Delayed Pituitary Adenylate Cyclase–Activating Polypeptide Delivery After Brain Stroke Improves Functional Recovery by Inducing M2 Microglia/Macrophage Polarization

Coralie Brifault, PhD; Marjorie Gras, BS; Donovan Liot, BS; Victor May, PhD; David Vaudry, PhD; Olivier Wurtz, PhD

Background and Purpose—Until now, except thrombolysis, the therapeutic strategies targeting the acute phase of cerebral ischemia have been proven ineffective, and no approach is available to attenuate the delayed cell death mechanisms and the resulting functional deficits in the late phase. Then, we investigated whether a targeted and delayed delivery of pituitary adenylate cyclase–activating polypeptide (PACAP), a peptide known to exert neuroprotective activities, may dampen delayed pathophysiological processes improving functional recovery.

Methods—Three days after permanent focal ischemia, PACAP-producing stem cells were transplanted intracerebroventricularly in nonimmunosuppressed mice. At 7 and 14 days post ischemia, the effects of this stem cell–based targeted delivery of PACAP on functional recovery, volume lesions, and inflammatory processes were analyzed.

Results—The delivery of PACAP in the vicinity of the infarct zone 3 days post stroke promotes fast, stable, and efficient functional recovery. This was correlated with a modulation of the posts ischemic inflammatory response. Transcriptomic and Ingenuity Pathway Analysis–based bioinformatic analyses identified several gene networks, functions, and key transcriptional factors, such as nuclear factor-κB, C/EBP-β, and Notch/RBP-J as PACAP's potential targets. Such PACAP-dependent immunomodulation was further confirmed by morphometric and phenotypic analyses of microglial cells showing increased number of Arginase-1+ cells in mice treated with PACAP-expressing cells specifically, demonstrating the redirection of the microglial response toward a neuroprotective M2 phenotype.

Conclusions—Our results demonstrated that immunomodulatory strategies capable of redirecting the microglial response toward a neuroprotective M2 phenotype in the late phase of brain ischemia could represent attractive options for stroke treatment in a new and unexploited therapeutic window. (Stroke. 2015;46:520-528. DOI: 10.1161/STROKEAHA.114.006864.)

Key Words: inflammation • microglia • pituitary adenylate cyclase-activating polypeptide • stroke

Stroke is a leading cause of death and long-term disabilities worldwide. Despite years of intense research and preclinical identification of numerous potential neuroprotective compounds, the only available treatment for brain ischemia relies on thrombolysis through injection of a recombinant tissue-type plasminogen activator. However, the treatment benefits to <10% of stroke victims because of a narrow therapeutic time window (<4.5 hours after stroke onset) and side effects.1 Consequently, there is a crucial need for the development of other strategies that could target later phases of the pathophysiological cascade of events after stroke.

Since its initial discovery, several studies have highlighted the neuroprotective effect of pituitary adenylate cyclase–activating polypeptide (PACAP)2 in vitro and in vivo models of neurodegenerative diseases.3,4 Administered either before or few hours after middle cerebral artery occlusion, PACAP reduces the infarct volume area and improves functional outcomes.5,6 Beside its well-known antiapoptotic activity, the neuropeptide PACAP exerts potent anti-inflammatory properties on innate immune compartment as illustrated by the decrease of the production of proinflammatory mediators interleukin (IL)-12, tumor necrosis factor (TNF)-α, and nitric oxide and the induction of the anti-inflammatory cytokine IL-10 in PACAP-treated macrophages stimulated by lipopolysaccharides.7–10 Whether PACAP acts directly by reducing apoptotic neuronal death11 or indirectly via modulation of...
the inflammatory processes is not fully understood yet. Nevertheless, there is growing evidence that PACAP could be of therapeutic value for the treatment of disorders involving apoptosis and neuroinflammation. However, its clinical use is restricted by its short half-life in systemic circulation (5–10 minutes in human blood)4,14 and in the context of brain stroke, by the difficulties in accessing the damaged cerebral tissues especially after cerebrovascular disruption. To circumvent these limitations, we propose a strategy of drug delivery based on the ability of genetically modified stem cells to migrate and locally deliver PACAP in the vicinity of the infarct area after brain transplantation. A key aspect of the experimental system is based on the use of stem cells and recipient mice of identical genetic background to obviate the immunosuppressive treatments that could interfere with the mechanisms under study. In this model, PACAP acts as a potent regulator of the microglial response in vivo at delayed time points after the stroke onset, leading to efficient functional recovery.

