potential antagonist, since D-SKIP did not mimic the SKIP effect. Furthermore, in some cases, D-SKIP acted as an inert compound not causing any effect, whereas in other cases it exacerbated the wildtype phenotype.

MATERIALS AND METHODS

Experimental Design

Animal group sizes were determined in a pilot study, and animals were randomly allocated into experimental groups before the experiment. Blinded experienced researchers performed independently the different methodologies described in the manuscript, and repeated these successfully, thus substantiating

the results. Biological replicates were used for all the *in vivo* procedures described in the manuscript. The exact experimental group allocations are included in each figure legend.

Animals

The *Adnp*$^{+/-}$ mice, on a mixed C57BL and 129/Sv background, were previously described (Pinhasov et al., 2003; Vulih-Shultzman et al., 2007). For continuous breeding, an ICR outbred mouse line was used (Malishkevich et al., 2015; Amram et al., 2016). Animals were housed in a 12-h light/12-h dark cycle animal facility, with free access to rodent chow and water. Genotyping was performed by Transnetyx (Memphis, TN, USA). Furthermore, for all *in vivo* behavioral procedures, animals were tracked, monitored and recorded using the EthoVision XT video tracking system and software (Noldus Inc., Leesburg, VA, USA). The MWM and open field *in vivo* tests were performed under bright illumination conditions (light levels of ∼500 lux), whereas the elevated plus-maze was performed with a dim light.

Peptide Synthesis and SKIP/D-SKIP Treatment

SKIP and D-SKIP were customarily synthesized by Hay Laboratories, Israel. Prior to beginning behavioral tests, intranasal treatment was administered twice daily, 5 days a week, over a 1-month period to 6-month-old male mice (2 μg/5 μl/mouse/dose), followed by a chronic daily administration regime of the peptides. For intranasal administration, the peptides were dissolved in a vehicle solution (Alcalay et al., 2004), termed DD, in which each milliliter included 7.5 mg of NaCl, 1.7 mg of citric acid monohydrate, 3 mg of disodium phosphate dihydrate, and 0.2 mg of benzalkonium chloride solution (50%). SKIP/D-SKIP or vehicle solution (DD) were administered to mice hand-held in a semi-supine position with nostrils facing the investigator. A pipette tip was used to administer 5 μl/mouse/dose. The mouse was handheld until the solution was totally absorbed (∼10 s). In days of scheduled behavioral tests, SKIP/D-SKIP were applied once a day, 2 h before the test.

Morris Water Maze (MWM)

The apparatus was a pool with a diameter of 140 cm, filled with opaque water (23–24°C). An escape platform (12 × 12 cm^2) was placed 0.5 cm below the water surface. Two daily tests, constituting two blocks of trials, 90 s each, were performed for five consecutive days (Gozes et al., 2000a). The platform location and the animal starting point were held constant within each pair of daily tests, but they were changed from day-to-day. The mice were allowed to stay on the platform for 20 s before and after each trial. The time taken for an animal to reach the platform (latency) was measured. The daily improvement (in seconds to reach the hidden platform) for each animal (in comparison to the starting day) was evaluated.

Elevated Plus-Maze (EPM)

The elevated plus maze (EPM) trial is used for testing anxiety, based on the assumption that animals suffer from fear of open spaces. The maze consists of two open arms and two closed arms (50 cm × 10 cm × 40 cm each). The arms of each type are opposite to each other. The maze is elevated to 50 cm height from the floor. Mice were placed onto the center of the maze, facing an open arm, and left free to explore it for 5 min. The time spent in the open and closed arms was recorded and compared. The data were analyzed using the following formula: D2 = (b − a)/(b + a), in which "a" designated the time spent in the open arms, and "b" designated the time spent in the closed arms (Alcalay et al., 2004). The longer stay in the closed arms reflects increased anxiety-like behavior (Pellow et al., 1985; Treit et al., 1993).

Open Field

The open field apparatus is a 50 × 50 cm square arena, with 30 cm high walls (white/black colored). Mice were individually placed in the open field and left to explore it freely for 15 min. The distance moved and time spent in the entire open field, as well as in its inner defined quadrants (center, border) were measured.

In silico Analysis

EB1 and EB3 homodimers (Honnappa et al., 2009; Bjelic et al., 2012) were used in order to predict the SKIP and D-SKIP conformations. Flexible docking was performed using Rosetta FlexPepDock (Raveh et al., 2010).

Statistical Analysis

Results are presented as means ± standard error of the mean (SEM). For two different categorical independent variables, two-way analysis of variance (ANOVA; for the elevated plus maze and open field tests) or two-way repeated-measures ANOVA (for the MWM test), followed by Tukey *post hoc* test were performed. Exact *p*-values for main effects are stated throughout the manuscript, whereas exact *post hoc p*-values are stated in the legends, with values smaller than 0.05 considered significant. Non-significant *P*-values for main effects are stated in the figure legends. For all *in vivo* procedures, all data were taken into consideration for the statistical analyses, conducted using either SigmaPlot software version 11 Inc. for Windows (Chicago, IL, USA), or GraphPad Prism versions 6 and 8 Inc. for Windows (La Jolla, CA, USA).

Study Approval

All procedures involving animals were conducted under the supervision and approval of the Animal Care and Ethics Committee of Tel Aviv University and the Israel Ministry of Health (M-15-059).

RESULTS

Adnp$^{+/-}$ Mice Display Spatial Learning Deficiencies in the MWM, With SKIP Providing Improvement, but Not D-SKIP

The MWM paradigm included two daily trials, aimed at assessing the impact of the *Adnp* genotype on spatial learning and memory abilities, as well as the effect of SKIP and D-SKIP. Latencies to find the hidden platform was measured daily, for 5 days, with the first daily trial measuring reference memory (trial 1), and the second daily trial evaluating short-term memory (trial 2; Gordon et al., 1995; Gozes et al., 2000a). Learning was defined as the

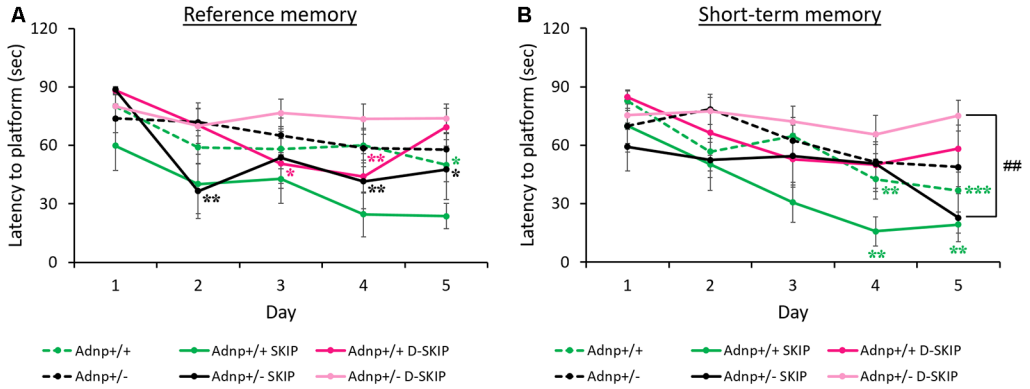

FIGURE 1 | Reference and short-term memory in the morris water maze (MWM): *Adnp*$^{+/+}$ mice learn the task, whereas *Adnp*$^{+/-}$ mice are impaired, with SKIP and D-SKIP displaying different behavioral outcomes. First and second daily water maze trials measuring reference and short-term memory are presented. To measure performance throughout the experimental days of the MWM, a two-way repeated-measures analysis of variance (ANOVA) with Tukey *post hoc* test was performed (*Adnp*$^{+/+}$ $N = 13$; *Adnp*$^{+/+}$ SKIP $N = 7$; *Adnp*$^{+/+}$ D-SKIP $N = 8$; *Adnp*$^{+/-}$ $N = 12$; *Adnp*$^{+/-}$ SKIP $N = 7$; *Adnp*$^{+/-}$ D-SKIP $N = 11$). The trials were implemented over five consecutive days. Latency measured in seconds to reach the hidden platform in its new daily location is depicted. **(A)** For Trial 1 [a non-significant value was observed for main interaction effect ($F_{(20,208)} = 1.0301$, $p = 0.427$)], Tukey *post hoc* tests showed that *Adnp*$^{+/+}$ male mice learned the task on day 5 (*$p = 0.034$), compared with the impaired *Adnp*$^{+/-}$ mice. D-SKIP treatment led to some improved performance on days 3–4 (*$p = 0.040$, **$p = 0.008$, respectively), which was then exacerbated on day 5. SKIP-treated *Adnp*$^{+/-}$ mice learned the task on days 2, 4–5 (**$p = 0.003$, **$p = 0.009$, *$p = 0.034$, respectively). **(B)** For Trial 2 [a non-significant value was observed for main interaction effect ($F_{(20,208)} = 1.119$, $p = 0.332$)], Tukey *post hoc* effects showed that vehicle-treated *Adnp*$^{+/+}$ male mice learned the task on days 4–5 (**$p = 0.003$, ***$p < 0.001$, respectively), compared with the impaired *Adnp*$^{+/-}$ mice. SKIP-treated *Adnp*$^{+/+}$ mice learned the task on days 4–5 (**$p = 0.004$, **$p = 0.008$, respectively). On day 5, a significant difference was found between SKIP- and D-SKIP treated *Adnp*$^{+/-}$ mice ($^{\#\#}p = 0.006$).

latency to find the hidden platform on a given experimental day, compared with the latency to find the platform on the first day.

In daily trial 1 (reference memory, **Figure 1A**), main effects for group ($F_{(5,208)} = 4.485$, $p = 0.002$) and day ($F_{(4,208)} = 9.124$, $p < 0.001$) were found. Specifically, vehicle-treated *Adnp*$^{+/+}$ mice learned the task on day 5 of the MWM, contrasting *Adnp*$^{+/-}$ mice. SKIP-treated *Adnp*$^{+/-}$ mice showed an unstable improvement on day 2, which was not observed on day 3, but significantly stabilized on days 4 and 5. D-SKIP did not show an effect on *Adnp*$^{+/-}$ mice. In *Adnp*$^{+/+}$ mice, while SKIP had no effect, D-SKIP-treatment showed a sporadic improvement on days 3–4, followed by an exacerbation on day 5.

In daily trial 2 (short-term memory, **Figure 1B**), main effects for group ($F_{(5,208)} = 4.303$, $p = 0.002$) and day ($F_{(4,208)} = 10.453$, $p < 0.001$) were found. Vehicle- and SKIP-treated *Adnp*$^{+/+}$ mice learned the task on days 4–5 of the MWM task, as opposed to *Adnp*$^{+/-}$ mice. Also, on day 5, a significant difference was found between SKIP- and D-SKIP treated *Adnp*$^{+/-}$ mice, with D-SKIP mice taking about 3-fold more time to find the hidden platform compared to SKIP-treated mice.

SKIP and D-SKIP Lead to Distinctive Anxiety-Related Behavioral Outcomes in the EPM and Open Field Tests

To evaluate anxiety-related behavior, the EPM and the open field behavioral tests were implemented. In the EPM test (**Figure 2A**), main effects for genotype ($F_{(1,90)} = 6.242$, $p = 0.014$) and treatment ($F_{(2,90)} = 21.109$, $p < 0.001$) were found. Specifically, SKIP-treated *Adnp*$^{+/+}$ mice spent significantly more time in the closed arms, compared with the vehicle- and D-SKIP-treated groups. In *Adnp*$^{+/-}$ mice, SKIP-treated animals spent significantly more time in the closed arms, compared with the D-SKIP-treated group only. In contrast, D-SKIP-treated *Adnp*$^{+/+}$ and *Adnp*$^{+/-}$ mice spent more time in the open arms, compared with vehicle-treated control groups, thus indicating a possible altered anxiety-related/increased risky behavior.

In the open field test (**Figure 2B**), the total distance traveled/locomotor activity was measured in all experimental groups, with a main treatment effect found ($F_{(2,63)} = 17.889$, $p < 0.001$). This parameter has been previously linked with positive symptoms of schizophrenia, and general psychotic behavior (van den Buuse et al., 2005; Merenlender-Wagner et al., 2010, 2014; van den Buuse, 2010). While no genotype-related phenotype was shown, SKIP-treated mice of both genotypes traveled a significantly decreased distance, compared with vehicle- and D-SKIP-treated groups, as in the case of *Adnp*$^{+/+}$ mice, or D-SKIP-treated group only, as in the case of *Adnp*$^{+/-}$ mice. Furthermore, D-SKIP-treated *Adnp*$^{+/+}$ and *Adnp*$^{+/-}$ mice traveled a longer distance, compared with the vehicle-treated control groups. When looking at the time spent in the center zone of the open field arena (**Figure 2C**), no significant differences were observed between the experimental groups.

SKIP and D-SKIP Interact With EB1 and EB3 Proteins in Distinctive Conformations

In silico analysis showed that the four-amino-acid peptides SKIP and D-SKIP bind EB1 and EB3 proteins using different residues, thus resulting in distinctive binding conformations. Specifically, in EB1, SKIP binds Tyrosine 217 (Y217), whereas D-SKIP binds

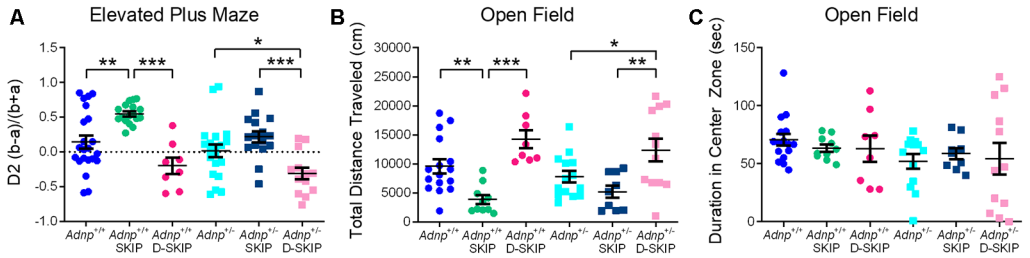

FIGURE 2 | SKIP treatment ameliorates the behavior of *Adnp*$^{+/-}$ male mice in the elevated plus-maze and open field, whereas D-SKIP aggravates. Two-way ANOVA with Tukey *post hoc* test was performed (*Adnp*$^{+/+}$ $N = 16–22$; *Adnp*$^{+/+}$ SKIP $N = 10–17$; *Adnp*$^{+/+}$ D-SKIP $N = 8$; *Adnp*$^{+/-}$ $N = 13–20$; *Adnp*$^{+/-}$ SKIP $N = 9–16$; *Adnp*$^{+/-}$ D-SKIP $N = 12–13$). **(A)** Elevated plus maze [EPM; A non-significant value was observed for main interaction effect ($F_{(2,90)} = 0.953$, $p = 0.389$)]: In *Adnp*$^{+/+}$ mice, SKIP-treated animals spent more time in the closed arms, compared with their D-SKIP- and vehicle-treated counterparts. In *Adnp*$^{+/-}$ mice, D-SKIP-treated animals spent more time in the open arms, compared with SKIP- and vehicle-treated mice. In *Adnp*$^{+/+}$ mice, significant Tukey *post hoc* differences were observed between SKIP- and both vehicle- and D-SKIP-treated mice (**$p = 0.002$, ***$p < 0.001$, respectively). In *Adnp*$^{+/-}$ mice, significant differences were observed between SKIP- and D-SKIP-treated mice (***$p < 0.001$), as well as between D-SKIP- and vehicle-treated mice (*$p = 0.028$). **(B)** Open field—Total Distance Traveled [non-significant values for main effects included genotype ($F_{(1,63)} = 0.467$, $p = 0.497$) and interaction ($F_{(2,63)} = 0.845$, $p = 0.434$) effects]: SKIP-treated *Adnp*$^{+/+}$ mice traveled a decreased distance, compared with their D-SKIP- and vehicle-treated counterparts. In addition, D-SKIP-treated *Adnp*$^{+/-}$ mice displayed an increased distance, compared with SKIP- and vehicle-treated mice. In *Adnp*$^{+/+}$ mice, significant Tukey *post hoc* differences were observed between SKIP- and both vehicle- and D-SKIP-treated mice (**$p = 0.008$, ***$p < 0.001$, respectively). In *Adnp*$^{+/-}$ mice, significant differences were observed between SKIP- and D-SKIP-treated mice (**$p = 0.002$), as well as D-SKIP- vs. vehicle-treated mice (*$p = 0.033$). **(C)** Open field—Duration in Center Zone [non-significant values for main effects included genotype ($F_{(1,62)} = 2.458$, $p = 0.122$), treatment ($F_{(2,62)} = 0.0601$, $p = 0.942$) and interaction effects ($F_{(2,62)} = 0.408$, $p = 0.667$)]: no significant *post hoc* differences were observed between the experimental groups.

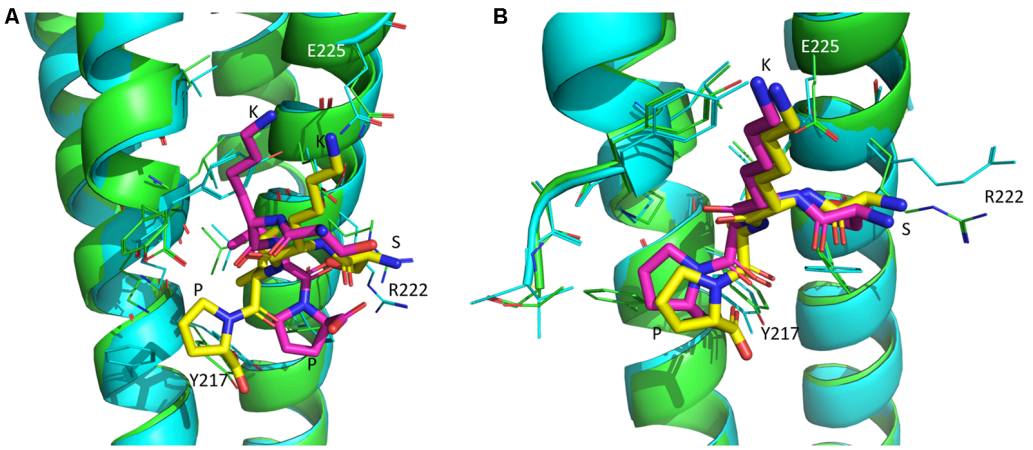

FIGURE 3 | SKIP and D-SKIP bind in silico EB1 and EB3 in distinct conformations. Three-dimensional (3D) structures of EB1 and EB3 homodimers (green and cyan) are shown as ribbons. SKIP (yellow) and D-SKIP (purple) peptides are shown as sticks. **(A)** In EB1, SKIP binds Tyrosine 217 (Y217), whereas D-SKIP binds Arginine 222 (R222). **(B)** In EB3, SKIP binds Glutamate 225 (E225), but D-SKIP does not. The figures were generated using Pymol. The best scoring conformations are shown.

Arginine 222 (R222; **Figure 3A**). Furthermore, in EB3, SKIP binds Glutamate 225 (E225), but D-SKIP does not (**Figure 3B**).

DISCUSSION

The current study has examined *in vivo* cognitive and anxiety-related behavioral aspects in the unique *Adnp*-haploinsufficient mouse model, when treated with either SKIP or D-SKIP peptides, both derived from the ADNP-snippet, NAP. The results indicated that *Adnp* deficiency impairs the cognitive state of the animals, and that this condition may be ameliorated by SKIP treatment, but not by D-SKIP. It should be noted that the study was performed on relatively aged animals, with treatment beginning at the age of 6 months, and behavioral tests performed at the age of 7–9 months. In this respect, the *Adnp*-deficient phenotype was previously shown to present increased age-dependent neurodegeneration and tauopathy (Vulih-Shultzman et al., 2007; Hacohen-Kleiman et al., 2018). This phenotype can also be explained by the highly important role of ADNP as vital for mammalian brain formation and function (Pinhasov et al., 2003; Vulih-Shultzman et al., 2007), and as a master-gene regulating hundreds of

key genes (Vulih-Shultzman et al., 2007; Amram et al., 2016). Interestingly, with PACAP, known to regulate ADNP (Zusev and Gozes, 2004), various similarities were observed between the *Adnp*-haploinsufficient mouse model and PACAP-deficient mice. These included slower weight gain during the first weeks of life, coupled with delayed neurobehavioral development (Farkas et al., 2017; Hacohen-Kleiman et al., 2018). Furthermore, dentition in old and young mice was associated with PACAP (Sandor et al., 2014, 2016), while tooth eruption in mice and children was associated with ADNP (Gozes et al., 2017b).

In terms of cognition, the evaluation of the *Adnp*-haploinsufficient mouse model by the MWM paradigm corroborated previous findings in male $Adnp^{+/-}$ mice, exhibiting spatial learning deficiencies (Vulih-Shultzman et al., 2007). The current results with SKIP in the MWM are supported by previous social behavioral tests, coupled with the social/olfactory abilities improved by SKIP, but not D-SKIP (Amram et al., 2016). The results are also supported by past findings with the parent peptide NAP, showing significant improvement in learning the MWM task (Vulih-Shultzman et al., 2007). Altogether, these findings provide evidence for the neuroprotective nature of SKIP, but not D-SKIP. Importantly, while the MWM test may partially rely on visual cues, mice are not considered "visual animals," but rather nocturnal, relying primarily on olfactory and auditory cues as well as touch/balance senses (use of whiskers for sensing the environment; Pinto and Enroth-Cugell, 2000). Specifically, the ICR background strain of the *Adnp*-deficient mouse model used here, as well as white mice, in general, may suffer retinal abnormalities, causing reduced visual abilities (Keeler, 1924). However, the degree of visual impairment is not the same within all albino mice (Wong and Brown, 2006). It should also be noted that for the MWM task, ICR mice were utilized before in multiple independent studies (Ge et al., 2013; Wang et al., 2014; Kim et al., 2015; Kitanaka et al., 2015).

An additional aspect examined was anxiety-related behavior, by implementing the EPM and open field behavioral tests. In the EPM, D-SKIP opposed the effects of SKIP, thus leading to a reduced anxiety-like phenotype and an increased altered/risky behavior. A similar finding was previously found in PACAP- and PAC1 receptor-null mice, thereby implying that the ADNP-regulating PACAP, plays a role in stress mechanisms (Hammack et al., 2009; Vaudry et al., 2009). In the open field test, SKIP led to a hypo-locomotive behavior, suggesting an increased anxiety-like state (Coelho et al., 2014; Park et al., 2018). This correlates the finding observed in SKIP-treated mice in the EPM (increased D2 score indicating an increased anxiety level), preserving a normal mouse phenotype. However, the effect of D-SKIP treatment contrasted SKIP, with a significantly increased total distance traveled (hyper-locomotion), previously linked with psychotic behavior (van den Buuse et al., 2005; Merenlender-Wagner et al., 2010, 2014; van den Buuse, 2010), compared with vehicle- and SKIP-treated mice. This finding of D-SKIP-treated mice being hyperactive in the open field could also offer a possible explanation to the reference memory result in the MWM, where it may seem that D-SKIP first improved and finally worsened reference memory in $Adnp^{+/+}$ mice. Importantly, there may be additional factors affecting open-field behavior (e.g., locomotor activity) including sex, strain and stress (Sestakova et al., 2013). In this respect, we have previously shown an increased locomotor activity in $Adnp^{+/-}$ female mice. This behavior was exacerbated by stress-challenge, with a further significant increase in locomotion, partially ameliorated by PACAP (Sragovich et al., 2019b). To summarize our mouse behavioral findings, it should be noted that there may be some data variability in this type of experiment, thus posing a certain limitation. Nevertheless, all the *in vivo* experiments implemented in the current study, have shown that the most highly repeated significant findings were the opposite phenotypic outcomes observed in SKIP- and D-SKIP-treated mice. Combined with rigorous statistical analyses, the validity of these experimental findings was confirmed.

Complementing the above *in vivo* behavioral results, a bioinformatics *in silico* analysis has suggested that SKIP and D-SKIP, both containing the SIP motif, which serves as the NAP EB binding site (Oz et al., 2014; Amram et al., 2016), interact with the tubulin/microtubule EB1 and EB3 proteins in distinctive conformations. The different *in silico* binding patterns observed between SKIP and D-SKIP to EB1/EB3 conform to the different interaction patterns previously found between these two peptides and NAP in NMR analysis, with SKIP, but not D-SKIP, interacting with NAP (Amram et al., 2016). Interestingly, a follow-up NMR analysis displayed an interaction between NAP and the neuroprotective peptide D-SAL (Gozes et al., 2016). This finding suggested a possible potential direct interaction of D-SAL with the endogenous ADNP, to provide microtubule fortification and neuroprotection, as was also previously shown *in vivo* (Shiryaev et al., 2011). Importantly, the protection by SKIP was thoroughly described from the *in vitro* level, in a cell culture model of zinc intoxication, to the *in vivo* social behavior and histological levels (Amram et al., 2016). This has proven that it is based on the NAP core sequence, and despite being shortened by half, the 4-amino-acid SKIP did not result in a loss of function (Amram et al., 2016). Furthermore, *in vitro* binding experiments performed for the neuroprotective parent peptides NAP (NAPVSIPQ) and NAPVSKIPQ, have shown an interaction with EB1/EB3, thus enhancing synaptic plasticity (Oz et al., 2014). When using affinity chromatography, SKIP was shown to enhance the interaction of the NAPVSIPQ motif of Adnp with EB3, suggesting augmentation of Adnp MT fortification under compromised situations, such as *Adnp* haploinsufficiency (Amram et al., 2016). NAP and SKIP were also shown to increase microtubule dynamics, through a mechanism of EB1/EB3 and Tau recruitment to microtubules, thereby providing neuroprotection (Ivashko-Pachima et al., 2017; Ivashko-Pachima and Gozes, 2019). Therefore, our findings may partially explain the distinctive natures of SKIP and D-SKIP, possibly underlying the *in vivo* behavioral outcomes presented in the current study. Importantly, it should be kept in mind that despite the increased accessibility of *in silico* technologies to scientists, allowing the enhancement of drug development processes, these are essentially predictive methods, the results of which should be interpreted cautiously (Valerio, 2009).

To conclude, as was previously shown (Amram et al., 2016), and here as well, binding inhibition may be caused in the case of SKIP, when replacing the L-amino-acids with D-amino-acids. Furthermore, it is possible that SKIP and D-SKIP mediate distinct actions in the brain. In this respect, we have previously shown in the hippocampus that the SKIP normalized the expression of the ASD-linked gene *Slc6a4*, encoding a serotonin transporter, whereas D-SKIP did not, thus providing a partial explanation to the distinctive *in vivo* behavior (Amram et al., 2016). Since the NAP-derived SKIP was previously presented as a novel future therapeutic option for potential ASD drug development (Amram et al., 2016), the current findings are of great importance. This is also in light of our recent results, showing that the $Adnp^{+/-}$ mouse mimics the human autism-like *ADNP* syndrome patient, and suggesting NAP as a drug candidate for treating this pathology (Hacohen-Kleiman et al., 2018). Therefore, the present study has practical pharmacological significance, as it is important to distinguish between agonists and antagonists, thus assisting at making crucial therapeutic decisions at an early stage of the drug development process.

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 Animal Care and Ethics Committee of Tel Aviv University and the Israel Ministry of Health (M-15-059).

AUTHOR CONTRIBUTIONS

SS provided input to project design, performed experiments, analyzed data, and wrote the article. NA performed experiments and analyzed data. AY performed the bioinformatics *in silico* analysis. Prof. IG led the entire project, provided funding, designed experiments, analyzed data, and wrote the article.

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The Role of Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Signaling in the Hippocampal Dentate Gyrus

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Pituitary adenylate cyclase-activating polypeptide (PACAP, *ADCYAP1*) dysregulation has been associated with multiple stress-related psychopathologies that may be related to altered hippocampal function. In coherence, PACAP- and PAC1 receptor (*ADCYAP1R1*)-null mice demonstrate changes in hippocampal-dependent behavioral responses, implicating the PACAPergic system function in this structure. Within the hippocampus, the dentate gyrus (DG) may play an important role in discerning the differences between similar contexts, and DG granule cells appear to both highly express PAC1 receptors and receive inputs from PACAP-expressing terminals. Here, we review the evidence from our laboratories and others that PACAP is an important regulator of activity within hippocampal circuits, particularly within the DG. These data are consistent with an increasing literature implicating PACAP circuits in stress-related pathologies such as post-traumatic stress disorder (PTSD) and implicate the hippocampus, and in particular the DG, as a critical site in which PACAP dysregulation can alter stress-related behaviors.

