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Augmented Cystine–Glutamate Exchange by Pituitary Adenylate Cyclase-Activating Polypeptide Signaling via the VPAC1 Receptor

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ABSTRACT In the central nervous system, cystine import in exchange for glutamate through system x_c^- is critical for the production of the antioxidant glutathione by astrocytes, as well as the maintenance of extracellular glutamate. Therefore, regulation of system x_c^- activity affects multiple aspects of cellular physiology and may contribute to disease states. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuronally derived peptide that has already been demonstrated to modulate multiple aspects of glutamate signaling suggesting PACAP may also target activity of cystine–glutamate exchange via system x_c^-. In this study, 24-h treatment of primary cortical cultures containing neurons and glia with PACAP concentration-dependently increased system x_c^- function as measured by radiolabeled cystine uptake. Furthermore, the increase in cystine uptake was completely abolished by the system x_c^- inhibitor, (*S*)-4-carboxyphenylglycine (CPG), attributing increases in cystine uptake specifically to system x_c^- activity. Time course and quantitative PCR results indicate that PACAP signaling may increase cystine–glutamate exchange by increasing expression of xCT, the catalytic subunit of system x_c^-. Furthermore, the potentiation of system x_c^- activity by PACAP occurs via a PKA-dependent pathway that is not mediated by the PAC1R, but rather the shared vasoactive intestinal polypeptide receptor VPAC1R. Finally, assessment of neuronal, astrocytic, and microglial-enriched cultures demonstrated that only astrocyte-enriched cultures exhibit enhanced cystine uptake following both PACAP and VIP treatment. These data introduce a novel mechanism by which both PACAP and VIP regulate system x_c^- activity. **Synapse 68:604–612, 2014.**

INTRODUCTION

In the central nervous system (CNS), cystine–glutamate exchange is critical for the maintenance of extracellular glutamate concentrations (Baker et al., 2002; Moran et al., 2005; Murphy and Baraban, 1990) as well as supplying cystine to astrocytes for glutathione production (Kranich et al., 1998; Sagara et al., 1993). The cystine–glutamate antiporter, system x_c^-, composed of the light chain xCT and heavy chain 4F2hc subunits, is a sodium independent amino acid transporter that exchanges one molecule of extracellular cystine for one molecule of intracellular glutamate (Bannai, 1986; Bannai and Kitamura, 1980; Sato et al., 1999). Nonvesicular extrasynaptic glutamate release by system x_c^- expressed on astrocytes may be important for activation of extrasynaptic NMDA and group II/III metabotropic glutamate receptors that, in turn, affect neuronal excitability (Kupchik et al., 2012; Moran et al., 2005; Pow, 2001). In support, nonvesicular glutamate released by the cystine–glutamate antiporter is reported to be an essential regulator of extrasynaptic glutamate concentrations in the CNS (Baker et al., 2002). Moreover, dysregulation of extrasynaptic glutamate concentrations resulting from altered system x_c^- activity has been implicated in glutamate excitotoxicity (Piani and Fontana, 1994) and psychiatric

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disorders (Baker et al., 2003, 2008). With the large array of pathological states in the CNS stemming from both insufficient glutathione synthesis and dysfunctional glutamate homeostasis, system x_c^- is positioned to heavily influence a variety of pathologies ranging from compulsive disorders to neurodegenerative disease (Berman et al., 2011; de Groot and Sontheimer, 2011; Grant et al., 2007, 2009; Knackstedt et al., 2009; Park et al., 2004; Sontheimer, 2011; Zhou and Kalivas, 2008). Unfortunately, the regulation of this heterodimeric amino acid transporter is poorly understood.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic neuropeptide with widespread expression throughout the CNS and periphery (Ghatei et al., 1993; Hannibal, 2002; Vaudry et al., 2009). In the CNS, substantial evidence has linked PACAP signaling to glutamate neurotransmission through its modulation of the ionotropic glutamate receptors NMDA and AMPA (Chen et al., 1999; Costa et al., 2009; Macdonald et al., 2005; Yaka et al., 2003). Furthermore, all PACAP receptors, including the high affinity PAC1 receptor as well as the vasoactive intestinal polypeptide (VIP) receptors VPAC1 and VPAC2, are reportedly expressed on astrocytes (Ashur-Fabian et al., 1997; Grimaldi and Cavallaro, 1999; Grimaldi and Cavallaro, 2000; Masmoudi-Kouki et al., 2007; Suzuki et al., 2003; Tatsuno et al., 1991) suggesting a significant involvement of PACAP signaling in the regulation of astrocyte function. This notion is further supported by PACAP treatment of primary astrocyte cultures producing increased expression and function of the glial glutamate transporters GLT-1 and GLAST (Figiel and Engele, 2000), which are needed for clearance of vesicular glutamate released by neurons from the synapse to prevent spillover of synaptic glutamate into extrasynaptic areas (Asztely et al., 1997; Diamond and Jahr, 2000; Zheng et al., 2008). Importantly, PACAP synthesis and transmission may be exclusively released from glutamate neurons in the CNS (Fahrenkrug and Hannibal, 2004; Hannibal et al., 2000) allowing PACAP to have a potential modulatory role on glutamate signaling at both the neuron as well as on the surrounding glia.

