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Design and biological assessment of membrane-tethering neuroprotective peptides derived from the pituitary adenylate cyclase-activating polypeptide type 1 receptor

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ABSTRACT

Background: The pituitary adenylate cyclase-activating polypeptide (PACAP) type 1 receptor (PAC1), a class B G protein-coupled receptor (GPCR), has emerged as a promising target for treating neurodegenerative conditions. Unfortunately, despite years of research, no PAC1-specific agonist has been discovered, as activity on two other GPCRs, VPAC1 and VPAC2, is retained with current analogs. Cell signaling is related to structural modifications in the intracellular loops (ICLs) of GPCRs. Thus, we hypothesized that peptides derived from the ICLs (called pepducins) of PAC1 might initiate, as allosteric ligands, signaling cascades after recognition of the parent receptor and modulation of its conformational landscape.

Methods: Three pepducins were synthesized and evaluated for their ability to 1) promote cell survival; 2) stimulate various signaling pathways associated with PAC1 activation; 3) modulate selectively PAC1, VPAC1 or VPAC2 activation; and 4) sustain mobility and prevent death of dopaminergic neurons in a zebrafish model of neurodegeneration.

Results: Assays demonstrated that these molecules promote SH-SY5Y cell survival, a human neuroblastoma cell line expressing PAC1, and activate signaling via $G\alpha_s$ and $G\alpha_q$, with distinct potencies and efficacies. Also, PAC1-Pep1 and PAC1-Pep2 activated selectively PAC1-mediated $G\alpha_s$ stimulation. Finally, experiments, using a zebrafish neurodegeneration model, showed a neuroprotective action with all three pepducins and in particular, revealed the ability of PAC1-Pep1 and PAC1-Pep3 to preserve fish mobility and tyrosine hydroxylase expression in the brain.

Conclusion: We have developed the first neuroprotective pepducins derived from PAC1, a class B GPCR.

General significance: PAC1-derived pepducins represent attractive templates for the development of innovative neuroprotecting molecules.

Abbreviations: ACN, acetonitrile; BBB, blood-brain barrier; Boc, tert-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; BRET, bioluminescence resonance energy transfer; DCM, dichloromethane; DIEA, N,N-diisopropylethylamine; DMEM, Dulbecco's Modified Eagle's Medium; DMF, dimethylformamide; dpf., days post-fertilization; FBS, fetal bovine serum; Fmoc, fluorenylmethyloxycarbonyl; GFP 10, green fluorescent protein 10; GPCR, G protein-coupled receptor; hpf., hours post-fertilization; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MO, medulla oblongata; PAC1, pituitary adenylate cyclase-activating polypeptide type 1 receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; P/S, penicillin/streptomycin; RlucII, *Renilla* luciferase II; RP-HPLC, reverse phase-high performance liquid chromatography; Tel, telencephalon; vDC, ventral diencephalon; VIP, vasoactive intestinal peptide; VPAC, vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide receptor.

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1. Introduction

The pituitary adenylate cyclase-activating polypeptide (PACAP) was discovered based on its capacity to stimulate potently the activity of adenylyl cyclase from rat anterior pituitary cells [1]. This peptide, which exists in two endogenous isoforms termed PACAP38 and PACAP27, mediates its effects via the activation of three G protein-coupled receptors (GPCRs), i.e. PAC1, VPAC1 and VPAC2 [2]. PACAP isoforms are peptide hormones belonging to the secretin/glucagon/vasoactive intestinal peptide (VIP) family and in particular, the 27-amino acid molecule shares up to 68% sequence homology with VIP. While VIP exhibits about 1000 times less affinity for PAC1 than both PACAP isoforms, these peptides bind VPAC1 and VPAC2 with similar potencies [3]. Biochemical and pharmacological studies have shown that PACAP and its receptors are widely distributed in the central nervous system (CNS) and in peripheral tissues [3]. Among its numerous biological activities, PACAP is involved in the development and the regulation of the central nervous [4,5], cardiac [6], inflammatory [7], skeletal [8] and reproductive systems [9]. In addition, it has been demonstrated that both isoforms show striking beneficial effects in various cell or animal models of neurodegenerative pathologies such as Parkinson's, Alzheimer's, and Huntington's diseases, as well as in ischemia, traumatic brain injury, diabetes, myeloma, kidney injury, headaches, cardiac injury, and retinopathy [10–17]. In the CNS, the neuroprotective actions of PACAP are mediated via PAC1 activation, which reduces neuronal apoptosis, and to a lesser extent through modulation of VPAC1 and VPAC2 receptor expression and stimulation, which regulates inflammatory processes [18,19]. Conversely, VPAC2 stimulation produces detrimental peripheral cardiovascular effects such as vasodilation and tachycardia [20]. Over the years, numerous studies have demonstrated that PAC1 represents an attractive and promising target for the treatment of neurodegenerative disorders. More specifically, PAC1 receptors, ubiquitously expressed in neurons and astrocytes [21], activate a transduction cascade of second messengers that ultimately blocks caspase activation and inhibits the expression of pro-apoptotic factors [22]. PACAP-mediated neuroprotection, through PAC1 activation, therefore appears to involve a wide network of anti-apoptotic and survival factors that could slow down the progression of neurodegenerative processes related to CNS diseases [11,16,17,23–25]. For instance, topical administration of TAT-conjugated PACAP attenuated ischemic retinal degeneration via PAC1 receptors [26]. Also, doxycycline, a small-molecule PAC1 allosteric modulator, was able to increase the neuroprotective action of PACAP through its PAC1 receptors. Unfortunately, despite studies conducted to understand the specific determinants involved in the recognition and activation of PAC1 by PACAP, no specific PACAP-derived PAC1 ligand has been discovered to date, although some selective compounds have been described [17,27–31].

It is well established that GPCRs can adopt multiple conformations and that those are further shaped by their interactions with ligands, partner receptors or regulatory proteins [32]. Still, ligand interactions with GPCRs, by altering receptor conformation at the level of the intracellular loops (ICLs), modulate the type and the extent of contribution of different effectors involved in signaling pathways [32]. Several studies have shown that pepducins, which are lipidated peptides derived from the ICLs or the C-terminal tail of a receptor of interest, can be rapidly designed and engineered to specifically target, activate or block a cognate receptor. Such an approach is particularly attractive when agonists and antagonists development remains exceedingly challenging, as observed for instance during drug discovery efforts related to ligands of the class B GPCR subfamily. Thus, demonstration that such ago-allosteric modulators derived for instance from the ICLs of a target receptor can promote its activation and subsequent signaling cascades has opened up new vistas in drug discovery [33]. Indeed, these derivatives have been developed from intracellular loops of several GPCRs such as the protein-activated receptor-1 (PAR1) and −2 (PAR2), the β_2-adrenergic receptor, the C-X-C chemokine receptor 4, the sphingosine-1-phosphate receptor, and the $P2Y_2R$ [34–39]. These compounds, which appear to be highly selective for their parent receptor, might function as allosteric agonists or positive/negative allosteric modulators, making them highly useful for the investigation of GPCR signaling [40]. While their mechanisms of action is not completely understood, it has been supposed that these lipidated peptides are anchored to the membrane and interact with their parent receptor by sequence homology recognition, thereby stabilizing different GPCR conformations and modulating the signaling pathways [41,42]. Their effects are quite striking and it is worth mentioning that some are currently going through clinical studies. This is the case with PZ-128, a pepducin derived from the third ICL of PAR1, that is currently evaluated in phase 1 clinical trials as an antiplatelet agent [43]. Hence, the development of pepducins derived from the ICLs of PAC1-null, the most abundant PAC1 isoform in the brain [44], represents an appealing strategy to target PAC1-associated neuroprotective actions, while avoiding significant activation of the VPAC receptors.