Keywords: pituitary adenylate cyclase-activating polypeptide, PAC1 receptor, hippocampus, dentate gyrus, contextual fear conditioning

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Pituitary adenylate cyclase-activating polypeptide (PACAP, *ADCYAP1*) dysregulation has been associated with multiple stress-related psychopathologies (Kormos and Gaszner, 2013; Hammack and May, 2015; Lutfy and Shankar, 2019) and we and others have reported that a single nucleotide polymorphism (SNP) in the PAC1 receptor gene (*ADCYAP1R1* rs2267735) as well as circulating PACAP levels were associated with post-traumatic stress disorder (PTSD; Ressler et al., 2011). These findings were consistent with a growing literature demonstrating central PACAP signaling as critical for stress responding and fear- and anxiety-like behavior in rodents. PTSD is characterized by several features, which may represent dysregulation in several distinct neural circuits. Notably, in the original report, Ressler et al. (2011) reported that the risk allele of rs2267735 was associated with hyper-arousal and dark-enhanced startles, which are behaviors that have been closely associated with activation of subregions of the bed nucleus of the stria terminals (BNST). These observations have corroborated several years of work in our laboratories demonstrating that BNST PACAP activation is necessary and sufficient for many of the behavioral sequelae produced by chronic stress (Hammack et al., 2010; Hammack and May, 2015), as well as cocaine self-administration (Miles et al., 2018, 2019).

However, dysregulated fear extinction and fear discrimination are also hallmark features of PTSD (see Jovanovic et al., 2012; Sangha et al., 2020), and rs2267735 was also associated with compromised extinction and reduced discrimination between fear-conditioned stimuli (Ressler et al., 2011; Pohlack et al., 2015; Mercer et al., 2016; Ramikie and Ressler, 2016). These data suggest that PACAP dysregulation has effects in multiple neural circuits associated with PTSD symptoms, which may include regions of the amygdala, medial prefrontal cortex (mPFC) and hippocampus. There are several lines of evidence suggesting that PACAP activation has distinct and interesting actions in the central nucleus of the amygdala (CeA; Missig et al., 2017; Meloni et al., 2019; Varodayan et al., 2020), basolateral amygdala (BLA; Cho et al., 2012; Schmidt et al., 2015), and mPFC (Kirry et al., 2018, 2019), and some of this work has been reviewed elsewhere (Miles and Maren, 2019). Here, we discuss potential roles of PACAP regulating activity within the hippocampus, as well as the behavioral consequences of such regulation.

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP)

PACAP is the archetypical member of the vasoactive intestinal peptide (VIP)-secretin-glucagon family of bioactive peptides and was isolated from ovine hypothalami based on its ability to stimulate adenylyl cyclase activity in anterior pituitary cells (Kimura et al., 1990; Miyata et al., 1990). Two α-amidated forms of PACAP arise from alternative posttranslational processing of the precursor molecule; PACAP38 has 38 amino acid residues [rat pro-PACAP(131-168)], while PACAP27 corresponds to the amino terminus of PACAP38 [proPACAP(131-157)]. Despite similarities in endoproteolytic processing by PC1 and PC2 (prohormone convertase 1 and 2, respectively) at dibasic amino acid processing sites, the levels of PACAP38 predominate in most tissues, although the ratio of the PACAP38: PACAP27 appears tissue-specific (Arimura et al., 1991). PACAP27 exhibits 68% amino acid identity with VIP (Kimura et al., 1990; Miyata et al., 1990); the 28-amino acid VIP peptide is also α-amidated but unlike PACAP, does not have alternatively processed forms. PACAP appears to represent the ancestral peptide and from gene duplications, VIP/PACAP and glucagon/GLP-1/GIP peptides appear to arise from different branches of the cladistic tree (Sherwood et al., 2000). PACAP peptides are well conserved among species and widely distributed among central and peripheral tissues to implicate their evolutionary importance in maintaining physiological homeostasis (Sherwood et al., 2000; Vaudry et al., 2009).

PACAP RECEPTORS: EXPRESSION AND SIGNALING

PACAP can bind to three Class B heptahelical G protein-coupled receptors (GPCR). The PAC1 receptor is selective for the two PACAP isoforms (PACAP27/PACAP38); the VPAC1 and VPAC2 receptors exhibit similar high affinities for PACAP and VIP peptides (Harmar et al., 2012; Blechman and Levkowitz, 2013). Unlike the VPAC1 and VPAC2 GPCRs which are preferentially coupled to Gαs and adenylyl cyclase activity, the PAC1 receptors can be dually coupled to Gαs and Gαq/11 to engage adenylyl cyclase and phospholipase C activities, respectively. In addition to these classical plasma membranes delimited signaling mechanisms, the PAC1 receptors have also been shown to internalize and transduce long term endosomal signaling, especially β-arrestin-mediated ERK activation, to deliver second messengers to intracellular sites with high spatial and temporal resolution (Calebiro et al., 2010; Scita and Di Fiore, 2010; McMahon and Boucrot, 2011; Irannejad et al., 2013). From these studies, the PAC1 receptor can activate a multitude and integrated sequelae of downstream signaling events for cellular responses. Adding to the complexity, PAC1 receptors are unique among the Class B receptors in that multiple receptor variants depend on the absence or presence of two 84-bp Hip and Hop cassettes that encode inserts into the third cytoplasmic loop of the GPCR. Hence the PAC1 receptor can be Null with neither Hip nor Hop inserts, just Hip alone, just Hop or HipHop (Spengler et al., 1993; Harmar et al., 2012; Blechman and Levkowitz, 2013). Depending on the cell type, the different PAC1 receptor isoforms can be differentially coupled to the diverse downstream signaling cascades. From receptor isoform analyses, all regions of the mammalian central nervous system, including humans, preferentially express the PAC1Null and PAC1Hop receptor variants; only postganglionic sympathetic neurons appear unique in the expression of just the PAC1Hop receptor variant (Braas and May, 1999).

In our work related to stress- and pain-responding, only BNST and CeA infusions with PACAP altered anxiety- or pain-related behaviors (Hammack et al., 2010; Missig et al., 2014, 2017; Roman et al., 2014); parallel studies with VIP infusions had no apparent effects suggesting PAC1 receptor signaling in these behaviors. In accord, BNST PACAP and/or PAC1 receptor expression was upregulated by chronic stress or pain states (Hammack et al., 2009, 2010; Lezak et al., 2014; Missig et al., 2017) whereas VIP or VPAC1/VPAC2 transcript levels in these regions were not sensitive to these conditions. The PACAP effects could be blocked by the PAC1 receptor antagonist PACAP(6-38); moreover, many of the effects of PACAP infusion in these areas were mimicked by the specific PAC1 receptor agonist maxadilan (Missig et al., 2014, 2017; Roman et al., 2014) to demonstrate definitively roles for PAC1 receptor signaling in these responses.

PAC1 receptors are abundant and widely distributed in the CNS (Hashimoto et al., 1996; Jaworski and Proctor, 2000). Within the hippocampus, early *in situ* hybridization studies demonstrated high levels of PAC1 receptor transcripts in the granule cell layer (GCL) of the dentate gyrus (DG). Indeed, DG PAC1 receptor transcript expression can be striking compared to other brain regions (Hashimoto et al., 1996; Jaworski and Proctor, 2000). Complementary studies using PACAP-Cre mice (Bradford Lowell, McLean Hospital, Harvard University) demonstrated PACAP fiber projections from hippocampal hilar mossy cells to the DG inner molecular layer (IML), which implicated the formation of short PACAPergic synaptic circuits between PACAP-expressing hilar mossy cells and the proximal

dendrites on PAC1 receptor-expressing DG granule cells (see below, Condro et al., 2016). PACAP may also bind to DG VPAC1 receptors; there is little VPAC2 receptor expression in this region (Vertongen et al., 1997). In sum, these observations implicated roles for PACAP/PAC1 receptor signaling in modulating hippocampal functions, including cognition, and learning and memory with potential consequences for contextually-mediated fear behaviors.

THE HIPPOCAMPUS, LEARNING, AND MEMORY

The functional anatomy of the hippocampus has been extensively reviewed (Schultz and Engelhardt, 2014). The axons from the entorhinal cortex (EC) layer 2 neurons project *via* the perforant pathway to the dendrites of granule cells in the outer molecular layers of the DG (Dolorfo and Amaral, 1998a,b). The mossy fibers of DG granule cells in turn project to the hippocampal CA3 region (Amaral et al., 2007; Blaabjerg and Zimmer, 2007), which then project to the CA1 region *via* Schaeffer Collaterals (Ishizuka et al., 1990). Pyramidal cells from CA1 then project back to layer 5 of the EC (Amaral and Witter, 1989). In the dorsal hippocampus, these circuits have been extensively studied for their role in the processing of contextual information. For example, several studies have determined distinct functional roles for the DG in contextual learning processes. Besnard et al. (2014) demonstrated enhanced DG activity following contextual conditioning as well as during the retrieval of contextual conditioning. Moreover, precise pharmacological inhibition of DG activity demonstrated the necessity of DG function in the expression of contextual fear at short time intervals (Hernández-Rabaza et al., 2008). Given that the overall activity of DG neurons is low under basal conditions, the DG may be preferentially activated in the acquisition and retrieval of contextual memories.

Using a transgenic mouse model where the expression of channelrhodopsin is under the control of the immediate-early gene c-fos promoter, Liu et al. (2012) reactivated the same population of DG neurons that were active at the time of contextual memory encoding. The reactivation of these DG neurons was able to enhance freezing behavior, presumably by stimulating the retrieval of the original contextual fear memory (Liu et al., 2012). Since this original report, many others have demonstrated that the reactivation of DG neuronal activity, hypothesized to be the encoding of context, is sufficient to retrieve contextual memories (Redondo et al., 2014; Ryan et al., 2015; Roy et al., 2016). Conversely, silencing DG neurons originally activated during memory encoding reduced freezing responses to a fearful context (Denny et al., 2014), suggesting that DG neuronal activity is necessary for retrieval. Hence, the population of DG neurons activated during the encoding of a contextual memory may represent the trace of that memory, also termed the memory engram (Ghandour et al., 2019). Bernier et al. (2017) further clarified the role of DG activity by demonstrating that DG inactivation significantly reduced contextual discrimination between similar contexts, while not affecting discrimination between very different contexts. As the DG may be critically involved in discerning the differences between similar contexts, altered hippocampal function may result in fear generalization across multiple contexts, which is a hallmark symptom of PTSD.

PACAP AND THE HIPPOCAMPUS

Several studies have demonstrated that PACAP systems regulate hippocampal activity. For example, the fEPSP response upon perforant pathway stimulation is enhanced in the presence of PACAP (Kondo et al., 1997), and in accord, Matsuyama et al. (2003) observed in both PACAP and PAC1 receptor knockout mice (Otto et al., 2001) a significant decrement in long term potentiation (LTP) following high-frequency stimulation of the perforant path. Otto et al. (2001) interpreted from receptor immunocytochemical data that the PAC1 receptors were presynaptic on DG mossy fibers and suggested an impairment of LTP at the mossy fiber/CA3 synapse following afferent stimulation in PAC1 receptor knockout mice. A few other studies have attempted to determine the role of PACAP in specific hippocampal subregions, especially the CA1. Hence, synaptic strength at the Schaeffer collateral synapse is sensitive to PACAP (Kondo et al., 1997; Roberto and Brunelli, 2000; Roberto et al., 2001; Ciranna and Cavallaro, 2003), which may be mediated by PACAP effects on AMPA currents (Costa et al., 2009; Toda and Huganir, 2015), NMDA currents (Macdonald et al., 2005), or other intrinsic channel mechanisms (Taylor et al., 2014). Behaviorally, PAC1 receptor knockout mice demonstrated decrements in several hippocampal-dependent tasks, such as foreground contextual fear conditioning (Sauvage et al., 2000), and background contextual fear conditioning (Otto et al., 2001). Moreover, PACAP infusions into CA1 enhanced the consolidation of contextual fear conditioning, and antagonism of the PAC1 receptor at CA1 impaired fear consolidation as well as subsequent fear extinction (Schmidt et al., 2015). Despite these studies, there is limited evidence of PACAP innervation of CA1 or CA3. Recent studies with PACAP-EGFP mice demonstrated sparse PACAPergic innervation of CA1 or CA3 (Condro et al., 2016), which appeared to complement the *in situ* hybridization data showing proportionately much less PAC1 receptor transcript expression in these areas than other regions of the hippocampus (Hashimoto et al., 1996; Jaworski and Proctor, 2000). Hence, whether the early observations on PACAP-mediated hippocampal function reflected VIP or PACAP signaling on VPAC receptors remained unclear. However, as a result of the availability of more recent tools and models, there has been a refocus of PACAP roles in the hippocampus.

PACAP AND THE DG: ANATOMY AND PHYSIOLOGY

In aggregate, the recent data from studies using the PACAP-EGFP and PACAP-Cre mice have been in alignment with previous PACAP and PAC1 receptor *in situ* hybridization

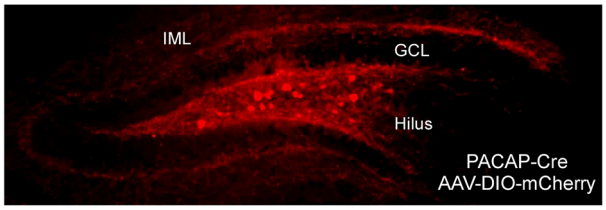

FIGURE 1 | Neuronal pituitary adenylate cyclase-activating polypeptide (PACAP) expression and projection in the dentate gyrus (DG). The DG in PACAP-Cre mice was infused with Cre-dependent mCherry reporter. In coherence with work in PACAP-EGFP mice and previous PACAP and PAC1 receptor *in situ* hybridization studies, PACAP expression was in apparent hilar mossy cells which elaborated axonal projections predominantly to the inner molecular dendritic layer of granule cells. GCL, granule cell layer; IML, inner molecular layer.

studies, demonstrating that hippocampal PACAP expression is largely from hilar mossy cells which send projections predominantly to PAC1 receptor-expressing DG granule cell dendrites at the IML (Condro et al., 2016). From *in situ* hybridization data, PAC1 receptor mRNA expression is extensive in DG granule cells suggesting a role for PACAP in regulating DG activity. Previous *in situ* hybridization studies also identified PACAP-expressing neurons in DG hilar cells, and in coherence from PACAP-EGFP and PACAP-Cre mouse data, we and others have observed dense PACAP-positive axonal projections to the DG IML, where PACAP afferents likely synapse onto the dendrites of PAC1 receptor-expressing DG granule cells (**Figure 1**, Condro et al., 2016). Together, the anatomy suggests that a primary role of hippocampal PACAP signaling appears to be in the regulation of DG granule cell activity, and DG-related behaviors.

Using whole-cell recordings in current-clamp mode, Johnson et al. (2020) have observed that PACAP enhances the excitability of DG granule cells assessed by determining the number of action potentials elicited by 1-s depolarizing current steps of increasing intensity (**Figure 2**). Thus, PACAP increased the slope of the excitability curve, an effect of PACAP that was observed in other neuron types (Cho et al., 2012; Gupte et al., 2016; May and Parsons, 2017). Moreover, this increase in excitability was associated with a negative shift in the threshold for action potential generation, observed in nearly all DG granule cell neurons, due to post-synaptic actions of PACAP, and appeared to be independent of changes in input resistance and changes in resting membrane potential (Johnson et al., 2020). Importantly, while DG neurons demonstrate VPAC1 and low levels of VPAC2 receptor expression, the excitatory actions of PACAP were not mimicked by VIP, suggesting that the PACAP regulation of DG activity was PAC1 receptor-dependent. From these observations, we propose that PACAP release could serve to increase the excitability of DG granules cells that have been activated by other means, i.e., PACAP alone would likely not have independent neuronal effects without concurrent synergistic excitatory inputs. Hence the primary consequence of DG PAC1 receptor signaling would be to amplify the responses of activated neurons.

Following previous work, we investigated the signaling mechanisms and intrinsic membrane currents potentially contributing to the PACAP/PAC1 receptor enhancement of hippocampal neuronal excitability (Johnson et al., 2020). As noted above, there are many second messenger cascades downstream of PAC1 receptor activation, including plasma membrane delimited Gαs-mediated AC/cAMP and Gαq/11-mediated PLC/DAG/IP_3 signaling. Also, both AC/cAMP and PLC/DAG/IP_3 signaling, along with β-arrestin-mediated endosomal signaling following receptor internalization, can activate MEK/ERK signaling cascades.

In peripheral parasympathetic postganglionic cardiac neurons, all of these signaling pathways appeared to contribute to neuronal excitability by modulating different ionic conductances (Parsons et al., 2016; Tompkins et al., 2016; May and Parsons, 2017). Cyclic AMP generation was shown to gate the nonselective cationic H-current (Merriam et al., 2004; Tompkins et al., 2009), cAMP-activation of PKA was implicated in the activation of T-type calcium currents (Tompkins et al., 2015) and ERK signaling was shown to enhance a voltage-dependent sodium current possibly through phosphorylation of Nav1.7 (Tompkins et al., 2016). However, conditions that blunt clathrin-mediated receptor endocytosis, including treatment with the clathrin inhibitor Pitstop2 or the dynamin inhibitor dynasore, as well as decreasing ambient temperature, essentially eliminated the PACAP effect on cardiac neuron excitability (Merriam et al., 2013; May et al., 2014; Tompkins et al., 2018; Parsons and May, 2019). Thus receptor internalization and recruitment of endosomal signaling was a critical mechanism. While receptor internalization has been associated primarily with desensitization mechanisms, these views are being reconsidered; these PACAP/PAC1 receptors signaling studies were unique and among those implicating receptor endosomal signaling mechanisms as important drivers of neuronal excitability.

The signaling mechanisms underlying PACAP-enhanced DG granule cell excitability appeared different from those in peripheral cardiac neurons. Interestingly, from inhibitor studies, neither the AC/cAMP/PKA nor PLC/DAG/IP_3 signaling appeared to contribute to PACAP modulation of DG cell excitability. In contrast, treatment with the MEK inhibitor, PD98059, virtually eliminated PACAP-enhanced induced excitability of DG granule cells (Johnson et al., 2020). Also, like cardiac neurons, the excitatory effects of PACAP on DG granule cells were essentially eliminated by treatment with the cell-permeable clathrin-mediated endocytosis inhibitor Pitstop2. Thus the results in aggregate suggested that for hippocampal DG cells, PAC1 receptor endosomal recruitment of MEK/ERK signaling represents the primary second messenger mechanism contributing to the PACAP modulation of neuronal excitability in DG granule cells (Johnson et al., 2020). These results appeared consistent with our other reports investigating PAC1 receptor signaling in other CNS pathways (Merriam et al., 2004; May et al., 2014; Missig et al., 2017; Miles et al., 2019). GPCR endosomal signaling, as opposed to membrane-bound activation of G-protein pathways, has been described to produced sustained long-lasting activation that is less sensitive to extracellular events that normally regulate signaling, such as transmitter diffusion

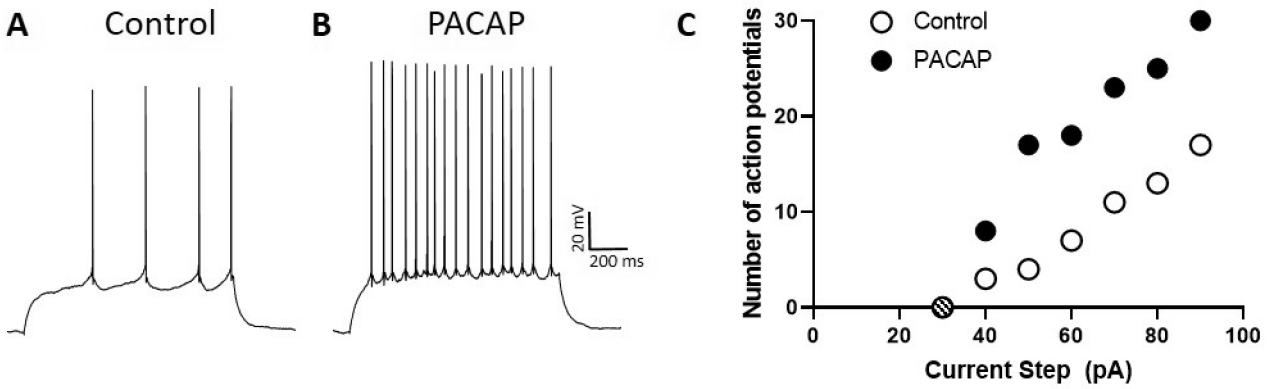

FIGURE 2 | PACAP enhances the excitability of DG neurons. Trace **(A)**, representative patch-clamp recording in current-clamp mode from DG neuron demonstrating action potential generation elicited by a 1 s, 50 pA current step. Trace **(B)**, same DG neuron as in **(A)** demonstrating increased action potential generation under the same conditions but in the presence of PACAP. Dentate granule cell spiking upon PACAP infusion was greater than that elicited before peptide exposure. **(C)** Excitability curves produced by plotting the number of action potentials elicited by 1-s current steps of increasing strength. Note that the excitability curve for neurons treated with PACAP (filled circles) is steeper than that for untreated control cells (open circles). The hashed circle indicated no action potentials were elicited by a 30 pA step.

and/or inactivation. Perhaps not surprisingly, the excitatory effects of PACAP in the *ex vivo* electrophysiological preparation were long-lasting even after peptide ligand washout. This mechanism for producing long-lasting changes in neuronal excitability may have important behavioral consequences, although the temporal dynamics of the behavioral effects of PACAP are largely unexplored. PACAP has been shown to alter the activity of several ion channels that regulate the excitability of neurons and ongoing studies focus on the identification of ionic currents in DG cells that potentially are the target of ERK phosphorylation.

PACAP AND THE DG: BEHAVIOR

As noted earlier, DG granule cells play important roles in contextual fear conditioning, acquisition and retrieval, and given that PACAP may produce long-lasting changes in neuronal excitability of DG neurons (Johnson et al., 2020), PACAP may play a role in regulating these processes. As PACAP appears to augment DG neuronal activity to excitatory inputs, PACAP administration before training may facilitate contextual fear acquisition by enhancing the rate of conditioning or altering freezing behaviors during extinction testing on subsequent days. Alternatively, DG PACAP infusions before re-exposure to conditioning context may enhance fear expression thereby decreasing the rate of extinction. Whether DG PACAP signaling affects fear conditioning acquisition and/or extinction is under study but preliminary results suggest PACAP has a more pronounced effect on the latter. In this particular paradigm, DG PACAP/PAC1 receptor activation before extinction may lead to persistent neuronal activity in the neurons that make up the contextual fear memory engram to delay extinction processes.

As noted above, a polymorphism in the PAC1 receptor has been associated with PTSD symptoms (Ressler et al., 2011) and carriers of the risk allele exhibit differential hippocampal-dependent function in a contextual conditioning task compared to subjects with the normal allele (Pohlack et al., 2015). A hallmark feature of PTSD is the compromised ability to extinguish fear memories, which may be consistent with the DG PACAP behavioral effects described above. Not surprisingly, the processing of contextual information is highly associated with fear extinction, where the context may be used to disambiguate between the original fear memory and the extinction memory. Hence, should PACAP alter the processing of contextual information, the original fear memory may persist contributing to the disorder. Hippocampal dysfunction has also been associated with fear generalization, a feature of PTSD where fear responses are expressed in situations or environments unrelated to the acquired context (Dunsmoor and Paz, 2015). DG PACAP signaling may also participate in this process. For example, if chronic stress-induced DG PACAP neuroplasticity and altered DG function were to obfuscate engrams encoding fear memories with non-threatening or safe engram representations, then a consequence may be behavioral abnormalities that participate in PTSD. As an illustration, if behavioral engram representations for celebratory fireworks were obfuscated or supplanted, from stress-mediated PACAP neuroplasticity, by engrams encoding fear from combat barrages, then the changes in DG function may reinforce other maladapted stress circuits leading to psychopathologies. These data, combined with data implicating the role of stress-related PACAPergic dysregulation in other limbic regions in the regulation of emotional behavior, continue to implicate maladaptations to central PACAP/PAC1 receptor systems in the development and expression of fear and anxiety-related behavioral disorders.

DISCUSSION

Neural circuits in the hippocampus have been highly implicated in the processing of contextual information,

including paradigms associated with emotional (fear) learning. In particular, processing in the hippocampal DG may be particularly critical for processing subtle differences between similar contexts. Anatomical data suggest that DG granule cells highly express PAC1 receptors and receive dense input from PACAP-expressing axon terminals in the IML. And, physiological data suggest that PACAP enhances the excitability of DG granule cells *via* PAC1 receptor endosomal signaling that activates MEK/ERK pathways to regulate the function of intrinsic membrane currents (Johnson et al., 2020). Consistent with this anatomy and physiology, PACAP infusions into the hippocampal DG appear to enhance the retention of a contextual fear memory. These data are consistent with previous reports implicating dysregulations in PACAP systems with PTSD symptoms and suggest that the hippocampal DG may be an important target for the effects of PACAP dysregulation. These data support an expanding literature that also implicates bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), hypothalamic subregions as other key structures where PACAP activation regulates emotional behavior. Together these data suggest that central PACAP activation is a key event that coordinates the activity of many circuits associated with the behavioral response to threatening stimuli; hence, PACAP systems may also play a key role when severe or chronic stress produces emotional pathology. These systems may be key targets for future therapeutic approaches to stress-related psychiatric disorders.

AUTHOR CONTRIBUTIONS

GJ performed some of the studies described in the manuscript. GJ, RP, VM, and SH contributed to some of the experimental designs, writing and editing of the manuscript.

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MINI REVIEW
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VIP Modulation of Hippocampal Synaptic Plasticity: A Role for VIP Receptors as Therapeutic Targets in Cognitive Decline and Mesial Temporal Lobe Epilepsy

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Vasoactive intestinal peptide (VIP) is an important modulatory peptide throughout the CNS acting as a neurotransmitter, neurotrophic or neuroprotective factor. In the hippocampus, a brain area implicated in learning and memory processes, VIP has a crucial role in the control of GABAergic transmission and pyramidal cell activity in response to specific network activity by either VIP-containing basket cells or interneuron-selective (IS) interneurons and this appears to have a differential impact in hippocampal-dependent cognition. At the cellular level, VIP regulates synaptic transmission by either promoting disinhibition, through activation of $VPAC_1$ receptors, or enhancing pyramidal cell excitability, through activation of $VPAC_2$ receptors. These actions also control several important synaptic plasticity phenomena such as long-term potentiation (LTP) and long-term depression (LTD). This paper reviews the current knowledge on the activation and multiple functions of VIP expressing cells in the hippocampus and their role in controlling synaptic transmission, synaptic plasticity and learning and memory processes, discussing also the role of $VPAC_1$ and $VPAC_2$ VIP receptors in the regulation of these different processes. Furthermore, we address the current knowledge regarding changes in VIP mediated neurotransmission in epileptogenesis and mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS), and discuss the therapeutic opportunities of using selective VIP receptor ligands to prevent epileptogenesis and cognitive decline in MTLE-HS.

Keywords: VIP, synaptic plasticity, interneurons, hippocampus, MTLE, cognition, VPAC1 receptors

INTRODUCTION

Vasoactive intestinal peptide (VIP), a 28 amino-acid residue peptide originally isolated from porcine duodenum by Mutt and Said (1974), owes its name to its powerful ability to cause vasodilatation (Said and Mutt, 1970), by promoting vascular smooth muscle relaxation in the gastrointestinal tract when released by peripheral nerves of the sympathetic nervous

system (Said and Rosenberg, 1976). In subsequent years, VIP was described in multiple peripheral and central neuronal control systems, where it acts as neurotransmitter, neurotrophic or neuroprotective factor (Borbély et al., 2013; Deng and Jin, 2017). Discovery of pituitary adenylate cyclase-activating polypeptide (PACAP) in the ovine hypothalamus (Miyata et al., 1989), where it acts as an endocrine regulator, brought additional complexity to the understanding of the actions of VIP, since these two peptides share common receptors and are often present together in the same brain regions (see below). The actions of PACAP on synaptic transmission, plasticity and cognition are reviewed in another paper in this research topic (Ciranna and Costa, 2019) and will be discussed here only when clarifying the duality of VIP vs. PACAP signaling. VIP is nowadays recognized as an important modulator of synaptic transmission and plasticity, network excitability as well as of learning and memory processes and has been associated with cognitive deficits in several central nervous system (CNS) diseases. This paper reviews the multiple roles of VIP in synaptic transmission, synaptic plasticity and hippocampal-dependent learning and memory processes, the role of VIP in hippocampal and cognitive disfunction in mesial temporal lobe epilepsy (MTLE) and the therapeutic opportunities that this presents.

