PACAP Promotes Matrix-Driven Adhesion of Cultured Adult Murine Neural Progenitors

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Abstract

New neurons are born throughout the life of mammals in germinal zones of the brain known as neurogenic niches: the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus of the hippocampus. These niches contain a subpopulation of cells known as adult neural progenitor cells (aNPCs), which self-renew and give rise to new neurons and glia. aNPCs are regulated by many factors present in the niche, including the extracellular matrix (ECM). We show that the neuropeptide PACAP (pituitary adenylate cyclase-activating polypeptide) affects subventricular zone-derived aNPCs by increasing their surface adhesion. Gene array and reconstitution assays indicate that this effect can be attributed to the regulation of ECM components and ECM-modifying enzymes in aNPCs by PACAP. Our work suggests that PACAP regulates a bidirectional interaction between the aNPCs and their niche: PACAP modifies ECM production and remodeling, in turn the ECM regulates progenitor cell adherence. We speculate that PACAP may in this manner help restrict adult neural progenitors to the stem cell niche *in vivo*, with potential significance for aNPC function in physiological and pathological states.

Keywords

adult neural progenitor cells, adult neurogenesis, cell adhesion, extracellular matrix, pituitary adenylate cyclase-activating polypeptide, pituitary adenylate cyclase-activating polypeptide

Introduction

The adult mammalian brain contains a population of quiescent cells known as adult neural progenitor cells (aNPC), which can give rise to new neurons and glia throughout the lifetime of the individual (Altman, 1962). These cells populate two areas of the brain—the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus. The SVZ is located between the striatum and the ependymal cell layer that lines the lateral ventricles (Doetsch et al., 1997). It has been firmly established in rodents that the SVZ aNPCs give rise to transit amplifying cells that proliferate and migrate into the olfactory bulb. They contribute to interneuron replacement in the granule cell layer (Lois and Alvarez-Buylla, 1994) and to the maintenance of cellular circuitry of the olfactory bulb (Cummings et al., 2014). The function of SGZ

progenitors is less clearly defined. They give rise to mature excitatory neurons in the granule layer of the hippocampus and have been implicated in some forms of learning and memory (reviewed in Deng et al., 2010). In addition to their roles in physiological brain plasticity, aNPCs have been implicated in neural repair following injury and in neurodegenerative conditions.

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Thus, high hopes have been placed in aNPCs as a potential source of new neurons for cell replacement therapies of neurodegenerative diseases and brain injury (recently reviewed in López-Bendito and Arlotta, 2012; Bellenchi et al., 2013; Miller and Gomez-Nicola, 2014; Ruan et al., 2014).

aNPCs are maintained in a so-called neurogenic niche, where specialized components of the extracellular matrix (ECM) and soluble factors secreted by the stroma contribute to the maintenance of "stemness," that is, self-renewal and multipotency, by these progenitors (Kazanis et al., 2007; Ninkovic and Götz, 2007; Kazanis, 2009; Mercier, 2016). How these different signals are integrated to contribute to the stem cell phenotype of aNPCs is, however, poorly understood.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a secreted peptide with pleiotropic functions in the central nervous system and beyond (reviewed in Moody et al., 2011; Nakamachi et al., 2011; Shen et al., 2013; Waschek, 2013). Specifically, it has been shown to regulate the proliferation and survival of neuroblasts in the embryonic and postnatal brain (Vaudry et al., 1999; Nicot and DiCicco-Bloom, 2001; Suh et al., 2001; Nicot et al., 2002; Niewiadomski et al., 2013). Moreover, PACAP regulates the differentiation of neural progenitors into different neuronal and glial lineages (Lee et al., 2001; Vallejo and Vallejo, 2002; Nishimoto et al., 2007; Watanabe et al., 2007; Ohtsuka et al., 2008; Hirose et al., 2011). It exerts its actions on the cells through one of three G-protein coupled receptors: PAC1, which is specific for PACAP; VPAC1 and VPAC2, which have an equal affinity for PACAP and a related neuropeptide VIP (Harmar et al., 1998; Vaudry et al., 2000). PACAP is expressed at the SVZ (Mercer et al., 2004), and aNPCs express PAC1 and VPAC2 receptors (Mercer et al., 2004; Ohta et al., 2006; Scharf et al., 2008). PACAP protects aNPCs from a variety of pro-apoptotic insults (Mansouri et al., 2012, 2016, 2017) and has been shown to promote the proliferation and prevent differentiation of aNPCs cultured in the absence of growth factors, both when the cells were maintained as clonally derived neurospheres (Mercer et al., 2004; Sievertzon et al., 2005) and when they were cultured as a monolayer on a poly-lysine-coated surface (Scharf et al., 2008). Moreover, PACAP promotes the proliferation of SVZ and SGZ cells *in vivo* (Mercer et al., 2004; Ohta et al., 2006). The proliferative effect of PACAP is synergistic with epidermal growth factor (EGF) and is dependent on the phospholipase C-protein kinase C pathway (Mercer et al., 2004). Notably, previous studies have examined the effects of PACAP on aNPCs in cultures lacking other growth factors known to be essential for the maintenance of their stem cell identity. These factors, which are likely to be present in addition to PACAP in the neurogenic niches, include ligands of epidermal growth factor (EGF) receptors (transforming growth factor α [TGFα] or EGF) and fibroblast growth factor (FGF) receptors (such as basic FGF [bFGF]; Enwere, 2004; Ghashghaei et al., 2007; Zhao et al., 2007; Deleyrolle and Reynolds, 2009). Previous studies of the effects of PACAP on aNPCs have focused on growth factor-independent functions of PACAP (Mercer et al., 2004; Sievertzon et al., 2005; Scharf et al., 2008). To mimic the composition of signals that the aNPCs may be exposed to in the stem cell niche *in vivo*, that is, under nondifferentiation conditions, we cultured them in the presence of EGF and bFGF. We show here that under such experimental conditions, treatment of the cells with PACAP induced their attachment to rigid surfaces and that this effect is mediated by secreted components of the ECM.

Materials and Methods

Isolation and Culture of SVZ aNPCs

aNPCs were isolated from the SVZs of 7- to 8-week-old male C57Bl/6 mice or PAC1^{-/-} mice (Jamen et al., 2000) as described (Deleyrolle and Reynolds, 2009). Briefly, the mice were sacrificed by pentobarbital injection, and their skulls were opened to expose the brain. The brain was cooled in ice-cold DMEM/F12 supplemented with HEPES. The rostral part of the brain was sectioned coronally on a mouse brain slicer, and the periventricular region was excised using a scalpel blade from two to three 1-mm thick sections, starting from the ventral-most section in which the ventricle was apparent. The periventricular tissue was cut into small pieces using fine scissors and transferred to a conical tube containing ice-cold DMEM/F12 with HEPES. The tissue was transferred to a biological safety cabinet and washed thrice with sterile Hank's buffered salt solution. This solution was replaced with DMEM/F12 supplemented with B-27 (Life Technologies) and gently triturated with a 1-mL pipette tip. Trypsin (Trypsin-EDTA solution, Life Technologies) was added to a final concentration of 1 mg/mL and DNAse I (Worthington) was added to a final concentration of 0.1 mg/mL to dissociate the cells. The tissue was allowed to incubate at 37°C for 30 min with manual agitation after every 10 min interval. The trypsin was inactivated by adding fetal bovine serum to a final concentration of 10%. The tissue was then triturated using two sterile diminishing-bore fire-polished glass Pasteur pipettes and passed through a 40-μm cell strainer (Corning Falcon). The cells were centrifuged at $200 \times g$ for 2 min and resuspended at 10^4 cells/mL in neurosphere media, containing Neurobasal, L-glutamine (0.5 mM), penicillin/streptomycin (1×), B-27 supplement (1×), EGF (10 ng/mL), bFGF (10 ng/mL), and heparin (2 μg/mL). All neurosphere media components were purchased from Life Technologies, except heparin and EGF,