Thus, the goal of this study was to investigate the propensity of pepducins derived from PAC1 intracellular loops to 1) promote human neuroblastoma cell (SH-SY5Y) survival, 2) activate PAC1-null and VPAC receptors and finally 3) exert *in vivo* neuroprotective actions. Accordingly, using PACAP38 as the control molecule, PAC1-derived pepducins were evaluated for their capacity to protect human neuroblastoma cells against 1-methyl-4-phenylpyridinium (MPP^+) toxicity, a toxin reproducing cellular damage found in Parkinson's disease (PD) and, using bioluminescence resonance energy transfer (BRET)-based biosensors, for their ability to promote G protein ($G\alpha_s$ and $G\alpha_q$) activation, two G proteins associated with PACAP/PAC1 neuroprotective action. In an *in vivo* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced model of neurodegeneration using zebrafish, we assessed the propensity of PAC1-derived pepducins to preserve the fish motility in water and tyrosine hydroxylase (TH) expression in the CNS of the animal. Altogether, our results reveal the capacity of PAC1-derived pepducins to protect neuroblastoma cells against MPP^+ toxicity and activate signaling pathways associated with PAC1 neuroprotection. Additionally, *in vivo*, we showed that these peptides, and particularly PAC1-Pep1, maintained partially zebrafish mobility and protected significantly dopaminergic neurons against MPTP toxicity. These tools represent innovative PAC1-targeted allosteric agonists with unique pharmacological profiles.

2. Materials and methods

2.1. Materials

The fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids, the Fmoc-Rink-amide AM-functionalized polystyrene resin and the coupling reagent (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) were purchased from Chem-Impex (Wood Dale, IL). Trifluoroacetic acid (TFA), methanol (MeOH), acetonitrile (ACN), diethyl ether, *N*,*N*-dimethylformamide (DMF), piperidine, dichloromethane (DCM) and bovine serum albumin (BSA) were obtained from Fisher Scientific (Nepean, ON). Prolume Purple (methoxy-e-coelenterazine) was purchased from NanoLight™ Technology (Pinetop, AZ). N,N-diisopropylethylamine (DIPEA), dimethyl sulfoxide (DMSO), 1,2-ethanedithiol (EDT), thioanisole, α-cyano-4-hydroxycinnamic acid, phenol, 1-methyl-4-phenylpyridinium (MPP^+), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-phenyl-2-thiourea (PTU) and all other chemicals were from Sigma-Aldrich (Mississauga, ON). Ham's F12, MEM medium, OptiMEM, fetal bovine serum (FBS), L-glutamine, D-glucose, penicillin and streptomycin (P/S) and all other cell culture reagents were from Wisent (St-Bruno, QC).

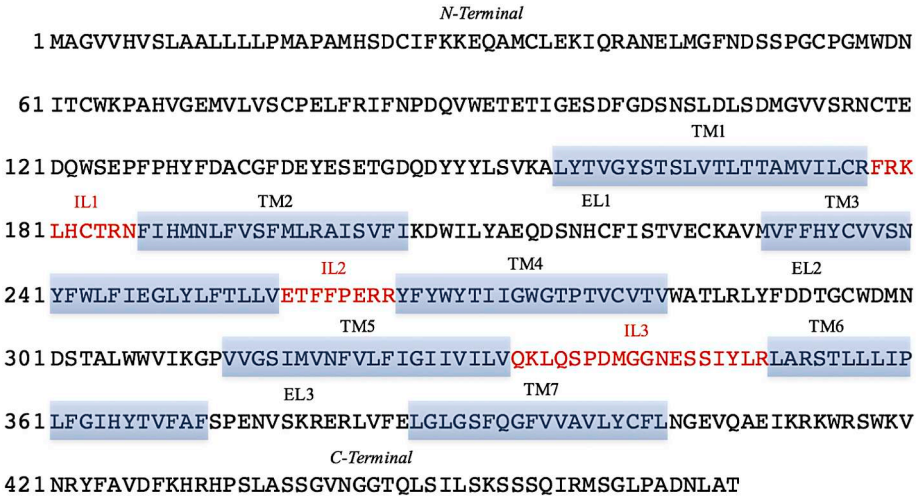

Fig. 1. Predicted PAC1-null transmembrane domains using TMPred. Each predicted transmembrane (TM) helix is shown with blue shading. Each predicted intracellular loop (IL) is shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Peptide synthesis

Pepducin sequences were derived from the sequence of the human PAC1-null receptor (Fig. 1 and Table 1). All peptides were synthesized using a commercially available Fmoc-Rink-amide MBHA resin and following Fmoc-based solid phase peptide synthesis procedures. All protected amino acids (3 equiv. based on the resin substitution) were coupled using an *in situ* activation step with BOP (3 equiv) and DIEA (5 equiv) in DMF for 45 min. At the end of the synthesis, using palmitic anhydride (3 equiv), a palmitoyl moiety was added to the N-terminus of each ICL-derived peptide to produce the related pepducin [45,46]. Deprotection of the Fmoc-protecting group was achieved in 15 min with 20% piperidine in DMF. Each coupling and deprotection step was monitored using a ninhydrin test. Peptide-resins were dried *in vacuo* and then cleaved with reagent K (TFA/water/phenol/thioanisole/EDT; 82.5:5:5:5:2.5). Peptides were purified by preparative reverse phase HPLC to attain a minimum of 95% purity and the molecular weight of each compound was verified by MALDI-TOF mass spectrometry using the α-cyano-4-hydroxycinnamic acid matrix. Peptides were lyophilized and kept at −20 °C until use.

2.3. Pepducin preparation

Stock solutions of PAC1-derived pepducins were prepared by solubilizing peptides in a DMSO/H_2O mixture (5%/95%) at a concentration of 1 mg/mL. At 10^{-7} M, which is the highest concentration used in most assays (except when assessing the pepducin cytotoxicity), our solution contains only 0.05% of DMSO. This DMSO concentration was therefore used in our assays as a control.

2.4. Cell culture

The neuroblastoma cell line SH-SY5Y, endogenously expressing human PAC1 and VPAC2 receptors but not VPAC1 [44], was grown in a 1:1 mixture of MEM medium and Ham's F-12 nutrient mixture containing 15% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 100 IU/mL of penicillin/streptomycin (P/S). Human embryonic kidney (HEK) 293 cells were grown in DMEM medium supplemented with 10% FBS, 4.5 g/L D-glucose, 2 mM L-glutamine and 100 IU/mL of P/S. All cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and passages were performed using trypsinization when cells reached about 80% confluence.

Table 1
PAC1-null-derived pepducin sequences.

Compounds	Sequence	MW (Da)	
		(Observed)	(Calculated)
PAC1-Pep1	Pal-FRKLHCTRN-amide	1411.6	1410.5
PAC1-Pep2	Pal-ETFFPERR-amide	1317.9	1317.5
PAC1-Pep3	Pal-QKLQSPDMGGNESSIYLR-amide	2261.4	2259.7

Pal, Palmitoyl moiety; MW, molecular weight.

2.5. Cytotoxicity assays

SH-SY5Y cells were seeded into 96-well plates at a density of 50,000 cells/well. After 24 h, cells were starved for 2 h and then incubated at 37 °C for 24 h with increasing concentrations of PAC1-derived pepducins (10^{-8}–10^{-5} M). Cell toxicity was assessed using a LDH cytotoxicity assay kit (BioChain®, Newark, CA) and a microplate reader FlexStation®3 (Molecular Devices, San Jose, CA) to determine formazan formation related to lactate dehydrogenase (LDH) activity. Positive (lysis solution) and negative (non-treated cells) controls were included in each experiment. These assays were performed in 3 independent experiments and at least in triplicate. Percentage of cytotoxicity for each compound was obtained using the formula:

$$\%Cytotoxicity = \frac{LDH\ activity\ of\ the\ studied\ compound - LDH\ activity\ of\ the\ negative\ control}{LDH\ activity\ of\ the\ positive\ control - LDH\ activity\ of\ the\ negative\ control} \times 100$$

2.6. Cell survival assays

SH-SY5Y cells, seeded into 96-well plates at a density of 30,000 cells/well, were incubated for 48 h. Cells were starved for 2 h and then incubated at 37 °C for 4 h with PACAP or pepducins (10^{-7} M) prior to the addition of MPP^+ (1.5 mM final concentration), as previously described [47]. Cell viability was assessed 24 h after the MPP^+ treatment using the cell titer Blue-cell viability assay kit (Promega, Madison, WI) and a microplate reader FlexStation®3 (Molecular Devices, San Jose, CA) to determine fluorescence intensity related to the conversion of resazurin into resoflurin. Positive control (MPP^+-treated cells) and negative control (non-treated cells) were included in each experiment. Cell survival assays were performed in 3 independent experiments and at least in triplicate.