VIP IN THE HIPPOCAMPUS

Upon its discovery, VIP expression was reported in the human hippocampus and the hippocampus of animal models (Emson et al., 1979; Lorén et al., 1979; Besson et al., 1984), where VIP was also shown to bind to hippocampal membranes (Taylor and Pert, 1979; Besson et al., 1984). Shortly after, it became evident that VIP expression was predominant in hippocampal GABAergic interneurons (Köhler, 1982, 1983; Léránth et al., 1984; Kosaka et al., 1985) and that modulation of GABAergic transmission was likely an important target for VIP action. VIP was also early recognized to have a crucial role in mnemonic processes and particularly in hippocampal-dependent memory traits (Cottrell et al., 1984; Flood et al., 1990; Glowa et al., 1992). Nevertheless, the first report of its physiological actions in the CNS described VIP excitation of hippocampal CA1 neurones (Dodd et al., 1979). This enhancement in pyramidal cell excitability was latter shown to occur essentially through reduction of the Ca^{2+}- and cAMP-dependent K^+-conductance, leading to a decrease of the long-lasting afterhyperpolarization (sAHP) and a reduction of the accommodation of firing (Haas and Gähwiler, 1992). This action was postsynaptic since it prevailed in low Ca^{2+} – high Mg^{2+} medium and was later demonstrated to depend on protein kinase A (PKA) activity (Haug and Storm, 2000). Later, the actions of VIP on hippocampal GABAergic transmission were described showing that VIP increases the frequency of miniature IPSCs in cultured pyramidal neurones without affecting their amplitude (Wang et al., 1997), which suggests a presynaptic facilitation of GABA release by VIP. This appeared contradictory since VIP actions would lead to opposing effects on pyramidal cell excitability. All these findings are summarized in **Table 1**. When the anatomy of VIP-expressing interneurons (*VIP*$^+$ *INs*) in the hippocampus was elucidated (Acsády et al., 1996a,b; Hájos et al., 1996) the different roles of VIP in modulation of hippocampal GABAergic transmission and regulation of pyramidal cell excitability began to be clarified.

Detailed immunohistochemistry studies fully characterized hippocampal *VIP*$^+$ *INs* dendritic trees and axon projections (Acsády et al., 1996a,b), allowing the classification of *VIP*$^+$ *INs* into two fundamental groups according to their targets: *VIP*$^+$ *basket cells* are responsible for somatic inhibition of pyramidal cells, are also immunoreactive for cholecystokinin (*VIP*$^+$- *CCK*$^+$ *BCs*, **Figure 1**) and do not express parvalbumin, as most *BCs* in the hippocampus. *VIP*$^+$ *INs* that selectively innervate other interneurons (*VIP*$^+$ *IS INs*) include two subtypes: (a) interneurons with cell bodies located at the *stratum pyramidale (SP)* or near and projecting to the *stratum Oriens/Alveus* border (*VIP*$^+$ *IS O/A INs* or type III IS cells, **Figure 1**), that also express the interneuron marker calretinin and target mostly somatostatin-expressing (SOM$^+$) *oriens lacunosum-moleculare (OLM)* interneurons innervating the distal dendrites of pyramidal cells at the *stratum lacunosum-moleculare (SLM)* and (b) *VIP*$^+$ *INs* that project their axons to the *stratum radiatum* (*SR*, *VIP*$^+$ *IS SR INs*, **Figure 1**), with cell bodies located either at the *SR/SLM* border (type II IS cells) or at *SR/SP* and targeting *interneurons* controlling synaptic transmission to proximal dendrites of pyramidal cells in the *SR* (Acsády et al., 1996a,b; Klausberger and Somogyi, 2008). In genetically modified VIP-eGFP mice, additional targets of *VIP*$^+$ *IS O/A INs* in the *O/A*, including bistratified cells and oriens–oriens INs, have been described and recently a new VIP expressing interneuron population located at the O/A (*VIP*$^+$ long-range projecting INs, VIP$^+$ *LRP INs*) was described targeting INs within the O/A in CA1 but also both *INs* and pyramidal cells within the *subiculum* (Francavilla et al., 2018). It is not clear if it is also present in the rat hippocampus.

Considering the early acquired knowledge (Acsády et al., 1996a,b) on the target selectivity of VIP-IS hippocampal interneurons in the rat, Yanovsky et al. (1997) studied the influence of VIP application to the *Oriens/Alveus* border and showed that in the absence of synaptic interactions, VIP increased the firing rate of these interneurons and decreased the slope of the fEPSPs recorded at the *SR* and *SLM*, thus decreasing excitatory synaptic transmission through an increase in inhibitory transmission (Yanovsky et al., 1997). These mechanisms could not account for the previously observed increase in synaptic transmission and pyramidal cell firing (Haas and Gähwiler, 1992). In fact, VIP-mediated concomitant pre and post-synaptic enhancement of GABAergic transmission generating disinhibition of synaptic transmission to pyramidal cell dendrites (Cunha-Reis et al., 2004) appears to coexist with direct VIP mediated actions on pyramidal cell bodies either promoting enhancement glutamatergic EPSCs (Ciranna and Cavallaro, 2003) or GABAergic currents (Cunha-Reis et al., 2004) in rat hippocampal slices. The physiological relevance of these conflicting observations remains poorly understood but may be relevant in distinct physiological conditions, depending on network and behavioral state-dependent activation of different interneuron populations (Tyan et al., 2014;

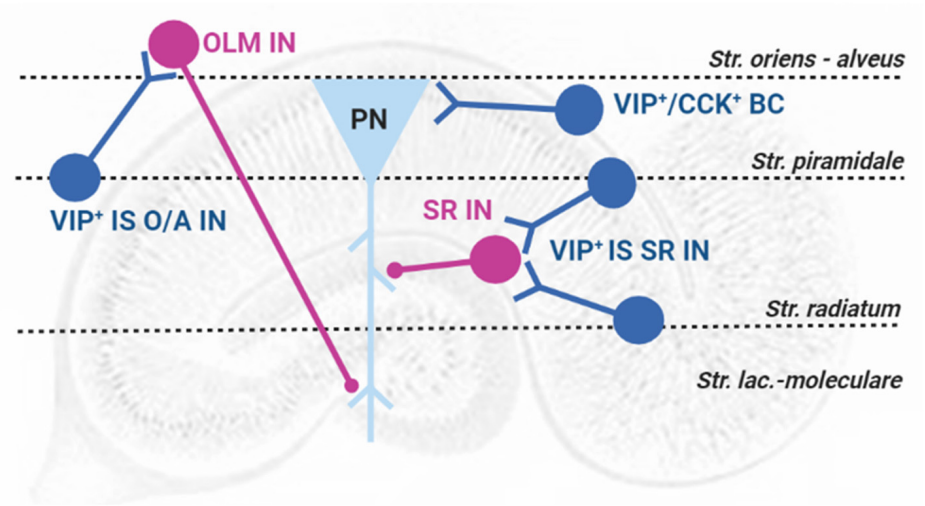

FIGURE 1 | Representation of VIP-containing interneurons in the rat hippocampus: layer location and target selectivity. PN, pyramidal neuron (triangle, light blue); Interneurons (circles, pink); VIP-containing interneurons (circles, blue); *VIP$^+$-CCK$^+$ BCs*: VIP-containing *basket cells*; *VIP$^+$ IS O/A IN*: VIP-containing interneuron-selective interneuron targeting the *stratum oriens/Alveus* and *VIP$^+$ IS SR IN*: VIP-containing interneuron-selective interneuron targeting the *stratum radiatum*; OLM IN – *Stratum oriens* interneuron projecting to the *Stratum lacunosum-moleculare*; SR IN – *Stratum radiatum* local interneurons. Str.: *stratum*.

Artinian and Lacaille, 2018; Francavilla et al., 2018; Turi et al., 2019; Luo et al., 2020).

VIP AND VIP RECEPTORS

VIP shows structural similarity to other neuroendocrine peptides, including secretin, glucagon, gastric inhibitory peptide, growth hormone releasing factor and PACAP (Deng and Jin, 2017); and belongs glucagon–secretin–VIP family of peptides targets (Clynen et al., 2014). VIP acts trough two high affinity receptors ($VPAC_1$ and $VPAC_2$) that belong to Group II receptor (GPCR) family and are encoded by two different genes sharing only 55% similarity. These have nearly the same affinity for VIP (in the low nanomolar range) (Yang et al., 2010; Harmar et al., 2012) and bind also PACAP with similar affinity, hence the name VPAC given to VIP receptors (Laburthe et al., 2002). The VIP receptor subfamily also includes a third receptor, PAC_1 (PACAP specific receptor), which binds VIP with low affinity (in the micromolar range) (Harmar et al., 2012). VPAC receptors exhibit multiple consensus sites for phosphorylation by intracellular kinases and *N*-glycosylation, but differences in the *N*-glycosylation are observed according to tissue and/or species (Laburthe et al., 2002).

Both VPAC and PAC_1 receptors are positively coupled to $G_{\alpha s}$ and stimulate the cAMP/PKA signaling pathway (see Laburthe et al., 2002; Harmar et al., 2012 for review). However, PAC_1 receptors additionally strongly stimulate $G_{\alpha q}$ and the phospholipase C (PLC)/PKC signaling pathway, while VPAC receptors activate it weakly (Harmar et al., 1998; Yang et al., 2010). However, $VPAC_1$ receptors can couple to $G_{i/o}$ proteins in the hippocampus (Shreeve, 2002) and VIP enhancement of $[Ca^{2+}]_i$ in astrocyte cultures depends on IP_3 turnover (Fatatis et al., 1994).

VIP RECEPTORS AND HIPPOCAMPAL NEUROTRANSMISSION

Although VIP and PACAP receptors have a widespread expression in the brain, $VPAC_1$ receptors are predominantly found in the hippocampus and cerebral cortex, while $VPAC_2$ receptors prevail in the thalamus and suprachiasmatic nucleus, showing lower expression in the hippocampus, spinal cord, dorsal root ganglia and brainstem (Harmar et al., 2012; Borbély et al., 2013). Not surprisingly, VIP and VIP receptors are involved in learning and memory processes (Yang et al., 2010; Borbély et al., 2013), yet, elucidating the differential involvement of each VIP receptor in the actions of VIP has proven very difficult until ligands with enough selectivity to discriminate between $VPAC_1$ and the $VPAC_2$ receptor were developed (Gourlet et al., 1997a,b,c; Moreno et al., 2000). This is particularly important in the hippocampus, where both receptors are expressed (Vertongen et al., 1997; Joo et al., 2004).

VIP receptors are unevenly distributed in different hippocampal layers. $VPAC_2$ receptors are more expressed in *SP* of the Ammon's Horn implying a key role in the modulation of hippocampal pyramidal cell activity, whereas $VPAC_1$ receptors are preferentially located in the *SO* and *SR* and partially co-localized with glial markers (Acsády et al., 1996a; Joo et al., 2004). No study has to date identified $VPAC_1$ receptors in hippocampal interneurons, yet the fact that VIP enhancement of synaptic transmission to CA1 pyramidal cells involves inhibition of GABAergic interneurons that control

TABLE 1 | Effects of VIP on hippocampal excitatory and inhibitory networks and VIP receptors involved.

Action	Target	Receptor	Species/preparation	References
Enhanced pyramidal cell excitability	CA1 PNs	Unknown	Rat hippocampal slices	Dodd et al., 1979
Enhanced synaptic transmission and pyramidal cell excitability and; Reduced the slow afterhyperpolarization (Ca^{2+}-dependent K^+ current)	CA1 PNs	Unknown	Male Wistar rats (young adult): hippocampal slices	Haas and Gähwiler, 1992
VIP application to the *O/A* in the absence of synaptic interactions, increased the firing rate O/A INs and; Decreased fEPSP slope rat the *SR* and *SLM*,	CA1 INs and PNs	Unknown	Male NMRI mice (young adult): hippocampal slices	Yanovsky et al., 1997
Increased the frequency of mIPSCs without affecting their mean magnitude	Hippocampal neurons	Unknown	Cultured hippocampal neurons	Wang et al., 1997
Enhanced EPSCs	CA1 PNs	Unknown	Juvenile male Wistar rats: hippocampal slices	Ciranna and Cavallaro, 2003
Enhanced synaptic transmission through disinhibition and pyramidal cell excitability Enhanced GABAergic currents Enhanced GABA release	CA1 PNs (dendrites and soma) CA1 INs and PNs GABAergic nerve terminals	Unknown	Male Wistar rats (young adult): hippocampal slices	Cunha-Reis et al., 2004
VIP enhanced synaptic transmission	CA1 PNs (dendrites)	$VPAC_1$ and $VPAC_2$ receptors	Male Wistar rats (young adult): hippocampal slices	Cunha-Reis et al., 2005
Enhances pyramidal cell excitability	CA1 PNs	$VPAC_2$ receptor	Male Wistar rats (young adult): hippocampal slices	Cunha-Reis et al., 2006
VIP enhanced the amplitude of NMDARs	CA1 PNs	$VPAC_1$/$VPAC_2$ receptors	Juvenile and young adult male wistar rats: isolated CA1 neurons and hippocampal slices	Yang et al., 2009
Endogenous VIP inhibits CA1 hippocampal LTP	CA1 PNs (dendrites)	$VPAC_1$ receptor	Male Wistar rats (young adult): hippocampal slices	Cunha-Reis et al., 2010
Endogenous VIP inhibits hippocampal CA1 LTD and depotentiation	CA1 PNs (dendrites)	$VPAC_1$ receptor	Juvenile and young adult male Wistar rats: hippocampal slices	Cunha-Reis et al., 2014
Enhances exocytotic GABA release and GAT-1 nerve terminal reversal Inhibits exocytotic GABA release	GABAergic nerve terminals	$VPAC_2$ receptor $VPAC_1$ receptor	Male Wistar rats (young adult): isolated nerve terminals	Cunha-Reis et al., 2017

CA1, hippocampus Cornus Ammonis 1; GAT-1, GABA transporter 1; INs, interneurons; NMRI, Naval Medical Research Institute; O/A, hippocampal Oriens-Alveus layer; PNs, Pyramidal neurons; SR, Stratum radiatum; SLM, Stratum lacunosum-moleculare; $VPAC_1$ and $VPAC_2$, VIP – Vasoactive intestinal peptide; VIP receptors 1 and 2.

pyramidal cell dendrites, leading to disinhibition (Cunha-Reis et al., 2004), an action mediated by activation of $VPAC_1$ receptors (Cunha-Reis et al., 2005) preferentially located in the *SO*, *SR* or *O/A* (Vertongen et al., 1997; Joo et al., 2004) suggests $VPAC_1$ receptors are in fact responsible for VIP actions on hippocampal interneurons. $VPAC_2$ receptors are the main mediators of VIP enhancement of pyramidal cell excitability (Cunha-Reis et al., 2006), and likely mediators of VIP enhancement of NMDA receptor currents in pyramidal cells (Yang et al., 2009), effects that are mostly post-synaptic and independent of GABAergic transmission (Ciranna and Cavallaro, 2003; Cunha-Reis et al., 2004), and that likely involve inhibition of the sAHP (Haas and Gähwiler, 1992) (see **Table 1**).

VIP modulation of hippocampal GABAergic transmission involves both presynaptic enhancement of GABA release and postsynaptic facilitation of GABAergic currents in interneurons (Wang et al., 1997; Cunha-Reis et al., 2004). We recently reported a dual opposing regulation of GABA release by VPAC receptors in isolated hippocampal nerve terminals (Cunha-Reis et al., 2017): $VPAC_1$ receptors inhibit and $VPAC_2$ receptors enhance GABA release. $VPAC_1$ receptor activation inhibits voltage-gated calcium channel (VGCC)-dependent GABA exocytosis through a $G_{i/o}$ and PKA-independent and partially PKC-dependent mechanism (Cunha-Reis et al., 2017). $VPAC_2$ receptor activation enhances VGCC-dependent GABA exocytosis by a G_s/PKA/PKC-dependent mechanism but also enhances GAT-1 carrier-mediated GABA outflow through a G_s/PKC-dependent mechanism. Given the asymmetry in $VPAC_1$ and $VPAC_2$ receptor location in different layers of Ammon's horn, VIP may differentially modulate GABA release to pyramidal cells and *INs*, and thus have distinct

consequences on synaptic transmission to pyramidal cell dendrites and pyramidal cell activity, suggesting several possible therapeutic applications.

VIP AND SYNAPTIC PLASTICITY

Synaptic plasticity relies on long-lasting, activity-dependent bidirectional changes in the strength of synaptic communication leading to long-term potentiation (LTP) and long-term depression (LTD) (Mellor, 2018) and is widely accepted as the cellular mechanism underlying memory storage (Bliss and Collingridge, 2019). LTP can be triggered by a single episode of high frequency stimulations (HFS), such as a tetanus or theta burst (Albensi et al., 2007; Larson and Munkácsy, 2015; Bliss and Collingridge, 2019), mimicking the firing of hippocampal principal cells during learning tasks, and was the first synaptic plasticity mode to be associated with hippocampal-dependent memory formation (Bliss and Collingridge, 2019). LTD can be elicited by low-frequency stimulation (LFS), mimicking hippocampal activity during delta waves, and is involved in hippocampal-dependent memory processes associated with behavioral flexibility like memory extinction, reversal learning, reformulation of previously formed memories, terminating/shifting attention and in stabilizing the effects of learning (Kitchigina et al., 1999; Albensi et al., 2007; Kemp and Manahan-Vaughan, 2007; Collingridge et al., 2010). Both LTP and LTD require the activation of NMDA receptors, and their stability or long-lasting expression is dependent on subsequent activation of multiple intracellular cascades (Collingridge et al., 2010; Bliss and Collingridge, 2019). LTP of glutamatergic transmission requires activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and recruitment and insertion of AMPA receptors into the postsynaptic membrane (early-LTP) (Park et al., 2018; Benke and Traynelis, 2019). Endurance and stability of LTP is believed to require synaptic contact enlargement and both PKA activity and *de novo* protein synthesis (late-LTP) (Park et al., 2018; Bliss and Collingridge, 2019).

Recent evidence supports the view that disinhibition plays a crucial role in regulating hippocampal synaptic plasticity (Artinian and Lacaille, 2018). Furthermore, Yang et al. (2009) showed that exogenously applied VIP enhances NMDA currents in CA1 pyramidal cells, an effect mimicked by $VPAC_2$ and to a lesser extent by $VPAC_1$ selective agonists. This suggests that either endogenous VIP or PACAP, the two endogenous agonists of this receptor, could contribute to NMDA-dependent hippocampal synaptic plasticity such as LTP, LTD and depotentiation. We recently described that endogenous VIP, through $VPAC_1$ receptor activation, modulates the NMDA receptor-dependent LTD and depotentiation in the CA1 area of the hippocampus (Cunha-Reis et al., 2014). Furthermore, disinhibition achieved through inhibition of $VPAC_1$ receptors was more efficient than blockade of $GABA_A$–mediated transmission in revealing LTD, suggesting that *SR* interneurons are fundamental in restraining synaptic adaptations underlying expression of LTD. $VPAC_1$ receptor activation by endogenous VIP also enhances hippocampal LTP induced by TBS, an action that is dependent on GABAergic transmission and involves phosphorylation of GluA1 AMPA subunit by CamKII, a fundamental mechanism for receptor synaptic recruitment (Cunha-Reis et al., 2010; Carmo and Cunha-Reis, 2011; Cunha-Reis and Carmo, 2011) (see **Table 1**). Activation of hippocampal $VPAC_2$ (but not $VPAC_1$) receptors also promotes phosphorylation of GluA1 at Ser845 (Toda and Huganir, 2015), a PKA target site that is implicated in LTP maintenance and late-LTP (Benke and Traynelis, 2019).

VIP and PACAP modulation of hippocampal principal cell activity targets (directly or indirectly) both the dendritic and somatic compartments implicating these peptides in regulation of both Hebbian and homeostatic plasticity (Wefelmeyer et al., 2016; Yee et al., 2017; Foncelle et al., 2018), yet the physiological and behaviorally relevant stimuli for this modulation are still largely uncovered. Recently, it was described that *VIP^+ IS INs* are activated by both Schaffer collateral and commissural excitatory fibers, being recruited fundamentally during theta oscillations but not during fast ripples (Luo et al., 2020), suggesting a fundamental role in information gating during spatial navigation and memory encoding. Accordingly, *VIP^+ IS INs* are targeted by *medium raphe* serotonergic and GABAergic projections and septal cholinergic fibers, fundamental for the pacing, engagement and suppression of hippocampal theta rhythm (Vinogradova et al., 1999; Borhegyi et al., 2004; Vandecasteele et al., 2014).

Release of large dense core vesicles containing neuropeptides is known to require high-intensity repetitive stimulation, unlike release of small synaptic vesicles containing fast transmitters such as glutamate or GABA (Ghijsen and Leenders, 2005). Firing of VIP-containing interneurons locked with theta rhythm may suffice to release endogenous VIP from hippocampal nerve terminals.

VIP IN COGNITIVE PROCESSES

Early from its discovery, VIP was described to have a crucial role in mnemonic processes and particularly in hippocampal-dependent memory traits. In particular, endogenous VIP was implicated in spatial learning in the Morris water maze (Glowa et al., 1992; Takashima et al., 1993a; Itoh et al., 1994), avoidance learning in the T-maze (Flood et al., 1990) or the shuttle box, together with reduced rearing exploratory behavior (Cottrell et al., 1984; Takashima et al., 1993b), suggesting that VIP is mainly involved in regulating motivated learning behavior. VIP has lateralized effects on the modulation of exploratory behavior and passive avoidance learning (Ivanova et al., 2008, 2009) and anxiolytic and anti-depressive effects (Ivanova et al., 2014), and rescues deficits in hippocampal-dependent passive avoidance learning tasks in a rat model of depression. Recently, VIP-mediated hippocampal disinhibition of pyramidal cell activity was shown to play a crucial role in goal-directed spatial learning tasks (Turi et al., 2019).

VIP-KO mice show decreased expression of $VPAC_2$ and to a lesser extent $VPAC_1$ receptors together with strong circadian rhythm disruption and enhanced arousal and hyperactivity in the open-field test (Girard et al., 2006). Furthermore, VIP-deficient mice shows impaired recall and reversal learning in a fear conditioning test and deficits in social behavior (Chaudhury et al., 2008; Stack et al., 2008). $VPAC_2$-KO mice display normal acquisition of fear conditioning, contextual and cued fear memory, but impaired extinction of cued fear memory (Ago et al., 2017).

VIP participates in the pathophysiology of several neurological disorders associated with cognitive disfunction, like depression (Ivanova et al., 2012), autism spectrum disorders, Alzheimer's disease (AD), Parkinson's disease (PD) and epilepsy (de Lanerolle et al., 1995; Hill, 2007; White et al., 2010). Due to its anti-apoptotic, anti-inflammatory and neuroprotective actions, VIP and its receptors constitute promising therapeutic targets in many of these pathologies (Gozes, 2001; Delgado et al., 2002; Yu et al., 2017).

VIP, SEIZURES, AND EPILEPSY

Epilepsy is the most common, chronic neurological disease (Devinsky et al., 2018) and is characterized by the incidence of recurrent, unprovoked seizures with associated cognitive, psychological and social disturbances (Clynen et al., 2014; Devinsky et al., 2018). According to its underlying causes epilepsy is classified into genetic or idiopathic. More than 500 genes are associated with predisposition to develop epilepsy (Devinsky et al., 2018). Idiopathic epilepsy, has unknown causes but often follows several possible precipitating events such as head trauma, stroke, brain hypoxia, infectious/autoimmune diseases, tumors or childhood febrile seizures (Clynen et al., 2014).

Mesial temporal-lobe epilepsy with hippocampal sclerosis (MTLE-HS), the most prevalent form of symptomatic focal epilepsy, is a heavy burden for the healthcare system. Many MTLE-HS patients are refractory to treatment with multiple anti-epileptic drugs, and amygdalohippocampectomy surgery is the last intervention to prevent complex partial seizures (Kuang et al., 2014). Declarative memory deficits (Helmstaedter and Kockelmann, 2006) are also a hallmark of MTLE-HS, that can be further aggravated by hippocampal removal. Most MTLE cases are idiopathic and evidence suggests that precipitating events trigger epileptogenesis by generating aberrant synaptic plasticity/neuronal excitability, excitotoxicity, secondary non-convulsive *status epilepticus*, inflammation and generation of reactive oxygen species (ROS) (Devinsky et al., 2018; Rana and Musto, 2018), ultimately leading to occurrence of spontaneous recurrent seizures. MTLE-HS is characterized by hippocampal sclerosis, massive neuronal loss and severe astrogliosis (Thom, 2014). Enhanced neurogenesis initially drives formation of new neural pathways in epileptogenesis (Beck and Yaari, 2008), but is impaired in MTLE-HS chronic phase (Zhong et al., 2016). Impaired LTP, due to pathological saturation (Beck et al., 2000), is a major cause for cognitive impairment in MTLE-HS, but changes in input/output neuronal electrical properties (El-Hassar et al., 2007a) and inhibitory/excitatory balance also occur from early in epileptogenesis (El-Hassar et al., 2007b). New drug targets able to control seizures or preventing epileptogenesis are an urgent need (Clynen et al., 2014).

Neuropeptides, such as VIP, are stored in large dense-core granules and are released during the sustained high-frequency activity (5–40 Hz) occurring during epileptiform activity, being implicated in regulation of seizure susceptibility, constituting appealing targets for the development of new AEDs, potentially less susceptible to side-effects (Clynen et al., 2014).

VIP is an important regulator of hippocampal activity through both direct actions on pyramidal cell excitability (Haas and Gähwiler, 1992) and by regulating synaptic transmission and synaptic plasticity to pyramidal cell dendrites through disinhibition (Cunha-Reis et al., 2004, 2010, 2014; Cunha-Reis and Carmo, 2011; Luo et al., 2020), actions that have a major impact on hippocampal-dependent learning and memory formation (Turi et al., 2019).

In human MTLE-HS, an up-regulation in VIP receptors in the seizure focus (hippocampus) was linked to the loss of principal neurons (i.e., granule cells and pyramidal neurons) without changes in the pattern and distribution of VIP^+ *INs* (de Lanerolle et al., 1995). Accordingly, an enhancement in VIP^+ *INs* has been described in a mouse model of temporal lobe epilepsy (TLE) (King and LaMotte, 1988) and decreased dendritic but not somatic GABAergic inhibition has been implicated in different animal models of experimental TLE (Sloviter, 1987; Cossart et al., 2001). Although an enhancement in disinhibition caused by VIP could be implicated in reduced seizure threshold in MTLE-HS, enhancement in VIP expression is more likely a compensatory mechanism for the selective loss of OLM interneurons in TLE, the main targets of VIP^+ *IS O/A INs*. Recently, it was described that while the overall density of the VIP^+ *IS O/A INs* was preserved, the number of their synaptic contacts in CA1 *O/A* was reduced in the pilocarpine model of TLE and was accompanied by significant alterations in their dendritic morphology and passive membrane properties (David and Topolnik, 2017).

Following kainic acid and pentylenetetrazole-induced seizures in rodents, an early short-term decrease in hippocampal VIP levels following the initial (precipitating) seizures was described (Marksteiner et al., 1989; Romualdi et al., 1992), suggesting that transient changes in VIP expression either contribute or counteract selective interneuron loss and plasticity changes during latent-period epileptogenesis. Preliminary studies *in vitro* show that changes in synaptic plasticity and synaptic plasticity markers following brief insults like hypoxia, bicuculine-induced seizures or inter-ictal like activity are either prevented or enhanced by a $VPAC_1$ receptor antagonist, suggesting that different epileptogenic events are differentially regulated by $VPAC_1$ receptor activity (Cunha-Reis, 2013; Carvalho-Rosa and Cunha-Reis, 2019).