While PACAP appears to be a critical signal for astrocytes (Masmoudi-Kouki et al., 2007) as well as a key modulator of glutamate signaling (Magistretti et al., 1998), the affects of PACAP signaling on system x_c^- function are unknown. To test whether PACAP may regulate the activity of the cystine–glutamate antiporter, we performed radiolabeled cystine uptake assays in primary cortical cultures following PACAP treatment. System x_c^- activity was potentiated following 24-h treatment with PACAP and subsequent experiments were performed to determine the specific mechanism mediating the increased cystine–glutamate exchange produced by PACAP application in these cultures.

MATERIALS AND METHODS

Materials

Timed pregnant female Swiss Webster mice (Charles River Laboratories; Wilmington, DE) were housed in a climate-controlled room with a 12 hr light/dark cycle. Animals had free access to standard diet (Harlan 8604 formulation) and water until preparation of cell cultures. All procedures using animals were approved by the Marquette University Institutional Animal Care and Use Committee. PACAP was obtained from California Peptide Research (Napa, CA), PACAP6-38 from Anaspec (Fremont, CA), and VIP, VIP6-28, PG 97-269, PG 99-465 from Bachem (Torrance, CA). (S)-4-carboxyphenyglycine (CPG) was purchased from Tocris Bioscience (R&D Systems; Minneapolis, MN), and H89 from Sigma (St. Louis, MO). All other common chemicals were purchased from Sigma (St. Louis, MO).

Cortical cell cultures

Primary mixed cortical cultures containing both neurons and glia were prepared from embryonic day 15-16 mouse pups as previously described (Lobner, 2000). Dissociated cortical cells suspended in Eagles' Minimal Essential Medium (MEM, Earle's salts, supplied glutamine-free) supplemented with 5% heat-inactivated horse serum (Atlanta Biologicals; Lawrenceville, GA), 5% fetal bovine serum (Atlanta Biologicals), 2 mM glutamine and glucose (total 21 mM) were plated on 24-well plates coated with poly-D-lysine and laminin. Neuronal-enriched cultures were prepared in an identical manner with the addition of 10 μM cytosine arabinoside 48 h after plating cultures to inhibit glial replication. Neuronal-enriched cultures using this method have astrocyte levels of $<1\%$ of the total cell population (Dugan et al., 1995; Rush et al., 2010). Astrocyte-enriched glial cultures were prepared using a similar protocol to that of the mixed cortical culture, however cortices were obtained from postnatal day 1-3 mouse pups (Choi et al., 1987; Rush et al., 2010; Schwartz and Wilson, 1992). To obtain microglial cultures, microglia were collected after shaking astrocyte-enriched glial cultures and then plated in media containing 10 ng/ml colony stimulating factor (CSF) (Barger and Basile, 2001). All cultures were maintained in humidified 5% CO_2 incubators at 37°C.

Radiolabeled cystine uptake

Radiolabeled cystine uptake was measured as previously described (Liu et al., 2009, 2012, 2014; Rush et al., 2012). Briefly, before treatment on in vitro day 15, cultures were washed in serum-free media and incubated for a specified period with or without drug.