2.7. Pepducin specificity

Using bioluminescence resonance energy transfer (BRET)-based

biosensors, activation of $G\alpha_s$ was monitored in HEK 293 cells transiently expressing PAC1, VPAC1 or VPAC2 or an empty plasmid. These receptors (pcDNA3.1_PAC1, pcDNA3.1_VPAC1 or pcDNA3.1_VPAC2) or the empty plasmid and the biosensor for $G\alpha_s$ (pcDNA3.1_$G\alpha_s$-RlucII, pcDNA3.1_$G\gamma1$-GFP_{10} and pcDNA3.1_$G\beta1$) were transiently co-transfected into HEK 293 cells using the TransIT-2020 transfection reagent. A 1.5:1 ratio of TransIT-2020 was used. On the day of transfection, cells were trypsinized and suspended at a density of 300,000 cells/mL in media supplemented with 2.5% FBS. DNA and TransIT-2020 were combined in OptiMEM, and the mixture was incubated at room temperature (RT) for 30 min before being added to the cells. Transfected cells were then seeded in 96-well plates at a density of 30,000 cells/well for the $G\alpha_s$ activation assays. After 24 h, medium was removed and changed with media supplemented with 5% FBS and 100 IU/mL of P/S before being incubated for another 24 h. Cells were washed with 120 μL PBS solution supplemented with 0.1% glucose, followed by an incubation with 80 μL of this solution for 2 h. Then, 10 μL of a 50 μM solution (prepared before use) of Prolume Purple (stock at 1 mg/mL in methanol) and 10 μL of the 10× concentration of peptide (5×10^{-6} M), in PBS containing 0.1% glucose, were added just before reading. BRET signals, measured on a TECAN Infinite 2000, were monitored for 45 min, every 5 min, after co-addition of the coelenterazine derivative and the peptide ligand. Filters were set at 410 nm and 515 nm to detect *Renilla* luciferase II (RlucII, donor) and green fluorescent protein 10 (GFP10, acceptor) light emission, respectively. BRET ratios were determined by calculating the ratio of light emitted by GFP10 over the light emitted by RlucII. ΔBRET were obtained by subtracting background measured for each receptor. BRET experiments were performed in 3 independent experiments and at least in duplicate.

2.8. Bioluminescence resonance energy transfer assays

Using bioluminescence resonance energy transfer (BRET)-based biosensors, the activation of $G\alpha_q$ and $G\alpha_s$ was monitored in SH-SY5Y cells. The pLVXi2H-Polycis_$G\alpha_q$-RlucII-GFP_{10}-$\gamma1$-$\beta1$ or pcDNA3.1_$G\alpha_s$-RlucII, pcDNA3.1_$G\gamma1$-GFP_{10} and pcDNA3.1_$G\beta1$ plasmids were transiently transfected into SH-SY5Y cells using the conditions described above. Then, 10 μL of the 10× concentration of peptide were added before resuming the incubation for 30 min at RT. After 25 min, 10 μL of a 50 μM solution (prepared before use) of Prolume Purple (stock at 1 mg/mL in methanol) in PBS containing 0.1% glucose was added to wells and BRET signals were monitored for 5 min after co-addition of the coelenterazine derivative and the peptide ligand. BRET was assessed as described above. Finally, BRET signals were normalized to the maximum signal measured with PACAP38 at 5×10^{-6} M. G protein activation assessments were performed in 3 independent experiments and at least in duplicate.

2.9. Zebrafish maintenance

All experimental procedures were performed in accordance with the regulations and ethical guidelines from the Canadian Council on Animal Care. We also received the approval from the institutional committee for animal care of the Institut National de la Recherche Scientifique.

Embryonic development was followed in hours post-fertilization (hpf) or days post-fertilization (dpf), as described previously [48]. Zebrafish (*Danio rerio)* embryos and larvae were maintained at 28 °C and were collected and staged using standard methods [48]. For tyrosine hydroxylase (TH) immunostaining, embryos were maintained in water containing 0.003% of 1-phenyl-2-thiourea (PTU) for depigmentation.

2.10. Treatment protocols with peptides and neurotoxic agent

Embryos were distributed in 6-well plates (20–25 embryos/well) containing fish water (5 mL) with or without the studied compound. First of all, embryos were exposed to 10^{-4} M of MPTP, 10^{-7} M PACAP38 or 10^{-7} M pepducin to assess the toxicity of each substance. At 1 dpf, embryos were manually dechorionated and then treated with a solution prepared in fish water containing either 10^{-4} M of MPTP, 10^{-7} M PACAP38 or 10^{-7} M pepducin or co-treatment with MPTP and peptides (Fig. 2). For control, fish water was replaced with fresh media at the same time as the treatment. Co-exposure of fish embryos to MPTP

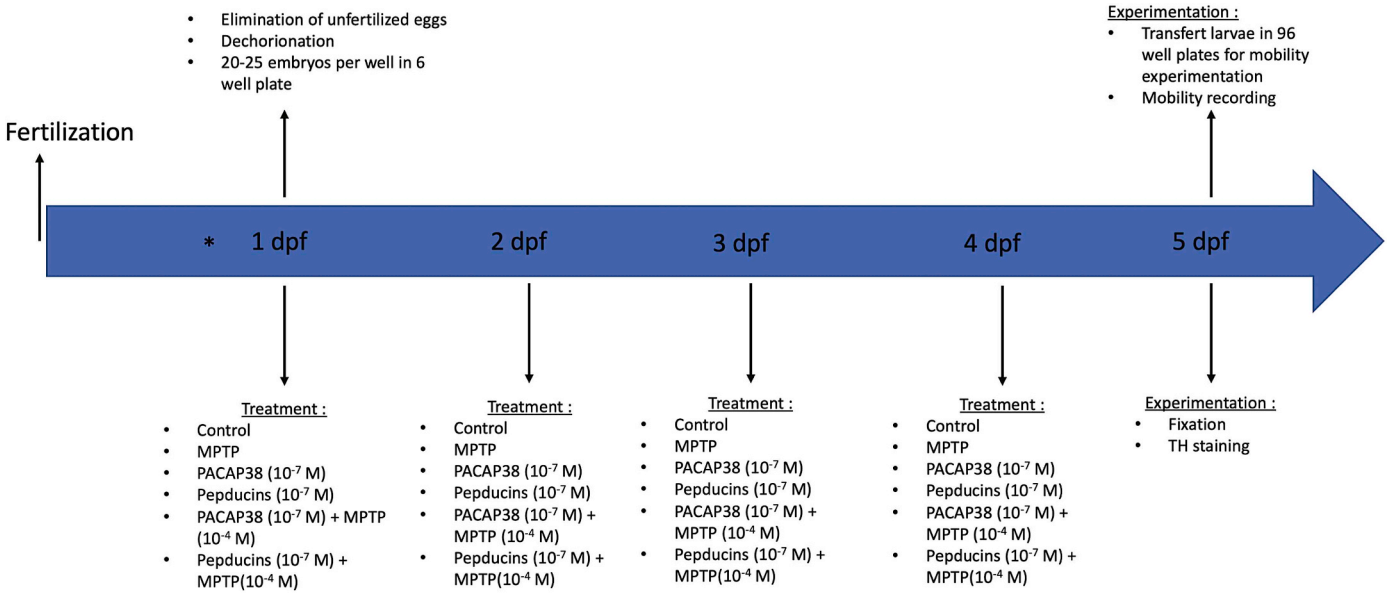

Fig. 2. Timeline of the zebrafish experimentation. From 1 dpf, larvae were treated every day for 5 days, as described in different groups. After 20 hpf, PTU was added in media to provoke larvae depigmentation. At 5 dpf, larvae were separated for mobility test and TH-positive neuron staining.