Materials and Methods

Materials and Methods are detailed in the online-only Data Supplement. All animal procedures were conformed to the French recommendations for the care and use of laboratory animals and approved by the regional ethics committee (authorization number, N/01-07-09/15/07-12).

Animal Procedures

Twelve- to 16-week-old male mice (129Sv mice or Cx3cr1^GFP transgenic mice) were anesthetized with isoflurane and submitted to focal permanent cerebral ischemia by electrocauterization of the right middle cerebral artery. Seventy-two hours after occlusion, ischemized mice were subjected to a stereotaxic intracerebroventricular injection of 5.10^6 embryonic stem (ES) or PACAP-expressing ES (ES-P) cells or saline solution according to experimental groups.

Behavioral Testing

Post-traumatic neurological impairment was analyzed using a mouse neurological severity score, as previously described.16 The hole-board test permitted an evaluation of the exploratory behavior and motor coordination of mice.17

Histopathologic Studies

At 7 and 14 dpi, animals were deeply anesthetized with pentobarbital and euthanized by transcardiac perfusion with 4% paraformaldehyde. Brain coronal slices of 30-µm-thick were cut using a vibrating blade microtome (VT1200S, Leica). The ischemic area was determined using a thionin-based morphometric analysis and 3-dimensional (3D) reconstruction using Imaris Software (Bitplane).

Immunohistochemistry Analysis

Free-floating 30-µm-thick sections were incubated in blocking buffer followed by incubation with mouse liver Arginase-1 (Arg-1) antibody (Abcam, reference ab60176) revealed by secondary Alexa 568 conjugated antibody (Invitrogen, reference A11057). Sections were mounted and viewed under a confocal laser scanning microscope (TCS SP5, Leica).

Morphological Analysis of Microglial Cells

Morphometric measurements of cell shape were performed on green fluorescent protein (GFP)–expressing microglia of Cx3cr1^GFP transgenic mice. Morphological parameters characterizing the microglial activation were determined using IMARIS software (Bitplane).

Gene Expression Analysis

At 7 and 14 dpi, total RNAs of mouse from contra- and ipsilateral hemispheres were extracted from a series of 40-µm-thick brain slices using TRI-reagent (Sigma) according to the manufacturer’s protocol. Total RNAs were reverse transcribed into single-stranded cDNA and TNF-α, IL-10, and Ym-1 encoding cDNA quantified by real-time polymerase chain reaction on an ABI Prism 7500 Sequence Detection System (Life Technologies) using GAPDH as a reference gene. For transcriptomic analysis, mRNAs extracted from ipsilateral hemispheres of saline- and ES mice were reverse transcribed into cDNA and loaded on a TaqMan® OpenArray® Mouse Inflammation Panel plate (Life Technologies, reference 4475393). Real-time polymerase chain reaction and quantitative gene expression were performed on a QuantStudio 12K Flex Real-Time Polymerase Chain Reaction System (Life Technologies).

Bioinformatic Analysis

The transcriptional signatures obtained from TaqMan® OpenArray® Gene Expression analysis were further analyzed through Ingenuity Pathway Analysis (IPA) software tools.

Statistical Analysis

All values were expressed as mean±SEM. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc.) and based on 1-way ANOVA followed by post hoc Tukey test for multiple comparisons. Statistical significance was set at P<0.05.

Results

Delayed and Targeted Delivery of PACAP Promotes Functional Recovery After Stroke

To study the functional recovery resulting specifically from delayed and targeted PACAP delivery, wild-type ES cells and ES-P cells, producing and releasing PACAP as demonstrated at the transcriptomic level by real-time polymerase chain reaction and at the protein level by Western blot, immunocytotoxicity, and radioimmunoassay (Figure I in the online-only Data Supplement), were injected intracerebroventricularly 3 days after permanent middle cerebral artery occlusion in mice. The functional deficits were assessed at 7 and 14 days post ischemia (dpi) using neurological severity scores and hole-board tests. Although the delayed injection of ES cells did not reduce the functional deficits after stroke, the graft of ES-P cells promoted fast and stable functional recovery as illustrated by the neurological severity score at 7 and 14 dpi when compared with saline and ES groups (Figure 1A, upper). Similarly, motor coordination, typically affected after brain ischemia, was restored by ES-P cell injection in contrast to ES-grafted cells to levels comparable with those in sham-operated animals as measured by stumble frequency in hole-board tests (Figure 1A, middle), whereas there were no differences in ambulatory times among the 4 experimental groups (Figure 1A, lower).