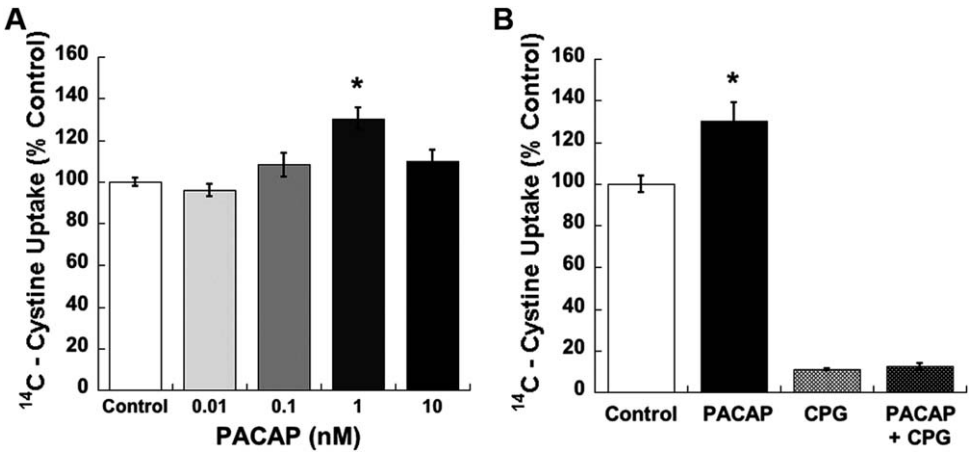

Fig. 1. PACAP increases cystine uptake through system x_c^- in a concentration-dependent manner. (**A**) Concentration response of 24-h PACAP treatment on primary mixed cortical cultures on radiolabeled cystine uptake (n = 6–12). (**B**) Inhibition of system x_c^- with (S)-4-carboxyphenylglycine (CPG; 200 μM) blocks cystine uptake in both control and PACAP (1 nM) treated primary mixed cortical cultures (n = 4–12). Data are expressed as mean ± SEM. *, $P < 0.05$ compared with control group.

Following incubation, cultures were then washed with HEPES buffered saline solution and immediately exposed to 0.3 μM ^{14}C-cystine (Perkin Elmer; Waltham, MA) for 20 minutes. This concentration of ^{14}C-cystine was used to simulate the estimated level of extracellular cystine found in the brain (Baker et al., 2003). Following ^{14}C-cystine exposure the cultures were washed with HEPES buffered saline solution and dissolved in 250 μl of 0.1% sodium dodecyl sulfate. Of the 250 μl sample, 200 μl were used for scintillation counting, with the remaining sample used for protein quantification using the Bio-Rad DC protein assay (Hercules, CA). Counts were normalized protein concentration in each well and expressed as a percentage of ^{14}C-cystine uptake in controls on the same experimental plate. For experiments using CPG, 200 μM CPG was added to the ^{14}C-cystine solution during the 20 minute uptake experiment.

Quantitative reverse transcription PCR

Cultures used for qRT-PCR were treated the same as for radiolabeled cystine uptake assays. However, following drug incubation, cells were harvested for total RNA extraction using TRiZOL (Life Technologies; Grand Island, NY). Single-stranded cDNA synthesis was performed with 1 μg total RNA using the Promega Reverse Transcription System (Madison, WI). Real-time quantitative PCR was performed with the StepOne real-time PCR system (Applied Biosystems; Carlsbad, CA) using PerfeCTa SYBR Green FastMix containing ROX (Quanta Biosciences; Gaithersberg, MD). Relative quantification of xCT transcripts was analyzed via the $\Delta\Delta C_t$ method normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers for each gene were designed to span an exon–exon junction and had efficiencies of approximately 95%. Product sizes for each primer set were 88 bp for xCT and 116 bp for GAPDH. Melt curve analysis of experiments confirmed a single product for each reaction. Primer sequences were as follows: xCT forward – AGG GCA TAC TCC AGA ACA CG; xCT reverse – GAC AGG GCT CCA AAA AGT GA; GAPDH forward – AAG GGC TCA TGA CCA CAG TC; and GAPDH reverse – GGA TGC AGG GAT GAT GTT CT.

Statistics

Data are presented as means ± standard errors of the mean, and were analyzed statistically by analysis of variance. Fischer LSD analysis was used for all post hoc group comparisons. Statistical analyses were performed using Sigma Plot 11 software (Systat Software Inc.; San Jose, CA). $P < 0.05$ were considered statistically significant.

RESULTS

The effect of PACAP on system x_c^- activity was initially tested in primary mixed cortical cultures following 24-h treatment of PACAP using concentrations ranging from 0.01 to 10 nM. Radiolabeled cystine uptake was significantly increased only at a concentration of 1 nM PACAP (Fig. 1A; $P < 0.01$). Consequently, we used the 1 nM concentration of PACAP for all future experiments examining cystine uptake following PACAP treatment unless otherwise stated. To determine whether the increased cystine uptake induced by PACAP could be attributed completely to increased system x_c^- activity, we coapplied the system x_c^- inhibitor CPG (200 μM) with the radiolabeled cystine during uptake experiments, as the use of CPG for inhibition of system x_c^- has been used previously in a similar manner without negatively affecting cell viability (Fogal et al., 2007; Liu et al.,