Table 2
Zebrafish groups according to their respective treatment.

Number	Group	Treatment
1	Control	No treatment
2	PACAP38	10^{-7} M PACAP38
3	PAC1-Pep1	10^{-7} M PAC1-Pep1
4	PAC1-Pep2	10^{-7} M PAC1-Pep2
5	PAC1-Pep3	10^{-7} M PAC1-Pep3
6	MPTP	10^{-4} M MPTP
7	MPTP + PACAP38	10^{-4} M MPTP + 10^{-7} M PACAP38
8	MPTP + PAC1-Pep1	10^{-4} M MPTP + 10^{-7} M PAC1-Pep1
9	MPTP + PAC1-Pep2	10^{-4} M MPTP + 10^{-7} M PAC1-Pep2
10	MPTP + PAC1-Pep3	10^{-4} M MPTP + 10^{-7} M PAC1-Pep3

and peptide was performed until day 5 to assess the neuroprotective action of the PAC1-derived pepducins. Fresh solutions containing MPTP and peptide were prepared every day. Different groups were evaluated, as described in Table 2, and compared to a control group (no exposure to any substances) or an MPTP-treated group.

2.11. Behavioral experiments

Zebrafish motor activity was analyzed at 5 dpf using a Basler GenIcam camera and a DanioVision recording chamber (Noldus, Wageningen, NL). Larvae were placed individually in a 96-well plate (1 embryo/well), acclimatized during 15 min in dark and then locomotor of larval zebrafish activity was recorded during a 2 h light period. Three independent experiments were performed using at least 10 fish per condition. Data analysis was performed using the Ethovision XT 12 software from Noldus to quantify the total distance swum.

2.12. Immunocytochemistry, microscopy and image analysis

At 5 dpf, zebrafish larvae were fixed in Dent's fixative (80% MeOH/20% DMSO) overnight (O/N) at 4 °C. Specimens were progressively rehydrated into PBS and then washed 3 times (for 30 min each time) with PBS containing 1% Triton X-100 (PBS-T), before being treated with 10% normal goat serum (NGS), 1% DMSO and 2% BSA in PBS-T (the blocking solution) for 1 h at RT. Thereafter, a primary monoclonal mouse anti-TH antibody (Millipore, MAB318), diluted at 1:200 in the blocking solution, was added and the incubation was resumed O/N at 4 °C. Specimens were washed in PBS-T at RT (4 times; 30 min each), blocked for 1 h with this solution and then incubated O/N at 4 °C in PBS-T containing a goat anti-mouse Alexa-488 conjugated secondary antibody (Invitrogen, Burlington, ON). Final washing steps were performed at RT with PBS-T (4 times; 30 min each), before collecting the specimens. Fish heads were dissected and mounted in 80% glycerol/PBS on microscope slides with glass spacers. Imaging was achieved with a Zeiss LSM780 system attached to a Zeiss Axio Observer Z1 device. An EC Plan-Neofluar 10× 0.3NA objective was used for the observations and a Plan-Apochromat 20× 0.8NA was utilized for quantification of the TH-positive neurons. Global count of TH-positive neurons, using the Zen lite blue edition software (Carl Zeiss Canada Ltd., Toronto, ON), was assessed in hindbrain region excluding TH-positive neurons of the medulla oblongata, which correspond to noradrenergic neurons [49]. This experiment was realized in 3 independent tests with at least 10 fish per condition.

2.13. Data analysis

Data, expressed as mean ± S.E.M., were analyzed with Prism software (Graphpad Software, San Diego, CA). Sigmoidal concentration-response curves with variable slope and one-site competition function were fitted to determine EC_{50}. A decrease in BRET signal was observed at high concentrations of PACAP resulting in bell-shaped curves. This phenomenon has been previously described in the literature and is explained by receptor desensitization [50]. Thereafter, E_{max} of each ligand was normalized with the highest value obtained for PACAP38. Statistical comparisons between PACAP38 and pepducins were analyzed by Student's *t*-Tests, and differences were considered significant when $^{*}P < 0.05$. Data of behavioral assays are depicted with column bar graphs and expressed as mean ± S.E.M. Data were analyzed with Prism software and statistical analyses were performed using Student's *t*-Tests, in comparison with control or MPTP-treated groups. Student's *t*-Tests were also performed in order to compare the data obtained for the larvae treated with MPTP alone and for those co-treated with MPTP and a peptide. Differences were considered significant when $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$. TH-positive neurons were counted using the Zen lite blue edition software (Carl Zeiss Canada Ltd., Toronto, ON) and data analysis was achieved with the Prism software. Statistical comparisons were analyzed with Student's *t*-Tests, and differences were considered significant when $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$.

3. Results

3.1. In vitro characterization

3.1.1. Cytotoxic activity

Due to their propensity to cross the plasma membrane [42], we first evaluated the potential cytotoxicity of the three PAC1-derived pepducins using SH-SY5Y cells. More precisely, we evaluated the ability of these pepducins to induce LDH release, generally associated with damaged cells. As shown in Fig. 3, at 10^{-5} M, PAC1-Pep2 and PAC1-Pep3 induced 35% and 45% LDH release, respectively. At 10^{-6} M, PAC1-Pep2 and PAC1-Pep3 were still weakly toxic. Interestingly, PAC1-Pep1, derived from the first ICL of PAC1-null, was unable to trigger LDH release at any of the tested concentrations. Thereby, for further experiments, a concentration of 10^{-7} M was chosen to avoid toxic effects.

3.1.2. Survival assays

Pepducins were initially evaluated for their ability to protect neuroblastoma SH-SY5Y cells against the neurotoxic effects of MPP^{+}, an active metabolite of MPTP that produces neuronal damages very similar to those observed in PD [51]. These cells, which endogenously express PAC1 and VPAC2 receptors but not VPAC1 [44], exhibit dopaminergic neuron characteristics [52] and have been largely used as an *in vitro* model of PD [17]. As observed in Fig. 4, all pepducins were able to significantly protect SH-SY5Y cells against MPP^{+} toxicity, whereas VIP failed to exert a similar effect. Interestingly, the neuroprotection level measured with PAC1-Pep1, PAC1-Pep2 and PAC1-Pep3 was comparable to that of PACAP38 used at the same concentration.

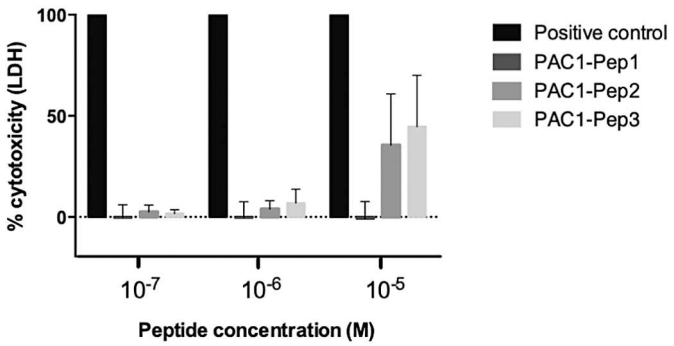

Fig. 3. LDH release by SH-SY5Y cells after a 24 h treatment with pepducins at increasing concentrations. Values represent the mean ± S.E.M. of 3–6 independent experiments performed in sextuplet.