In MTLE patients, the up-regulation of VIP receptors observed chronically is consistent with an increase in surviving neurons and levels of reactive glia (de Lanerolle et al., 1995;

Clynen et al., 2014), suggesting that VPAC receptors (especially $VPAC_1$) are promising targets for preventing epileptogenesis, a process that extends beyond the initial latent period (Devinsky et al., 2018). Given their role in the control of hippocampal synaptic plasticity they constitute also excellent candidates for prevention or attenuation of cognitive decline in MTLE. Furthermore, the dual role of $VPAC_1$ and $VPAC_2$ receptors in the control of hippocampal GABA release makes them the perfect targets for development of drugs aiming to control the imbalance in GABAergic and glutamatergic transmission associated with TLE (Schousboe et al., 2014; Cunha-Reis et al., 2017).

CONCLUDING REMARKS

In conclusion, the importance of VIP, acting through $VPAC_1$ or $VPAC_2$ Rs, either to the control of hippocampal disinhibition leading to enhanced synaptic transmission or promoting a direct enhancement of pyramidal cell excitability suggests that VIP can have a differential impact in hippocampal-dependent cognition, and its possible therapeutic applications should be explored. The up-regulation of VIP receptors observed in MTLE patients and the finding obtained in animal models that the interneuron targets of VIP-containing interneurons are particularly susceptible to epileptic damage, suggest that VPAC receptors (especially $VPAC_1$) are promising targets for epileptogenesis prevention and for prevention or attenuation of cognitive decline in MTLE.

AUTHOR CONTRIBUTIONS

AC-R: writing – review and editing. DC-R: resources, supervision, funding acquisition, project administration, and writing – original draft, review and editing.

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CORRECTION
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Corrigendum: VIP Modulation of Hippocampal Synaptic Plasticity: A Role for VIP Receptors as Therapeutic Targets in Cognitive Decline and Mesial Temporal Lobe Epilepsy

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Keywords: VIP, synaptic plasticity, interneurons, hippocampus, MTLE, cognition, VPAC1 receptors

A Corrigendum on

VIP Modulation of Hippocampal Synaptic Plasticity: A Role for VIP Receptors as Therapeutic Targets in Cognitive Decline and Mesial Temporal Lobe Epilepsy
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In the original article, there was a mistake in **Figure 1** as published. Circuits on the left side of the Figure moved down and stretched and do not represent what is described in the text for VIP O/A interneurons and their targets, OLM cells that should have their cell body in the stratum oriens. The corrected **Figure 1** appears below.

Additionally, in the original article, there was an error in the identification of type II IS cells (a different nomenclature for a subpopulation (and not all) of VIP+ interneurons projecting to the stratum radiatum).

A correction has been made to section *VIP in the Hippocampus*, Second *Paragraph.*

The corrected paragraph is shown below.

Detailed immunohistochemistry studies fully characterized hippocampal *VIP*$^+$ *INs* dendritic trees and axon projections (Acsády et al., 1996a,b), allowing the classification of *VIP*$^+$ *INs* into two fundamental groups according to their targets: *VIP*$^+$ *basket cells* are responsible for somatic inhibition of pyramidal cells, are also immunoreactive for cholecystokinin (*VIP*$^+$-*CCK*$^+$ *BCs*, **Figure 1**) and do not express parvalbumin, as most *BCs* in the hippocampus. *VIP*$^+$ *INs* that selectively innervate other interneurons (*VIP*$^+$ *IS INs*) include two subtypes: (a) interneurons with cell bodies located at the *stratum pyramidale (SP)* or near and projecting to the *stratum Oriens/Alveus* border (*VIP*$^+$ *IS O/A INs* or type III IS cells, **Figure 1**), that also express the interneuron marker calretinin and target mostly somatostatin-expressing (SOM$^+$) *oriens lacunosum-moleculare* (*OLM*) interneurons innervating the distal dendrites of pyramidal cells at the *stratum lacunosum-moleculare (SLM)* and (b) *VIP*$^+$ *INs* that project their axons to the *stratum radiatum* (*SR*, *VIP*$^+$ *IS SR INs*, **Figure 1**), with cell bodies located either at the *SR/SLM* border (type II IS cells) or at *SR/SP* and targeting *interneurons* controlling synaptic transmission to proximal dendrites of pyramidal cells in the *SR* (Acsády et al., 1996a,b; Klausberger and Somogyi, 2008). In genetically modified VIP-eGFP mice, additional targets of *VIP*$^+$ *IS O/A INs* in the *O/A*,

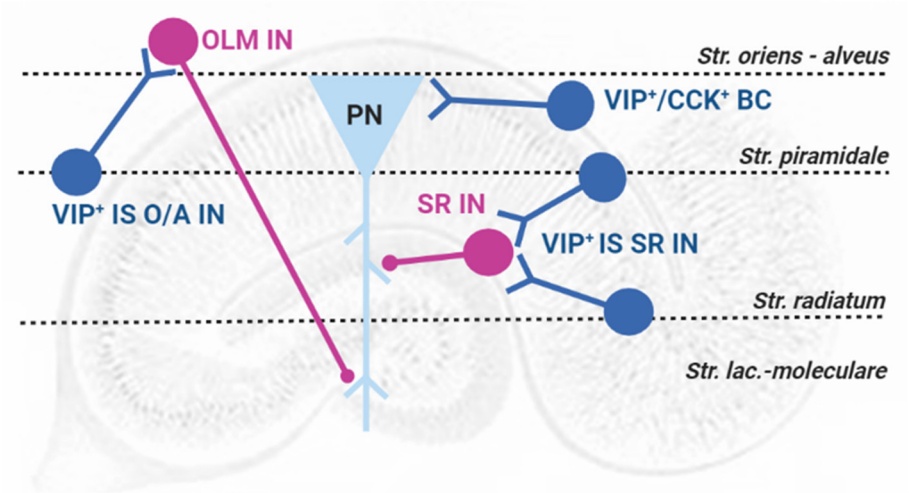

FIGURE 1 | Representation of VIP-containing interneurons in the rat hippocampus: layer location and target selectivity. PN, pyramidal neuron (triangle, light blue); Interneurons (circles, pink); VIP-containing interneurons (circles, blue); *VIP$^+$-CCK$^+$ BCs*: VIP-containing *basket cells*; *VIP$^+$ IS O/A IN*: VIP-containing interneuron-selective interneuron targeting the *stratum oriens/Alveus* and *VIP$^+$ IS SR IN*: VIP-containing interneuron-selective interneuron targeting the *stratum radiatum*; OLM IN – *Stratum oriens* interneuron projecting to the *Stratum lacunosum-moleculare*; SR IN – *Stratum radiatum* local interneurons. Str.: *stratum*.

including bistratified cells and oriens–oriens INs, have been described and recently a new VIP expressing interneuron population located at the O/A (*VIP$^+$* long-range projecting INs, *VIP$^+$ LRP INs*) was described targeting *INs* within the O/A in CA1 but also both *INs* and pyramidal cells within the *subiculum* (Francavilla et al., 2018). It is not clear if it is also present in the rat hippocampus.

The authors apologize for these errors and state that they do not change the scientific conclusions of the article in any way. The original article has been updated.

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REVIEW
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Protective Effects of Pituitary Adenylate Cyclase-Activating Polypeptide and Vasoactive Intestinal Peptide Against Cognitive Decline in Neurodegenerative Diseases

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Cognitive impairment is one of the major symptoms in most neurodegenerative disorders such as Alzheimer's (AD), Parkinson (PD), and Huntington diseases (HD), affecting millions of people worldwide. Unfortunately, there is no treatment to cure or prevent the progression of those diseases. Cognitive impairment has been related to neuronal cell death and/or synaptic plasticity alteration in important brain regions, such as the cerebral cortex, substantia nigra, striatum, and hippocampus. Therefore, compounds that can act to protect the neuronal loss and/or to reestablish the synaptic activity are needed to prevent cognitive decline in neurodegenerative diseases. Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are two highly related multifunctional neuropeptides widely distributed in the central nervous system (CNS). PACAP and VIP exert their action through two common receptors, VPAC1 and VPAC2, while PACAP has an additional specific receptor, PAC1. In this review article, we first presented evidence showing the therapeutic potential of PACAP and VIP to fight the cognitive decline observed in models of AD, PD, and HD. We also reviewed the main transduction pathways activated by PACAP and VIP receptors to reduce cognitive dysfunction. Furthermore, we identified the therapeutic targets of PACAP and VIP, and finally, we evaluated different novel synthetic PACAP and VIP analogs as promising pharmacological tools.

Keywords: Alzheimer's disease, Parkinson's disease, Huntington's disease, cognition, synaptic plasticity, PAC1, VPAC1, VPAC2

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INTRODUCTION

Cognition is the result of the formation of functional neuronal circuits in many cerebral areas governed by a dynamic phenomenon named synaptic plasticity. People suffering from neurodegenerative diseases commonly present cognitive impairment, such as dementia, deficits in learning and attention, or incomplete executive function among others, affecting the

daily life of patients and their families. This impairment is mediated by the extracellular or intracellular accumulation of protein aggregates that disrupt synaptic plasticity leading to neuronal dysfunction and/or neuronal death. Several mechanisms have been involved in neuronal plasticity disturbance and neuronal cell death, such as inflammation, excitotoxicity, oxidative stress, and neurotrophic deprivation. These mechanisms affect genetic expression through the activation/inhibition of some pathways and transcription factors. The result of altered genetic expression is the disruption of structural and functional synaptic plasticity and/or the viability of neurons. Thus, molecules able to promote synaptic plasticity or neuronal viability by the interaction and inhibition of these mechanisms in brain areas related to learning and memory could be interesting therapeutic compounds to stop the cognitive decline in neurodegenerative diseases.

Pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) are well-known neuropeptides that exert a potent neuroprotective effect through the activation of different signaling pathways and transcriptional-genetic activity (Dejda et al., 2005; Vaudry et al., 2009). Some data in animal models show that the lack of PACAP and VIP is associated with a cognitive decline. Indeed, PACAP deficient mice display impaired recognition memory (Shibasaki et al., 2015) and VIP deficient mice exhibit cognitive deficits (Chaudhury et al., 2008). Recently, it has been shown that VIP positive interneurons in the CA1 hippocampal area are necessary for spatial learning (Turi et al., 2019). Interestingly, in rat hippocampal slices, it has been observed that PACAP and VIP exert a direct effect in synaptic transmission (Ciranna and Cavallaro, 2003). This context and the capacity of PACAP and VIP to cross the blood-brain barrier suggest that these neuropeptides and their receptors-mediated signaling could have promising therapeutic activity in neurodegenerative diseases. In this review, we summarize the pieces of evidence showing that PACAP and VIP administration help to preserve cognitive function in different preclinical models of Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). We also identify the main molecular mechanisms by which PACAP and VIP promote neuronal survival and synaptic plasticity in neurons to preserve cognitive performances. Finally, we review novel VIP and PACAP synthetic derivatives which could represent promising therapeutic tools for the treatment of neurodegenerative diseases.

PACAP AND VIP PROMOTE COGNITIVE FUNCTION IN NEURODEGENERATIVE DISEASE MODELS

Alzheimer's Disease

Alzheimer's disease is the most common form of dementia characterized by the irreversible deterioration of cognitive function (Alzheimer's Association, 2015) associated with the accumulation of amyloid-β (Aβ) plaques and neurofibrillary tangles in the cognitive brain areas (Hou et al., 2019).

The potential of PACAP as a therapeutic agent in AD neuropathology has been studied for many years in different experimental models (**Table 1**). *In vitro* studies have reported that PACAP protects against Aβ-mediated toxicity (Onoue et al., 2002; Han et al., 2014b). This neuroprotective effect of PACAP is associated with an improvement of the cognitive function in AD mice models. A long-term daily intranasal PACAP administration ameliorates the performance in the novel object recognition test in the AD transgenic mice model overexpressing the amyloid precursor protein (APP; Rat et al., 2011). In these animals, the rescue of memory deficits was linked with an increase of brain-derived neurotrophic factor (BDNF) and the enhancement of the non-amyloidogenic pathway of APP (Rat et al., 2011). Also, PACAP reduced the inflammatory response (Rat et al., 2011). Thus, PACAP is suggested to prevent cognitive decline in AD exerting neurotrophic, neuroprotective, and anti-inflammatory effects. Interestingly, the capacity of PACAP to counteract cognitive decline was also proved in SAMP8 mice, another AD model expressing the Aβ (Nonaka et al., 2012). In this study, PACAP alone or together with cyclodextrins was given by intranasal administration. PACAP was found in all brain regions, but some regions such as the occipital cortex and striatum incorporated much more PACAP than others. This pattern of distribution of the labeled PACAP is different from the one found when the peptide is given by intravenous injection, in which the highest uptakes are found in the hippocampus and hypothalamus (Nonaka et al., 2002). The addition of cyclodextrins can increase or decrease the uptake of PACAP in different brain regions depending on the cyclodextrin used. Such treatment could thus be used to promote neuropeptide uptake toward specific brain regions and was shown to improve the learning memory of SAMP8 mice in the T-maze task (Dogrukol-Ak et al., 2009). Because of these promising results, it was proposed that the downregulation of PACAP may be involved in AD neuropathology. Indeed, a comparative analysis of gene expression showed decreased protein levels of PACAP and BDNF in three different models of AD with Aβ deposition (Wu et al., 2006). Years later, PACAP expression was found decreased and inversely correlated with Aβ and Tau protein levels in a triple transgenic mouse model of AD (3xTG, Psen1/APPSwe/TauP301L; Han et al., 2014b). According to these findings, studies with AD patients demonstrated that PACAP levels were reduced in cortical areas such as the entorhinal cortex, the middle temporal gyrus, the superior frontal gyrus, and the primary visual cortex (Han et al., 2014a). Interestingly, PACAP deficits in humans were associated with clinical severity of AD (Han et al., 2015) and inversely correlated with Aβ plaques and neurofibrillary tangles (Han et al., 2014b). Altogether, these results point out that reduced levels of PACAP may contribute to the pathological process of AD and could have an important implication in the cognitive decline.

Different studies using animals and cell culture models of AD have revealed that VIP also acts as a neuroprotective agent against the pathogenesis of this disorder (**Table 1**). A recent study using 5XFAD mice, an AD model with massive cerebral Aβ deposits, shows that VIP intraperitoneal chronic

TABLE 1 | Beneficial effects of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) in cellular and animal models of different neurodegenerative diseases.

		PACAP	VIP
Alzheimer's disease	Animal model	Improves performance in the novel object recognition test in APP[V717I]-transgenic mice (Rat et al., 2011). Improves learning in T-maze task in SAMP8 mice (Dogrukol-Ak et al., 2009).	Decreases Aβ accumulation and atrophy in the hippocampus and cortex in 5XFAD mice (Korkmaz et al., 2019). Reduces inflammation and attenuates amyloidosis in the hippocampus in PS1/APP transgenic mice (Song et al., 2012). Ameliorates the cognitive performance in the water maze test in ApoE mice (superactive VIP derivate; Gozes et al., 1997).
	Cellular model	Protects against Aβ-mediated toxicity in PC12 cells (Onoue et al., 2002) and primary neuronal cell culture (Han et al., 2014b).	Protects against Aβ-induced cell death in PC12 cells (Onoue et al., 2002) and cerebral cortex cell cultures (Gozes et al., 1996).
Parkinson's disease	Animal model	Improves learning and memory in three different paradigms of water-maze task in MPTP-injected mice (Deguil et al., 2010). Prevents from motor and behaviour deficits in 6-OHA lesioned rats (Reglodi et al., 2004a,b). Improves locomotor function and behaviour alterations in rotenone-induced snails (Maasz et al., 2017).	Prevents from oxidative stress and apoptosis in 6-OHDA-lesioned rats (Tunçel et al., 2012). Enhances the spine density and prevents from dopaminergic cell loss in 6-OHDA lesioned rats (Korkmaz et al., 2012). Supresses microglial activation in MPTP- induced mice (Delgado and Ganea, 2003) and astrogliosis in 6-OHDA lesioned rats (Yelkenli et al., 2016). Reverses the rotational deficits in a 6-OHDA lesioned rats (Tunćel et al., 2005).
	Cellular model	Protects against 6-OHDA (Takei et al., 1998), MPTP (Chung et al., 2005), rotenone (Wang et al., 2005), salsolino (Brown et al., 2013) and paraquat (Hajji et al., 2019).	Protects against dopamine and 6-OHDA toxicity in PC12 and neuroblastoma cells (Offen et al., 2000).
Huntington's disease	Animal model	Reduces the loss of striatal neurons and attenuates behavioural disturbances in striatum-lesioned mice by quinolonic acid injection (Tamás et al., 2006). Improves the performance in the novel object recognition test and the T-maze spontaneous alternation task in R6/1 transgenic mice (Cabezas-Llobet et al., 2018).	
	Cellular model	Induces neuritic branching in R6/1 hippocampal primary culture (Cabezas-Llobet et al., 2018).	

administration significantly decreases Aβ accumulation and reduces the atrophy in brain regions involved in cognition, such as hippocampus and cortex (Korkmaz et al., 2019). Importantly, VIP protection against Aβ-induced cell death was also observed in experiments using PC12 cells and cortical cell cultures (Gozes et al., 1996; Onoue et al., 2002). However, the survival effect of VIP against Aβ-induced cell death seems to be less effective than PACAP protective activity. This result could be due to the growing evidence that the neuroprotective effect of VIP is mainly through its action on microglial cells. In microglia/neuron co-cultures, some studies showed that the presence of VIP protects from Aβ-induced neurodegeneration, inhibiting the secretion of pro-inflammatory interleukins and neurotoxic agents from microglia and inducing Aβ phagocytosis (Delgado et al., 2008; Song et al., 2012). Interestingly, both immunosuppressive and anti-amyloidosis effects of VIP have been confirmed *in vivo*. Constitutive overexpression of VIP in the hippocampus reduces inflammation and attenuates amyloidosis in transgenic PS1/APP mice, which present increased β-amyloid production associated with behavioral abnormalities (Song et al., 2012). Inflammatory reactions and microglia activation are well known to contribute to neuronal degeneration and to be associated with dementia

in AD. Therefore, neuroprotective and immunomodulatory capacities of VIP make this neuropeptide an attractive candidate for the treatment of cognitive deficits in AD. However, the implication of VIP expression in AD pathology is still unclear. A study using human samples did not show any differences in VIP expression in different cognitive-related regions such as the hippocampus, amygdala, thalamus, and striatum of patients with AD (Ferrier et al., 1983). In contrast, a significant reduction of VIP immunoreactivity was found in the cerebral cortex of AD patients (Arai et al., 1984). In AD animal models, the role of VIP in the neuropathology has been poorly studied. Only in the AD mice model deficient in apolipoprotein E (ApoE), a reduction in VIP transcription was reported (Gozes et al., 1997). Interestingly, chronic intranasal administration of a superactive VIP agonist ameliorates the learning and memory deficits of these animals in water maze test (Gozes et al., 1997), suggesting that the peptide could at least be of therapeutical interest for the treatment of the disease.

Parkinson's Disease

Parkinson's disease is the second most common neurodegenerative disorder after AD (Kalia and Lang, 2015). PD neuropathology is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the subsequent loss of their projections to the striatum (Bezard et al., 2003). Although PD has long been characterized by motor disturbances, cognitive dysfunction is common in patients and can range from mild impairment to dementia (Caviness et al., 2007). Nowadays, the relationship between the neurodegenerative process and cognitive decline in PD remains unclear. While some authors point out a frontal lobe dysfunction because of the affection in the mesocortical dopaminergic system, others suggest a deficit in the cortico-basal ganglia circuit due to the nigrostriatal dopaminergic degeneration (Sawamoto et al., 2007). Besides, some clinical studies indicate that hippocampus atrophy could also contribute to memory impairment in PD (Brück et al., 2004).

The neuroprotective action of PACAP in PD has been well-established in both *in vitro* and *in vivo* models (**Table 1**, Reglodi et al., 2017, 2018). PACAP has proved to be neuroprotective in different cell cultures and explant models of PD, using 6-hydroxydopamine (OHDA), 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, salsolinol and paraquat neurotoxic agents (Takei et al., 1998; Chung et al., 2005; Wang et al., 2005; Brown et al., 2013; Lamine-Ajili et al., 2016; Hajji et al., 2019). These *in vitro* results are in line with findings observed in animal models, which have demonstrated the therapeutic action of PACAP in motor and behavioral disturbances of PD (Reglodi et al., 2004a,b; Maasz et al., 2017). The capacity of PACAP to prevent the cognitive impairment in PD was showed using three specific cognitive processes in an MPTP mouse model: habit learning, working memory, and spatial reference in learning and memory, which depend on the integrity of the striatum, frontal cortex, and hippocampus, respectively. PACAP could improve learning and memory functions in those three different paradigms (Deguil et al., 2010). Hence, the authors suggest that PACAP could not only restore the dopaminergic neurotransmission but also the hippocampal cholinergic transmission, as other authors had seen before (Masuo et al., 1993; Wang et al., 2008; Maasz et al., 2017). The fact that PACAP was able to protect the three neuronal cell types makes it a promising therapeutic agent to fight against cognitive decline in PD. Also, recent results obtained in different animal models of PD showed the dysregulation of PACAP and its receptors in brain areas related to cognition. A specific reduction of PAC1 receptor was reported in the basal ganglia of MPTP-induced parkinsonian macaques (Feher et al., 2018). Likewise, PACAP-knockout mice were shown to be more vulnerable to paraquat (a pesticide that increases the risk of PD) than wild-type (Watson et al., 2013). However, there are no studies relating to a decrease of PACAP or PAC1 receptor level with impairment of cognitive functions in PD. Therefore, further research is needed to establish if endogenous PACAP and its receptors are involved in the progression of PD, and specifically in cognitive symptoms.

In different PD neuronal and animal models, VIP has proved to protect either directly or indirectly (**Table 1**, Korkmaz and Tunçel, 2019). VIP was found to protect directly against dopamine and 6-OHDA toxicity in PC12 and neuroblastoma cells (Offen et al., 2000). VIP also exerted a direct effect preventing neurons from oxidative stress and apoptosis in a 6-OHDA murine model (Tunçel et al., 2012). Moreover, VIP was found to enhance the spine density and act as a neurotrophic factor in the striatum of parkinsonian rats (Korkmaz et al., 2012; Yelkenli et al., 2016). VIP could also indirectly exert protection through the suppression of both microglial activation and astrogliosis in PD (Delgado and Ganea, 2003; Yelkenli et al., 2016). Besides this, some authors support that VIP neuroprotective effect is probably mediated by masts cells, suggesting a VIP immunomodulatory action (Tunćel et al., 2005). Also, VIP was found to reverse the rotational deficits in a 6-OHDA rat model of PD (Tunćel et al., 2005), indicating a potential therapeutic activity. Unfortunately, no studies have been conducted regarding the specific effect on animals' cognitive function. Moreover, in contrast to PACAP, there is no evidence that VIP participates in PD neuropathology. VIP levels were found unaltered in the brains of both demented and non-demented parkinsonian patients (Jégou et al., 1988).

Huntington's Disease

Huntington's disease is a hereditary autosomal neurodegenerative disorder characterized by cognitive, psychiatric, and motor dysfunction. The genetic cause of HD is an abnormal expansion of CAG in the gene encoding for the protein huntingtin (htt; MacDonald et al., 1993). The resulting mutant htt (mhtt) protein causes a severe degeneration of striatal neurons that leads to motor disabilities. Also, mhtt promotes synaptic dysfunction in some cortical and hippocampal neuronal populations, which has been associated with the cognitive decline in HD (Giralt et al., 2012).

There are only two studies that explore the therapeutic potential of PACAP in the treatment of HD (**Table 1**). However, the results are promising. First, the effect of PACAP was studied in an excitotoxic model of HD. In HD-induced rats, PACAP treatment reduces the loss of striatal neurons and attenuates behavioral disturbances (Tamás et al., 2006). Years later, a second study found that PACAP can counteract the hippocampal-dependent cognitive decline in a transgenic HD mouse model by the analysis of novel object recognition test and the T-maze spontaneous alternation task (Cabezas-Llobet et al., 2018). This cognitive improvement is associated with an increased expression of proteins related to synaptic plasticity, such as BDNF, and the recovery of synaptic particles in the hippocampus. Moreover, in HD hippocampal primary cultures, PACAP treatment increases the number and length of neurites (Cabezas-Llobet et al., 2018). Thus, PACAP is suggested to improve the cognitive function by enhancing synaptic plasticity in the hippocampus. Interestingly, PACAP treatment also restores the PAC1 receptor protein level, which is altered in the hippocampus of HD mice from the onset of cognitive decline. Therefore, the beneficial effects of PACAP are suggested to be through the stimulation of the PAC1 receptor (Cabezas-Llobet et al., 2018).

Very few authors have studied the possible role of VIP in HD pathology. The first study regarding VIP and HD, showed no changes in VIP protein levels in the frontal cortex and basal ganglia in human HD post-mortem samples (Emson et al., 1979). However, some other studies indicate that VIP expression is altered in brain areas related to non-motor symptoms in HD. A post-mortem analysis of HD patients revealed a reduction of VIP immunoreactivity in the central nucleus of the amygdala (Zech et al., 1986). Recently, a decreased expression of VIP receptors was also found in the hippocampus of a transgenic HD mouse model (Cabezas-Llobet et al., 2018). Therefore, the impaired VIPergic signaling found in these regions could be involved in neuropathology which leads to cognitive and neuropsychiatric symptoms in HD (Fahrenkrug et al., 2007). Unfortunately, there are no other references regarding the relationship between VIP and non-motor disturbances in HD, and no information concerning the VIP therapeutic potential on the symptomatology.

Amyotrophic Lateral Sclerosis and Multiple Sclerosis

Amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) are two diseases that affect both the brain and spinal cord nervous tissue. ALS is marked by progressive degeneration of upper and lower motor neurons while MS is characterized by demyelination due to an autoimmune response. Although ALS and MS have been traditionally viewed as diseases of the motor system, cognitive impairment also occurs in these pathologies (Chiaravalloti and DeLuca, 2008; Benbrika et al., 2019). It has been suggested that VIP and PACAP may contribute to ALS and MS non-motor symptomatology (Staines, 2008). In ALS patients, VIP levels were found decreased in cerebrospinal fluid (CSF) while PACAP and PAC1 receptor had an altered expression in the motor cortex (Werdelin et al., 1989; Bonaventura et al., 2018). Similarly, in MS patients VIP and PACAP CSF levels are significantly diminished (Andersen et al., 1984; Baranowska-Bik et al., 2013). In animals, it has been shown that a synthetic modified analog of VIP exerts an anti-inflammatory effect improving motor function and increasing life-span in a ALS rat model (Goursaud et al., 2015). In an MS-related mouse model, PACAP ameliorated both clinical and pathologic manifestations of experimental autoimmune encephalomyelitis (EAE), while treatment with VIP reduced incidence and severity of EAE by an anti-inflammatory action (Kato et al., 2004; Fernandez-Martin et al., 2006). Moreover, PACAP deficient mice exhibited exacerbated EAE, a phenotype associated with increased inflammatory response, suggesting a protective role of the endogenous source of this neuropeptide in the disease (Tan et al., 2009, 2013). Unfortunately, no studies are exploring the capacity of PACAP and VIP to fight the cognitive decline in ALS and MS.

MECHANISMS OF ACTION: FROM RECEPTOR (PAC1, VPAC1 AND VPAC2) ACTIVATION TO MOLECULAR MECHANISMS

PACAP/VIP Receptors and Its Distribution in the Central Nervous System

The beneficial effects of PACAP and VIP on cognitive disturbances that occur in neurodegenerative diseases are due to the expression of their receptors in affected brain areas. PACAP has a high and specific affinity for the PAC1 receptor (PAC1R; Harmar et al., 2012). Additionally, PACAP and VIP share two receptors: VPAC1 receptor (VPAC1R) and VPAC2 receptor (VPAC2R), thanks to their high homology of structure (∼68% amino acid identity).