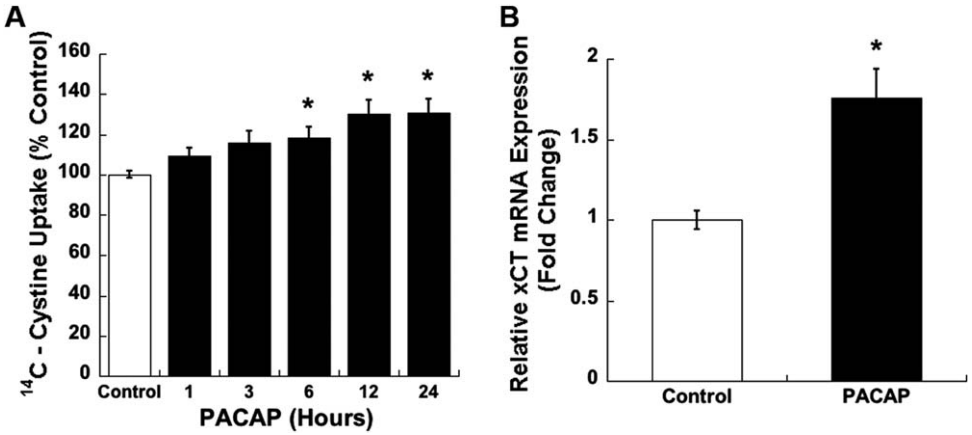

Fig. 2. Time course of cystine uptake and quantitative PCR for xCT mRNA expression following PACAP treatment. (**A**) A time course revealed a delayed effect of PACAP (1 nM) on cystine uptake in primary mixed cortical cultures. PACAP treatment for 6, 12, and 24 h yielded significant increases in radiolabeled cystine uptake (n = 6–8). (**B**) Quantitative PCR from RNA isolated from control and 24-h PACAP (1 nM) treated primary mixed cortical cultures (n = 7–8). Data are expressed as mean ± SEM. *, $P < 0.05$ compared with control group.

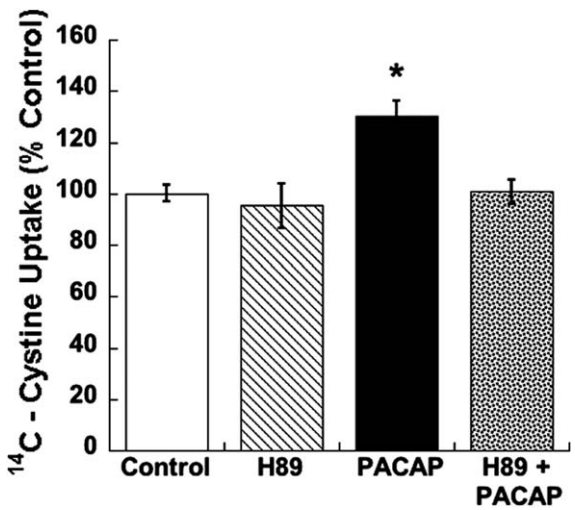

Fig. 3. PACAP increases cystine uptake via a PKA-dependent pathway. 10 μM of the PKA inhibitor H89 completely blocked increases in cystine uptake induced by 24-h PACAP (1 nM) treatment in primary mixed cortical cultures (n = 8–16). Data are expressed as mean ± SEM. *, $P < 0.05$ compared with control group.

2012, 2014). CPG inhibited approximately 90% of radiolabeled cystine uptake in control cells as well as the increase in system x_c^- activity following 24-h PACAP treatment (Fig. 1B; $P < 0.05$).

To determine the optimal incubation period for PACAP treatment needed to augment system x_c^- activity, mixed cortical cultures were incubated with 1 nM PACAP for 1, 3, 6, 12, and 24 h before the radiolabeled cystine uptake assay. PACAP treatment produced significant increases in cystine uptake following incubation times ranging from 6 to 24 h (Fig. 2A; $P < 0.05$). The necessity for long-term treatment with PACAP to significantly increase cystine uptake in primary mixed cortical cultures suggests that PACAP may be involved with the transcriptional regulation of the cystine–glutamate antiporter. Using quantitative real-time PCR (qPCR) we measured mRNA expression of the catalytic subunit of system x_c^-, xCT. Relative quantification for xCT mRNA was performed on total RNA extracted from control or PACAP-treated primary mixed cortical cultures for 24 h normalized to the housekeeping gene GAPDH. PACAP treatment for 24 h produced significantly increased xCT mRNA levels in these cultures (Fig. 2B; $P < 0.01$).

PACAP signaling through its G protein-coupled receptors often utilizes Gαs to increase adenylyl cyclase, a process leading to cyclic AMP formation and increased activation of protein kinase A (PKA) (Dickson and Finlayson, 2009). To determine whether the PKA pathway contributed to augmented system x_c^- activity by PACAP, mixed cortical cultures were treated with 10 μM H89 (Figiel and Engele, 2000), a PKA inhibitor, during the 24-h PACAP treatment. Inhibition of PKA activity by H89 completely blocked the increase in radiolabeled cystine uptake induced by PACAP treatment (Fig. 3; $P < 0.05$).