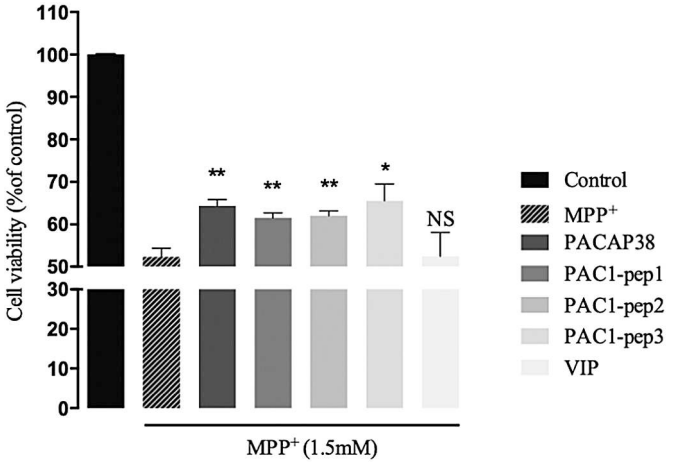

Fig. 4. Pepducins and PACAP38 protect SH-SY5Y neuroblastoma cells against MPP^+ toxicity. Effects of MPP^+ (1.5 mM) on cell viability following a 4 h pretreatment with PACAP38 or pepducins (10^{-7} M). Values represent the mean ± S.E.M. of 3 to 6 independent experiments performed in sextuplet. Statistical analyses were performed using the Student's *t*-Test *versus* the percentage of cell viability measured in presence of MPP^+ alone (NS, non-significant; $^*P \leq 0.05$; $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$).

3.1.3. Pepducin selectivity

Pepducin selectivity was evaluated using HEK 293 cells transiently transfected with or without PAC1, VPAC1 or VPAC2 receptors. As shown in Fig. 5a, after 45 min, neither PACAP38 nor pepducins were able to induce $G\alpha_s$ activation in absence of receptors. However, in HEK 293 cells transiently expressing PAC1-null, VPAC1, or VPAC2, PACAP38 induced a sustained $G\alpha_s$ activation right after cell treatment (Fig. 5b, c, and d). Then, following an analysis of the kinetics of activation with HEK 293 cells expressing PAC1-null, it has been shown that PAC1-Pep1, PAC1-Pep2 and PAC1-Pep3 induced after 45 min a steeper $G\alpha_s$ activation that reached 26%, 65% and 43% of PACAP-mediated $G\alpha_s$ activation, respectively (Fig. 5d). Interestingly, also after 45 min, none of the pepducins stimulated $G\alpha_s$ in HEK 293 cells transiently transfected with VPAC1, while only PAC1-Pep3 induced a weak VPAC2-dependent $G\alpha_s$ activation that reached 16% of the PACAP-associated VPAC2 stimulation (Fig. 5d). Altogether, these results suggest that PAC1-Pep1 and PAC1-Pep2 are able, at least in HEK 293 cells, to stimulate $G\alpha_s$ through a PAC1-null receptor-dependent action.

3.1.4. Pharmacological characterization of PAC1-derived pepducins

It has been demonstrated that PACAP-mediated PAC1 activation stimulates multiple signaling pathways following $G\alpha_q$ and $G\alpha_s$ triggering, and that this activation is associated with inhibition of neuronal apoptosis [53]. Using BRET-based biosensors, we investigated, in SH-SY5Y cells, the propensity of PAC1-derived pepducins to activate $G\alpha_q$ and $G\alpha_s$. As shown in Table 3, PAC1-Pep1 (pEC_{50} = 9.54 ± 0.83) and PAC1-Pep2 (pEC_{50} = 9.15 ± 0.76), derived from the first and second ICL of the receptor, stimulated $G\alpha_q$ with an apparent higher potency (albeit not significant) than PACAP38, whereas PAC1-Pep1 exhibited a lower efficacy than PACAP38 (E_{max} = 59 ± 11% *versus* E_{max} = 79 ± 11%). Interestingly, PAC1-Pep3, in contrast to PACAP38, was not able to stimulate $G\alpha_q$ but was almost as potent and efficient at stimulating $G\alpha_s$ as PACAP38 (pEC_{50} = 6.92 ± 0.40 *versus* pEC_{50} = 6.88 ± 0.56; E_{max} = 72 ± 16% and 94 ± 17%, respectively). PAC1-Pep1 (pEC_{50} = 5.92 ± 0.32) and PAC1-Pep2 (pEC_{50} = 5.87 ± 0.30), although they were less potent than PACAP38 (pEC_{50} = 6.92 ± 0.40), appeared to be more effective (E_{max} = 212 ± 47% and 234 ± 52%, respectively) than PACAP38 (94 ± 17%) (Table 3).

3.2. In vivo characterization

Parkinson's disease (PD) is a neurodegenerative disorder characterized by tremor, bradykinesia and muscle rigidity, as well as verbal disturbance and deficits in attention [51]. These symptoms are mainly associated with a loss of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc), thereby leading to a decrease of dopamine release. Many studies have shown that this disease is associated with multiple etiological factors such as exposure to environmental toxins or genetic mutations, which lead to mitochondrial damage, oxidative stress, inflammation and excitotoxicity [51,54–57]. With the growing aging population, the prevalence of PD is becoming a major public health issue. At present, symptomatic treatments like dopamine substitution, anticholinergic medications, immunotherapies and surgical procedures are available, but none of them are disease-modifying [51]. Therefore, there is an urgent need to develop new ways to slow down or even stop neuronal loss associated with PD. In order to confirm that PAC1-derived pepducins can, as PACAP38, promote neuroprotective actions *in vivo*, their ability to preserve fish mobility and prevent the death of dopaminergic neurons was evaluated following a treatment of zebrafish larvae with the neurotoxic agent MPTP [58]. This translational model, which has never been used in the context of PACAP research, displays conserved biochemical and neurobehavioral features of the phenomenology in humans [59]. Moreover, it represents a great model for drug screenings as well as for studying the mechanisms underlying cell death of dopaminergic neurons in PD [60]. PAC1-derived pepducins and PACAP38, at the tested concentration, did not induce larval zebrafish death (data not shown).

3.2.1. Locomotor activity

Total swimming distance was assessed during a 2 h period with untreated and MPTP-treated zebrafish larvae following exposure to PACAP38 and pepducins. Of note, during this 2 h test period, the larvae exhibited a consistent frequency in their movements. As shown in Fig. 5, treatments with PACAP38 or PAC1-pepducins (10^{-7} M) did not affect the swimming capacity of untreated zebrafish larvae. Previous studies have demonstrated that MPTP, at 10^{-4} M, is sufficient to produce a consistent locomotor deficit in 5 dpf zebrafish [61–63]. Accordingly, using the same concentration of MPTP, we noticed a 90% decrease of the total swimming distance in 5 dpf zebrafish compared to that measured with untreated larvae (Fig. 6). Co-treatments with MPTP and PACAP38, PAC1-Pep1 or PAC1-Pep3 retained partial (approximately 25%) swimming abilities in zebrafish larvae. Surprisingly, despite its potency to stimulate the $G\alpha_q$ and $G\alpha_s$ signaling, PAC1-Pep2 failed to maintain some mobility in the MPTP-treated fish group.

3.2.2. Neuroprotective action on dopaminergic neurons

TH expression, which is a marker of dopamine (DA) production, was appraised in MPTP-treated or untreated zebrafish brains following treatment with the pepducins. At 5 dpf, TH-positive neurons in ventral diencephalon (vDC), telencephalon (Tel) and medulla oblongata (MO) (Fig. 7a) were observed. Considering that the TH-positive neuronal cells localized in the MO are noradrenergic neurons [49], they were excluded from global counting. Embryos treated with MPTP (10^{-4} M) showed a loss of TH-positive neurons in vDC and a reduced dopaminergic neuron levels in Tel (Fig. 7b), which is consistent with previous reports [63]. Addition of PACAP38 or PAC1-derived pepducins during the MPTP treatment produced a protection of TH-positive neurons, particularly in Tel (Fig. 7c, d, e and f). Interestingly, the pepducins displayed a better neuroprotection in vDC than PACAP38 (Fig. 7c, d, e and f). A global count of TH-positive neurons in Tel and vDC demonstrated that embryos treated with MPTP exhibited a highly significant loss of total dopaminergic neurons while, PACAP38, PAC1-Pep1 and PAC1-Pep3 substantially protected zebrafish embryos against MPTP toxicity (Fig. 8), promoting around 75% of neuronal survival. Finally, PAC1-Pep2 also protected zebrafish DA neurons against the