which were from Sigma, and bFGF, which was from Peprotech. The cells were placed in non tissue culture-treated 25-cm^2 flasks at 7×10^4 cells per flask (Nunc) for 7 days, and EGF and bFGF were readded at 5 ng/mL each on Day 3 and 5 of culture. The first neurospheres started appearing after 3 to 5 days and were fully grown by Day 7. For replating, the spheres were centrifuged at $200 \times g$ for 5 min, and the media was replaced with 500 µL Accutase (Life Technologies). The spheres were incubated for 5 min at 37°C, triturated with a 200 µL pipette tip, incubated for 10 more min at 37°C, and triturated with a small-bore fire-polished pipette until very few visible spheres remained. The suspension was centrifuged at $200 \times g$ for 5 min, and the cell pellet was resuspended in neurosphere media. The remaining spheres were removed from the suspension by passing it through a 40-µm cell strainer, the cells were then counted and replated at 10^4 cells/mL in nontissue culture-treated flasks (7×10^4 cells per flask). Experiments were performed on cells from Passage 3 to 6. All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of California Los Angeles (Protocol Number: 93-302, IACUC A3196-01).

Measurement of Cell Attachment

Neurospheres were dissociated in Accutase as described earlier, and plated into wells of a six-well cell culture plate (Nunc) at a 10^4 cells/mL in 2 mL of neurosphere media per well supplemented with PACAP-38 (referred to as PACAP in the text; Merck Millipore) and other peptides/drugs at indicated concentrations. For experiments involving forskolin (FSK; Sigma), all wells not treated with FSK were supplemented with 0.25% DMSO to control for DMSO used as FSK vehicle. The cells were incubated at 37°C for 1 week, and were supplemented with EGF, bFGF, PACAP, VIP, PHI, and FSK at half the initial concentration on Day 3 and 5. After 1 week, the neurosphere suspension was gently transferred to an Eppendorf tube, and the cells were centrifuged and dissociated using trypsin. The remaining attached cells were trypsinized in the well and then transferred to an Eppendorf tube. Trypsin was neutralized using 20% fetal bovine serum in DMEM. Trypsinized cells were triturated to obtain a single-cell suspension and the cells in each fraction (suspension vs. attached) were counted.

aNPC Differentiation

aNPCs were cultured for 7 days in complete neurosphere media until well-developed neurospheres formed. For anti-nestin staining, neurospheres were dissociated with Accutase and plated on poly-l-ornithine and fibronectin-coated glass coverslips in 24-well plates in neurosphere media containing EGF and bFGF. PACAP was added to selected wells and the cells were cultured for 7 days with a media change on Day 4. For all other immunofluorescence procedures, whole neurospheres (approximately 5×10^4 cells per well) were plated in neurosphere media in poly-l-lysine- and laminin-coated coverslips in 24-well plates. The cells were allowed to adhere to coverslips for 2 days in the presence or absence of 100 nM PACAP. Afterwards, neurosphere media was replaced with differentiation media containing Neurobasal, L-glutamine (0.5 mM), penicillin/streptomycin (1×), B-27 supplement (1×), heparin (2 µg/mL), and 1% fetal bovine serum without PACAP. The cells were allowed to differentiate for 5 days with half of the media changed every other day.

Immunofluorescence Staining

The cells were fixed in 4% paraformaldehyde for 5 min at room temperature, washed thrice for 5 min in phosphate-buffered saline (PBS). The cells were stained with the following antibodies: mouse anti-MAP2ab (1:500 cat.#M1406; Sigma), rabbit anti-glial fibrillary acidic protein (GFAP; 1:2000, cat.# Z0334; Dako), for 1 hr at room temperature, and secondary AlexaFluor488, AlexaFluor594, or AlexaFluor647-labeled donkey antibodies. Anti-nestin staining was performed overnight using the anti-nestin antibody (1:50 cat.# 556309, BD Pharmingen) followed by FITC-conjugated secondary anti-mouse antibody. Imaging was performed on a Zeiss AxioImager Z2 LSM 700 laser scanning confocal microscope using the Plan-Apochromat 20×/0.8 M27 air-immersion objective.

Live/Dead Cell Assay

aNPCs were dissociated with Accutase and plated on poly-l-lysine- and laminin-coated 6-well plates at 10^5 cells per well. They were allowed to grow in the presence or absence of PACAP for 5 days, then detached from the wells using Accutase for 3 min at 37°C. Cell dissociation was stopped using Neurobasal containing $1 \times$ B-27 supplement and the cells were centrifuged at $200 \times g$ for 5 min and resuspended in PBS. The cells were incubated for 5 min in propidium iodide solution, and propidium iodide uptake was measured by flow cytometry on the BD LSRFortessa instrument (Beckton Dickinson) using the 488 nm excitation laser and 610/20 nm band pass emission filter. Results were analyzed using FACSDiva 6.2 software.

Cell Cycle Assay

aNPCs were dissociated with Accutase and plated on poly-l-lysine- and laminin-coated 6-well plates at 10^5

cells per well. They were allowed to grow in the presence or absence of PACAP for 5 days, then detached from the wells using Accutase for 7 min at 37°C. Cell dissociation was stopped using Neurobasal containing $1 \times$ B-27 supplement and the cells were centrifuged at $200 \times$ g for 5 min. The cells were resuspended in PBS and then centrifuged at $2500 \times$ g for 5 min. The cells were resuspended in 300 μL of PBS, and 700 μL of −20°C ethanol was added. Ethanol-fixed cells were stored at −20°C. On the day of the assay, the cells were centrifuged at $300 \times$ g for 5 min at 4°C, the supernatant was decanted, and the cells were resuspended in 1 mL of 4°C PBS. The cells were re-centrifuged at $300 \times$ g for 5 min and resuspended in 0.5 mL of 4°C PBS. They were incubated in a solution containing saponin, propidium iodide, and RNAse for 30 min, and DNA content of cells was measured by flow cytometry on the BD LSRFortessa instrument as described for the live/dead cell assay.

Immunoblot

aNPCs were dissociated with Accutase and plated on poly-l-lysine- and laminin-coated 24-well plates at 5×10^4 cells per well. They were allowed to grow in the presence or absence of PACAP for 4 days and then washed with PBS and lysed with RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2% Igepal CA-630, 0.25% sodium deoxycholate, 1 mM NaF, $1 \times$ SigmaFAST protease inhibitor cocktail, and 1 mM DTT). Protein concentration was measured using the BCA assay and equal amounts of protein were loaded onto an SDS-PAGE gel. Following transfer, nitrocellulose membranes were blocked with the blocking buffer containing 5% bovine serum albumin in tris-buffered saline + 0.05% Tween-20 and incubated overnight at 4°C with anti-phospho-protein kinase A (PKA) substrate antibody (Cell Signaling cat. #9624), anti-phospho-PKC substrate antibody (Cell Signaling cat. #2261), or anti-α-tubulin antibody (Sigma cat. T6199) diluted to 1 μg/mL in the blocking buffer, followed by washes and incubation with HRP-conjugated secondary antibodies. Chemiluminescent signal was detected using the Amersham Imager 600RGB and densitometric measurements were performed in ImageJ.