To identify the PACAP receptor type mediating the effects on cystine–glutamate exchange, cortical cultures were coincubated with antagonists for the PAC1 receptor or VPAC receptors. Inhibition of the PAC1 receptor with 100 nM PACAP6-38 did not block PACAP facilitated radiolabeled cystine uptake, but instead potentiated the effects of PACAP treatment alone (Fig. 4A; $P < 0.05$ compared with PACAP treatment). The nonspecific VPAC receptor antagonist VIP6-28 at a concentration of 100 nM was effective in attenuating the increased cystine uptake following 24 h 1 nM PACAP treatment (Fig. 4B; $P < 0.05$). To

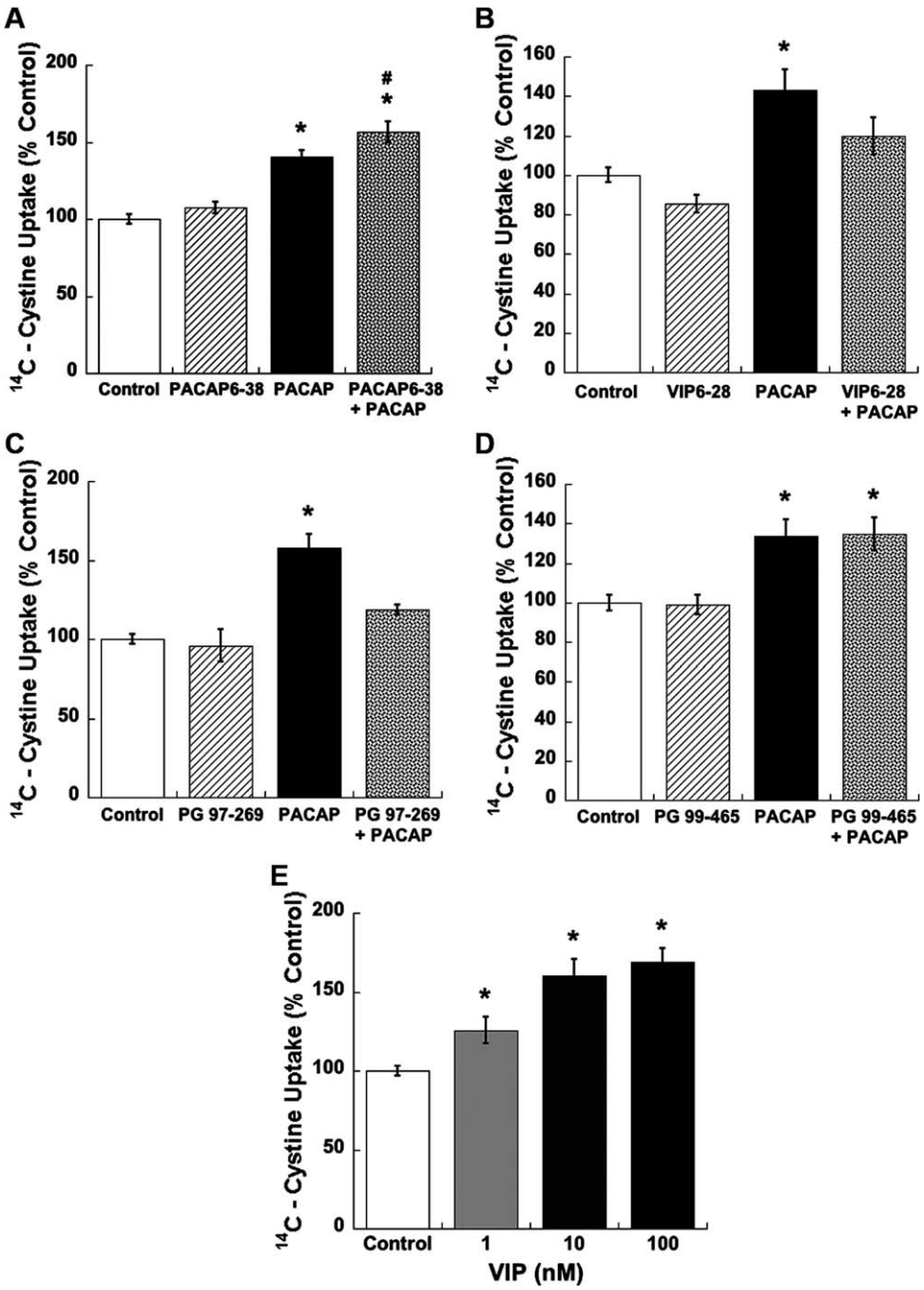

Fig. 4. Increases in cystine uptake induced by PACAP are mediated by the VPAC1R. (**A**) The PAC1R antagonist PACAP6-38 (100 nM) caused a potentiation of 1 nM PACAP on cystine uptake (n = 16–24). (**B**) The VIP receptor antagonist VIP6-28 (100 nM) significantly attenuated the increased cystine uptake caused by PACAP treatment (n = 7–16). (**C**) Treatment with 100 nM PG 97-269 (specific VPAC1R antagonist) abolished the 1 nM PACAP effect on cystine uptake (n = 8–16), while (**D**) 100 nM PG 99-465 (VPAC2R antagonist) had no effect (n = 8–12). (**E**) A concentration response of 24-h VIP treatment showed that VIP increases cystine uptake at a similar concentration to PACAP, as well as at the higher concentration ranges where PACAP was ineffective (n = 8–16). Data are expressed as mean ± SEM. *, $P < 0.05$ compared with control group. #, $P < 0.05$ compared with PACAP group.