PAC1R is much more expressed in the whole Central Nervous System (CNS) than VPAC1R and VPAC2R transcripts (Basille et al., 2000; Jolivel et al., 2009). The dentate gyrus of the hippocampus, the supraoptic nucleus of the hypothalamus, cerebral cortex, and olfactory bulb are regions where PAC1R is most highly expressed (Hashimoto et al., 1996; Nomura et al., 1996; Zhou et al., 2000). PAC1R mRNA high expression levels are also present in cingulate, entorhinal and piriform cortices; pyramidal and nonpyramidal cells of the hippocampal formation; amygdaloid nuclei; centromedial, mediodorsal, and ventromedial nuclei of the thalamus; hypothalamus; nucleus accumbens; superior colliculus, central gray and SNc; pontine raphe nuclei and cerebellum (**Figure 1**; Hashimoto et al., 1996; Shioda et al., 1996; Zhou et al., 2000). Concerning VPAC1R and VPAC2R, studies indicate a different but complementary distribution of their mRNA localization throughout the brain. VPAC1R is expressed in the hippocampus and the cerebral cortex, while VPAC2R highest expression is found in the thalamus, the hypothalamus, the hippocampus, the amygdala and the pontine nuclei (**Figure 1**; Vaudry et al., 2009). Both VPAC receptors have low expression in the olfactory bulb (**Figure 1**). In the cerebral cortex, VPAC1R is abundant in layers

III and V, while on the other hand, VPAC2R is rather localized in layer VI (Usdin et al., 1994). Importantly, despite the distinctive mRNA distribution, protein expression of PAC1R, VPAC1R, and/or VPAC2R have been found in all cognition-related regions (Joo et al., 2004).

As all three receptors belong to the superfamily of G protein-coupled receptors, the beneficial effects of PACAP and VIP are associated to the activation of their related signaling second messengers: cAMP/adenylyl cyclase (AC)/protein kinase A (PKA), phospholipase (PLC)/protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K)/Akt transduction pathways (Dickson and Finlayson, 2009; Vaudry et al., 2009). Expression and activity dysregulation of these proteins and related signaling mechanisms are involved in cognitive disturbances that occur in neurodegenerative diseases. Thus, the activation of these pathways and their related transcriptional machinery in brain areas related to learning and memory function has been proposed to favor the cognitive functions by promoting neuronal viability or synaptic plasticity (Giralt et al., 2012; Rai et al., 2019; Ureshino et al., 2019). Importantly, the actions of PACAP and VIP among these proteins can be direct or indirect, as receptors are not only present in neurons but are also expressed in glial cells (Ashur-Fabian et al., 1997; Grimaldi and Cavallaro, 1999).

The anti-Apoptotic and Neurotrophic Functions of PAC1R

PAC1 Receptor in Anti-apoptotic Signaling

The antiapoptotic effects of PACAP in neurons are mainly mediated through PAC1R that activates pathways and genes downregulated in cognitive brain areas of neurodegenerative diseases. In immature cerebellar granule neurons, PACAP-PAC1R stimulation protects against many pro-apoptotic insults such as hydrogen peroxide, ethanol, and C2-ceramide (Vaudry et al., 2002b,c; Falluel-Morel et al., 2004; Aubert et al., 2006). This effect is associated with the AC/PKA pathway and phosphorylation of the extracellular signal-regulated kinase (ERK). Hence, PACAP action is blocked by PKA or MAPK inhibitors and mimicked by raising cAMP levels (Vaudry et al., 1998, 2000b; Falluel-Morel et al., 2004). Also, PAC1R activation promotes the transcription of the immediate-early gene c-fos, which induces B-cell lymphoma 2 (Bcl-2) expression. In the mitochondria, Bcl-2 blocks the cytochrome c release into the cytosol, preventing caspase-3 activation (Falluel-Morel et al., 2004; Aubert et al., 2006). Inhibition of mitochondrial apoptotic pathway by PACAP-PAC1R stimulation has been demonstrated *in vivo* using an AD mouse model overexpressing the APP. In these animals, PACAP intranasal administration increases both PACAP and PAC1R mRNA expression as well as the Bcl-2 protein levels (Rat et al., 2011). In addition to these findings, *in vitro* studies have demonstrated PACAP protective effect against Aβ-mediated toxicity *via* the inhibition of caspase-3 activity in PC12 cells (Onoue et al., 2002) or *via* the enhancement of the mitochondrial function in primary neuronal cell culture (Han et al., 2014b). Interestingly, in PC12 cells, it has been shown that PACAP-PAC1R activation also protects against rotenone-induced apoptosis, a PD cellular model, through a mitochondrial-independent pathway (Wang et al., 2005). Importantly, the molecular mechanisms involve the activation of MAPK by PKA leading to the inhibition of caspase-3 activity (Wang et al., 2005). PACAP also activates various genes through the PKA pathway (Vaudry et al., 2002a), some of which such as peroxiredoxin 2 (Botia et al., 2008), tissue plasminogen activator (tPA; Raoult et al., 2011), stathmin (Dejda et al., 2010) and Serpin b1a (Seaborn et al., 2014), being involved in its neuroprotective effect.

Besides AC/PKA and MAPK, some other pathways are suggested to be involved in the PAC1R-mediated anti-apoptotic action. For instance, PAC1R stimulation has demonstrated to prevent cerebellar granule neurons from caspase-3 activation *via* PKC transduction pathway (Vaudry et al., 2000a). In olfactory neurons, PAC1R also inhibits apoptosis *via* activation of the PLC (Han and Lucero, 2006). Moreover, PACAP protects against KCl-induced apoptosis of cerebellar granule neurons by the activation of the PI3K/Akt pathway (Bhave and Hoffman, 2003). In conclusion, PAC1R acts *via* different but sometimes complementary signaling pathways to block neuronal apoptosis mainly through the inhibition of caspase-3 activity (Seaborn et al., 2011; **Figure 2**). However, more data are needed to associate the activation of these pathways with the PACAP-mediated cognitive enhancement, especially since besides its direct neuroprotective effect, PACAP may act indirectly on neuronal survival *via* activation of PAC1R expressed by astrocytes. Indeed, PACAP stimulates endozepine release from cultured astrocytes *via* a PAC1R/PKA pathway (Masmoudi et al., 2003) and intracerebroventricular injection of the endozepine octadecaneuropeptide (ODN) prevents the degeneration of dopaminergic neurons in an *in vivo* model of PD (Bahdoudi et al., 2018). Interestingly ODN protects neurons from apoptosis through inhibition of the oxidative stress (Kaddour et al., 2013), which is often responsible for cell death in neurodegenerative diseases (Niedzielska et al., 2016).

PAC1 Receptor in Neurotrophic Signaling

PACAP/PAC1R stimulation has been found to enhance the expression of neurotrophins and related receptors in neuronal cells, promoting survival, synaptic plasticity, proliferation, and/or differentiation. Several *in vitro* and *in vivo* studies have shown that PAC1R stimulation can increase BDNF transcription and expression. BDNF is a key regulator of the maintenance of neuronal populations in the CNS and plays an important role in synaptic plasticity and synaptogenesis (Bramham and Messaoudi, 2005; Cohen-Cory et al., 2010; Bathina and Das, 2015). It has been demonstrated that BDNF protein level is positively correlated with the number of dendritic spines in the dentate granular neurons of mice (Stranahan, 2011). Importantly, BDNF expression is downregulated in many neurodegenerative diseases (Bathina and Das, 2015). It has also been shown a reduced expression of BDNF in PAC1R deficient-mice (Zink et al., 2004). Moreover,

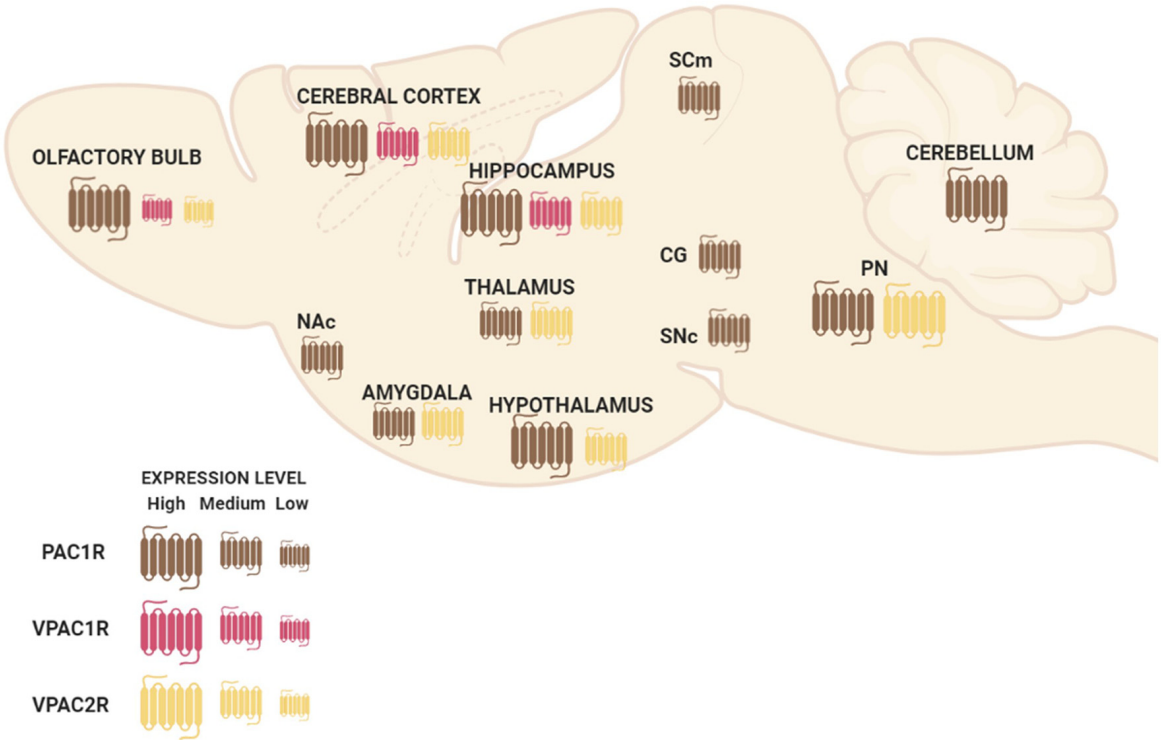

FIGURE 1 | Schematic representation of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) receptors' mRNA distribution in a sagittal view of the mouse brain. Abbreviations: Nac, nucleus accumbens; SCm, superior colliculus; CG, central gray; SNc, substantia nigra pars compacta; PN, pontine raphe nuclei.

in AD and HD murine models and PD cellular models, beneficial effects of PACAP have been related to the increase of BDNF gene expression and protein levels (Rat et al., 2011; Brown et al., 2013, 2014; Cabezas-Llobet et al., 2018). Interestingly, some *in vitro* experiments have allowed the characterization of the molecular mechanisms involved. In rat cortical and hippocampal cultured neurons PACAP-PAC1R stimulation induced BDNF transcription through the activation of AC/PKA signaling pathway (Yaka et al., 2003; Dong et al., 2010). AC/PKA together with MAPK is known to act through phosphorylation of cAMP response element-binding protein (CREB), which enhances BDNF signal transduction (Impey et al., 1998; Tao et al., 1998). Accordingly, PAC1R activation has been shown to enhance CREB phosphorylation and BDNF expression in *in vitro* and *in vivo* neurodegenerative models (Rat et al., 2011; Brown et al., 2013; Cabezas-Llobet et al., 2018). Therefore, BDNF enhanced expression has been proposed to be a key mechanism underlying neuroprotection and cognitive improvement induced by PACAP-PAC1R stimulation.

It has also been described that the capacity of PACAP-PAC1R to induce BDNF can be exerted through the glutamatergic N-methyl-D-aspartate (NMDA) receptor. NMDA receptors play an essential role in synaptic plasticity and synaptogenesis (Nicoll and Malenka, 1999). Dysregulation of NMDA receptors activity is associated with cognitive impairment in AD (Avila et al., 2017), PD (Vanle et al., 2018), and HD (Giralt et al., 2012) among others. Because their function is regulated by different cell modifications, the effect of PAC1R signaling on NMDA receptors activity has been studied. In cortical and hippocampal neurons PAC1R activation potentiates NMDA receptors, which induces BDNF expression (Pellegri et al., 1998; Yaka et al., 2003; Dong et al., 2010). It is described that NMDA receptors activation can promote the synthesis and release of BDNF (Marini et al., 1998). Therefore, PACAP/PAC1R has been proposed to regulate NMDA receptor activity promoting a trophic activity through the action of BDNF. Importantly, PAC1R related signaling can also activate directly the BDNF Tyrosine kinase B (TrkB) receptor, as observed in primary cultures of hippocampal neurons (Lee et al., 2002). Altogether, these results strongly suggest that PACAP-PAC1R enhances in a direct and/or indirect manner BDNF expression and trophic signaling responsible for neuroprotection, synaptic plasticity, and cognitive improvement (**Figure 3**).

PAC1R activation has also been shown to promote proliferation in neurogenic adult brain regions. This fact is interesting because, in the early stages of many neurodegenerative diseases, such as AD, HD, and PD, adult neurogenesis decreases, which is associated with non-motor symptoms such as memory impairment and cognitive decline (Winner et al., 2011). In adult mice, PAC1R stimulation was found to enhance the proliferation in lateral ventricle and dentate

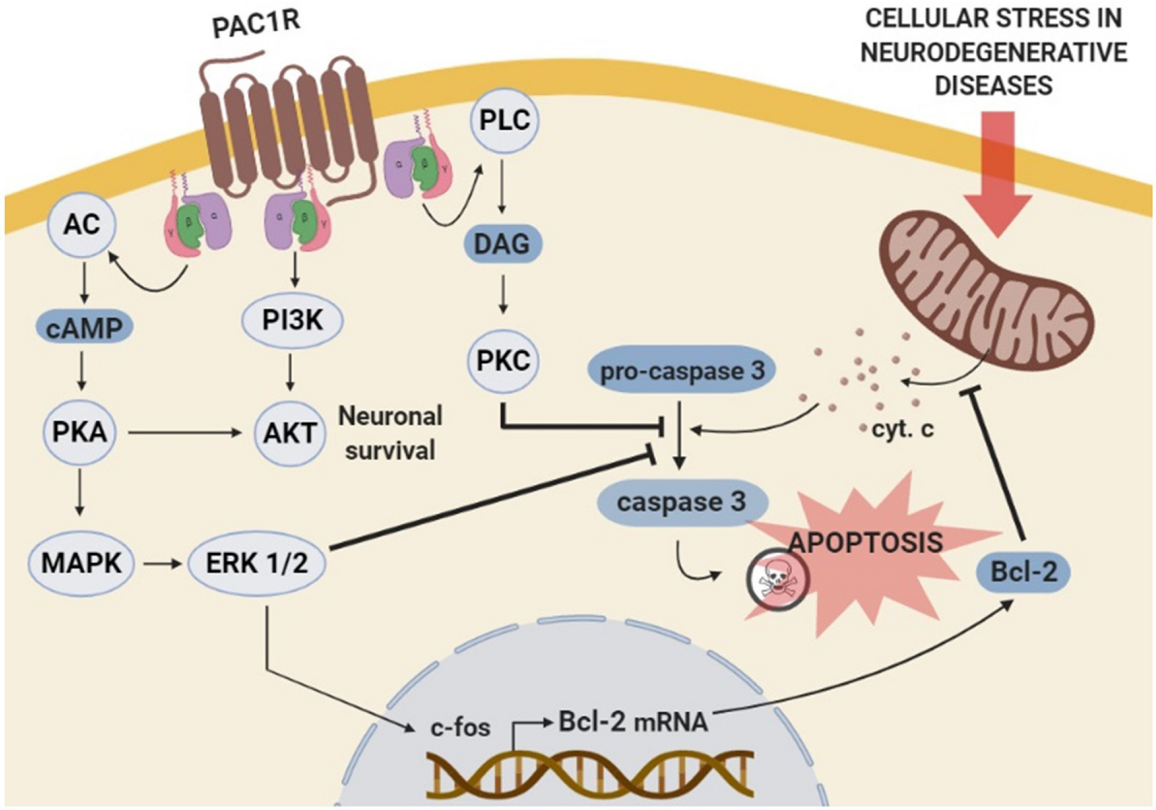

FIGURE 2 | Schematic representation of the molecular mechanisms involved in PAC1R mediated anti-apoptotic effect. The stimulation of PAC1R activates protein kinase A (PKA) cascade signaling, leading to extracellular signal-regulated kinase (ERK) phosphorylation. Activated ERK induces the transcription of neuroprotective genes such as c-fos and Bcl-2, which finally inhibits the mitochondrial apoptotic pathway and blocks pro-caspase-3 activation. The inhibitory effect of the PACAP-PAC1R system on pro-caspase-3 cleavage can be induced via additional phosoholipase C (PLC)/protein kinase C (PKC) pathway. Moreover, PAC1R can also activate phosphatidylinositol 3 kinase (PI3K)/Akt system and promote neuronal survival to counteract the apoptotic process.

gyrus of the hippocampus, two neurogenic regions sensitive to neurodegeneration. The authors defined PKA as the principal signaling pathway involved (Mercer et al., 2004). In contrast, in immature cerebellar granule cells, PAC1R was suggested to inhibit proliferation *via* the AC/PKA pathway (Obara et al., 2007) responsible for the increase of Lot-1 expression (Contestabile et al., 2005; Fila et al., 2009). In cerebellar granule cells, PC12, and neuroblastoma cells, PAC1R activation promotes differentiation with neurite initiation and elongation (Deutsch et al., 1993; Hernandez et al., 1995; Gonzalez et al., 1997). Results of these studies point out an AC dependent but PKA independent signaling pathway in this action (Deutsch et al., 1993; Hernandez et al., 1995; Ravni et al., 2006, 2008). Moreover, a specific analysis of the transduction pathways involved in cell differentiation induced by PACAP concluded that PKC is not implicated (Vaudry et al., 1998). Downstream AC, PACAP would activate ras-proximate-1 (Rap1) and MAPK to induce early growth response protein 1 (Egr1) expression and in turn neuroblast differentiation (Ravni et al., 2008). The ability of PACAP to promote dendritic spine remodeling in cultured hippocampal neurons is mediated among others by the disintegrin and metalloproteinase 10 (ADAM10; Gardoni et al., 2012) and the microRNA-132 upregulation (Hayata-Takano et al., 2019). Taken together, these results indicate that, PAC1R activation coupled to AC and their downstream transduction pathways (Ravni et al., 2006) are determinant for the control of neuroblast proliferation and differentiation in brain regions affected by neurodegenerative diseases.

Neuroprotective Actions Related to VPAC1R and VPAC2R

VPAC Receptors in Neurogenesis and Synaptic Function

VPAC receptors are considered dynamic regulators of postnatal and adult hippocampal neurogenesis by regulating survival, proliferation, and differentiation of precursor cells in the CNS. It has been shown that VPAC2R maintains stem cells population by supporting their survival and preventing neuronal or glial differentiation. In contrast, VPAC1R activation has been found to promote neurogenic granule cell fate (Zaben et al., 2009). Interestingly, both VPAC receptors were demonstrated to be determinant for the synaptic function in the hippocampus (Cunha-Reis et al., 2005). The activity of VPAC receptors

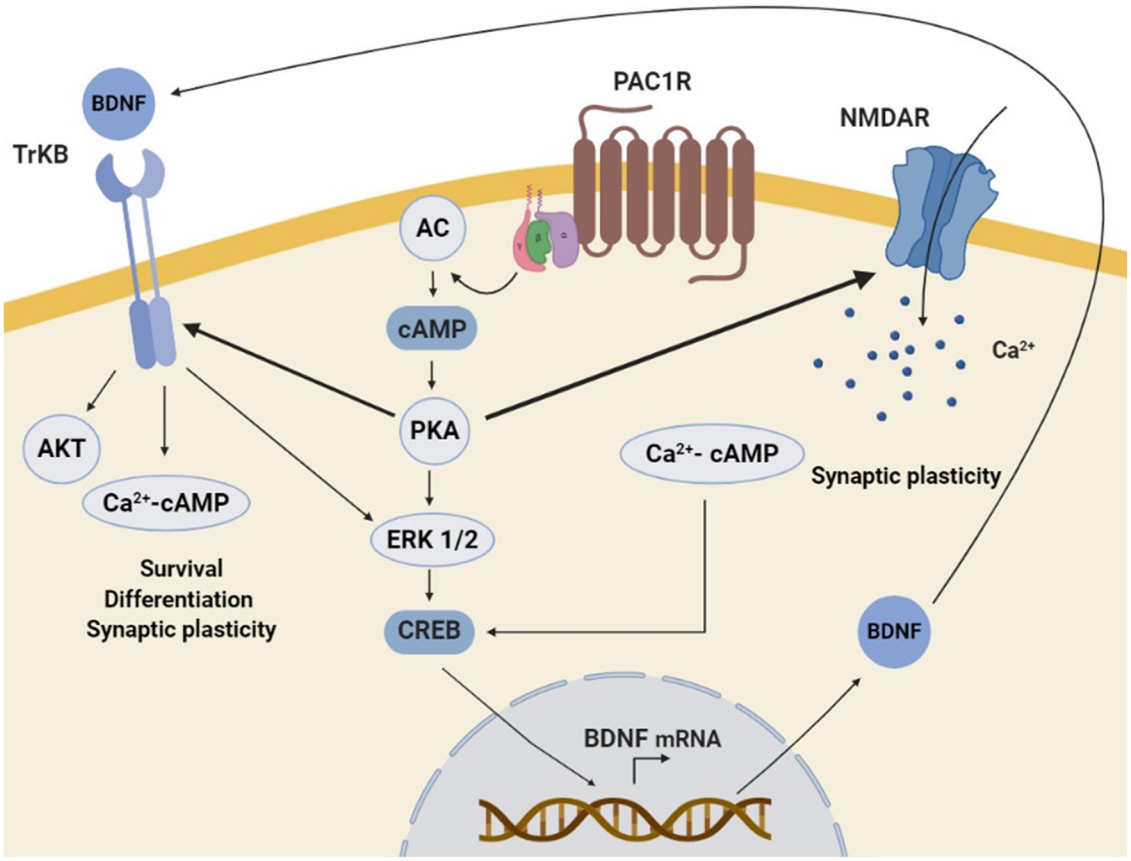

FIGURE 3 | Schematic representation of the molecular mechanisms involved in PAC1R mediated neurotrophic effect. The stimulation of PAC1R by PACAP activates the protein kinase A (PKA) signaling pathway, which can potentiate N-methyl-D-aspartate (NMDA) receptors and/or induce downstream the phosphorylation of extracellular signal-regulated kinase (ERK). In both cases, CAMP response element-binding protein (CREB) is activated and enhances brain-derived neurotrophic factor (BDNF) transcription. Increased BDNF protein levels activate the TrkB receptor, which can also be potentiated by PKA. Finally, TrkB associated trophic signaling pathways promote neuronal survival, differentiation, and synaptic plasticity.

has been associated with the PKA signaling pathway (Vaudry et al., 2000b) which leads to ERK activation, which finally promotes cell proliferation and/or differentiation (Langer, 2012). Moreover, the VPAC1R-mediated synaptic plasticity in the hippocampus is specifically mediated through the PKC pathway (Cunha-Reis et al., 2005).

VPAC Receptors in Glia Mediated Effects

Although some authors have found VIP direct effect in neurons, there is increasing evidence that most neuroprotective actions promoted by VPAC receptors in neurodegenerative diseases involve glial cells.

In astrocytes, it has been demonstrated that VPAC receptors activation induces the secretion of neurotrophic and neuroprotective factors (**Figure 4**). For instance, activity-dependent neuroprotective protein (ADNP) and activity-dependent neurotrophic factor (ADNF) are released after VPAC receptors stimulation from astrocytes (Brenneman and Gozes, 1996; Bassan et al., 1999). ADNP and ADNF regulate brain development and promote neuronal survival and plasticity (Brenneman and Gozes, 1996; Gozes and Spivak-Pohis, 2006). Importantly, these functions are mainly related to their most active fragments, NAP (NAPVSIPQ) and ADNF-9 (SALLRSIPA), respectively, which are demonstrated to be neuroprotective against multiple toxins *in vitro* (Gozes et al., 2003; Lagrèze et al., 2005; Smith-Swintosky et al., 2005). For instance, both ADNF9 and NAP protect against dopamine and 6-OHDA toxicity in PC12 and neuroblastoma cell models of PD (Offen et al., 2000). Interestingly, VIP-stimulated astrocytes can also secret neurotrophin-3 (Brenneman et al., 1997, 2003; Dejda et al., 2005). Specific involvement of VPAC1R and VPAC2R in astrocyte-mediated neuroprotection has been poorly studied, but some results suggest that VPAC2R plays the main key role. It has been found that VIP/VPAC2R system is neuroprotective by promoting the release of ADNP and BDNF in cultured astrocytes (Zusev and Gozes, 2004; Passemard et al., 2011). Moreover, the increased expression of glutamate transporters associated with reactive astrocytes could prevent cultured neurons against excitotoxicity (Nishimoto et al., 2011). Importantly, astrocytic-dependent neuroprotection has been

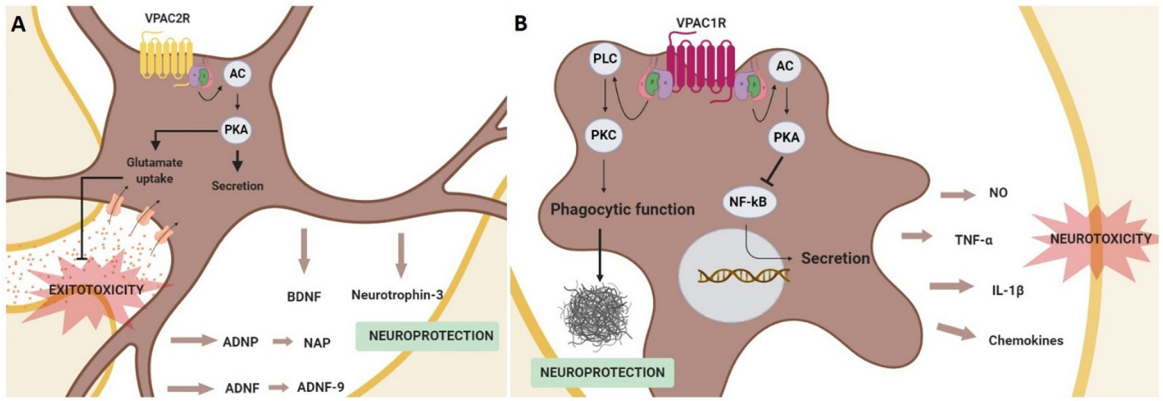

FIGURE 4 | Schematic representation of neuroprotective effects mediated by the activation of the VPAC receptors in glial cells. In astrocytes **(A)** VPAC2R activation promotes the secretion of neurotrophic and neuroprotective factors, in addition to prevent neurons from excitotoxicity by enhancing the glutamate re-uptake in astrocytes. These actions are associated with adenylyl cyclase (AC)/protein kinase A (PKA) activation. In microglia **(B)** the stimulation of VPAC1R inhibits the secretion of pro-inflammatory agents through the activation of the PKA signaling pathway and protects against β-amyloid fibrils enhancing the microglia phagocytic function *via* protein kinase C (PKC) activation.

related to the cAMP/PKA pathway (Nishimoto et al., 2011; Passemard et al., 2011; **Figure 4**).

It has also been described that VIP can regulate the release of proinflammatory cytokines from microglia. This action of VIP on microglial cells has been studied in neurodegenerative diseases, as excessive microglial activation contributes to the physiopathology of AD, PD, and HD, among others (Subhramanyam et al., 2019). In AD and PD models, it has been shown that VIP exerts a neuroprotective effect through the inhibition of tumor necrosis factor-α (TNFα), interleukin 1β (IL-1β), nitric oxide (NO) and different chemokines release (Delgado, 2002; Delgado and Ganea, 2003; Delgado et al., 2008; Song et al., 2012). Importantly, this VIP effect has been related specifically to VPAC1R activation coupled to the cAMP/PKA pathway and associated to the inhibition of NF-κB activation, a transcription factor of several genes encoding neurotoxic mediators (Delgado, 2002; Delgado and Ganea, 2003; Delgado et al., 2008). Interestingly, in a AD *in vivo* model, VPAC1R stimulation also enhances microglial phagocytosis of fibrillar beta-amyloid, but in this case through the activation of the PKC pathway (Song et al., 2012; **Figure 4**).

Even if the above information suggests a key role of VPAC receptors coupled to the cAMP/PKA pathway in the regulation of adult neurogenesis most of the actual investigations are restricted to their function during development of the SNC. Consequently, more studies are needed to clarify and understand the contribution of VPAC receptors in the context of cognitive dysfunction in neurodegenerative diseases. Currently, it is considered that in the context of neurodegenerative diseases, the VIP-VPACR system exerts its neuroprotective action mainly indirectly through astroglial and microglial cells. In astrocytes, VPAC2R has a key role in promoting the secretion of different neuroprotective and neurotrophic factors, besides preventing from glutamate excitotoxicity. On the other hand, VPAC1R in microglia acts as an inflammatory mediator and protects against β-amyloid fibrils.