Conditioned Media-Mediated Cell Attachment

PAC1-null aNPC neurosphere cultures were obtained from mice lacking the PAC1 receptor (Jamen et al., 2000), as detailed earlier. Wild-type (WT) mouse-derived neurospheres were treated with the indicated doses of PACAP for 7 days, and the conditioned media was collected from the culture and sterile-filtered to remove any remaining WT cells. The WT-conditioned media was placed in wells of a six-well plate for 5 days, and then removed. The wells were rinsed three times with sterile PBS. PAC1-null aNPC neurospheres were dissociated into a single-cell suspension using Accutase, and the cells were placed in conditioned media-treated wells in neurosphere media without PACAP. The cells were allowed to grow for 3 days, and their attachment to the well surface was examined using phase-contrast microscopy.

DNA Microarray Experiments

RNA was isolated using TRIzol from all cells (both floating and attached) treated for 24 hr or 96 hr with either vehicle (control) or PACAP (10 nM). RNA from five to six independent samples per group was pooled and cleaned using RNeasy spin columns (Qiagen), yielding ~10 μg RNA per treatment. The samples were submitted to the UCLA DNA Microarray Facility for hybridization with Affymetrix GeneChip Mouse Genome 430 2.0 arrays. The.CEL files obtained from each array scan were analyzed using the Affymetrix Expression Console suite (build 1.4.1.46) with the Robust Multichip Analysis algorithm to subtract background and normalize data. The obtained normalized $\log_2$ expression values for control samples at a given time point were subtracted from $\log_2$ PACAP-treated sample values from the same time point to obtain $\log_2$(PACAP/control) ratio values for each time point. Log_2 ratios of more than 1 (over 2-fold increase) or less than −1 (over twofold decrease) were considered significant. If a gene was represented by more than one probe set on the array, it was considered significantly changed if at least one of the probe sets showed up- or downregulation by more than two-fold. Raw microarray data (.CEL files) and robust multichip analysis results were submitted to Gene Expression Omnibus (GEO; accession number GSE66193).

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

aNPCs were dissociated with Accutase and plated on poly-l-lysine- and laminin-coated 24-well plates at 5×10^4 cells per well. They were allowed to grow in the presence or absence of PACAP for 4 days, then RNA was isolated using TRIzol. Reverse transcription was performed using the AMV First strand synthesis kit (NEB) with random primers. Real-time quantitative-polymerase chain reaction (PCR) was performed on the LightCycler 480 instrument using Roche LightCycler SYBR Green I Master Mix and the following primers: Lgals3 forward: CAGTGAAACCCAACG CAAAC, Lgals3 reverse: TTCCTTTCCCCAGTTATT GTCC; Tgfbr2 forward: CAAGTTTTGCGATGTG AGACTG, Tgfbr2 reverse: CCGTCTCCAGAGTAATG TTCTTG; Spon1 forward: TGTGGGAGTATGGAGG GTATG, Spon1 reverse: CTGGGCTTTGGCTTTGATG; GAPDH forward: GGCCTTCCGTGTTCCTAC,

GAPDH reverse: TGTCATCATACTTGGCAGGTT; Adamts6 forward: GAAGAGGAAACACTGGACT ACG, Adamts6 reverse: TCCGATGCTTGAATCC TGAAC; Fbln2 forward: AAATACCCCAGCATCC ACAG, Fbln2 reverse: CAACAGGTCTCGATT AGGTCC; Sparc forward: ATTGGCGAGTTTGAGAA GGT, Sparc reverse: TTGCATGGTCCGATGTAGTC; Olfml3 forward: TTTTGTCATGGACGGGACC, Olfml3 reverse: CTACTCTGATCCTGGCATTGG; Sulf1 forward: CTTCCAAACGACACAATCCAC, Sulf1 reverse: TCCC CTCACTTCCCTTAAATTC; Adcyap1r1 forward: AAAT GAGTCTTCCCCAGGTTG, Adcyap1r1 reverse: CCCC TATGGTTTCTGTCATCC; Vipr1 forward: CCCATC CTCATCTCCATCTTG, Vipr1 reverse: GAGGG ATGAGCAGAAGTGTG; Vipr2 forward: ACCTTGG GCTACAGTGTTTC, Vipr2 reverse: CCTTGACCAGC ACAGAGATG; Ecm1 forward: CTGTGGAAGT GGAAGGGTC, Ecm1 reverse: CAGAGGTTCTCG ATGAAGGC; Col6a1 forward: CTGGTGAAGGAGAA CTATGCAG, Col6a1 reverse: GTCTAGCAGGATGGT GATGTC; Npnt forward: TGCCCTATCGTGTTC CATG, Npnt reverse: ACTCTTCCAGTCGCACATTC.

Relative quantification of gene expression was performed using the $\Delta\Delta Cp$ method using GAPDH as the housekeeping gene.

Gene Ontology Analysis of DNA Microarray Results

Significantly upregulated or downregulated genes obtained from the DNA microarray experiments were submitted to the DAVID 6.7 online tool for the selection of significantly up- or downregulated functional gene groups (based on Gene Ontology [GO]) and group clusters using the RDAVIDWebService library for R. The GO terms used belonged to three annotation categories: GO_CC_FAT (cellular compartments), GO_BP_FAT (biological process), and GO_MF_FAT (molecular function). The three categories were selected to filter out overly broad GO terms.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism, R, and Microsoft Excel. Significant differences of mean cell numbers among multiple treatments were assessed using analysis of variance, and the post hoc Tukey's test was used to determine the statistical significance of pairwise mean differences. A value of $p < .05$ was considered significant.

Results

PACAP Induces Surface Attachment of aNPCs

To determine the effects of PACAP on aNPCs in the presence of growth factors, we cultured adult mouse-derived neurospheres in media containing EGF and bFGF. Under these conditions, PACAP induced attachment of the neurospheres to uncoated plastic surface of the dishes in a dose-dependent fashion (Figure 1(a, b)). This phenotype was not associated with cell differentiation because virtually all untreated and PACAP-treated cells expressed the aNPC marker nestin when cultured in neurosphere media in the presence of EGF and bFGF (Figure 1(c)). Consistent with the undifferentiated aNPC phenotype, the attached cells could form secondary and tertiary neurospheres upon dissociation and replating regardless of PACAP addition (Figure 1(d)). A small fraction (<5%) of cells in PACAP-treated but not in control cells stained positive for the astrocyte marker GFAP when plated as monolayers in the same growth medium on poly-L-lysine- and laminin-coated coverslips, suggesting that PACAP can promote astroglial differentiation of aNPCs even in the presence of EGF and bFGF (Figure 1(e)). However, even in PACAP-treated wells GFAP-positive cells were restricted only to some areas, especially those with highest cell densities. Both the cell cycle analysis and the live/dead cell assay revealed only very modest differences between PACAP-treated and untreated cells, suggesting that PACAP does not greatly affect cell proliferation or death (Figure S1). PACAP-treated cells, like control cells, are able to differentiate into MAP2ab-positive neurons and GFAP-positive astrocytes, which implies that PACAP does not limit the differentiation potential of aNPCs (Figure 1(f)). Nevertheless, astrocytes generated from PACAP-treated aNPCs have a mostly stellate appearance with many thin projections, whereas control astrocytes are flat and epitheloid in shape.