further investigate which VPAC receptors were involved in the augmentation of system x_c^- activity, primary mixed cortical cultures were pretreated with specific antagonists for VPAC1R and VPAC2R. A 100 nM concentration of the VPAC1R antagonist PG 97-269 blocked the increased cystine uptake produced by 24 h PACAP (1 nM) treatment in mixed cortical cells (Fig. 4C; $P < 0.05$), while similar treatment with the VPAC2R antagonist PG 99-465 had no effect (Fig. 4D; $P < 0.05$). Finally, a concentration response using

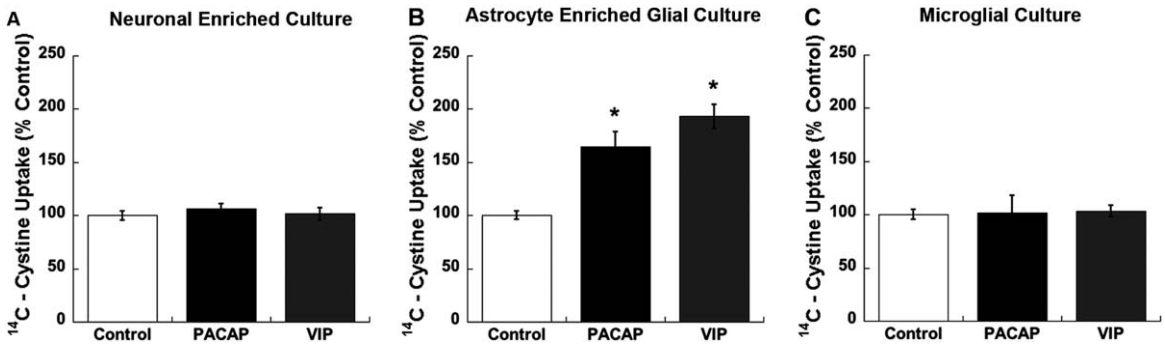

Fig. 5. Cell type specific analysis of PACAP and VIP treatment on cystine uptake. (**A**) Cystine uptake was unaffected following PACAP (1 nM) and VIP (10 nM) treatment in neuronally enriched cultures (n = 6–16). (**B**) Both PACAP and VIP significantly increased cystine uptake in astrocyte-enriched cultures (n = 8–16). (**C**) There was no affect of PACAP or VIP on microglial cystine uptake (n = 5–8). Data are expressed as mean ± SEM. *, $P < 0.05$ compared with control group.

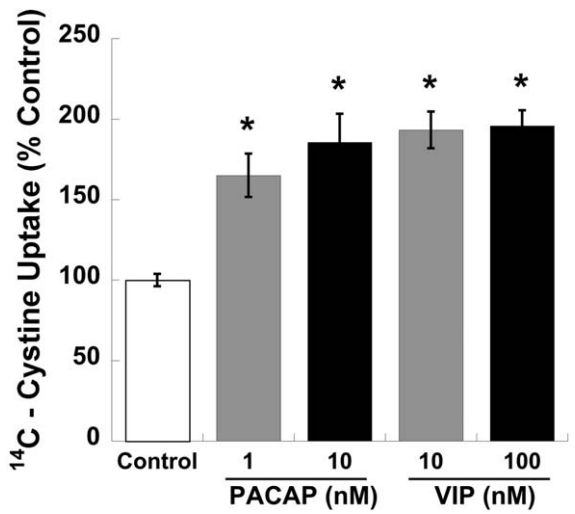

Fig. 6. Cystine uptake in astrocyte-enriched glial cultures following treatment with increased concentrations of PACAP and VIP. In contrast to mixed cortical cultures, radiolabeled cystine uptake in astrocyte-enriched glial cultures was increased by both 1 and 10 nM PACAP, while VIP treatment again showed significantly increased uptake at both 10 and 100 nM similar to mixed culture treatment (n = 616). Data are expressed as mean ± SEM. *, $P < 0.05$ compared with control group.