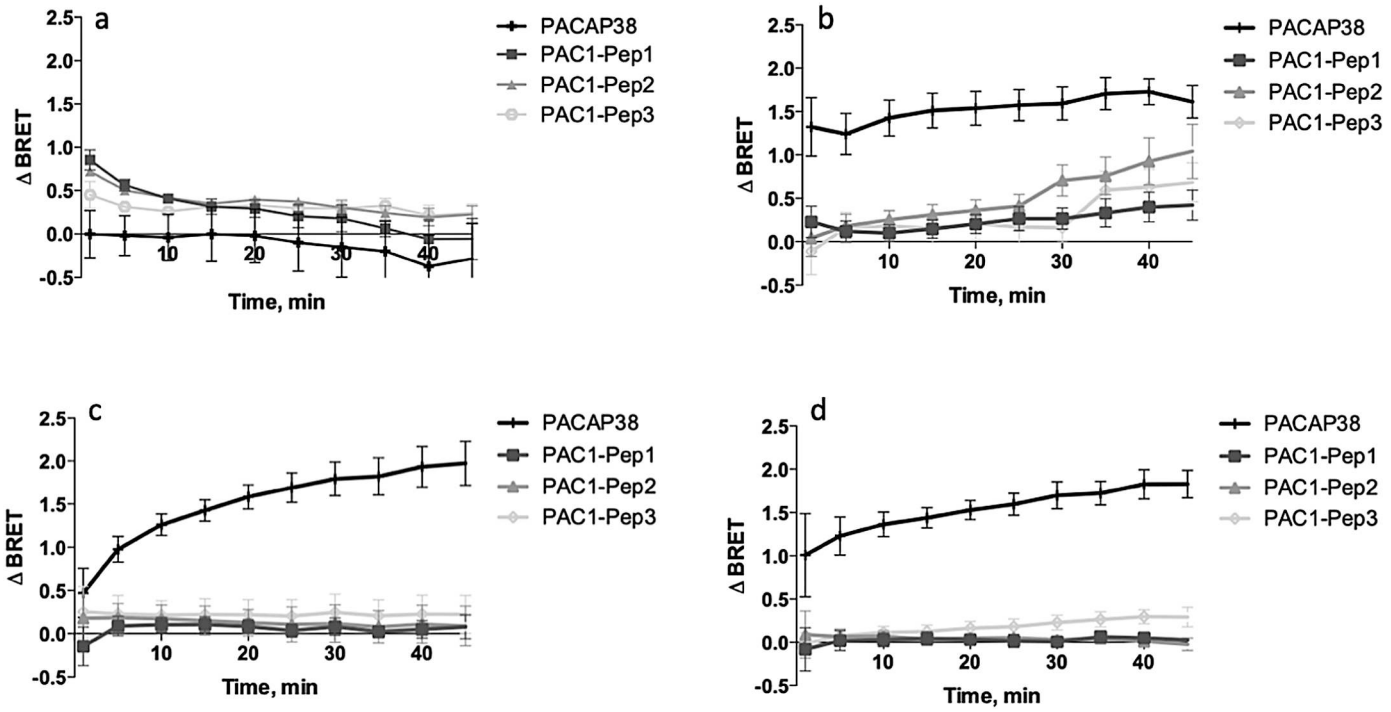

Fig. 5. PAC1-Pep1 and PAC1-Pep2 selectively trigger PAC1-mediated $G\alpha_s$ activation. HEK 293 cells co-tranfected with empty vector, pcDNA 3.1_PAC1, pcDNA 3.1_VPAC1 or pcDNA 3.1_VPAC2 and biosensor pcDNA3.1_$G\alpha_s$-RlucII and pcDNA3.1_$G\gamma1$-GFP_{10} were pre-incubated with Prolume purple for 2 min and stimulated with 5×10^{-7} M of pepducins or 5×10^{-7} M of PACAP38. Changes in BRET were monitored every 5 min during a 45 min recording period. Data represent the mean ± S.E.M. of 3 to 6 independent experiments performed in duplicate. a. PACAP38 and pepducin responses in HEK 293 cells in the absence of PAC1; b. PACAP38 and pepducin responses in HEK 293 transiently transfected with PAC1; c. PACAP38 and pepducin responses in HEK 293 cells transiently transfected with VPAC1; d. PACAP38 and pepducin responses in HEK 293 cells transiently transfected with VPAC2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Agonist activity of PACAP38 and PAC1-derived pepducins.

Compound	$G\alpha_q$			$G\alpha_s$		
	n	pEC_{50} [a]	E_{max} (%) [b]	n	pEC_{50} [a]	E_{max} (%) [b]
PACAP38	4	7.64 ± 0.57	79 ± 11	4	6.92 ± 0.40	94 ± 17
PAC1-Pep1	4	9.54 ± 0.83	59 ± 11	4	5.92 ± 0.32	234 ± 52*
PAC1-Pep2	4	9.15 ± 0.76	91 ± 16	5	5.87 ± 0.30	212 ± 47*
PAC1-Pep3	4	Inactive		5	6.88 ± 0.56	72 ± 16

[a] Negative logarithm of the concentration producing 50% of the maximal effect.

[b] Maximum efficacy is expressed as a percentage of the activation induced by the related endogenous ligand PACAP. Our analysis takes into account the bell-shape effect observed at high concentrations of ligands. Statistical analysis was performed using a Student's *t*-Test *versus* the value obtained with native PACAP38 (*$P \leq 0.05$). Each replicate (n) was conducted on different cell passages.

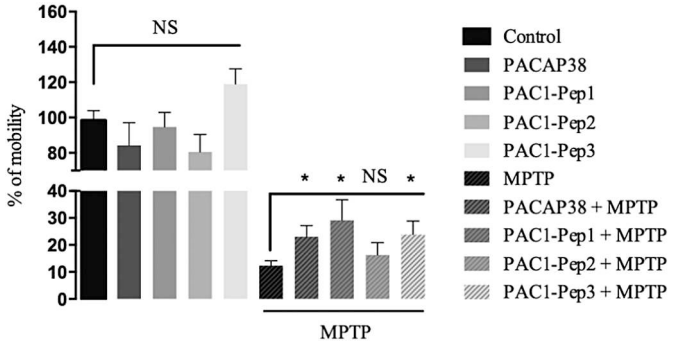

Fig. 6. PAC1-Pep1 and PAC1-Pep3 sustain partially zebrafish mobility in a PD model. Effect of MPTP (100 µM) on zebrafish mobility after a daily 2 h co-treatment with PACAP38 or pepducins (10^{-7} M), performed at 5 dpf. Results with peptides alone are displayed as solid bars while co-treatments are represented by striped bars. Values represent the mean ± S.E.M. of 3 to 8 independent experiments performed with 16 to 32 individual zebrafish. Statistical analyses were performed using the Student's *t*-Test *versus* the percentage of mobility in zebrafish control (NS, non-significant) or *versus* the percentage of mobility in zebrafish measured in presence of MPTP alone (NS, non-significant; *$P \leq 0.05$).

neurotoxicity of MPTP (up to 54%), but to a lower extent than the other peptides.