PACAP-Induced Cell Adhesion is Mimicked by VIP and PKA Activation

Because PACAP shares two receptor subtypes (VPAC1 and VPAC2) with another secreted polypeptide—VIP—we wanted to verify whether VIP was also able to induce aNPC attachment to dish surface in the presence of growth factors. VIP did induce some degree of adhesion, but it was significantly less potent than PACAP (Figure 2(a), (c), and (e)). A different PACAP/VIP receptor ligand, peptide histidine-isoleucine, showed no effect on aNPC attachment (Figure 2(a)). We tested the expression of the three PACAP receptor types at the mRNA level and found that the PACAP-specific PAC1 receptor was the dominant receptor subtype in aNPCs, with VPAC2 showing lower detection and VPAC1 undetectable. PACAP-mediated aNPC adhesion was also mimicked by FSK, an adenylate cyclase activator, suggesting that this effect is mediated through the adenylate cyclase-PKA pathway (Figure 2(c, e)). Consistent with this hypothesis, we found that
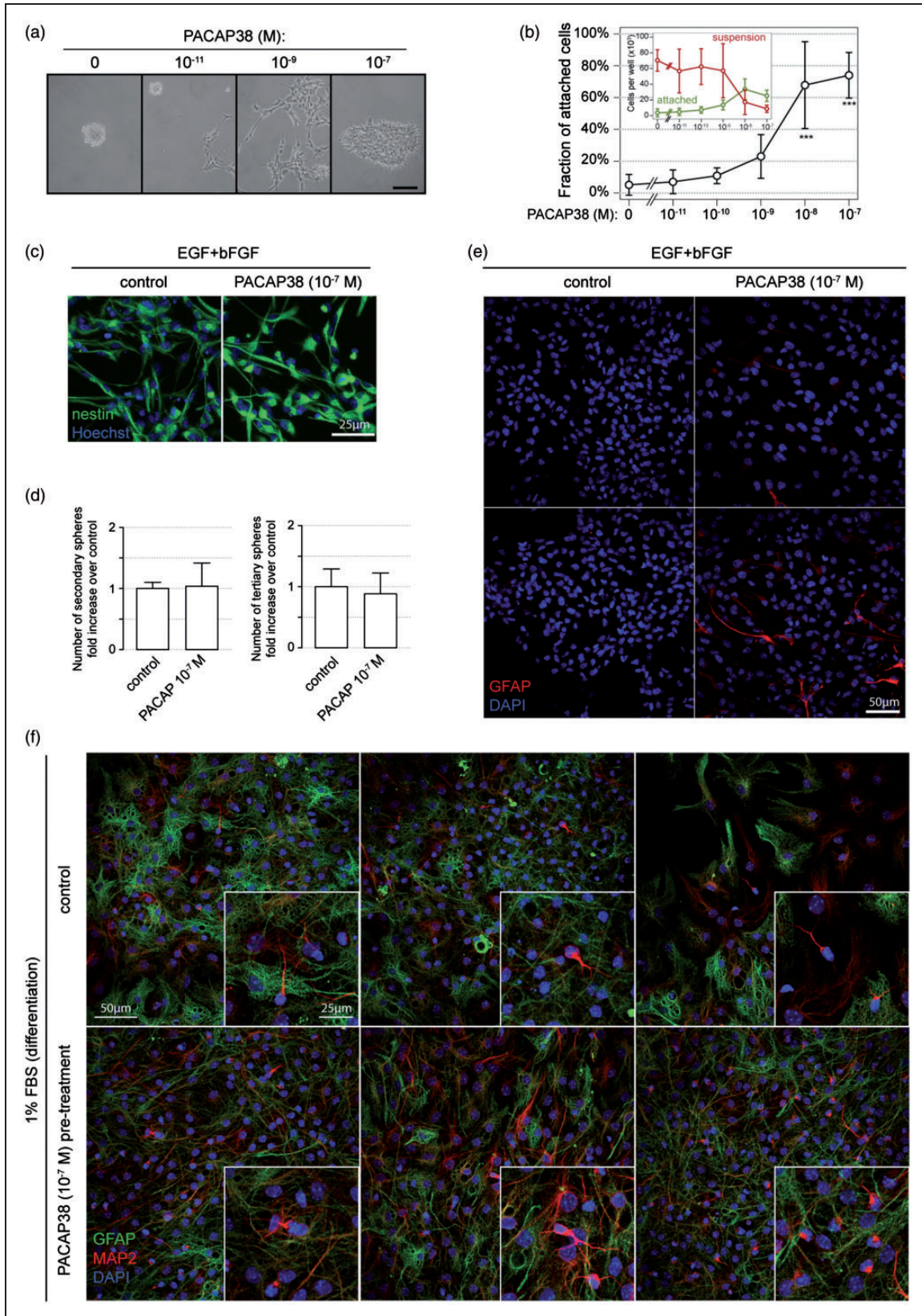
Figure 1. PACAP induces surface adhesion of aNPCs in the presence of growth factors. (a) aNPCs were grown in the presence of bFGF and EGF for 7 days on noncoated cell culture flasks in the absence or presence of indicated concentrations of PACAP38. Representative micrographs of cells are shown. Scale bar—50 µm. (b) aNPCs were grown in the presence of growth factors and the indicated concentrations of PACAP38 in six-well plates. Attached and unattached cells were counted from $n = 5$ wells. The graph shows mean fraction of attached cells $\pm$ standard deviation (SD) from one representative experiment out of three. PACAP concentration is placed on a logarithmic scale; *** $p < 0.001$ vs. control for Tukey's multiple comparison post hoc test. Inset: number of attached (green) and nonattached (red) cells

phosphorylation of PKA targets, but not targets of protein kinase C (PKC) was increased in PACAP-treated cells (Figure 2(d)).

PACAP Affects the Transcription of ECM Components and ECM-remodeling Enzymes in aNPCs

Because PACAP treatment of aNPCs increases attachment of spheres to the bottom of plastic dishes, we hypothesized that PACAP may affect the secretion or processing of ECM components in these cells. To test this hypothesis, we performed genome-wide transcriptional profiling of aNPCs untreated or treated with 10 nM PACAP for 1 or 4 days. Genes that were up- or downregulated more than two-fold by PACAP were then subjected to further analyses. PACAP upregulated the expression of 163 genes after 24 hr of treatment (Table S1). Eighty-two genes were upregulated at 96 hr, including 46 of those that were already induced after 1 day of PACAP treatment (Figure 3(a), Table S2). For some of the genes that were up-or downregulated by PACAP, we confirmed our microarray analysis results by performing quantitative real-time reverse transcription (RT)-PCR on independent samples of aNPCs that were cultured as a monolayer on poly-l-lysine- and laminin-coated plates. Consistent with our microarray analysis, PACAP (100 nM) treatment increased the expression of galectin 3 (Lgals3), TGFβ receptor 2 (Tgfbr2), sulfatase 1 (Sulf1), osteonectin (Sparc), fibulin 2 (Fbln2), ADAM metalloproteinase with thrombospondin Type 1 motif 6 (Adamts6), ECM protein 1 (Ecm1), collagen type VI α1 (Col6a1), and nephronectin (Npnt), and decreased the expression of F-spondin (Spon1; Figure 3(c)). Of the genes that we tested only fibronectin (Fn1) showed altered expression in microarray but not in RT-PCR

assays (not shown), suggesting that our microarray results are robust.

We used Ingenuity Pathway Analysis to suggest what upstream mediators were responsible for the effects of PACAP on gene transcriptions. Consistent with our findings suggesting the involvement of the cyclic adenosine monophosphate (cAMP)/PKA pathway in the effects of PACAP on aNPCs (Figure 2), the upstream regulator with the lowest p value (7×10^{-22}) at 24 hr of PACAP treatment was CREB, a known effector of PKA. The second highest ranked regulator was TGFβ (p value 2×10^{-19}). Moreover, one of the TGFβ receptors, TGFBR2, was upregulated by PACAP after 24 hr of treatment. At 96 hr of treatment, TGFβ was the most probable upstream mediator of the transcriptional effects of PACAP, suggesting that at least some of the effects of PACAP treatment are indirect, and depend on the upregulation of TGFβ signaling by the initial PACAP signal.