VIP concentrations ranging between 1, 10, and 100 nM also produced significant increases in radiolabeled cystine uptake (Fig. 4E; $P < 0.05$).

To isolate which cell type in a mixed cortical culture preparation primarily contributed to the increased radiolabeled cystine uptake following incubation with PACAP or VIP, we performed similar assays using neuronal, astrocyte, and microglia-enriched cultures. Optimal concentrations determined from previous concentration response experiments in mixed cortical cultures resulted in the use of 1 nM PACAP (Fig. 1A) and 10 nM VIP (Fig. 4E) concentrations to test cell type specific effects on cystine uptake. Incubation with either PACAP or VIP produced significant increases in radiolabeled cystine uptake in astrocyte-enriched cultures only (Fig. 5B; $P < 0.01$), with no changes detected in neuronal or microglia-enriched cultures. Furthermore, vehicle treatment of astrocyte-enriched glial cultures exhibited much higher radiolabeled cystine uptake (6.48 ± 0.33 cpm/μg protein) compared with vehicle treated neuronal (1.54 ± 0.07 cpm/μg protein) and microglial-enriched (2.45 ± 0.30 cpm/μg protein) cultures. Increased concentrations of PACAP (10 nM) and VIP (100 nM) included in astrocyte-enriched culture experiments from Figure 5B also produced significantly increased cystine uptake (Fig. 6; $P < 0.05$).

DISCUSSION

Previous reports have indicated that both neuropeptides and growth factors may be potential regulators of the cystine–glutamate antiporter, system x_c^- (Johnson and Johnson, 1993; Liu et al., 2012). The present study demonstrates that the pleiotropic neuropeptide PACAP is a novel regulator of system x_c^- activity. PACAP treatment of primary mixed cortical cultures in vitro for 24 h significantly increased radiolabeled cystine uptake. Inhibition of system x_c^- via coadministration of CPG with radiolabeled cystine eliminated cystine uptake almost completely in both control and PACAP-treated cultures indicating that PACAP facilitated cystine uptake was mediated specifically through system x_c^-. Furthermore, the increases in cystine–glutamate exchange appear to be mediated, in part, by increased transcription of the specific light chain subunit of system x_c^-, xCT. This induction of cystine–glutamate exchange activity appears to be driven primarily via VPAC1R signaling through a PKA-dependent pathway that is specific to astrocytes in our primary cortical cultures.

A concentration-response of PACAP treatment on primary mixed cortical cultures for 24 h produced a unique response curve of cystine uptake with a significant increase at the optimal concentration of 1 nM, while a concentration of 10 nM PACAP did not

produce any changes in cystine uptake (Fig. 1A). Of note, PACAP treatment can produce proliferation of astrocytes in vitro and a similar bell-shaped response curve was reported in these experiments (Hashimoto et al., 2003; Tatsuno et al., 1996); however, our data involving radiolabeled cystine uptake were normalized to total protein concentration in each well therefore accounting for any changes in astrocyte populations. In addition, the increase in cystine uptake in primary mixed cortical cultures at 1 nM PACAP was relatively modest compared with increases observed in astrocyte-enriched glial cultures that possess both astrocytes and microglia and lack neurons (Fig. 5B) suggesting that PACAP-mediated regulation of system x_c^- is primarily driven by signaling on astrocytes. In support, the use of microglial cultures showed no significant increases in cystine uptake from either PACAP or VIP treatment (Fig. 5C) ruling out significant contributions of microglia within the astrocyte-enriched cultures. Interestingly, large increases in cystine uptake were observed at the 10 nM concentration of PACAP in astrocyte-enriched cultures (Fig. 6), which did not occur in mixed cultures indicating that PACAP stimulation of neurons may evoke release of other factors capable of affecting cystine–glutamate exchange via system x_c^- on astrocytes.