4. Discussion

Over the last decade, several reports showed the ability of pepducins, *i.e.* N-terminally lipidated peptides derived from the ICLs or C-terminal tail of a GPCR, to activate selectively signaling pathways associated with a target receptor [35,37,38,64,65]. While in the present report, and due to common usage, only palmitoylation has been used to promote the translocation of the peptide to the inner leaflet of the plasma membrane, other N-terminal hydrophobic lipid tethers, including myristic or lithocholic moiety, have been also used for the conception of pepducins [42,46,64,66]. Hence, these compounds, which might function as allosteric agonists or modulators, are highly useful for the investigation of GPCR signaling [40], as demonstrated with studies of the protease-activated receptors, chemokine receptors, and β-adrenergic receptors [67]. As well, their properties make them attractive for the elaboration of treatments for various disorders including inflammatory diseases, cardiovascular pathologies, and cancer [68]. To the best of our knowledge, no pepducin has ever been developed for a class B receptor and more particularly for PAC1. Hence, based on the ICL sequences of the human PAC1-null receptor, the most

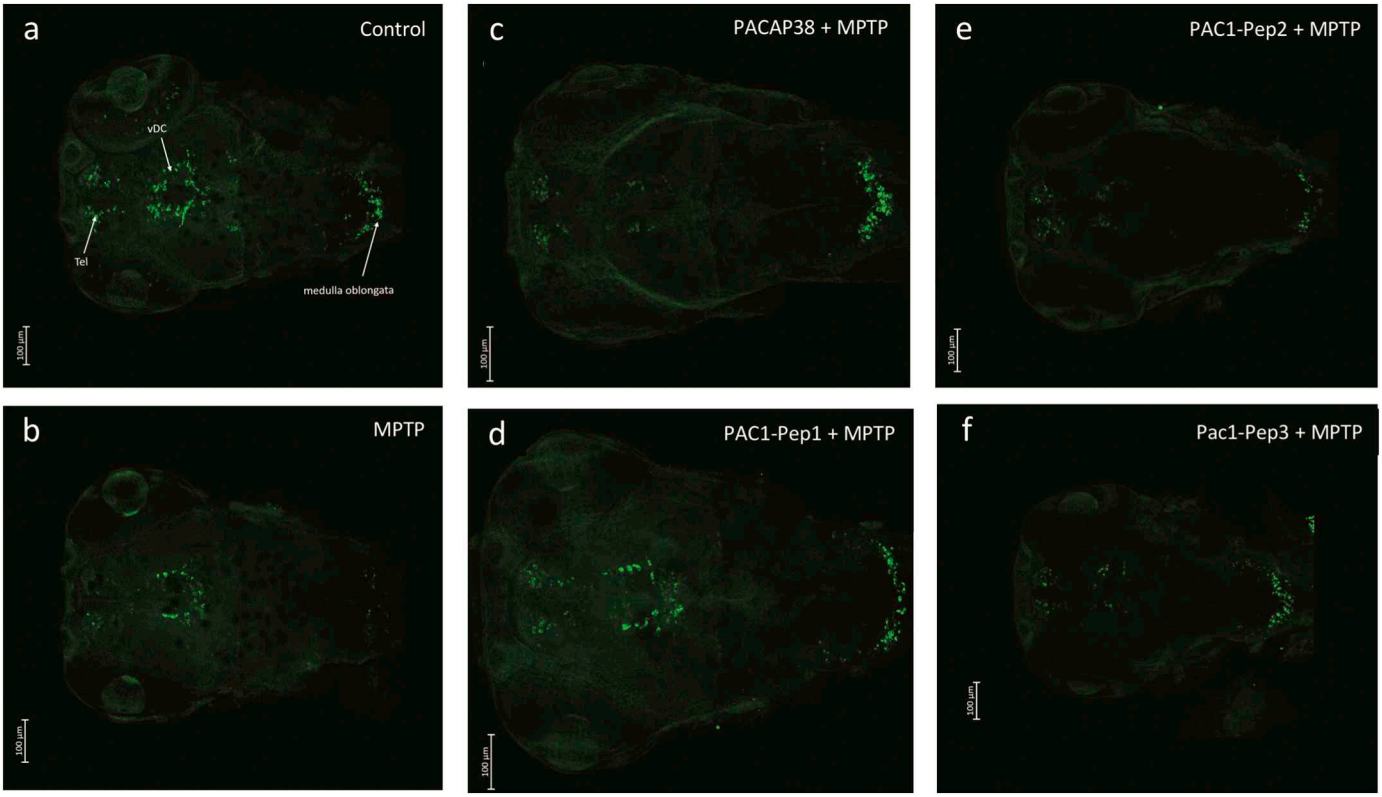

Fig. 7. Representative images at 5 dpf of tyrosine hydroxylase (TH)-positive neurons after co-treatment with MPTP (100 µM) and peptides (10^{-7} M). a, Control; b, MPTP; c, PACAP38 and MPTP; d, Pep1 and MPTP; e, Pep2 and MPTP; f, Pep3 and MPTP.

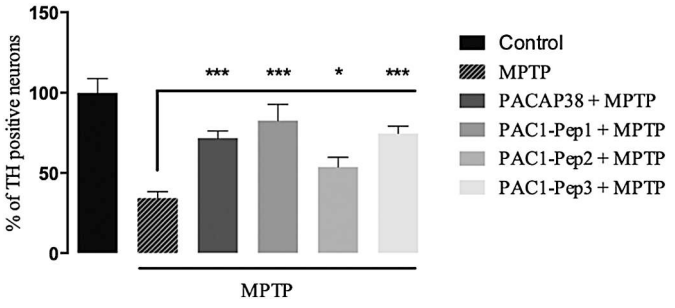

Fig. 8. Pepducins protect tyrosine hydroxylase (TH)-positive neurons against MPTP toxicity. Effect at 5 dpf of MPTP (100 µM) on TH expression in zebrafish midbrain after co-treatments with PACAP38 or pepducins (10^{-7} M). Values represent the mean ± S.E.M. of 6–8 independent experiments. Statistical analyses were performed using the Student's *t*-Test *versus* the percentage of TH-postitive neurons in zebrafish, measured in presence of MPTP alone (***$P \leq 0.001$).

abundant PAC1 isoform in the brain, we have synthesized and characterized the first set of pepducins related to this class B GPCR.

Over the past 30 years, the PACAPergic system has been of particular interest because of its remarkable neuroprotective effects in various cellular and animal models [17,53,69–71]. As part of this study, we have investigated the propensity of PAC1-derived pepducins to exert, *in vitro* and *in vivo*, PAC1-dependent neuroprotective action, in comparison to that of PACAP38, the most abundant isoform of this peptide in the brain [72]. We observed that pepducins derived respectively from the 1st, 2nd or 3rd ICL of PAC1-null protected, *in vitro*, SH-SY5Y neuroblastoma cells against MPP^+ toxicity. *In vivo*, only PAC1-Pep1 and PAC1-Pep3 were able to significantly sustain fish mobility and promote TH-positive cell survival in a zebrafish model of neurodegeneration. On the one hand, similarly to PACAP38 that exerts its neuroprotective action through the activation of two different G proteins, *i.e.* $G\alpha_q$ and $G\alpha_s$, [53], PAC1-Pep1 and PAC1-Pep2 were able to trigger $G\alpha_q$ and $G\alpha_s$ activation, albeit to different extents (Table 3). On the other hand, PAC1-Pep3, which exhibited neuroprotective action *in vitro* and *in vivo*, was unable to trigger $G\alpha_q$ activation, thereby suggesting 1) that this pathway is not involved in its neuroprotective effect and 2) that other signaling features are probably involved in the neuroprotective action of the three pepducins. Indeed, PACAP exerts its neuroprotection through various pathways including PKA/MAPK activation, caspase-3 inhibition, BDNF up-regulation, and proapoptotic factors or anti-inflammatory process inhibition, which all arise from G protein- and/or β-arrestin-dependent activations [53,73]. Also, it has been demonstrated that PAC1-dimer endocytosis produced anti-apoptotic activity in CHO transfected cells [74], and that β-arrestins play a role in PAC1 signaling, particularly through ERK phosphorylation [75]. Considering all these observations, a deeper characterization of the proximal and distal signaling effectors activated by PAC1-pepducins will have to be performed to thoroughly depict their mechanisms of action and the anti-apoptotic pathways by which they exert neuroprotection.