DESIGNING PACAP AND VIP SYNTHETIC DERIVATES TO IMPROVE THE THERAPEUTIC ACTIVITY IN NEURODEGENERATIVE DISEASES

The therapeutic application of PACAP and VIP neuropeptides for the treatment of neurodegenerative diseases shows some limitations. Importantly, both neuropeptides are susceptible to be rapidly degraded. In humans, PACAP38 isoform has a plasma half-life of less than 5–10 min (Ramos-Álvarez et al., 2015) while VIP shows a plasmatic half-life of approximately 1 min (Domschke et al., 1978). Additionally, the widespread distribution of PACAP/VIP receptors in the CNS and the peripheral tissues can cause many side effects. Therefore, recent developed synthetic analogs showing increased plasmatic half-life and displaying receptor selectivity have been proposed as therapeutic agents.

PACAP is metabolized mainly by dipeptidyl peptidase IV (DPP IV; Zhu et al., 2003), which promotes the formation of dipeptides from the N-terminus (Mentlein, 1999). As the PACAP amino-terminal domain is essential for the activation of its receptors (Gourlet et al., 1996), the cleavage by DPP IV suppresses PACAP biological activity (Gourlet et al., 1996). Moreover, PACAP can also be cleaved by endopeptidases, that recognize two dibasic pairs (Arg14-Lys15 and Lys20-Lys21), and carboxypeptidases, which action depends on the C-terminal segment (Bourgault et al., 2009). Therefore, chemical strategies aimed to modify the putative DPP IV, endopeptidase, and carboxypeptidase sites of cleavage offer the possibility to obtain more stable PACAP-analogues. For instance, the addition of an acetyl or hexanoyl group at the N-terminus and the inversion

of chirality of the Ser residue in position 2 are known to offer stability to PACAP by completely suppressing the action of DPP IV (Bourgault et al., 2008). Additionally, different amino acids have been determined as crucial for the specific binding of PACAP to its receptors, including His1, Asp3, and Phe6 (Lamine et al., 2016). As PACAP neuroprotective effects are mainly related to PAC1R, it has been proposed synthetic PAC1R agonists as interesting therapeutic molecules (Ramos-Álvarez et al., 2015). The Acetyl-[Ala15, Ala20] PACAP38 propylamine was designed to be a potent PAC1R agonist, in addition, to be more metabolically stable than native PACAP (Bourgault et al., 2008). Recently, the effects of this analog on memory and post-learning BDNF expression have been studied in Wistar rats. Surprisingly, the administration of acetyl-(Ala15, Ala20) PACAP38-propyl amide did not improve the cognitive function of rats, and accordingly, did not induce BDNF expression (Ladjimi et al., 2019). However, the authors showed that this analog could increase the activity of antioxidative enzymes in the neocortex of rats (Ladjimi et al., 2019). Unfortunately, the effect of acetyl-(Ala15, Ala20) PACAP38-propyl amide has not been tested in neurodegenerative models. The Ac-[Phe(pI)6, Nle17] PACAP (1–27) is another analog designed to specifically activate PAC1R/VPAC1R without affinity to VPAC2R. This is important because the activation of VPAC2R is associated with peripheral side effects such as vasodilation, an increase of heart rate, and water retention (Warren et al., 1992; Tsutsumi et al., 2002; Farnham et al., 2012). Interestingly, Ac-[Phe(pI)6, Nle17] PACAP (1–27) presents resistance against dipeptidyl peptidase IV activity, increasing its stability in human plasma (Lamine et al., 2016). This analog protects from MPP^+-induced toxicity and appeared to be as efficient as PACAP (Lamine et al., 2016). Moreover, it was found to exert reduced cardiovascular side effects after treatment in an *in vivo* model of PD. However, the capacity of Ac-[Phe(pI)6, Nle17] PACAP (1–27) analog on cognitive deficits in neurodegenerative models has not been evaluated yet. Shorter fragments such as the amidated PACAP23 have shown their neuroprotective action *in vitro* (Lamine et al., 2019) but it remains to investigate if they keep their activity *in vivo*, in particular after intranasal injection. Indeed, the C terminal part of PACAP seems to be important for its transportation across the blood-brain barrier (Banks et al., 2006). Thereby, novel PACAP-TAT peptide with enhanced ability to cross biological barriers (Yu et al., 2012) and gH625-liposomes (Iachetta et al., 2019) may help to deliver PACAP derivatives to the brain.

Regarding VIP, its susceptibility to endopeptidases is also well known (Deng and Jin, 2017). To overcome this metabolic limitation, the addition of an N-terminal stearic acid (lipophilic) to different VIP short fragments was tested. The sequence stearyl–Lys–Lys–Tyr–Leu–NH2 (stearyl–KKYL–NH2) was found to have higher stability than VIP, providing the main neurotrophic and neuroprotective actions of the peptide (Gozes et al., 1999). Moreover, replacing the methionine in position 17 with a norleucine (Nle), resulted in a superactive VIP analog. Named stearyl-Nle17-VIP (SNV), it is 100-fold more potent than VIP in promoting neuronal survival, acting at femtomolar-picomolar concentrations in rat cerebral cortical cultures (Gozes et al., 1995; Ashur-Fabian et al., 1999). Interestingly, the beneficial effects of SNV in cognitive function have been tested *in vivo*. Using two rat models of developmental retardation, water maze experiments showed that SNV improves cognitive functions (Gozes et al., 1998). Similarly, chronic treatment with SNV was demonstrated to improve learning and memory functions in ethylcholine aziridium treated animals (Gozes et al., 1996) and in an apolipoprotein E (ApoE) deficient mice (Gozes et al., 1997), used as models of AD. For its part, the VPAC2R agonist D-p-Cl-Ac-Phe(pI)6, Leu17-VIP slows the pathogenesis of PD through modulation of the inflammatory response (Olson et al., 2015).

Altogether, several analogues more stable and selective than PACAP and VIP have been designed to increase the therapeutic activity and applicability. However, their role in cognitive dysfunction in neurodegenerative diseases is poorly known. Therefore, further research and some clinical trials are needed to ensure the therapeutic potential of these promising analogs.

AUTHOR CONTRIBUTIONS

IS-T, NC-L, and XX wrote the manuscript. IS-T, DV, and XX revised the literature. Supervision and conceptualization was performed by DV and XX. Editing was performed by XX.

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Physical Activity Protects the Pathological Alterations of Alzheimer's Disease Kidneys via the Activation of PACAP and BMP Signaling Pathways

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Alzheimer's disease (AD) is a neurodegenerative disorder with typical amyloid beta (Aβ) aggregations. Elimination of the Aβ precursors *via* the kidneys makes the organ a potential factor in the systemic degeneration leading to AD. Pituitary adenylate cyclase-activating polypeptide (PACAP) exerts neuroprotective effects in AD and plays a protective role in kidney pathologies. Increased physical activity is preventive of the formation of AD, but its detailed mechanism and possible connections with PACAP have not been clarified. In the kidneys of AD mice, the effects of physical activity were investigated by comparing wild-type and AD organs. Aβ plaque formation was reduced in AD kidneys after increased training (TAD). Mechanotransduction elevated PACAP receptor expression in TAD mice and normalized the protein kinase A (PKA)-mediated pathways. BMP4/BMPR1 elevation activated Smad1 expression and normalized collagen type IV in TAD animals. In conclusion, our data suggest that elevated physical activity can prevent the AD-induced pathological changes in the kidneys *via*, at least in part, the activation of PACAP–BMP signaling crosstalk.

Keywords: PACAP, Alzheimer's disease, BMP signaling, physical activity, collagen type IV

INTRODUCTION

Alzheimer's disease (AD), a degenerative process in the central nervous system (CNS), is the most frequent cause of dementia in elderly patients, resulting in neuronal loss and leading to progressive cognitive deficit (Burns and Iliffe, 2009; Dong Y. et al., 2019; Nazarian et al., 2019; Cervellati et al., 2020; Pomilio et al., 2020). Because of its increased prevalence, AD is one of the major priorities

Abbreviations: Aβ, amyloid beta; AD mouse, Alzheimer's disease mouse; APP, amyloid precursor protein; BMP, bone morphogenetic protein; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; CREB, cAMP response element-binding protein; HE staining, hematoxylin and eosin staining; KO mouse, knockout mouse; mRNA, messenger ribonucleic acid; PAC1, pituitary adenylate cyclase-activating polypeptide type I receptor; PACAP, pituitary adenylate cyclase activating polypeptide; PKA, protein kinase A; P-PKA, phosphorylated protein kinase A; Smad, SMA ("small" worm phenotype) and *Drosophila* MAD ("Mothers Against Decapentaplegic") TAD mouse, trained Alzheimer's disease mouse; VPAC, vasoactive intestinal polypeptide receptor; WT mouse, wild-type mouse.

of healthcare systems, and various attempts have been made in the last decades to find reliable prevention methods or novel therapeutic approaches (Belghali et al., 2017; Panda and Jhanji, 2019; Whitehouse, 2019). One of the first signs of AD formation and manifestation is the pathological aggregation of amyloid beta (Aβ) deposits and the abnormal accumulation of tau proteins followed by inflammation (Tiraboschi et al., 2004). Amyloidogenesis is a well-controlled process in healthy tissues, but pathologic amyloid plaques can accumulate in various tissues in Alzheimer's disease (Whelly et al., 2012; Reglodi et al., 2018c). Accumulation of amyloid plaques has also been identified in peripheral organs, resulting in a systemic disorder (Morris et al., 2014; Reglodi et al., 2018c; Pomilio et al., 2020). Among others, Aβ aggregation pathologically alters pancreatic function (Kawarabayashi et al., 1996; Arbor et al., 2016; Tavares et al., 2017) and can negatively affect renal function (Kheirbakhsh et al., 2018).

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a 38-amino acid C-terminally α-amidated peptide that was first isolated in 1989 from an ovine hypothalamic extract by Miyata and colleagues (Miyata et al., 1989). PACAP belongs to the vasoactive intestinal polypeptide (VIP)–secretin–growth hormone-releasing hormone (GHRH)–glucagon superfamily. PACAP has three major G protein-coupled seven-transmembrane receptors: PAC1, VPAC1, and VPAC2. PAC1 is responsible for specific PACAP binding, while the VPAC1 and VPAC2 receptors have equal affinity to bind PACAP and VIP (Laburthe et al., 2002). PACAP and its receptors have been detected in the CNS and many peripheral tissues such as the respiratory tract, urinary tract, and digestive system (Vaudry et al., 2009), or in bone and chondrogenic cultures (Juhasz et al., 2015a; Jozsa et al., 2018). The protective/preventive role of PACAP has been proven in several age-related disorders, and lack of PACAP leads to accelerated aging (Reglodi et al., 2018a; Szegeczki et al., 2019). PACAP has been shown to attenuate the severity of AD in different animal models and protects from Aβ toxicity (Han et al., 2014a; Maasz et al., 2017). We have previously described that PACAP-deficient mice are prone to develop a presenile systemic amyloidosis, with peripheral organs severely affected (Reglodi et al., 2018c). The most dramatic amyloid deposition was found in the kidneys of aging PACAP knockout mice, where 100% of the animals developed a severe glomerular infiltration causing functional failure (Gronewold et al., 2017; Reglodi et al., 2018c). In addition, PACAP has a nephroprotective role in various kidney pathologies, such as toxic nephropathies and ischemia/reperfusion-induced kidney injury of rats (Horvath et al., 2019; Laszlo et al., 2019). Binding to its receptors, PACAP initiates the activation of various signaling pathways *via* the activation of intracellular messengers such as adenylate cyclase, which triggers protein kinase A (PKA), and subsequent activation of the phosphorylate transcription factors such as CREB and mediates different gene expressions (Kienlen Campard et al., 1997; Juhasz et al., 2014b; Dong Q. et al., 2019). PACAP has a modulatory role in several signaling cascades such as Notch and sonic hedgehog signaling (Sandor et al., 2014; Fulop et al., 2019), Sox transcription factor activation (Szegeczki et al., 2019), and has an important balancing function in bone morphogenetic protein (BMP) signaling (Jozsa et al., 2019; Laszlo et al., 2019).

BMP is a member of the transforming growth factor beta (TGF-β) protein family predominantly detected in bone and has several functions, from the embryonic development to the homeostasis of adult tissues (Bandyopadhyay et al., 2013). BMPs can bind to serine/threonine protein kinase receptor (BMPR1), the downstream targets of which bind the corresponding DNA sequence through the Smad cascade and regulate the expression of certain genes, such as those coding for collagens (Von Bubnoff and Cho, 2001). BMP4 is one of the major cytokines which regulate proper kidney development (Nishinakamura and Sakaguchi, 2014; Mills et al., 2017). BMP receptor activation *via* BMP4 influences the expression of basement membrane components, such as collagen type IV (Matsubara et al., 2015; Laszlo et al., 2019), which also plays an important role in proper filtration. Alterations in BMP4 expression have also been demonstrated in AD (Li D. et al., 2008). BMP and PACAP signaling crosstalk has already been shown in various processes. PACAP has been demonstrated to play a role as an antagonist of BMP4 in *Xenopus* early development (Otto et al., 2000), and BMPs can modulate PACAP function in peptidergic system formation (Pavelock et al., 2007). The expression of BMPs is altered in bone development processes (Jozsa et al., 2018) and shows a shifted expression pattern in the callus formation of PACAP knockout (KO) mice (Jozsa et al., 2019). The addition of PACAP increased BMP4 expression in the UMR-106 osteogenic cell line (Juhasz et al., 2014b). Moreover, we have demonstrated that PACAP addition elevated BMP4 expression in ischemia-induced kidney injury (Laszlo et al., 2019).

For detailed investigation of AD formation, various *in vivo* and *in vitro* experiments have been performed (Wu et al., 2019) in order to follow the molecular biological alterations. There are a few genetically modified mice with tau and Aβ overexpression showing macroscopical and molecular biological disorders of AD (Radak et al., 2010; Abraham et al., 2019). In these models, it has been proven that physical activity was able to postpone the manifestation of AD, but its detailed mechanism has not yet been investigated (Radak et al., 2010). As PACAP has an important protective role in the kidneys and has a direct protective role in AD, it could be one of the target molecules to positively influence Aβ elimination *via* the kidneys (Leckstrom et al., 1997).

In our experiments, we present evidence that physical activity has direct effects on AD in the kidneys of Alzheimer's disease mouse models. Furthermore, we show that PACAP and BMP signaling are also involved in the prevention of illness formation *via* physical activation.

MATERIALS AND METHODS

Animals

Male Alzheimer transgenic [B6C3-Tg(APPswe,PSEN1dE9)85Dbo/J] (n = 5) mice were used to follow the effects of AD. Three-month-old wild type (WT; no transgenic modulation and no training, n = 5), Alzheimer transgenic mice (AD, n = 5), and trained Alzheimer's disease

mice (TAD, $n = 5$) were kept under light/dark cycles of 12:12 h with food and water *ad libitum*. Alzheimer transgenic mice were trained on a treadmill four times per week for 1 h divided into 10 sessions. One session contained 2 min of low-intensity running (10 m/min) and 4 min of high-intensity running (20 m/min). The study was carried out in accordance with ethical guidelines (ethical permission number: PEI/001/2105-6/2014, Semmelweis University, Hungary). Genotyping was performed using a Phire Animal Tissue Direct PCR Kit (Thermo Fischer Scientific, Waltham, MA, United States) according to the manufacturer's instructions.

Light Microscopical Morphology

Kidneys were washed in phosphate-buffered saline (PBS) three times and fixed in a 4:1 mixture of absolute ethanol and 40% formaldehyde, then embedded in paraffin. Serial sections were made and hematoxylin–eosin (HE) staining (Sigma-Aldrich, MO, United States) for morphological analysis and Congo red staining (Abcam, Cambridge, United Kingdom) for Aβ accumulation were performed. The staining protocols were carried out according to the manufacturer's instructions. Photomicrographs were taken using a DP74 camera (Olympus Corporation, Tokyo, Japan) on an Olympus Bx53 microscope (Olympus Corporation, Tokyo, Japan). For measurement of the staining intensity, we used ImageJ 1.40g freeware. Pixel density analysis was performed from at least three photos of five independent experiments.

Immunohistochemistry

Immunohistochemistry was performed on WT, AD, and TAD mice kidney tissue samples to visualize the localization of CREB, Smad1, and collagen type IV (Col. IV). The kidneys were fixed in a 4:1 mixture of absolute ethanol and 40% formaldehyde and washed in 70% ethanol. After embedding, serial sections were made and deparaffinization was then followed by rinsing in PBS (pH 7.4). Non-specific binding sites were blocked with PBS supplemented with 1% bovine serum albumin (Amresco LLC, Solon, OH, United States), then the samples were incubated with polyclonal CREB (Millipore, MO, United States) at a dilution of 1:600, Smad1 (Cell Signaling, Danvers, MA, United States) at a dilution of 1:500, or Col. IV (Abcam, Cambridge, United Kingdom) antibody at a dilution of 1:600 at 4°C overnight. For visualization of the primary antibodies, anti-rabbit Alexa Fluor 555 secondary antibody (Life Technologies Corporation, Carlsbad, CA, United States) was used at a dilution of 1:1,000. The samples were mounted in a Vectashield mounting medium (Vector Laboratories, Peterborough, United Kingdom) containing 4',6-diamidino-2-phenylindole (DAPI) for nuclear DNA staining. For the negative controls, anti-rabbit Alexa Fluor 555 was used without the primary antibodies. For the detection of Col. IV, photomicrographs were taken using the DP74 camera (Olympus Corporation, Tokyo, Japan) on an Olympus Bx53 microscope (Olympus Corporation, Tokyo, Japan). Images were acquired using cellSense Entry 1.5 software (Olympus, Shinjuku, Tokyo, Japan) with constant camera settings to allow comparisons of the fluorescent signal intensities. For CREB and Smad1, an Olympus FV1000S confocal microscope (Olympus Co., Tokyo, Japan) was used applying × 60 oil immersion objective (NA: 1.3). For excitation, laser lines of 543 nm were used. The average pixel time was 4 μs. Z image series of 1 μm optical thickness were recorded in sequential scan mode. Images of Alexa555 and DAPI were overlaid using Adobe Photoshop version 10.0 software. Contrast of images was equally increased without changing constant settings.

RT-PCR Analysis

The kidneys of WT ($n = 5$), AD ($n = 5$), and TAD ($n = 5$) mice were mechanically ground and were dissolved in Trizol (Applied Biosystems, Foster City, CA, United States), after 30 min incubation on 4°C, and total RNA was isolated. RNA was harvested in RNase-free water and stored at -70°C. Reverse transcription was performed by using High-Capacity RT kit (Applied Biosystems, Foster City, CA, United States). For the sequences of primer pairs and details of the polymerase chain reactions (see **Table 1**). Amplifications were performed in a thermal cycler (Labnet MultiGene 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ, United States) as follows: 95°C, 2 min, followed by 35 cycles (denaturation, 94°C, 30 s; annealing for 45 s at optimized temperatures as given in **Table 1**; and extension, 72°C, 90 s) and then 72°C, 7 min. Actin was used as the internal control. PCR products were analyzed using a 1.2% agarose gel containing ethidium bromide. The optical densities of the PCR product signals were determined by using ImageJ 1.40g freeware.

Western Blot Analysis

The kidneys of WT ($n = 5$), AD ($n = 5$), and TAD ($n = 5$) mice were washed in physiological saline and stored at -70°C. The samples were mechanically disintegrated with a tissue grinder in liquid nitrogen. Then, they were collected in 100 μl of homogenization RIPA (radioimmunoprecipitation assay) buffer (150 mM sodium chloride, 1.0% NP40, 0.5% sodium deoxycholate, 50 mM Tris, pH 8.0) containing protease inhibitors (10 μg/ml aprotinin, 5 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 1 mM PMSF, 5 mM EDTA, 1 mM EGTA, 8 mM Na-fluoride, and 1 mM Na-orthovanadate). The suspensions were sonicated by pulsing burst for 30 s at 40 A (Cole-Parmer, IL, United States). The total cell lysates for Western blot analyses were prepared. Forty micrograms protein was separated in 7.5% SDS–polyacrylamide gels for the detection of PAC1, VPAC1, VPAC2, PKA, P-PKA, CREB, P-CREB, BMP2, BMP4, BMPR1, Smad1, Col. IV, and actin. The proteins were transferred electrophoretically to nitrocellulose membranes and exposed to the primary antibodies overnight at 4°C in the dilution, as given in **Table 2**. After washing for 30 min with PBS Tween (PBST), the membranes were incubated with the peroxidase-conjugated secondary antibody anti-rabbit IgG in a 1:1,500 (Bio-Rad Laboratories, CA, United States) or anti-mouse IgG in a 1:1,500 (Bio-Rad Laboratories, CA, United States) dilution. The signals were detected with enhanced chemiluminescence (Advansta Inc., Menlo Park, CA, United States) according to the instructions of the manufacturer. Actin was used as an internal control. The signals were developed with a gel documentary system (Fluorchem E, ProteinSimple, CA,

TABLE 1 | Nucleotide sequences, amplification sites, GenBank accession numbers, amplimer size, and PCR reaction conditions for each primer pair.

Gene	Primer	Nucleotide sequence (5′→3′)	Genbank ID	Annealing temperature (°C)	Amplimer size (bp)
PAC1 (ADCYAP1R1)	Sense	TATTACTACCTGTCGGTGAAG (912–932)	NM_007407.4	52	213
	Antisense	ATGACTGCTGTCCTGCTC (1107–1124)			
VPAC1 (VIPR1)	Sense	TTT GAG GAT TTC GGG TGC (974–991)	NM_011703.4	53	266
	Antisense	TGG GCC TTA AAG TTG TCG (1222–1239)			
VPAC2 (VIPR2)	Sense	CTC CTG GTA GCC ATC CTT (805–822)	NM_009511.2	53	149
	Antisense	ATG CTG TGG TCG TTT GTG (936–953)			
PKA (Prkaca)	Sense	GCAAAGGCTACAACAAGGC (847–865)	NM_008854	53	280
	Antisense	ATGGCAATCCAGTCAATCG (1109–1126)			
CREB (Creb1)	Sense	AGA TTG CCA CAT TAG CCC (95–112)	NM031017.1	52	441
	Antisense	GCT GTA TTG CTC CTC CCT (518–535)			
BMP2 (Bmp2)	Sense	AAG CCA GGT GTC TCC AAG (697–714)	NM_017178.1	53	209
	Antisense	AAG TCC ACA TAC AAA GGG TG (886–905)			
BMP4 (Bmp4)	Sense	TAG TCC CAA GCA TCA CCC (876–893)	NM_012827.2	53	294
	Antisense	TCG TAC TCG TCC AGA TAC AAC (1149–1169)			
BMPR1 (Bmpr1a)	Sense	CCA TTG CTT TGC CAT TAT (240–257)	NM_009758.4	47	487
	Antisense	TTT ACC AAC CTG CCG AAC (709–726)			
Smad1 (Smad1)	Sense	AGC ACC TAC CCT CAC TCC C (935–953)	NM_013130.2	56	306
	Antisense	GAA ACC ATC CAC CAA CAC G (1222–1240)			
Collagen type IV (Col4a1)	Sense	TCG GCT ATT CCT TCG TGA TG (4963–4982)	NM_007735.2	56	209
	Antisense	GGA TGG CGT GGG CTT CTT (5154–5171)			
Actin (Actb)	Sense	GCCAACCGTGAAAAGATGA (419–437)	NM_007393.5	54	462
	Antisense	CAAGAAGGAAGGCTGGAAAA (861–880)			

United States). The optical densities of the signals were measured by using ImageJ 1.40g freeware.

Statistical Analysis

All data are representative of at least five independent experiments. For all figures, the samples of the same WT, AD, and TAD animals were chosen with their inner control for a better comparison. Changes were based on the five results, but only one demonstrative photo from the same animal group was used in every figure. Statistical analysis was performed by ANOVA and unpaired Student's *t*-test. The threshold for the statistically significant differences as compared to the control samples was set at $^{*}p < 0.05$ and to the AD samples at $^{\#}p < 0.05$.

TABLE 2 | Antibodies used in the experiments.

Antibody	Host animal	Dilution	Distributor
Anti-BMP4	Rabbit, polyclonal	1:600	Cell Signaling, Danvers, MA, United States
Anti-BMPR1	Mouse, monoclonal	1:600	Abcam, Cambridge, United Kingdom
Anti-Smad1	Rabbit, polyclonal	1:600	Cell Signaling, Danvers, MA, United States
Anti-CREB	Rabbit, polyclonal	1:800	Millipore, Billerica, MA, United States
Anti-P-CREB	Rabbit, polyclonal	1:800	Millipore, Billerica, MA, United States
Anti-Col. IV	Mouse, monoclonal	1:500	Abcam, Cambridge, United Kingdom
Anti-PKA	Rabbit, polyclonal	1:800	Cell Signaling, Danvers, MA, United States
Anti-P-PKA	Rabbit, polyclonal	1:600	Cell Signaling, Danvers, MA, United States
Anti-BMP2	Mouse, monoclonal	1:500	Abcam, Cambridge, United Kingdom
Anti-actin	Mouse, monoclonal	1:10,000	Sigma-Aldrich, St. Louis, MO, United States

RESULTS

Reduced Aβ Accumulation After Physical Activity in AD Kidneys

In WT mice, a normal kidney cortex morphology was demonstrated (**Figure 1A**), without any Congo red positivity (**Figure 1C**). On the contrary, Aβ plaque accumulation was detectable in AD mice (**Figure 1A**). Homogenous eosinophilic deposits were visible, which are typical features of Aβ appearance in the interstitium of AD kidneys (**Figure 1A**). Strong Aβ positivity could be seen in the proximal and distal tubules and the Bowman capsule of AD kidneys with Congo red staining (**Figure 1C**). Moreover, the tubular systems of AD kidneys showed a strong eosinophilic appearance. Pixel analysis revealed a significantly elevated staining intensity in AD kidneys compared with the WT kidneys (**Figure 1B**). Interestingly, increased physical activity diminished the eosinophilic deposits in the interstitium and also reduced Aβ accumulation in the cortical tubules (**Figures 1A,C**, respectively). Physical activity decreased the elevated staining intensity in TAD animals compared with the AD samples (**Figures 1A,B**).