Effects produced by PACAP signaling are often attributed to PAC1R activation including the enhancement of glial sodium-dependent excitatory amino acid transporters (EAAT) following PACAP treatment of astrocyte cultures (Figiel and Engele, 2000), however, in our studies the PAC1R antagonist PACAP6-38 actually potentiated the effect of 1 nM PACAP treatment in mixed cultures on cystine uptake (Fig. 4A). Instead, the nonspecific VIP receptor antagonist VIP6-28 and the VPAC1R antagonist PG 97-269 both blocked PACAP-induced increases in cystine uptake suggesting that regulation of system x_c^- in mixed cortical cells is achieved through VPAC1R signaling. Furthermore, a concentration-response of VIP treatment did not produce the same response profile for cystine uptake in primary mixed cortical cultures as PACAP (Fig. 4E), indicating that VIP-neuron interactions do not suppress system x_c^- activity, and because VIP does not bind to PAC1R, perhaps demonstrating a role for neuronal PAC1R activation in reducing cystine–glutamate exchange during prolonged treatments in vitro.

PACAP and VIP are neurotrophic peptides that have numerous effects on both astrocyte function and glutamate homeostasis (Arimura et al., 1994; Brenneman et al., 1998; Figiel and Engele, 2000; Hannibal et al., 2000; Magistretti et al., 1998; Martin et al., 1995; Masmoudi-Kouki et al., 2011; Morio et al., 1996; Seaborn et al., 2011; Yuhara et al., 2001). As an excitatory neuropeptide expressed in glutamatergic neurons, PACAP enhances both NMDA and AMPA currents in the hippocampus through PAC1R signaling (Costa et al., 2009; Hannibal et al., 2000; Macdonald et al., 2005; Yaka et al., 2003). In addition, VIP and PACAP also convey signals of metabolic need to astrocytes by induction of glycogenolysis (Magistretti et al., 1998). Thus, regulation of cystine–glutamate exchange activity is yet another mechanism by which PACAP and/or VIP can modulate glutamate signaling through control of astrocyte function. Considering PACAP and VIP ameliorate glutamate toxicity and oxidative stress (Brown, 2000; Masmoudi-Kouki et al., 2011; Morio et al., 1996; Said et al., 1998; Shintani et al., 2005; Vaudry et al., 2002), their signaling may act through augmenting system x_c^- activity to increase extrasynaptic glutamatergic tone on the presynaptic group II/III metabotropic glutamate autoreceptors, dampening the potentially harmful excessive synaptic release of neurotransmitter.

Primary cortical neurons express PAC1 and VPAC2 receptor subtypes, while primary cortical astrocytes express all three receptor subtypes including VPAC1 (Grimaldi and Cavallaro, 1999). It is still unclear how treatment with PACAP for periods of 24 h or more affects PACAP receptor signaling and expression, however, in this experiments augmented system x_c^- activity occurs via activation of the VPAC1 receptor after six or more hours of treatment. In vivo expression of the VPAC1R is distributed throughout the CNS of rodents, with very high levels of immunoreactivity reported in cortical layers (Joo et al., 2004). Notably, aged rats display decreased VPAC1R expression in cortex, hippocampus, and amygdala (Joo et al., 2005), and coincidentally these same brain regions are also areas that are high risk for neurodegeneration, which can contribute to cognitive decline. Glutathione availability has also been shown to decline with age, and is thought to underlie the development of disease due to oxidative stress (Liu et al., 2004; Suh et al., 2004). Therefore, one contributing factor to the decline of glutathione production and increased incidence of cognitive decline with age may be the decreased expression of VPAC1R causing less cystine–glutamate exchange in the cognitive centers of the brain.

Alterations in system x_c^- activity and expression have been implicated in various disease states such as gliomas, schizophrenia, and drug abuse (Baker et al., 2003, 2008; Ye et al., 1999). Oxidative stress and glutamate dysfunction lead to the development of numerous pathologies, demonstrating that system x_c^- may be an attractive target for drug development. However, it should be noted that conflicting evidence exists regarding the phenotype of xCT knockout mice, where spontaneous deletion of the xCT gene characterized in sut/sut mutant mice results in

significant brain atrophy (Shih et al., 2006), the genetically engineered xCT knockout mice do not appear to have significant behavioral or morphological deficits (Lewerenz et al., 2013). Furthermore, increased system x_c^- activity may produce excitotoxicity under certain circumstances such as in primary brain tumors (de Groot and Sontheimer, 2011), interleukin-1β mediated hypoxic neuronal injury (Fogal et al., 2007; Jackman et al., 2010), and long-term treatment with fibroblast growth factor-2 (FGF-2) in vitro (Liu et al., 2014).

This study identifies a novel neuropeptide signal that regulates the function of the cystine–glutamate antiporter. PACAP/VIP signaling may critically modulate system x_c^- activity throughout the CNS conveying signals to astrocytes of increased activity and oxidative stress at the synapse. Future studies are needed to examine the potentially harmful affects of abnormal PACAP/VIP signaling on system x_c^- function, as well as the therapeutic potential of targeting these neuropeptides to drive system x_c^- activity for the treatment of disease.

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