PACAP isoforms belong to the secretin/glucagon/VIP peptide family. In particular, VIP exhibits 68% homology with PACAP27. Nonetheless, the former is a selective ligand of VPAC1/VPAC2 receptors [3], as it shows a very poor affinity for PAC1, the receptor related to PACAP neuroprotection. Each pepducin was derived from the sequence of the ICLs of PAC1-null, an abundant PAC1 isoform in the brain [44] and, as PACAP38, they all protected neuronal cells against MPP^+ toxicity, a property not shared with VIP. This result strongly suggests that the pepducin-induced neuroprotective action observed *in vitro* with SH-SY5Y cells is probably dependent on PAC1 receptor activation (Fig. 4). Among the three pepducins, PAC1-Pep2 was the most potent activator of $G\alpha_s$ in HEK 293 transiently transfected with the PAC1-null receptor, while it was the less potent in SH-SY5Y. Likewise,

in HEK 293 cells, PAC1-Pep2 reached only 44% of PACAP38 activation after 30 min while its E_{max} attained 234% in SH-SY5Y after the same period of time, demonstrating that cell context is extremely important when establishing pharmacological features. Indeed, it is well known that changes in overall signaling profiles can be observed in different cell context but ligands characterized by distinct signaling signatures retained their uniqueness across different cell backgrounds [76]. In our study, such characterization was performed in HEK 293 cells over-expressing the PAC1-null isoform or in SH-SY5Y neuroblastoma expressing numerous PAC1 isoforms [44]. It is now proven that different receptor isoforms can induce distinct patterns of intracellular signaling, which might explain the observed discrepancy. In this perspective, the later cell type appeared to be a highly appropriate tool for the pharmacological characterization of the pepducins because it gives representative PACAP/PAC1 actions.

Over the years, several PACAP analogs have been developed but their lack of specificity toward PAC1 remains a major obstacle in a therapeutic perspective due to related side effects, including VIPoma-like symptoms mediated by the activation of VPAC receptors [77]. Sequence analysis reveals that PAC1-ICL1 is strictly conserved between PAC1, VPAC1 and VPAC2, whereas PAC1-ICL2 exhibits 80% and 0% identity with VPAC1 and VPAC2, respectively, and PAC1-ICL3 shows 71% and 69% identity with VPAC1 and VPAC2, respectively (Table 4). It is now well established that the PACAP/VIP receptors are mainly coupled to the G-protein, $G\alpha_s$ [78]. In HEK 293 cells transiently transfected with one or the other of the PACAP receptors, the inability of PAC1-Pep1 to promote $G\alpha_s$ activation following VPAC1 or VPAC2 stimulation suggests its specificity for PAC1. However, while PAC1, VPAC1, and VPAC2 receptors can also form homodimers, VPAC1 and VPAC2 can form heterodimers with receptor activity-modifying proteins (RAMPs), a state leading to modulation of receptor activation [79]. The current knowledge suggests that receptor-pepducin interaction would simulate receptor dimerization [45]. Hence, upon different cellular backgrounds, variation in PAC1, VPAC1, VPAC2 monomers/homodimers ratios and/or RAMP expressions could therefore arise, thereby modulating receptor recognition and activation by pepducins. Therefore, the possibility that PAC1-pepducins can also trigger VPAC1 and VPAC2 activation cannot be excluded. Further investigations of the pepducin-receptor recognition process, and more particularly of PAC1-Pep1, will have to be conducted. Nevertheless, the data already suggest that peptide sequence is not the only parameter driving receptor activation.

To appraise the neuroprotection potential of PAC1-derived pepducins in an *in vivo* model, we have used zebrafish, which is a proven alternative to mammalian paradigms [59]. Numerous studies have already demonstrated the efficacy of MPTP to induce a neurodegeneration patterns in zebrafish larvae [58,61–63]. Moreover, Fradinger et al. have shown that the first and second ICLs of PAC1 are highly conserved between species and that only one amino acid, *i.e.* Ile^{362} in the zebrafish PAC1 receptor and Met^{362} in the human isoform, differs in ICL3, suggesting that the *in vivo* results would not be influenced by sequence differences between human and zebrafish PAC1 receptors [80]. Although PACAP itself crosses efficiently the blood-brain barrier (BBB) [81], this ability has not yet been shown with PAC1-derived pepducins. However, it is important to highlight that experiments were carried out by adding the neurotoxic agent and the pepducins directly into the aqueous environment of the animals. Moreover, at 3 dpf, it is known that the BBB is partially formed and that molecular exclusion is size-dependent. For example, at this step of development, sulfo-NHS-biotin (562 Da) can cross the BBB but, according to the literature, molecules over 4 kDa cannot [82]. PAC1-derived pepducins, which have molecular weights between 1200 Da and 2500 Da, are therefore expected to reach the fish CNS but this assertion will need to be confirmed. Also, at this stage of development, dopaminergic neurons are expressed in vDC and Tel, while noradrenergic neurons are found in the MO [49]. As previously reported for zebrafish, MPTP induces functional damages in dopamine transporter-positive neurons of the vDC and, more acutely, of the Tel [49]. These alterations precede TH-positive dopaminergic neuronal death [83]. In agreement with these observations, our results confirmed that MPTP resulted in a complete loss of TH-positive neurons in Tel and reduced significantly dopaminergic neuron expression in vDC (Fig. 7). The basis of this difference remains unknown at present and represents a complementary study area. A global count of dopaminergic neurons in the hindbrain demonstrated a very significant neuroprotective action of PACAP38, PAC1-Pep1 and PAC1-Pep3, with 72%, 83% and 74% TH-positive neurons, respectively (Fig. 8). Surprisingly, PAC1-Pep3 had a similar effect than PACAP38 but PAC1-Pep1 was more potent, an observation that could be correlated with its ability to trigger efficiently $G\alpha_s$ activation. These data support the previous observation that PACAP protected dopaminergic neurons against 6-OHDA and restored dopamine production [25]. However, due to the fact that PACAP-associated protection appears to be mediated by multiple downstream effectors, the observed differences between each pepducin cannot be restricted to their distinctive ability to activate $G\alpha_s$. We also observed the capacity of PACAP38, PAC1-Pep1 and PAC1-Pep3 to preserve a significant level of mobility of zebrafish larvae treated with the neurotoxic agent MPTP. Also, in contrast with the *in vitro* results showing neuroprotection, PAC1-Pep2 failed to improve zebrafish movement reduced by MPTP. While PAC1-Pep2 protected significantly TH-positive neurons, it is possible that this pepducin failed to protect or restore neuronal connectivity and motor circuit, thus explaining the absence of locomotor activity when larvae were treated with this molecule. However, other considerations such as its inability to cross efficiently complex biological membranes including the BBB or a poor metabolic stability could also be considered. Hence, additional investigations are required to assess the various particularities. Altogether, our results confirmed the capacity of PAC1-related pepducins, especially PAC1-Pep1 and PAC1-Pep3, to protect neurons against MPTP toxicity, for at least 5 days.

5. Conclusion

To conclude, we have developed a PAC1-derived pepducin, *i.e.* PAC1-Pep1, which exerts, both *in vitro* and *in vivo*, neuroprotective actions similar to that of PACAP. Taken together, our results demonstrate that PAC1-Pep1, derived from the ICL1 of PAC1, acts as an allosteric agonist, thereby engaging different signaling pathways following the activation of at least PAC1. This new derivative represents

Table 4
Intracellular loop sequences of PAC1, VPAC1 and VPAC2 receptors, and percentage of identity of VPAC ICLs *versus* those of PAC1.

Receptor	ICL1	ICL2	ICL3
PAC1-null	F^{178}RKLHCTRN^{185}	E^{257}TFFPERR^{264}	Q^{333}KLQSPDMGGNESSIYLR^{350}
VPAC1	R^{168}KLHCTR^{174}	Y^{241}TLLAVSFFSERKY^{254}	R^{317}ILLQKLRPPDIRKSDSSPYSRLAR^{341}
	100%	**58%**	**71%**
VPAC2	R^{152}KLHCTR^{158}	H^{228}TLLVAMLPPRRC^{240}	R^{304}ILLQKLTSPDVGGNDQSQYKRLAK^{328}
	100%	**0%**	**69%**

Predicted intracellular loop sequences of PAC1, VPAC1 and VPAC2 obtained using the NCBI website. Sequence alignment was done using NCBI standard protein BLAST tool. In bold, the percentage of identity of VPAC ICLs *versus* those of PAC1.

an innovative tool to study PAC1 activation in the context of neurodegeneration and opens up new vistas for the development of molecules with disease-modifying features ideal for the treatment of neurodegenerative disorders. In the continuity of this work, a special focus will have to be applied on the precise mechanism of action by which PAC1-Pep1 promotes PACAP receptors activation and neuroprotection.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

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