To determine if the observed PACAP-induced changes in gene expression were dependent on the presence of growth factors in the media, we compared our dataset to that of Sievertzon et al. (2005; ArrayExpress accession number E-MEXP-322), who also looked at the effect of PACAP on aNPCs, but in the absence of growth factors. Importantly, the sets of genes that were significantly regulated by PACAP in the presence of growth factors (this study) showed little overlap with the genes regulated by PACAP in the absence of growth factors (Sievertzon et al., 2005; Figure 3(d)). Specifically, the genes that were upregulated by PACAP in our study were equally likely to overlap with genes that were upregulated as with those that were downregulated by PACAP in the absence of growth factors. This analysis suggests that PACAP activates a different gene expression program depending on the presence or absence of growth factors.

Figure 1. Continued
per well from the same experiment expressed as mean ± SD. (c) PACAP does not induce differentiation of aNPCs. aNPCs were seeded on poly-L-ornithine- and fibronectin-coated coverslips and cultured for 7 days in neurosphere media in the absence or presence of PACAP. Undifferentiated aNPC marker nestin (green) was detected by immunofluorescence and nuclei were stained with the Hoechst 33342 dye (blue). (d) PACAP does not affect neurosphere formation by aNPCs. aNPCs were cultured as monolayers on poly-l-lysine- and laminin-coated plates in the absence or presence of PACAP for 2 days. Afterwards, they were dissociated and replated in neurosphere media without PACAP. For secondary sphere formation assay, the cells were plated at a density of three cells per well in 96-well plates and allowed to grow for 5 days. The number of spheres that grew in each well were counted using phase contrast microscopy. For the tertiary sphere formation assay, the cells were replated at 10^5 cells/well in a 12-well plate, and were dissociated and replated after 5 days into 96-well plates at a density of three cells per well as described earlier. Data are mean fold increase of sphere formation over control ± SD from $n = 4$ independent samples, each sample representing an average count of at least 10 wells. (e) PACAP increases the number of GFAP-positive cells in aNPC cultures. aNPC neurospheres were plated on poly-l-lysine- and laminin-coated coverslips and cultured in growth-factor containing neurosphere media in the presence or absence of PACAP for 2 days. Astrocyte marker GFAP (red) was detected by immunofluorescence and nuclei were stained with DAPI (blue). Two representative micrographs at low (upper) and high (bottom) cell densities are shown for each condition. (f) PACAP-treated and -untreated cells generate astrocytes and neurons upon differentiation. aNPC neurospheres were plated as in (e), but after 2 days, the growth factor- or PACAP-containing media was withdrawn and replaced with differentiation media containing 1% fetal bovine serum. After 5 days, the astrocyte marker GFAP (green) and the neuronal marker MAP2ab (red) were detected by immunofluorescence and nuclei were stained with DAPI (blue). Three representative micrographs are shown for each condition. Insets contain magnified fragments from each parent micrograph.
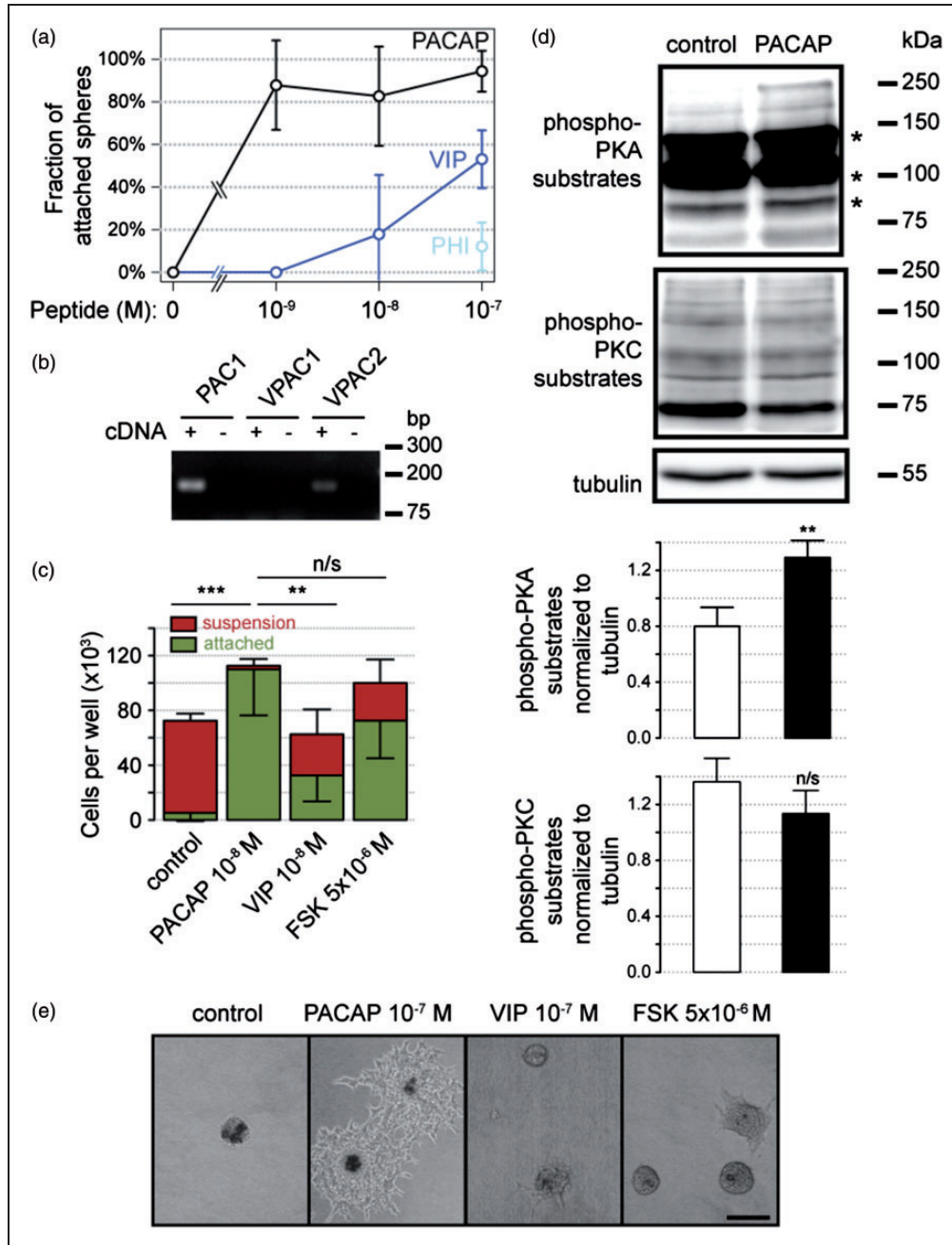
Figure 2. The effect of PACAP on aNPC attachment depends on a PACAP-selective receptor and the cAMP/protein kinase A pathway. (a) aNPCs were cultured in the presence of EGF and bFGF and the indicated concentrations of PACAP (black), VIP (dark blue), and PHI (light blue) for 7 to 8 days. Attached and nonattached neurospheres were counted in five fields of view at low magnification. The percentage of attached neurospheres $\pm$ SD from three independent experiments is shown. (b) Expression of genes for each of the three PACAP receptor types was analyzed by RT-PCR on mRNA from aNPCs cultured for 4 days on poly-lysine- and laminin-coated dishes. (c) Cultures of aNPCs were incubated in the presence of EGF and bFGF and the indicated peptides or forskolin (FSK) for 7 days. Attached and nonattached cells were counted as in Figure 1(b) from $n = 5$ wells. The stacked bar plot shows mean numbers of attached (green) and nonattached (red) cells per well $\pm$ SD. Fraction of attached cells was analyzed statistically using analysis of variance followed by the Tukey's post hoc multiple comparison test. *** $p < .001$, ** $p < .01$, n/s—nonsignificant. (d) Phosphorylation of protein targets by PKA and PKC was measured by immunoblot in lysates from cells cultured on poly-lysine- and laminin-coated dishes for 4 days in the absence or presence of 100 nM PACAP. Bar graph shows quantification of the intensity of phospho-PKA substrate and phospho-PKC substrate signal normalized to tubulin in $n = 3$ samples from each group $\pm$ SD. ** $p < .01$ in a two-sided t test. Asterisks indicate strong nonspecific bands in the phospho-PKA substrate immunoblot, which were excluded from the analysis. (e) aNPCs were grown for 5 days on nontreated cell culture flasks in the absence or presence of indicated concentrations of peptides and drugs. Representative micrographs of cells are shown. Scale bar—50 μm.
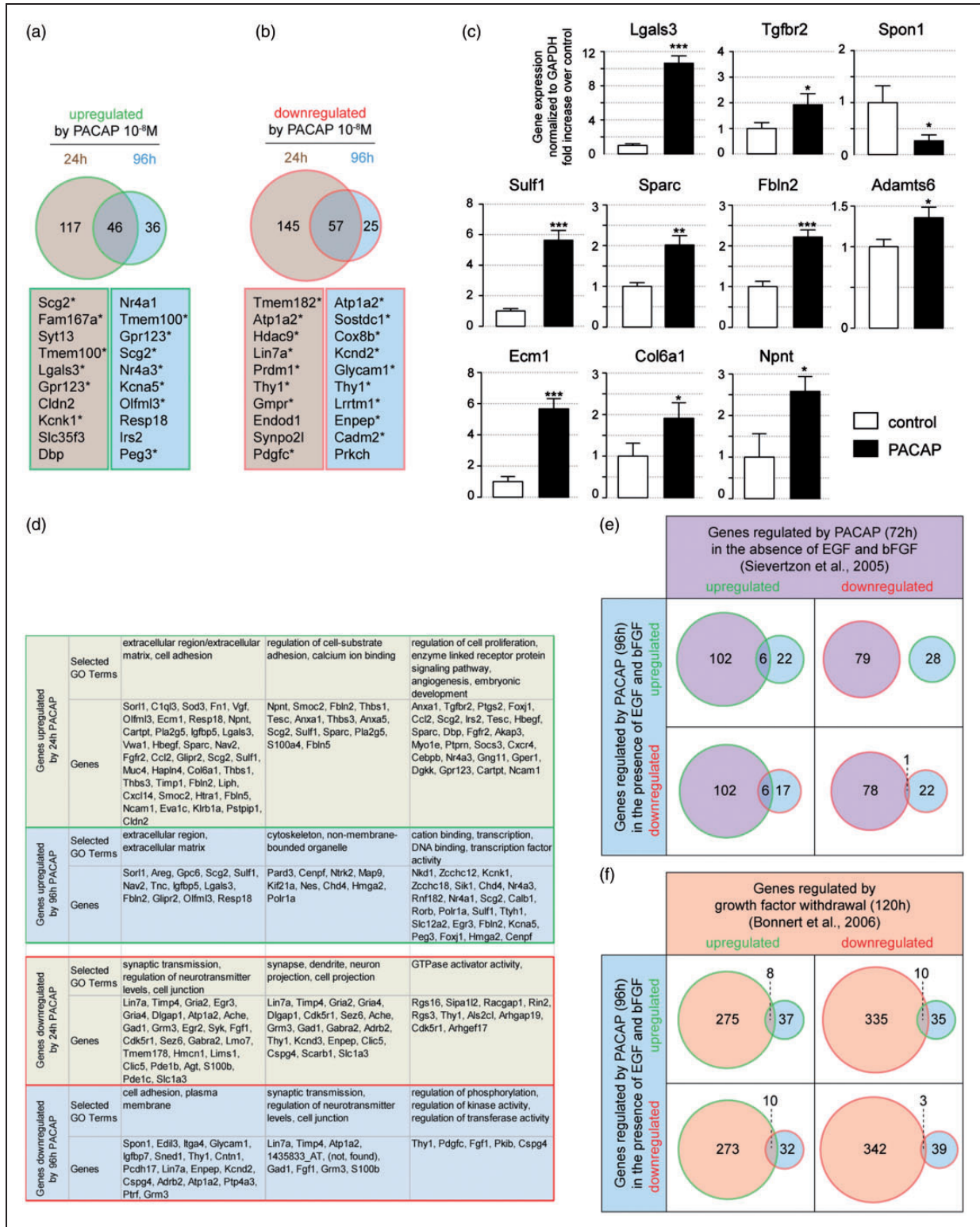
Figure 3. PACAP affects the gene expression program in aNPCs, but does not induce terminal differentiation. (a, b) Venn diagrams of genes up- and downregulated ((a) and (b), respectively) in aNPCs by 10^{-8} M PACAP after 24 hr (left, brown background) and 96 hr (right, blue background) of treatment. Top 10 up- and downregulated genes are enumerated for each treatment time. Genes that are up- or downregulated at both treatment times are marked with an asterisk. (c) Real-time quantitative RT-PCR analysis of the expression of selected genes that were up- or downregulated by PACAP. aNPCs were grown in monolayer in the absence (control) or presence of 100 nM PACAP for 4 days. Graphs show mean mRNA expression (arbitrary units) of selected genes $\pm$ SD from $n = 3$ independent samples;