Improvement of Functional Recovery Is Not Associated With a Reduction of the Lesion Size

Because the reduction of functional deficits could result from a decrease of tissue damages or cell replacement after stem cell transplantation, we measured the infarct and edema volumes using thionin-based morphometric analysis (Figure 1B–1D). Volumetric analyses of the ischemic lesions in the fronto-parietal cortex (Figure 1B) failed to reveal differences...
in lesion size ($P>0.05$; Figure 1C) or edema/atrophy ($P>0.05$; Figure 1D) between the different experimental groups at 7 and 14 dpi. These results suggest that the functional recovery associated with the local and delayed delivery of PACAP must be correlated with modulation of late pathophysiological processes.

**Delayed Local Delivery of PACAP Dampens Inflammatory Responses**

To determine whether the local delivery of PACAP modulates the postischemic inflammation, we performed a transcriptomic analysis on an OpenArray® platform using a panel of 632 genes involved in inflammation. This analysis was conducted at 7 dpi when functional recovery induced by ES-P–grafted cells was already effective. The differential transcriptomic profiles between ES-P and ES cell groups were then analyzed using IPA bioinformatic tools to determine regulated critical pathways that may participate in the neuroprotective action of PACAP. IPA analysis identified 5 main regulated gene networks, 4 of which having a significant score $>30$ (Figure 2). These 4 networks were related to the inflammatory response and encompassed genes involved in infectious disease, cellular movement, hematologic system development and function, immune cell trafficking, and cell death and survival (Figure 2A–2D). Interestingly, the results showed that compared with ES cell injections, ES-P cell grafts induced a modulation of chemotactic responses as illustrated by the downregulated expression of $Ccl2$, $Ccl3$, $Ccl4$, and $Ccr2$, with a concomitant upregulated expression of $Ccl2$, $Ccl3$, and $Ccr2$ (Figure 2A). In addition, IPA analyses highlighted a decrease of proinflammatory mediators as indicated by the downregulation of TNF (Figure 2D), interferons (Figure 2A), and IL-1 (Figure 2C) networks, in conjunction with an inhibition of the nuclear factor-$\kappa$B pathway (Figure 2D). In parallel, the genes more likely involved in the resolution of the inflammatory process, such as $Ptges$, $Pparg$, $Tgfb1$, $Hmox1$, and $Twix1$ (Figure 2B), and $Tnfaip3$ (Figure 2D), are upregulated.

**PACAP-Dependent Immunomodulation Is Rapid and Stable**

To confirm these results, we determined the expression levels of mRNAs encoding the proinflammatory cytokine...
TNF-α, the immunosuppressive cytokine IL-10, and the neuroprotective factor Ym-1 in ipsilateral hemispheres. These analyses were conducted at 7 and 14 dpi to study the evolution of the inflammatory response (Figure 3). Validating our panel-based transcriptomic results at 7 dpi, it was found that the expression level of TNF-α is significantly upregulated in the ischemic tissues of mice injected with saline or ES cells compared with that of sham-operated animals, whereas the TNF-α levels are significantly reduced in the brain of mice transplanted with ES-P cells (Figure 3A, left). Likewise, the expression levels of IL-10 and of Ym-1 are significantly increased in the ES-P group compared with other experimental groups (Figure 3B and 3C, left). Interestingly, at 14 dpi, the expression of TNF-α and IL-10 is maintained,
IPA identified 5 main activated transcription factors (Table), including CCAAT/enhancer-binding protein beta (CEBPB, \( z \)-score=2.129), early growth response protein 1 (EGR1, \( z \)-score=2.879), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA, \( z \)-score=2.233), signal transducer and activator of transcription 3 (STAT3, \( z \)-score=2.364), and 1 inhibited transcription factor recombination signal binding protein for immunoglobulin kappa J region (RBP-J, \( z \)-score=−2.417).

**Discussion**

Cerebral ischemia initiates a complex set of pathophysiologic events that evolve over time and space, leading to a massive and progressive neurodegeneration promoting severe functional recovery observed after delayed PACAP delivery correlates with polarization of microglial cells toward a M2 phenotype.