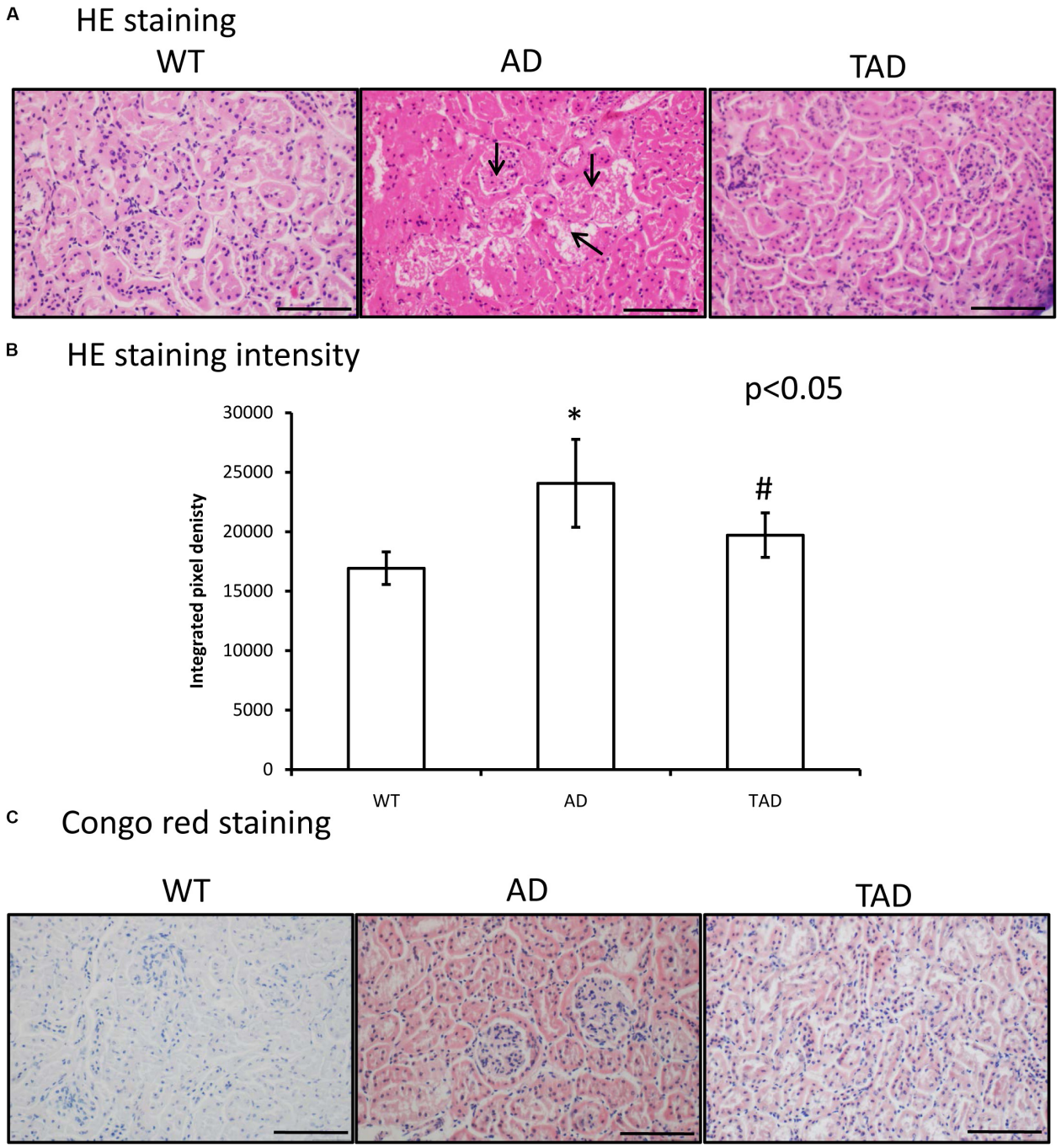

FIGURE 1 | (A) Representative microphotograph of wild-type (WT), Alzheimer's disease (AD), and trained Alzheimer's disease (TAD) kidneys stained with hematoxylin and eosin. Overview of the kidney cortex. Original magnification was × 20. *Scale bar*, 50 μm. *Arrows* show a representative area for amyloid beta (Aβ) plaque formation. **(B)** Staining intensity analysis. Representative data of five independent experiments. The staining intensities are presented as bar graphs ± SEM. *Asterisks* indicate significant ($^{*}p < 0.05$) differences in the staining pixel intensity compared to WT and ($^{\#}p < 0.05$) compared to AD. **(C)** Representative microphotographs of WT, AD, and TAD kidneys stained with Congo red. *Red colors* show Aβ accumulation. Original magnification was × 20. *Scale bar*, 50 μm.

Normalized Receptors of PACAP Signaling in TAD Animals

In WT kidneys, the messenger RNAs (mRNAs) of all PACAP receptors were detectable (**Figure 2A**). Some alterations were shown with the RT-PCR reactions in AD mice, which were normalized in TAD animals (**Figures 2A,C**). The protein expressions of PAC1, VPAC1, and VPAC2 receptors were equally demonstrated in WT kidneys (**Figures 2B,C**). On the contrary,

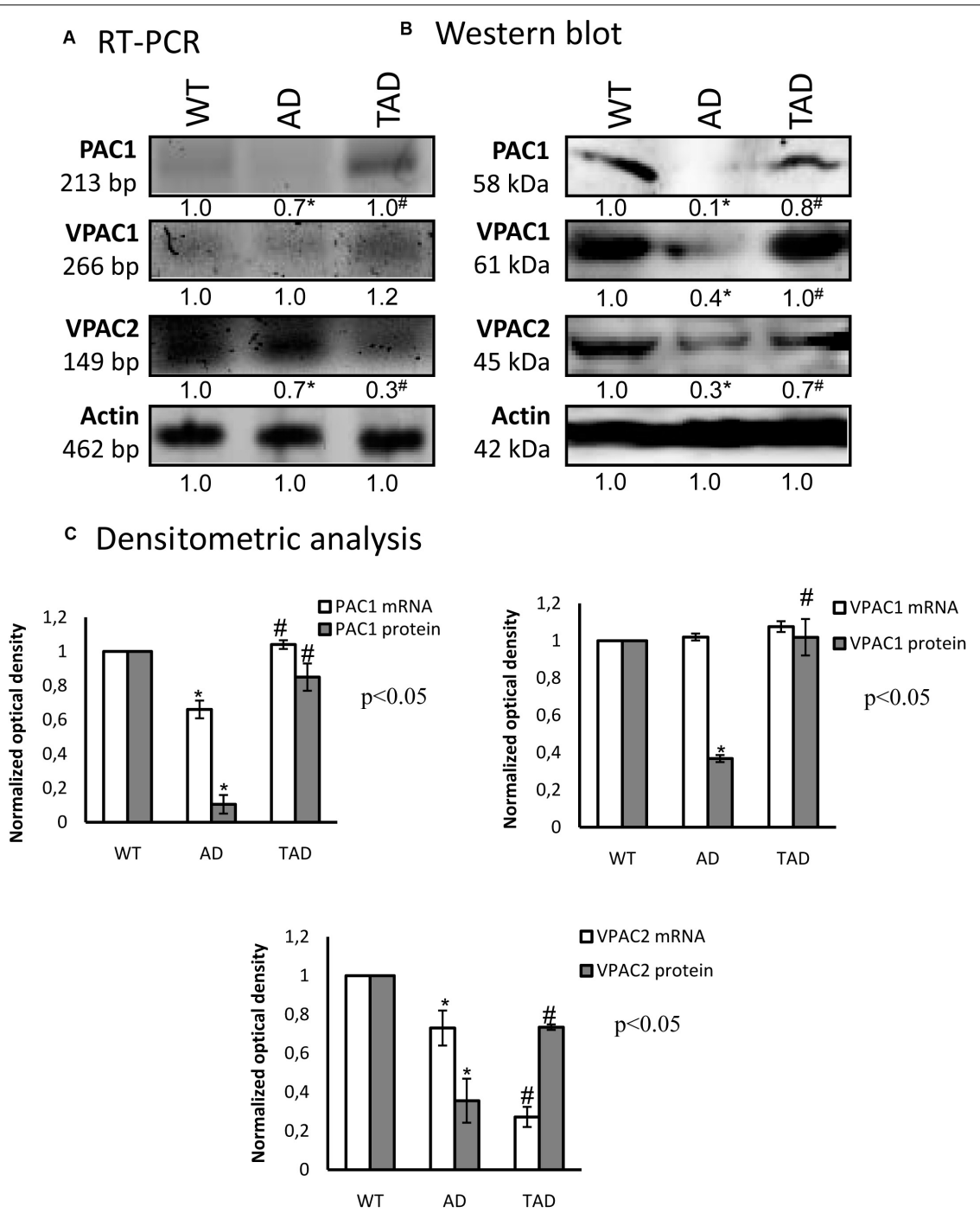

FIGURE 2 | mRNA **(A)** and protein **(B)** expressions of pituitary adenylate cyclase-activating polypeptide (PACAP) receptors in the kidneys. The optical density of the signals was measured and the results were normalized to the optical density of the wild type (WT). For **(A,B)**, the *numbers below the signals* represent the integrated densities of the signals determined by ImageJ software. *Asterisks* indicate significant (*$p < 0.05$) alterations of expressions as compared to the WT and ($^{\#}p < 0.05$) compared to Alzheimer's disease (AD). Representative data of five independent experiments. For reverse transcription PCR (RT-PCR) and for Western blot, actin was used as the control. **(C)** Statistical analysis of the RT-PCR and Western blot data. All data presented are the averages of at least five different experiments. Statistical analysis was performed with Student's *t*-test. All data were normalized on actin and are expressed as the mean ± SEM.

PAC1 receptor protein expression was almost undetectable, and strongly reduced VPAC1 and VPAC2 protein expressions were demonstrated in AD kidneys (**Figures 2B,C**). Physical activity increased the expressions of PACAP receptors in TAD kidneys (**Figures 2B,C**). Most dominantly, the protein expressions of PAC1 and VPAC1 receptors were elevated (**Figures 2B,C**).

Altered Canonical Signaling Pathways of PACAP in AD

To demonstrate the involvement of PACAP signaling in AD kidneys, first, we investigated the canonical downstream targets. PKA is activated by the elevated adenylate cyclase activity; subsequently, the phosphorylation of PKA is increased and triggers the transcriptional activity of CREB. In all experimental groups, the mRNAs of PKA and CREB were demonstrated without significant alterations (**Figures 3A,C**). In AD kidneys, a diminished PKA protein expression was detected, which was increased in TAD animals (**Figures 3B,C**). In TAD kidneys, the PKA expression almost normalized and reached the basal level, but the more active phosphorylated form of PKA after a strong reduction in AD mice did not re-increase (**Figures 3B,C**). Similarly, CREB protein expression was reduced in AD kidneys; moreover, its phosphorylated, thus more active, form was undetectable in AD (**Figures 3B,C**). The increased activity of AD mice augmented the expression of CREB and elevated the phosphorylation level of the transcription factor (**Figures 3B,C**). CREB expression and specific localization in proximal tubules have been demonstrated earlier (Taub, 2018). In WT kidneys, CREB appears in the cytoplasm and shows an apical accumulation in proximal tubules; only cytoplasmic signals appeared in distal tubules and barely detectable CREB was visible in the Bowman capsule (**Figure 3D**). CREB signal diminished and no subcellular specificity could be analyzed with this method in AD kidneys (**Figure 3D**). Interestingly, strong apical signals were demonstrated in proximal tubular cells of TAD kidneys, but no further alterations were shown in distal tubules and in renal corpuscles (**Figure 3D**).

Modified BMP Signaling in AD

For the investigation of PACAP signaling crosstalk, we followed the BMP signaling pathways. First, the mRNAs of BMP2 and BMP4 were detected in all experimental groups without significant alterations (**Figures 4A,C**). BMPR1 and Smad1 mRNA expressions were reduced in AD kidneys, but elevated in TAD kidneys (**Figures 4A,C**). The protein expression of BMP2 diminished in AD kidneys and did not increase in TAD animals (**Figures 4B,C**). Furthermore, AD kidneys showed a decreased BMP4 expression, but it was augmented in TAD animals (**Figures 4B,C**). A similar pattern was demonstrated in the expression of BMPR1, which normalized in TAD kidneys after a strong AD reduction (**Figures 4B,C**). The protein expression of Smad1 is strongly reduced in AD kidneys, and a lower but significant elevation was detected in TAD animals (**Figures 4B,C**). With immunohistochemistry, a strong cytoplasmic signal of Smad1 was demonstrated in WT kidneys (**Figure 4D**). In AD animals, Smad1 reduction was visible in the tubular system, but it increased in the proximal tubules of TAD kidneys (**Figure 4D**).

Altered Expressions of Basement Membrane Components in AD

As we have published earlier, the common target of BMP and PACAP signaling can be collagen type IV, which was also altered in ischemic conditions of the kidneys (Laszlo et al., 2019). The mRNA expression of collagen type IV was undetectable in AD kidneys, while it was demonstrated in TAD kidneys (**Figures 5A,C**). The protein expression of this collagen showed a prominent reduction in AD kidneys, but it was significantly elevated in TAD animals (**Figures 5B,C**). On the other hand, the immunopositivity of collagen type IV was decreased dominantly around the tubules of AD kidneys, while no such strong reduction was detected around the renal corpuscles (**Figure 5D**). In TAD animals, the signals of collagen type IV were elevated around the tubules and renal corpuscles (**Figure 5D**).

DISCUSSION

Numerous molecular and neuropathological disorders have been described in AD, such as tau hyperphosphorylation (Sierra-Fonseca and Gosselink, 2018), modification of Ca^{2+} signaling pathways (Glaser et al., 2019), and Aβ accumulation (Naseri et al., 2019), leading to neuronal and synaptic disorders (Cardozo et al., 2019). In Alzheimer's disease, several peripheral organs are also involved, such as the testis (Tavares et al., 2017), pancreas (Kulas et al., 2017), and kidneys (Paterson et al., 2017), indicating a complex systemic disease (Morris et al., 2014). Pathological Aβ accumulation in the kidneys (Gronewold et al., 2017) may result in filtration disorder. Although various signaling molecules have been identified as possible targets in the treatment of this neurodegeneration process (Loera-Valencia et al., 2019; Patel et al., 2019), the complexity of AD makes drug development difficult. Limited data show the importance of physical activity in postponing the manifestation of characteristic AD (Radak et al., 2010; Abraham et al., 2019). One of the possible protective agents could be PACAP, which has been described to have a preventive effect on AD formation (Han et al., 2014b). PACAP has also been shown to modify amyloid aggregation (Reglodi et al., 2018c) and has important preventive functions in aging (Reglodi et al., 2018a; Szegeczki et al., 2019). The general protective role of the neuropeptide has been demonstrated in various disorders, such as retinopathy (Vaczy et al., 2018), diabetes (Banki et al., 2014), Parkinson's disease (Reglodi et al., 2006; Reglodi et al., 2017), and inflammatory processes (Sakamoto et al., 2015). It has been shown that PACAP diminishes the harmful effects of oxidative stress (Juhasz et al., 2014a), ischemic conditions (Reglodi et al., 2018d), and mechanical overload (Juhasz et al., 2015b). Although the positive function of PACAP in AD has been published, a detailed analysis in peripheral organs has still not been completely performed.

In an AD mouse model system, we investigated the kidneys and the effects of physical exercise in connection to PACAP signaling pathways. Similarly to previous results, we identified a strong Aβ accumulation and intensive eosinophilia in AD kidneys (Gronewold et al., 2017). In contrast, the microscopical signs of Aβ deposits were not detectable after physical activity. Although cortical accumulation of Aβ has not been altered in high-intensity training (Frederiksen et al., 2019), it has also been published that training and running have a protective effect in the hippocampus of mice in AD (Schimidt et al., 2019).

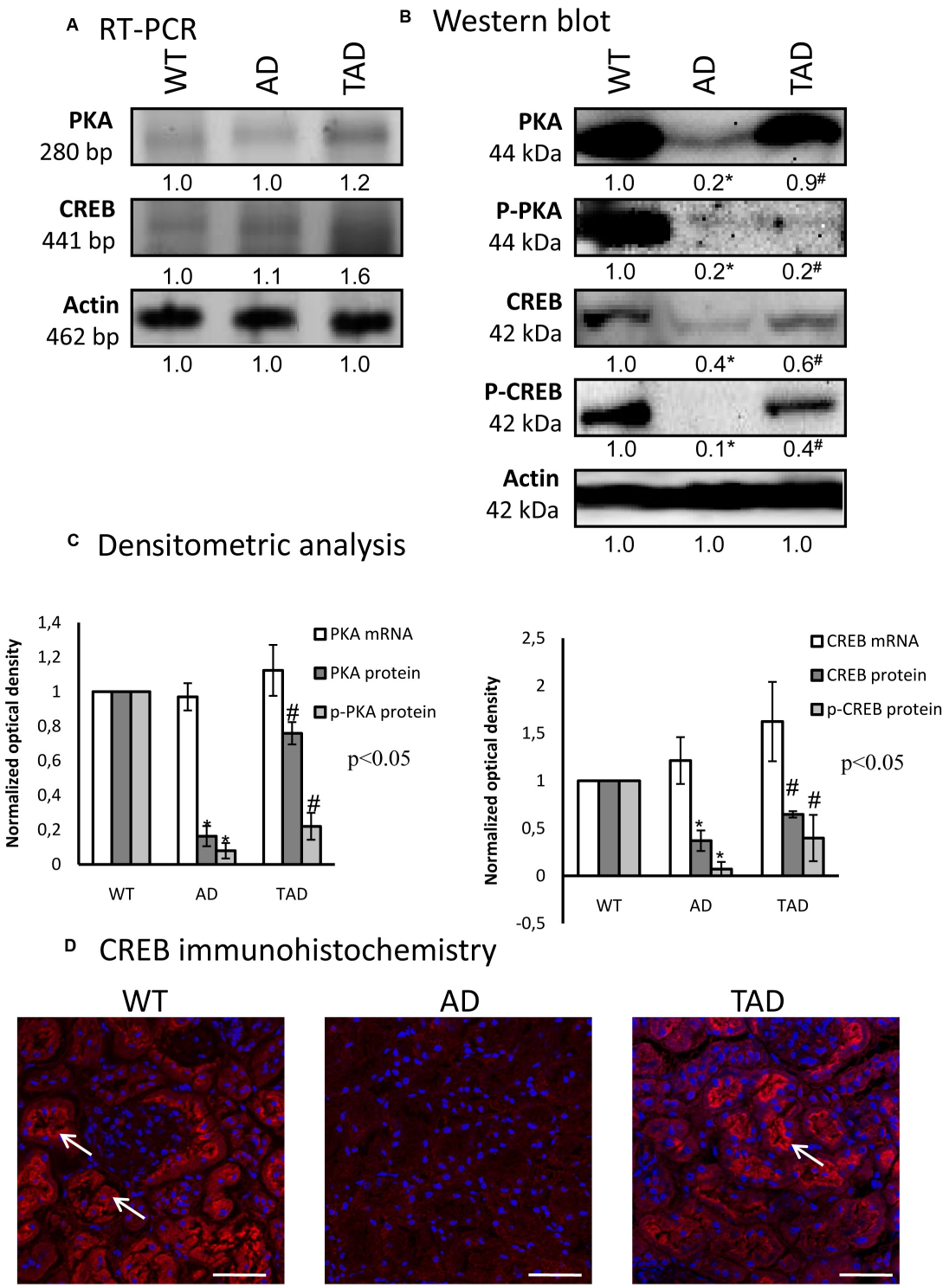

FIGURE 3 | mRNA **(A)** and protein **(B)** expressions of pituitary adenylate cyclase-activating polypeptide (PACAP) signaling in the kidneys. The optical density of the signals was measured and the results were normalized to the optical density of the controls. For **(A,B)**, the *numbers below the signals* represent the integrated densities of the signals determined by ImageJ software. *Asterisks* indicate significant ($*p < 0.05$) alterations of expressions as compared to the wild type (WT) and ($^{\#}p < 0.05$) compared to Alzheimer's disease (AD). Representative data of five independent experiments. For reverse transcription PCR (RT-PCR) and for Western blot, actin was used as the control. **(C)** Statistical analysis of the RT-PCR and Western blot data. All data presented are the averages of at least five different experiments. Statistical analysis was performed with Student's *t*-test. All data were normalized on actin and are expressed as the mean ± SEM.
(D) Immunohistochemistry of CREB in the cortex of the kidneys. *Arrows* show the apical part of the proximal tubules. Magnification was made with × 60 objective. *Scale bar*, 20 μm.

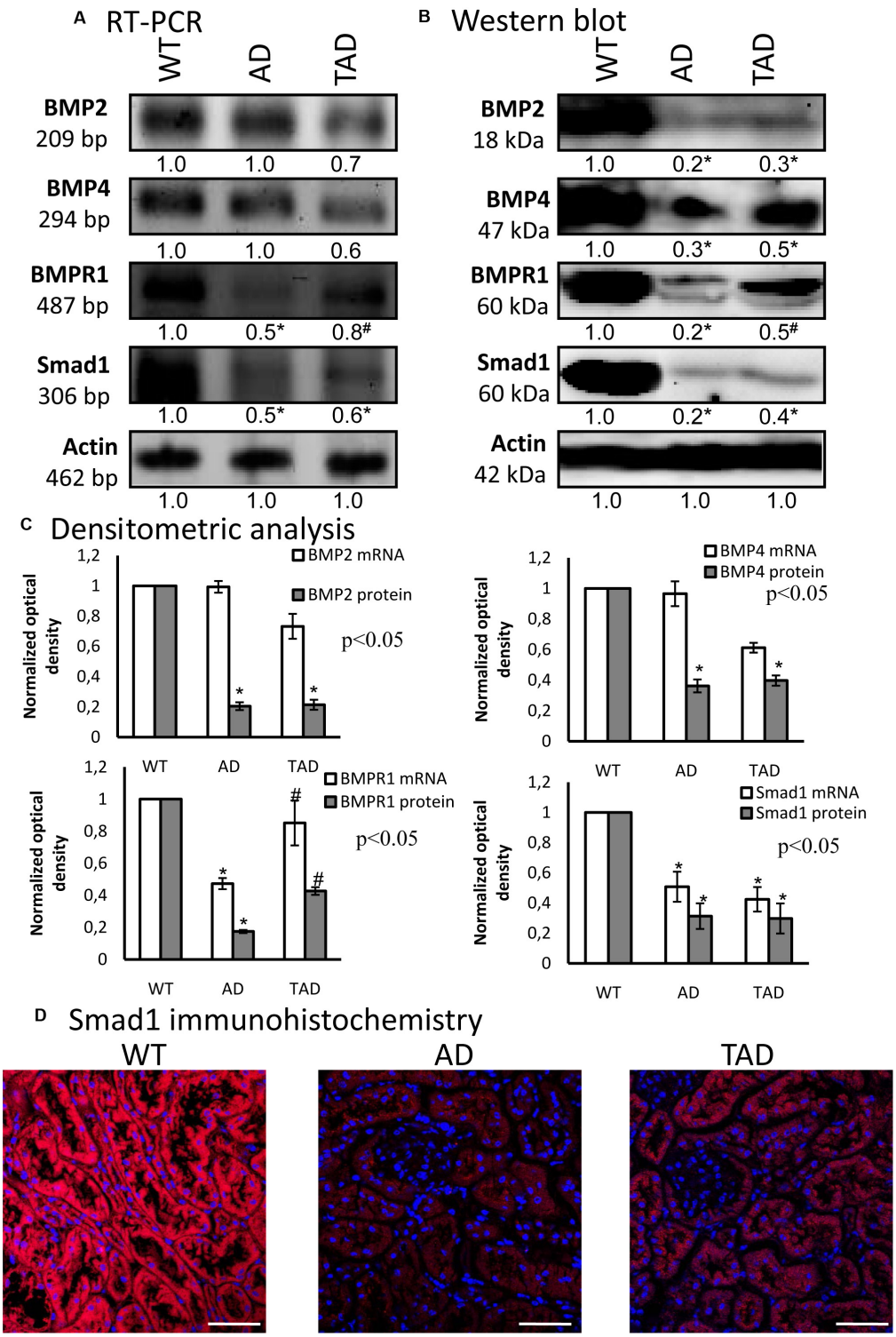

FIGURE 4 | mRNA **(A)** and protein **(B)** expressions of bone morphogenetic protein (BMP) signaling in the kidneys. For reverse transcription PCR (RT-PCR) and for Western blot, actin was used as the control. The optical density of the signals was measured and the results were normalized to the optical density of the controls. For panels **(A)** and **(B)**, the *numbers below the signals* represent the integrated densities of the signals determined by ImageJ software. *Asterisks* indicate significant ($^{*}p < 0.05$) alterations of expressions as compared to the wild type (WT) and ($^{\#}p < 0.05$) compared to Alzheimer's disease (AD). Representative data of five independent experiments. **(C)** Statistical analysis of the RT-PCR and Western blot data. All data presented are the averages of at least five different experiments. Statistical analysis was performed with Student's *t*-test. All data were normalized on actin and are expressed as the mean ± SEM. **(D)** Immunohistochemistry of Smad1 in the cortex of the kidneys. Magnification was made with × 60 objective. *Scale bar*, 20 μm.

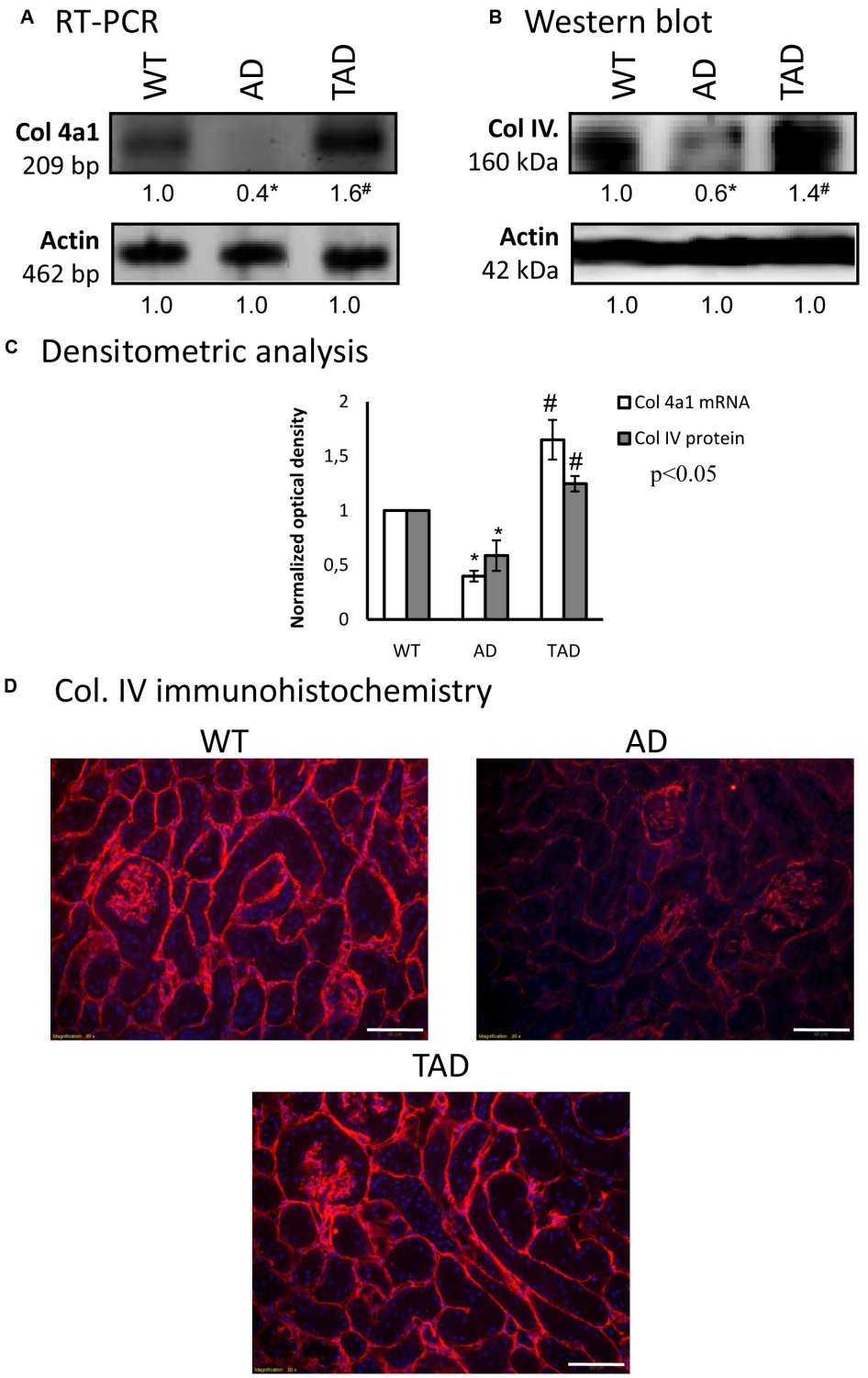

FIGURE 5 | mRNA **(A)** and protein **(B)** expressions of collagen type IV in the kidneys. For reverse transcription (RT-PCR) and for Western blot, actin was used as the control. The optical density of the signals was measured and the results were normalized to the optical density of the controls. For **(A,B)**, the *numbers below the signals* represent the integrated densities of the signals determined by ImageJ software. *Asterisks* indicate significant ($^{*}p < 0.05$) alterations of expressions as compared to the wild type (WT) and ($^{\#}p < 0.05$) compared to Alzheimer's disease (AD). Representative data of five independent experiments. **(C)** Statistical analysis of the RT-PCR and Western blot data. All data presented are the averages of at least five different experiments. Statistical analysis was performed with Student's *t*-test. All data were normalized on actin and are expressed as the mean ± SEM. **(D)** Immunohistochemistry of collagen type IV in the cortex of the kidneys. Magnification was made with × 20 objective. *Scale bar*, 50 μm.