Because aNPC adhesion is often associated with their differentiation, we wanted to more definitively rule out the possibility that PACAP-treated cells were losing their stem-like character. We thus compared our lists of up- and downregulated genes to the genes regulated in aNPCs by growth factor withdrawal (Bonnert et al., 2006; GEO accession number GSE4496; Figure 3(e)), and found no correlation between gene regulation by PACAP treatment and that of growth factor withdrawal. This finding is consistent with results suggesting that PACAP did not induce terminal differentiation of aNPCs in culture (Figure 1(c)).

We then grouped PACAP up- and downregulated genes (24 hr time point) based on their GO categories using the on-line DAVID tool (Database for Annotation, Visualization, and Integrated Discovery; Huang et al., 2009a, 2009b). Strikingly, the top two clusters of GO categories that resulted from this analysis included categories related to ECM, carbohydrate binding, and cell adhesion, which were consistent with the phenotype that we observed in PACAP-treated aNPCs (Figure 3(a, d), Table S3). The most significantly downregulated category clusters were related to synaptic transmission, suggesting that PACAP treatment inhibited neuronal differentiation of aNPCs (Figure 3(b, d), Table S4).

The Effects of PACAP Are Mediated Through Secreted Components

In aNPCs, PACAP upregulated the production of many secreted ECM components, such as ECM protein 1, collagen VI α1, hyaluronan and proteoglycan link protein 4, von Willebrand factor A domain containing 1, nephronectin, galectin 3, osteonectin, and fibulin 2. We therefore hypothesized that these components are responsible for the attachment phenotype seen in PACAP-treated aNPCs, as opposed to a direct effect of PACAP on the intrinsic ability of aNPCs to adhere. To validate this hypothesis, we tested the ability of conditioned media from PACAP-treated aNPCs (PACAP-CM) to induce adhesion of untreated cells. To that end, we preconditioned the wells with PACAP-CM and then removed

PACAP-CM, washed the wells with PBS and plated aNPCs in fresh neurosphere media without PACAP. In this way, only highly adhesive media components, like the ECM, remained in the conditioned wells, and PACAP itself, present in PACAP-CM, was washed out. To further rule out any effect of residual PACAP or autocrine PACAP secretion in this assay, we tested the effects of well preconditioning on cells isolated from mice lacking the PAC1 receptor (PAC1$^{-/-}$;Jamen et al., 2000). We found that PACAP-CM-treated wells induce the adhesion of PAC1$^{-/-}$ aNPCs (Figure 4(a)), which is consistent with a model of PACAP-mediated aNPC adhesion that involves the secretion of ECM components to the media (Figure 4(b)).