Because transcriptomic and bioinformatic analyses demonstrate a correlation between functional recovery and modulation of the local inflammatory responses, we examined microglia/macrophage dynamics that are central to this process. We used Cx3cr1-GFP heterozygous transgenic mice to explore specifically the activation status of microglial cells. Morphometric analysis of the GFP+ cells at 7 dpi in 2 peri-infarct regions (Figure 4A) revealed that the delayed PACAP delivery induced a significant decrease in the number of microglia/macrophage cells (Figure 4B, upper). Moreover, the area of the cell soma was reduced (Figure 4B, upper), whereas the number and area of fiber-like extension processes per cell (Figure 4B, lower left and right) were increased, suggesting that the phenotype of activated microglial/macrophage cells was altered. To confirm the PACAP-dependent skewing of the microglial response, we performed immunohistofluorescence labeling for the M2-phenotypic marker Arg-1 (Figure 4C and 4D). Because no GFP+ ES or ES-P cells seem Arg-1+ 1 week after transplantation in nontransgenic C57Bl/6 mice (Figure II in the online-only Data Supplement), we assume that the observed GFP+/Arg-1+ double positive cells derived only from host cells and not from the cells transplanted in the Cx3cr1-GFP model. The quantification of Arg-1+/GFP+ cells revealed that 18.9±0.6% of GFP+ microglial/macrophage cells in the ES-P group are Arg-1+, whereas double-labeled cells represent only 6.1±0.6% and 6.3±0.8% of microglial/macrophage cells in the saline and ES cell–treated groups, respectively (*\( P < 0.05 \); Figure 4D). The increased number of M2-phenotypic marker Arg-1 expressing microglial/macrophage cells in the border zone of the infarct area in mice transplanted with ES-P cells compared with the other groups (Merge channel; Figure 4A) revealed that the delayed PACAP delivery induced a significant decrease in the number of microglia/macrophage cells (Figure 4B, upper). Moreover, the area of the cell soma was reduced (Figure 4B, upper), whereas the number and area of fiber-like extension processes per cell (Figure 4B, lower left and right) were increased, suggesting that the phenotype of activated microglial/macrophage cells was altered. To confirm the PACAP-dependent skewing of the microglial response, we performed immunohistofluorescence labeling for the M2-phenotypic marker Arg-1 (Figure 4C and 4D). Because no GFP+ ES or ES-P cells seem Arg-1+ 1 week after transplantation in nontransgenic C57Bl/6 mice (Figure II in the online-only Data Supplement), we assume that the observed GFP+/Arg-1+ double positive cells derived only from host cells and not from the cells transplanted in the Cx3cr1-GFP model. The quantification of Arg-1+/GFP+ cells revealed that 18.9±0.6% of GFP+ microglial/macrophage cells in the ES-P group are Arg-1+, whereas double-labeled cells represent only 6.1±0.6% and 6.3±0.8% of microglial/macrophage cells in the saline and ES cell–treated groups, respectively (*\( P < 0.05 \); Figure 4D). The increased number of M2-phenotypic marker Arg-1 expressing microglial/macrophage cells in the border zone of the infarct area in mice transplanted with ES-P cells compared with the other groups (Merge channel; Figure 4C and 4D) further confirmed skewing of the microglial response.
functional deficits. Except for thrombolysis, therapeutic strategies targeting the acute phase of stroke are still ineffective in patients. Thus, it becomes essential to develop new approaches targeting new therapeutic windows to significantly improve the processes of functional recovery.

The beneficial effects of PACAP infusion and stem cell transplantation are well documented for stroke treatment. Nevertheless, there are several hurdles that need to be addressed before these approaches can be used in therapy. To circumvent these limitations, we integrated the 2 approaches by establishing a stem cell line designed to produce and release PACAP (Figure I in the online-only Data Supplement). From the capacity of transplanted stem cells to migrate toward ischemic area, the local PACAP delivery skirts issues related to PACAP stability and targeting to damaged brain regions where vascularization has been compromised. Because PACAP also has differentiation promoting properties, its paracrine and autocrine actions may reduce stem cell tumorigenic potential and associated risks (Figure I in the online-only Data Supplement).