Although physical activity generally decreases the amount of Aβ deposits or concentration in the blood and cerebrospinal fluid (Brown et al., 2019), no data are available to clarify the mechanism in the periphery. We can conclude that elevated physical activity protects the kidneys from Aβ accumulation. We have demonstrated that PACAP has a direct connection with mechanotransduction (Juhasz et al., 2015b; Szentleleky et al., 2019). Therefore, we investigated the possible alterations of the expressions of PACAP receptors in the kidneys of AD and trained AD animals. The expressions of all PACAP receptors were demonstrated in the kidneys, with a significant decrease in the AD samples. The protein expressions of all PACAP receptors recovered in TAD animals. VPAC1 has already been shown to be expressed in the highest level in proximal tubules (Eneman et al., 2016), and the expression of the PAC1 receptor was demonstrated in the HK-2 cell line (Li et al., 2010). Direct binding of PACAP in kidney diseases has been proven both on PAC1 and VPAC1 receptors (Li M. et al., 2008), suggesting their equal importance in AD-related disorders. Reduction of the PAC1 receptors has been demonstrated in the CNS of the AD models (Han et al., 2014a), and mechanical stimulation increased the expression in chondrogenic cells (Juhasz et al., 2015b). The importance of the PACAP signaling pathway is also hallmarked by the elevated expression of the VPAC receptor in TAD, demonstrating the VIP/PACAP-related signaling crosstalk in AD (Dogrukol-Ak et al., 2004). Subsequently, we conclude that training and increased physical activity has a direct effect on AD *via* the activation of PACAP signaling. The most described PACAP-mediated signaling pathway is regulated by PKA (Vaudry et al., 2009), and its activity is reduced in AD (Kumar and Singh, 2018). Moreover, it has been published that the increased activity of PKA *via* CREB phosphorylation can regulate and suppress Aβ secretion in platelets (Sepulveda et al., 2019). We also detected PKA and CREB expression reductions in AD kidneys, while in TAD kidneys their expressions normalized. On the other hand, the autophosphorylated PKA is not affected by physical activity, suggesting a PACAP-independent regulation of PKA phosphorylation (Smith and Scott, 2018). Autophosphorylation of PKA is not necessary for the phosphorylation of CREB and its activity is not in ratio with its phosphorylation, but other signaling elements can be partly involved in the increased CREB phosphorylation, such as ERK activation (Racz et al., 2006). CREB is one of the transcription factors which can be phosphorylated by PKA (Zakany et al., 2002), and its activation in synapses is important in memory-related gene expression (Teich et al., 2015). CREB phosphorylation can be harmed in AD and result in alterations of synaptic plasticity (Teich et al., 2015). CREB is an essential factor in the regulation of the Na-K ATPase function in kidney proximal tubules (Taub, 2018); subsequently, it regulates their filtration function. Elevated physical exercise increased the expression and phosphorylation of this transcription factor in AD kidneys. Notably, CREB localization was detected in the apical part of the proximal tubular cells in WT animals. This apical accumulation diminished in AD, but was expressed in the proximal tubular cells of the TAD groups. This phenomenon further strengthened the hypothesis that CREB regulates proximal tubule function and physical activity may rescue the normal filtration in AD. These facts suggest that PKA activation *via* PACAP signaling is one of the molecular pathways involved in the physical activity-mediated protection mechanisms in AD.

PACAP signaling has various molecular crosstalks (Juhasz et al., 2015a), such as BMP signaling (Jozsa et al., 2018), sonic hedgehog signaling (Juhasz et al., 2015b), or Notch signaling (Fulop et al., 2019). The connection of PACAP signaling and BMPs has been shown in bone formation (Juhasz et al., 2014b; Jozsa et al., 2018, 2019) and cartilage development (Juhasz et al., 2014a). Moreover, PACAP has an effect on BMP signaling in the kidneys in ischemic–reperfusion conditions (Laszlo et al., 2019). It has also been shown that BMP expression and function can be modified in AD in glial cell differentiation (Kwak et al., 2014). In this neurodegeneration, Aβ can modify the BMP2 and BMP4 release (Kwak et al., 2014) and has an effect on other BMPs, such as increased BMP6 expression (Crews et al., 2010). BMP2 and BMP4 mRNA expressions were not altered. We demonstrated similar results in the femurs (Jozsa et al., 2018), testis (Reglodi et al., 2018b), and various other organs of PACAP KO mice (Reglodi et al., 2018c), where the Western blot and mRNA levels were not in correlation. In the UMR-106 cell line, PACAP did not alter the mRNA expression, but the protein expression was changed (Juhasz et al., 2014b). In high-density cell cultures, the addition of PACAP did not significantly modulate the mRNA expressions of certain matrix-producing enzymes or matrix proteins (Juhasz et al., 2014a, 2015b). These findings indicate that PACAP signaling exerts more pronounced effects on posttranscriptional events and cellular protein metabolism, and it has less influence on the gene expression of the targeted downstream molecules. Although the BMP2 protein expression was not altered in TAD animals, we detected an elevation in the BMP4 protein expression after training. This suggests that physical activity induces the activation of BMP signaling partly *via* PACAP receptor activation. BMPR1 induces the activation of Smad transcription factors and can regulate the expression of various genes, such as basement membrane components (Villacorte et al., 2016). Interestingly, the expression of BMPR1 was strongly decreased in AD kidneys, but recovered in TAD animals. The ratio of Smad1 reduction and its elevation was slightly lower than that of BMPR1, but the immunopositivity of elevated Smad1 in TAD kidneys was visible in the proximal tubules. It strongly suggests a direct communication between PACAP and BMP signaling in AD kidneys. We conclude that physical activity mediates the PACAP–BMP4 signaling axis to protect the function of kidneys in AD.

We have already demonstrated that BMP signaling activated the expression of collagen type IV in ischemic reperfusion injury (Laszlo et al., 2019). It has also been discussed that PACAP has an effect on basement membrane expression, especially collagen type IV, in the testes of PACAP KO mice (Reglodi et al., 2018b). Furthermore, PACAP was able to elevate collagen secretion in the cartilage (Juhasz et al., 2014a). Components of the basement membrane have been altered around the microvessels of the cortex in AD (Christov et al., 2008); subsequently, the blood–brain barrier can be compromised. After physical activity, we detected an elevated collagen type IV expression in AD animals.

Collagen type IV has been shown to inhibit Aβ plaque formation (Kiuchi et al., 2002) and normalize the function of the blood–brain barrier. Therefore, the elevated collagen type IV expression in TAD kidneys indicates a normalized basement membrane formation, which can play a role in Aβ elimination in the kidneys.

CONCLUSION

In conclusion, our data suggest that physical activity in AD can decrease the Aβ plaque formation and that elevated physical exercise affects the PACAP/BMP-mediated signaling pathway and CREB activation in the kidneys. The latter can regulate the Na-K ATPase in proximal tubules and the expression of collagen type IV, which in turn normalize the kidney tubular system function. Therefore, the systemic effect of physical activity can postpone the formation of AD *via* normalization of amyloid elimination *via* the kidneys.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by PEI/001/2105-6/2014, Semmelweis University, Hungary, 1091 Budapest, Üllõi út 93. fsz. 2.

AUTHOR CONTRIBUTIONS

TJ, DR, and GH contributed to the study conception and design. ZR and DÁ established the physical activity model, animal care, and operations were established. HP, VS, BH, and TJ performed the molecular biological analysis. BH, HP, and VS performed immunohistochemistry and histology. VS and TJ performed the statistical analysis. HP, BH, VS, and AT did the acquisition of data. TJ, AT, RZ, and DR analyzed and interpreted the data. HP, RZ, TJ, GH, and DR participated in drafting the manuscript. All authors contributed to the article and approved the submitted version.

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Pituitary Adenylate Cyclase-Activating Polypeptide in Learning and Memory

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Pituitary adenylate cyclase-activating polypeptide (PACAP) is a highly conserved neuropeptide that regulates neuronal physiology and transcription through Gs/Gq-coupled receptors. Its actions within hypothalamic, limbic, and mnemonic systems underlie its roles in stress regulation, affective processing, neuroprotection, and cognition. Recently, elevated PACAP levels and genetic disruption of PAC1 receptor signaling in humans has been linked to maladaptive threat learning and pathological stress and fear in post-traumatic stress disorder (PTSD). PACAP is positioned to integrate stress and memory in PTSD for which memory of the traumatic experience is central to the disorder. However, PACAP's role in memory has received comparatively less attention than its role in stress. In this review, we consider the evidence for PACAP-PAC1 receptor signaling in learning and plasticity, discuss emerging data on sex differences in PACAP signaling, and raise key questions for further study toward elucidating the contribution of PACAP to adaptive and maladaptive fear learning.

Keywords: PACAP, learning, working memory, fear, anxiety, cognition, sex, PTSD

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INTRODUCTION

The salient experiences of our daily life create memories that form the narrative of our self. These memories enrich our connections with others and guide our decisions and behavior, but if born out of pain and trauma, can be debilitating. Intrusive, terrifying memories that are difficult to extinguish or that generalize to non-threatening situations are the hallmark of post-traumatic stress disorder (PTSD), a complex disorder with a lifetime prevalence of about 8% (Kessler et al., 1995; Kilpatrick et al., 2013). PTSD is characterized by altered stress reactivity, generalized fear to non-threatening cues and situations, and is more prevalent in females (Kessler et al., 1995; Kilpatrick et al., 2013). Recently, the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) has been linked to PTSD (Ressler et al., 2011; Almli et al., 2013; Uddin et al., 2013). This link has been attributed to the role that PACAP plays in regulating the somatic and affective components of chronic stress, discussed in excellent reviews (Hammack and May, 2015; King et al., 2017b; Miles and Maren, 2019). However, PACAP's role in memory has received comparatively less attention yet may also contribute to PTSD for which memory of the traumatic experience is central to the disorder. Here, we briefly review the literature supporting a role for PACAP in memory formation and discuss avenues for further investigation.

PACAP, AN OVERVIEW

Pituitary adenylate cyclase-activating polypeptide is a highly conserved pleiotropic neuropeptide in the vasoactive intestinal peptide (VIP)/secretin/glucagon family that modulates several physiological functions in the periphery and central nervous system *via* class B G-protein coupled receptors (Miyata et al., 1989, 1990; Arimura et al., 1991; Piggins et al., 1996; Arimura, 1998; Vaudry et al., 2009). In the brain, the 38-amino acid form of PACAP predominates (Arimura, 1998), and PACAP-38 and its receptors are widely expressed in circuits involved in memory, stress, and affect (Shioda et al., 1997; Hannibal, 2002; Joo et al., 2004; Condro et al., 2016). PACAP modulates neuronal function *via* the PACAP-specific high-affinity receptor PAC1 and the VPAC1 and VPAC2 receptors, which have similar affinity for PACAP and VIP. These receptors are coupled to Gαs, but PAC1 can also signal through Gαq (Spengler et al., 1993; Dickson and Finlayson, 2009; Vaudry et al., 2009; Harmar et al., 2012). Thus, PACAP can regulate the neuronal excitability and synaptic plasticity underlying memory and cognition through a diverse set of cAMP-mediated intracellular signaling (for a review see Johnson et al., 2019).

DISTRIBUTED MEMORY SYSTEMS

A role for PACAP in learning and memory was evident from early examinations of mice lacking the PAC1 receptor (PAC1R). Genetic deletion of PAC1R either globally or in the forebrain produced mild to severe impairments in certain forms of hippocampus-dependent learning (Sauvage et al., 2000; Otto et al., 2001a,b). Contextual fear conditioning, an associative paradigm in which subjects learn to associate the spatial configuration of environmental cues with a footshock, was impaired, while other hippocampus-dependent learning tasks, the Morris water maze and social transmission of food preference were unaffected. Hippocampus-independent cued fear conditioning was also unaffected by the loss of PAC1R signaling in the same study. Global knockouts displayed anxiety-like behavior and altered stress reactivity, which is consistent with PACAP's role in HPA axis regulation, but which could affect the conditional response of freezing used to assess memory in rodent fear conditioning. Thus, it is important that the forebrain-specific knockouts, which showed the same memory deficit, did not differ from wild-type controls in locomotor activity or anxiety-like activity in the open field or elevated plus maze (Otto et al., 2001b). Mice lacking the PACAP peptide also showed impaired contextual fear memory as well as deficits in novel object recognition (Takuma et al., 2014). However, these mice exhibit a wide range of altered behavior (reviewed in Hashimoto et al., 2006). Some of these behaviors could be ameliorated with environmental enrichment early in life, but not in adulthood, suggesting that PACAP's role in neural development may contribute to abnormal behaviors in PACAP deficient mice (Ishihama et al., 2010; Takuma et al., 2014). Nonetheless, exogenous delivery of PACAP intracerebroventricularly into adult rats enhanced the consolidation of a passive avoidance memory at low doses (Sacchetti et al., 2001) and temporarily impaired contextual fear memory at high doses (Meloni et al., 2016, 2018). These studies demonstrate a role for forebrain PACAP in contextual fear learning and suggest that PAC1R signaling may be preferentially engaged by aversive events.

More recently, behavioral pharmacology studies have identified specific brain regions where PACAP contributes to aversive memory or its extinction. These include the hippocampus, amygdala, and prelimbic cortex. In the hippocampus, the consolidation of contextual fear memory was enhanced by PACAP and impaired by the PAC1R antagonist PACAP6-38, when injected immediately after training (Schmidt et al., 2015). Hippocampal PACAP also contributes to fear extinction, while amygdala PACAP is needed for contextual fear memory but not its extinction (Schmidt et al., 2015). The subregional specificity of PACAP's effects in the hippocampus remain to be determined. The hippocampus is functionally heterogeneous with unique output connectivity along its dorsal-ventral axis (Dong et al., 2009). These studies targeted dorsal hippocampus and given the importance of ventral hippocampus to affective behavior and learning, future work should examine ventral hippocampal PACAP. PACAP also participates in the formation of trace fear memory, a form of cued fear learning dependent on the prelimbic cortex and hippocampus in addition to the amygdala (Kirry et al., 2018). Trace conditioning requires the association of a cue and shock separated in time, and linking these events requires sustained neuronal activity in prelimbic cortex (Baeg et al., 2001; Gilmartin and McEchron, 2005; Gilmartin et al., 2013b, 2014). Prelimbic injection of PACAP6-38 prior to training impaired the formation of the cued memory in females, but not males (Kirry et al., 2018). This sex difference, discussed below, may provide insight into the genetic link between PAC1R and PTSD, for which women with a genetic polymorphism in the PAC1R gene exhibit enhanced reactivity to threat-predictive cues (Ressler et al., 2011). The prefrontal cortex is also needed for aspects of contextual fear learning (Gilmartin and Helmstetter, 2010; Gilmartin et al., 2013a; Rozeske et al., 2015; Heroux et al., 2017; Twining et al., 2020), but PAC1R antagonism did not affect the contextual fear memory formed alongside the cued fear memory in trace conditioning (Kirry et al., 2018). Nor did the manipulation affect a non-aversive spatial-working memory T-maze task in either sex. These data suggest that PAC1R signaling contributes to prefrontal mechanisms of working memory or sustained attention required for predicting threat based on available cues. Together, these studies demonstrate a role for PACAP signaling in learning and memory and point to site-specific engagement of PACAP in cued and contextual fear.

CELLULAR AND SYNAPTIC PHYSIOLOGY

The regulation of synaptic glutamatergic signaling and the production of new proteins for long-term synaptic stabilization

are the basis of the cellular consolidation of memory, a process that generally concludes within a few hours after training (McGaugh, 2000; Asok et al., 2019). Adenylate cyclase/cAMP-driven intracellular signaling leading to CREB-mediated gene transcription is critical for the formation of long-term memories (Kandel et al., 2014; Asok et al., 2019). PACAP's namesake ability to activate these signaling cascades underlies its role in memory. In hippocampal circuits, PACAP modulates synaptic and evoked NMDA- and AMPA-mediated currents *via* PKA or PLC/PKC (Roberto and Brunelli, 2000; Roberto et al., 2001; Ciranna and Cavallaro, 2003; Yaka et al., 2003; Macdonald et al., 2005; Costa et al., 2009; Pecoraro et al., 2017). PACAP-dependent phosphorylation of NMDA receptor subunits promotes mossy fiber long-term potentiation (LTP), a cellular correlate of learning and memory, and LTP is impaired in mice lacking PACAP or PAC1R (Otto et al., 2001a; Matsuyama et al., 2003). PACAP's effects on plasticity are dose-dependent, with higher doses exerting inhibitory effects on hippocampal synaptic transmission *via* VPAC signaling (Costa et al., 2009), which reflects its dose-dependent effects on hippocampus-dependent fear memory (Sacchetti et al., 2001; Meloni et al., 2016). It is important to note that temperature is an important factor in the physiological investigation of PACAP. While lower temperatures (e.g., 21–24°C) are useful for slowing down the fast ion channel kinetics linked to PAC1R activation (Johnson et al., 2019), they can alter other measures of excitability and interfere with PAC1R endosomal signaling (Merriam et al., 2013). Moreover, the majority of existing hippocampal work has focused on CA1 and CA3, but PAC1R is expressed also in the dentate gyrus (Jaworski and Proctor, 2000; Joo et al., 2004). Recent work has shown that PACAP-PAC1R activation drives CREB-mediated transcription and promotes excitability of DG granule cells (Johnson et al., 2019; Johnson R. et al., 2020; Johnson R. L. et al., 2020).

Pituitary adenylate cyclase-activating polypeptide has dose-, receptor-, and circuit-specific effects on physiology in other brain areas, which highlights the potential ways in which PACAP can affect learning within a distributed network. In the amygdala, PACAP increases AMPA-mediated currents at BLA-CeA synapses *via* VPAC1 (Cho et al., 2012) and increases GABA release *via* PAC1R (Varodayan et al., 2019), mechanisms that underlie affective behavioral responses to chronic stress or pain. In the central nucleus of the amygdala, fear conditioning drives expression of the plasticity-related protein Arc and intracerebroventricular infusion of PACAP enhances this expression (Meloni et al., 2018). Arc, whose translation is regulated by PKA activity (Bloomer et al., 2008), is critical to several cellular processes supporting memory and cognition (e.g., Nikolaienko et al., 2018). PACAP's contribution to synaptic plasticity in cortical systems is not clear. Given the selective role for prelimbic PAC1R signaling in trace cued, but not contextual, memory (Kirry et al., 2018), PACAP may act on working-memory or sustained attention mechanisms. One candidate mechanism is the regulation of GluN2B-containing NMDARs, which promote recurrent activity in cortical circuits (Wang et al., 2013) and which are needed for trace cued, but not contextual fear learning in the prefrontal cortex and hippocampus (Gao et al., 2010; Gilmartin et al., 2013a). PACAP has been shown to phosphorylate the GluN2B subunit in the hippocampus and hypothalamus to regulate glutamatergic signaling (Yaka et al., 2003; Resch et al., 2014).

Pituitary adenylate cyclase-activating polypeptide can also influence memory *via* modulation of intrinsic physiology (see Open Questions) and *via* developmental maturation of memory circuits (reviewed in Shen et al., 2013). For example, developmental knockout of VPAC2 which is sensitive to both VIP and PACAP prevented the formation of fear extinction memory in adulthood (Ago et al., 2017). Importantly, VPAC2-KO mice had reduced cell size and dendritic branching in the prelimbic cortex, morphological changes similar to those observed after chronic corticosterone exposure and chronic stress, conditions which also produce fear extinction deficits (Wellman, 2001; Radley et al., 2004, 2006; Moench and Wellman, 2017; reviewed in Wellman et al., 2020). Whether impaired extinction in VPAC2-KO mice is a consequence of altered prefrontal morphology or lack of PACAP/VIP signaling at VPAC2 in the prefrontal-amygdala circuit during extinction learning remains to be determined. Nonetheless, these neuropeptides are necessary for proper neural maturation, and pathological disruptions in PACAP signaling during critical developmental windows could thus affect adult cognition by altering the development of memory-related circuits. Although we are only beginning to elucidate the diverse mechanisms by which PACAP affects learning and memory, the significant work on the diversity of PACAP signaling in stress-related behaviors provides a useful foundation for rapid progress in this effort (Hammack and May, 2015; Johnson et al., 2019; Ferrara and Gilmartin, 2020).

SEX DIFFERENCES

The work above implicates PACAP and PAC1R in neuroplasticity and memory and suggests that endogenous PACAP released during salient, aversive events contributes to memories for threat-predictive cues and contexts. Therefore, vulnerabilities in PACAP signaling may contribute to pathological fear in PTSD through its roles in learning and in mediating traumatic stress responses. Further investigation of the PACAP-PTSD link requires inclusion of female subjects, not only because females have largely been excluded from preclinical study, but also because of a sex-specific link between PACAP and PTSD. A single-nucleotide polymorphism (SNP) in the *adcyap1r1* gene encoding PAC1R is associated with symptom severity in women, but not men; altered DNA methylation of the same gene is associated with PTSD in both sexes (Ressler et al., 2002; Almli et al., 2013). The risk allele for PTSD is also associated with symptom severity in GAD females, but not males (Ross et al., 2020). The SNP is located in an estrogen-response element (ERE), and the nucleotide change interferes with estradiol-estrogen receptor alpha binding the ERE, leading to a decrease in receptor expression (Mercer et al., 2016). This points to a potential mechanism of vulnerability in women. Indeed, several labs are actively examining how PACAP in the

BNST and hypothalamic stress circuits regulates the affective response to stress in male and female rodents (King et al., 2017a,b; Ramikie and Ressler, 2018), and additional mechanistic insight may come from examination of sex differences in autonomic regulation by PACAP (Nakamachi et al., 2016). This work complements that of Bangasser, Valentino, and others detailing sex differences in the corticotropin-releasing factor (CRF) system and brainstem arousal systems [for excellent reviews, see Bangasser and Valentino (2014); Valentino and Bangasser (2016); Bangasser et al. (2019)].

Little is known about sex differences in PACAP's contribution to memory. Recent work showed that prelimbic PACAP participates in trace cued fear learning in females, but not males, and that mRNA levels for PAC1R are higher in females than males and further modulated by the estrous cycle (Kirry et al., 2018). Recently, Rajbhandari et al. (2021) reported increased fear generalization and impaired extinction in males, but not females, following viral deletion of PAC1R in the medial intercalated cells of the amygdala, a region involved in the suppression of fear following extinction (Duvarci and Pare, 2014). Females in that study showed a reduced asymptotic level of fear during acquisition. These behavioral results suggest that PACAP signaling exerts sex and region-specific modulation of fear memories. In humans, the PTSD-related PAC1R risk allele is associated with enhanced startle to threat-related cues, impaired fear and safety discrimination, and altered hippocampal and amygdala reactivity in fear conditioning (Ressler et al., 2011; Jovanovic et al., 2013; Stevens et al., 2014). In children, females with the risk allele showed enhanced fear responding to threat-related cues 1 year after conditioning (Jovanovic et al., 2020). While these clinical studies implicate associative learning processes in the PAC1R-PTSD link, it is difficult to distinguish the unique contributions of associative learning vs stress reactivity. Preclinical investigations are critical in this endeavor.

OPEN QUESTIONS

Pituitary adenylate cyclase-activating polypeptide contributes to learning and memory under salient, usually aversive conditions. The diversity of its neural function places this pleiotropic signaling peptide in the company of several peptide factors, such as CRF and estradiol, that have a wide range of function beyond that for which they were initially characterized (Hupalo et al., 2019; Taxier et al., 2020). This diversity likely underlies the influence that PACAP dysregulation appears to have in psychiatric illness, but also makes it challenging to determine the nature of that relationship. Here, we raise a few open questions to guide further investigation.

Open Question 1: Is PACAP's Role in Learning Selective for Aversive Episodic Memory?

Disruption of PAC1R signaling affects the formation of associative fear memories dependent on episodic memory systems, such as contextual fear or trace fear conditioning, but leaves standard delay cued conditioning and spatial learning largely intact. Interestingly, the *amnesiac* gene in drosophila, which codes for a putative PACAP homolog, AMN, is necessary for odor-shock associative memory (Quinn et al., 1979; Feany and Quinn, 1995; Turrel et al., 2018). While the receptor target(s) by which AMN mediates memory is unclear, the parallels in these behavioral observations raise the possibility that PACAP is selective for aversive learning; however, its role in appetitive or other non-aversive learning is largely untested. Thus, to determine PACAP selectivity to certain forms of memory, its contribution to non-aversive memory needs to be clarified. Importantly, PACAP and PAC1R manipulations in the mature circuit are needed to rule out learning deficits due to aberrant neural development of healthy memory circuits for which PACAP is implicated. To this point, the role for AMN in the development of associative memory systems was recently dissociated from its role in adult learning (Turrel et al., 2018).

Open Question 2: What Aspect of an Aversive Experience Recruits PACAP Signaling?

Pituitary adenylate cyclase-activating polypeptide is mobilized by repeated or chronic stressors or by persistent neuropathic pain states (Dickinson and Fleetwood-Walker, 1999; Mustafa, 2013), but release conditions in learning networks during relatively brief aversive events in fear conditioning are unknown. PACAP-expressing cells are found throughout learning circuits (Hannibal, 2002; Condro et al., 2016), many of which robustly respond to shock delivery. One possibility is that the aversive shock reinforcer may trigger the co-release of PACAP at glutamatergic terminals, facilitating cAMP-mediated signaling in support of robust fear memory. Alternatively, any sufficiently salient or arousing experience may mobilize PACAP and promote the consolidation of memory. The development of tools that allow accurate measurement of peptide release *in vivo* provide the circuit-level resolution needed to determine when and where PACAP is released during learning (Muller et al., 2014; Al-Hasani et al., 2018).

Open Question 3: What Are the Unique Peptide-Receptor Signaling Contributions to Learning and Memory?

The unique contributions of PACAP and VIP at PAC1 and VPAC1/2 receptors in learning and memory are poorly understood. For instance, VPAC2 receptors have partially overlapping distribution patterns in several regions important for learning and memory (Lee et al., 2010), and developmental manipulation of VPAC2 affects extinction learning in adulthood (Ago et al., 2017). Pharmacological dissociation of PACAP actions at PAC1R and VPAC2 is difficult as both are a target of the PAC1R antagonist PACAP6-38. Plus, as mentioned earlier, mechanistic dissection of these peptides *in vitro* is sensitive to experimental parameters such as temperature. Thus, new small molecule antagonists as well as gene-editing tools will

be invaluable in revealing the rich complexity of mnemonic regulation by these peptides.

Open Question 4: Does PACAP Bias Allocation of Specific Circuits Into Memory?

One intriguing, but speculative, role for PACAP in memory is to bias the allocation of cells or circuits into memory as a consequence of psychological stress or aversive experience (Kondo et al., 1997). Neuronal excitability at the time of learning determines which neurons are allocated to the memory trace (Yiu et al., 2014; Cai et al., 2016; Sehgal et al., 2018; Josselyn and Tonegawa, 2020). In the amygdala, cells with reduced afterhyperpolarization (AHP) and increased CREB phosphorylation immediately prior to training are recruited into a fear memory (Yiu et al., 2014). PACAP reduces the slow AHP calcium-activated potassium current sI_{AHP} *via* cAMP/PKA to increase excitability and action potential firing in hippocampal CA1 and neocortical neurons (Hu et al., 2011; Taylor et al., 2014). PACAP-elicited firing and PKA-dependent phosphorylation of CREB drive CREB-mediated transcription (Baxter et al., 2011). PACAP affects excitability and firing in a circuit-specific manner (e.g., Hurley et al., 2019) and intrinsic properties can be modified by experience (Sehgal et al., 2013; Dunn and Kaczorowski, 2019). Thus, PACAP released during stressful events or elevated PACAP following chronic stress or pain could influence which circuits are recruited into a memory trace. Addressing this possibility will shed light on how altered PACAP-PAC1R signaling in susceptible individuals corresponds with altered threat-associated memory in PTSD.

CONCLUDING REMARKS

Pituitary adenylate cyclase-activating polypeptide is a pleiotropic neuropeptide whose diverse signaling underlies its diversity of function including neural development, neuroprotection, stress regulation, autonomic activation, affective behavior, and memory. This in turn highlights the therapeutic potential of this peptide and receptor modulations, a potential that is discussed in this Research Topic and reflected in recent efforts to develop non-peptide small molecule compounds to selectively target PACAP receptors (Beebe et al., 2008; Takasaki et al., 2018). The link between PACAP-PAC1R and pathological fear learning and stress dysregulation in PTSD suggests another potential therapeutic use for such treatments. Realization of this potential requires continued efforts to address the role of PACAP in learning and in the complex interactions of stress and sex on memory.

AUTHOR CONTRIBUTIONS

MG and NF wrote and edited the manuscript. Both authors contributed to the article and approved the submitted version.

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