Discussion

The neuronal population in the postnatal brain had long been thought to be static, with no new neurons being generated past a certain age. The discovery of adult neurogenesis (Altman, 1962) not only broke this long-standing dogma but also raised new hopes for regenerative medicine. However, the use of the organism's intrinsic ability to generate new neurons in replacement therapy for neurodegenerative diseases and acute injuries has so far proved to be an elusive goal (Lindvall and Kokaia, 2010). The main problem appears to be the incomplete understanding of the regulation of adult neurogenesis in physiological and pathological conditions *in vivo*. Therefore, significant effort is being made to decipher the signaling pathways that regulate aNPC self-renewal, migration, and differentiation.

PACAP is a short polypeptide initially discovered as a regulator of pituitary function in mammals. It has been shown to have significant potential as a neuroprotective agent *in vitro* and *in vivo* (Waschek, 2013; Mansouri et al., 2016, 2017). Importantly, PACAP is upregulated during brain ischemia (Stumm et al., 2007; Riek-Burchardt et al., 2010; Lin et al., 2015) and in the cortex following traumatic brain injury (Skoglösa et al., 1999), further supporting the notion that it may be one of the endogenous signals that promote repair during neurodegenerative insults. In addition to its neuroprotective

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Figure 3. Continued

$*p < .05$, $**p < .01$, $***p < .001$ in a two-sided t test. The genes examined in the assay correspond to the following proteins: Lgals3—galectin 3, Tgfbr2—TGFβ receptor 2, Spon1—F-spondin, Sulf1—sulfatase 1, Sparc—osteonectin, Fbln2—fibulin 2, Adamts6—ADAM metallopeptidase with thrombospondin type 1 motif 6, Ecm1—extracellular matrix protein 1, Col6a1—collagen type VI α1, Npnt—nephronectin. (d) Top three categories (clusters) of genes up- or downregulated in aNPCs by PACAP treatment. (e, f) Venn diagrams comparing genes up- and downregulated by PACAP in the presence of growth factors (96 hr treatment) with those up- and downregulated by PACAP in the absence of growth factors for 72 hr (Sievertzon et al., 2005), (d) and those up- and downregulated in cells induced to differentiate by 120-hr long growth factor withdrawal (Bonnert et al., 2006). (e) For (d) and (e), only genes that are represented in both datasets were used for the analysis; hence, different total numbers of genes up- and downregulated by PACAP in the presence of growth factors between panels (a), (b) and (d), or (e).
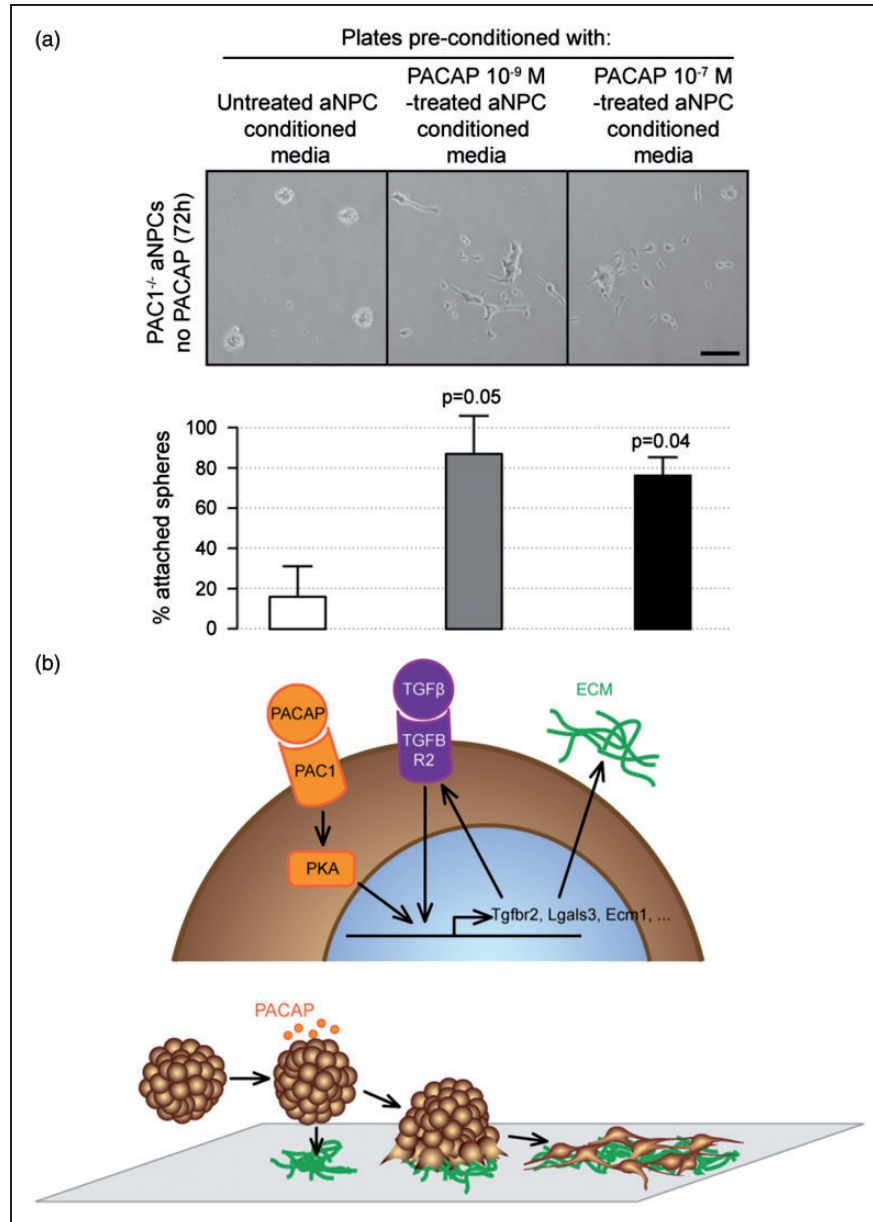
Figure 4. PACAP regulation of the extracellular matrix deposition alters aNPC cell adhesion. (a) PAC1$^{-/-}$ aNPCs were seeded on plates preconditioned with media from WT aNPCs treated for 1 week with the indicated concentrations of PACAP or with vehicle. The PAC1$^{-/-}$ aNPCs were subsequently cultured for 72 hr in the absence of PACAP. Representative images of cells are shown. Scale bar—50 μm. (b) Model of the role of PACAP in inducing adhesion of aNPC neurospheres to surfaces. PACAP binds to PAC1 receptors on the surface of aNPCs and stimulates the cAMP/PKA pathway. This results in the production of ECM components, and also of the TGFβ receptor 2. We speculate that TGFβ1 constitutively secreted by aNPCs activates TGFβR2 in an autocrine and paracrine manner. Both the PAC1/PKA pathway and the TGFβR2 pathway further increase the production of ECM by aNPCs. ECM components are secreted to media and adhere to the surface of the culture dish, which creates an adhesive surface to which aNPCs can attach.

potential, PACAP and its PAC1 receptor have been shown to affect neural progenitors in the embryonic and adult nervous system (Mercer et al., 2004; Ohta et al., 2006; Ago et al., 2011). Extending these published data, our results suggest that during neurodegeneration, PACAP might also be useful in replacing lost neurons by regulating the extracellular molecular environment of endogenous neurogenic niches.

Despite a well-established influence of PACAP on embryonic and adult neural progenitors, its mechanisms

of action are unclear. Previous work that attempted to map PACAP-induced transcriptional changes in aNPCs failed to uncover novel regulatory mechanisms, most likely due to the fact that the study was conducted under conditions of growth factor withdrawal (Sievertzon et al., 2005). In contrast, we show here that in the presence of two growth factors, EGF and bFGF, PACAP promotes aNPC adhesion by modifying their transcriptional output. Interestingly, when similar studies were conducted on embryonic rather than adult neural progenitors cultured as neurospheres in the presence of growth factors, no such PACAP-dependent cellular attachment was reported (Ohta et al., 2006). In embryonic neural progenitors, PACAP and PAC1 have moreover been reported to regulate cell migration *in vitro* and *in vivo* (Toriyama et al., 2012; Adnani et al., 2015, p. 1). This implies that the effects of PACAP on neural progenitors are varied and dependent on the developmental stage.

The fact that VIP is less potent than PACAP at inducing attachment of aNPCS strongly suggests that the principal receptor that mediates the effects of PACAP on aNPCs is the PACAP-preferring PAC1 receptor, which has approximately 1,000-fold higher affinity for PACAP than for VIP or PHI (Harmar et al., 1998) and which is abundantly expressed in aNPCs (Mercer et al., 2004; Scharf et al., 2008; Mansouri et al., 2012). Based on the transcriptional profile of PACAP-treated aNPCs, the downstream effectors of PAC1 in this context appear to be the cAMP-PKA pathway and, indirectly, the TGFβ pathway. aNPCs have been shown to express TGFβ1 (Klassen et al., 2003), suggesting that they may undergo autocrine and paracrine regulation by the TGFβ pathway. Importantly, this signaling pathway is known to affect both adult neurogenesis (Buckwalter et al., 2006; Wachs et al., 2006; Kandasamy et al., 2014) and extracellular protein secretion of neural cells (Hellbach et al., 2014).

We discovered that the enhanced aNPC attachment is mediated by factors that are secreted to conditioned media by PACAP-treated cells. We also identified a large number of ECM components, ECM modifying enzymes, and their inhibitors whose expression is regulated by PACAP in aNPCs. Taken together, this data strongly suggest that PACAP affects ECM production and modification by aNPCs.

The ECM has a well-established role in the maintenance of the neurogenic niches in both the embryonic and adult nervous system (Kazanis and ffrench-Constant, 2011; Theocharidis et al., 2014; Reinhard et al., 2016). However, very few ECM components have been studied in detail in the context of adult neurogenesis. The genes differentially regulated by PACAP in aNPCs include several integrin substrates (nephronectin and collagen VI) and other ECM glycoproteins (fibulins 2 and 5, mucin

4, olfactomedin-like 3) as well as glycoprotein-binding proteins Hapln4 and two lectins: galectin 3 and Nkrp1a. Interestingly, galectin 3 has been recently implicated in neuroblast migration from the SVZ to the olfactory bulb (Comte et al., 2011) and a related lectin galectin 1 was shown to play a key role in SVZ neurogenesis (Ishibashi et al., 2007, p. 1). In addition, PACAP increased the production of multiple so-called matricellular proteins (Bornstein and Sage, 2002): osteonectin and the related protein F-spondin, Smoc2, thrombospondin 3, and tenascin C. These proteins do not fulfill the typical ECM functions of mechanical scaffolding, but rather are modulators of cell-ECM interactions. Of these matricellular proteins, only tenascin C has been implicated to some degree in adult neurogenesis (Kazanis et al., 2007).

Besides regulating ECM component expression, PACAP might direct the remodeling of ECM by aNPCs through the modulation of expression of extracellular ECM-modifying enzymes and their inhibitors. The serine protease HtrA1 and tissue inhibitor of metalloproteinase (TIMP) 1 was upregulated, whereas TIMP 4 and several members of the ADAM/ADAMTS family of metalloproteinases were downregulated in PACAP-treated cells. Moreover, by downregulating the expression of heparan sulfate 3-O-sulfotransferase 2 and upregulating the expression of sulfatase 1, PACAP may affect the metabolism of heparan sulfate proteoglycans, which had been suggested to affect aNPC fate decisions (Chipperfield et al., 2002) and participate in the formation of specialized ECM niche structures known as fractones (Mercier et al., 2002; Mercier, 2016).

Very little is currently known about the ability of neural progenitors to remodel their niche through the production or degradation of ECM. It has mostly been assumed that, with few exceptions (Kazanis et al., 2010), the ECM in the niche is a product of cells that surround the NSCs rather than NSCs themselves. Our study suggests that neural progenitors derived from the adult brain also have a rich and regulatable secretome, which in turn feeds back into their behavior in an autocrine and/or paracrine manner.

An important question going forward is whether PACAP itself is in fact required for the ECM-mediated maintenance of adult neurogenesis *in vivo*. Some behavioral effects of PAC1 and PACAP deficiency have been reported in mice, but their link to adult neurogenesis is unclear (Hannibal, 2002; Mustafa et al., 2015).

Moreover, it will be interesting to determine which cells secrete PACAP at the neurogenic niche and at the site of injury. A recent study suggests that PACAP released from sites of ischemic stress may promote stem cell migration toward hypoxic lesions (Lin et al., 2015), and the involvement of ECM remodeling in this process will require further investigation. Future studies using

conditional knockout animals should bring us closer to resolving these important issues.

Other than the identity of cells that produce PACAP, it will be important to determine what classes of cells respond to PACAP treatment both within neurospheres and *in vivo*. Neurospheres are known to be composed of a heterogeneous population of cells at various stages of differentiation, from multipotent stem cells, through partially differentiated progenitors, all the way to some postmitotic glial and neuronal cells (Suslov et al., 2002; Parmar et al., 2003; Reynolds and Rietze, 2005; Jensen and Parmar, 2006). In our hands, cells derived from cultured neurospheres exhibited essentially uniform staining for nestin, a neural progenitor marker (Figure 1(c)), suggesting that few of the cells in the spheres were terminally differentiated. However, nestin is expressed both in multipotent neural stem cells and in partially differentiated progenitors in the adult central nervous system (Doetsch et al., 1997; Imayoshi et al., 2011), and we show that PACAP promotes the expression of the astrocyte marker GFAP in cells cultured in the presence of EGF and bFGF. Moreover, PACAP changes the phenotype of astrocytes that are generated from aNPCS from epitheloid (Type I-like) to stellate (Type II-like; Raff et al., 1983). Therefore, we cannot exclude the possibility that PACAP affects the "stemness" or differentiation potential of at least some neural progenitor classes. Future work should focus on determining which cells within the neurospheres are responsible for the observed increase in ECM component expression and whether PACAP affects the expression profile of these cells or the relative abundances of progenitor populations within neurospheres. An especially promising avenue of research would be to perform single-cell transcriptomics on aNPCs cultured in the presence or absence of PACAP.

Finally, our study shows that the "neurosphere assay" as a proxy for "stemness" in neural progenitors and cancer cells (Cohen et al., 2010) comes with serious caveats, as has been discussed before (Reynolds and Rietze, 2005). Specifically, PACAP causes aNPCs to no longer grow as neurospheres, but does not induce changes suggestive of the loss of stemness. We should therefore approach conclusions based on whether or not cells grow as floating nonadherent neurospheres with caution, and use multiple secondary assays to validate the self-regenerative potential of a putative stem cell population. Moreover, the neurospheres have only limited resemblance to the *in vivo* neurogenic niche, and therefore it will be important to find out, using targeted loss-of-function experiments, which of the PACAP-regulated genes are in fact expressed by neural progenitors *in vivo* and how each of them individually affects the behavior of adult brain stem cells in physiological states and in disease.

Author Contributions

J. A. W. and P. N. conceived the study; P. N., G. C. C., and J. R. C. performed experiments; J. A. W., P. N., G. C. C., J. R. C., and T. P. analyzed data and cowrote the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Supplementary material

Supplementary material for this paper can be found at http://journals.sagepub.com/doi/suppl/10.1177/1759091417708720

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