Numerous studies report beneficial effects of stem cell transplantation after stroke because of their ability to produce anti-apoptotic, neurotrophic, and immunomodulatory factors. In our model, the transplantation of nonmodified stem cells does not promote functional recovery at 7 and 14 dpi, suggesting that these cells and their secreted products, transplanted at 3 dpi, are ineffective in reducing the functional deficits. On the contrary,
delayed injection of PACAP-expressing stem cells promotes fast, stable, and efficient functional recovery, indicating that the neurological improvements result specifically from PACAP. In this experimental model of brain ischemia, the maximal infarct volume is reached 24 hours after middle cerebral artery occlusion and remains unchanged over time. As expected, the observed functional recovery did not correlate with a reduction of the lesion size or accelerated resorption of the edema. Moreover, the beneficial effects of ES-P cell transplantation do not rely on cell replacement by grafted cells. Indeed, many grafted cells easily visualized in the vicinity of the infarct zone at 7 dpi (Figure III in the online-only Data Supplement) were no longer detectable by day 14. Even in the absence of graft rejection reaction and local PACAP release, the survival of stem cells transplanted at 3 dpi seems strongly compromised, suggesting that the neuroprotective effect of the delayed PACAP delivery is not linked to its anti-apoptotic properties. Despite the rapid disappearance of ES-P cells, the fast and stable neurological improvements observed after local but transient PACAP delivery suggest that PACAP is able to quickly and stably restore a cerebral environment compatible with neuronal survival and functions.

Among the pathophysiological events induced by brain ischemia, inflammatory processes contribute to neurological deficits by generating a neurotoxic environment and altering the activity of still viable neuronal networks. The present experimental model is based on stem cell transplantation in syngeneic animals to obviate immunosuppressive treatment and allow a reliable evaluation of the immunomodulatory properties of PACAP. Our results show that on delayed administration, stem cell–mediated PACAP delivery can still modulate the ongoing inflammatory response. As reported in previous work, the local delivery of PACAP decreases the expression of proinflammatory mediators, such as TNF-α and IL-1β, and increases the expression of factors related to the resolution of inflammation, such as IL-10, transforming growth factor-β, IL1R antagonist, or Ym-1, clearly establishing PACAP as a potent immunomodulator. Bioinformatic analysis of gene regulations from a panel of 632 genes involved in inflammatory processes has revealed networks of regulated genes that support the anti-inflammatory activity of the peptide. Noticeably, immune cell trafficking and hematological system development and function are significantly affected processes. PACAP has already been shown to modulate the profile of chemokines produced by activated immune cells and consequently to modulate differentially the recruitment of various subtypes of lymphocytes. The coordinate regulation of expression of Cxcl9, Cxcl11, and Ccl22, for example, could result in reduced Th1 and Th2 cell infiltration. Relatedly, the upregulation of Ccr7 expression and the local increase of transforming growth factor-β and IL-10 expression suggest the preferential recruitment of Treg cells that could account for some of the observed neuroprotective effects of PACAP. Similarly, based on the differential transcriptional signatures between ES-P and ES cell–injected groups, IPA transcriptomic analyses reveal an increase of angiogenesis-related processes, specifically in PACAP-treated animals. This PACAP effect is correlated with the increase of vascular endothelial growth factor C expression (fold change=66.7) in line with previous work, showing indirect PACAP-mediated control of endothelial cell proliferation through the induction of vascular endothelial growth factor production. Because CD11b+ cells represent the major source of vascular endothelial growth factor C in the brain parenchyma, our data indicate that the beneficial effects of delayed PACAP delivery could rely on PACAP-induced vascular endothelial growth factor C–producing microglia/macrophages, thus promoting postischemic neovascularization. This hypothesis is reinforced by the increased Arg-1 labeling in vessel-like structures, illustrating an increased neoangiogenesis in ES-P cell–transplanted mice specifically. The fact that the local delivery of PACAP at 3 dpi could target the microglia/macrophage compartment, which mainly supports the early phase of the posts ischemic inflammatory response, is also substantiated by our transcriptomic analysis reporting that local PACAP release increases phagocytic activity and survival of microglia/macrophages (Table I in the online-only Data Supplement). This was further confirmed by our immunohistofluorescence experiments, demonstrating morphological changes of ischemia-activated microglia/macrophages and increased number of cells expressing the M2-phenotypic marker Arg-1 in the infarct border zone, specifically in ES-P–grafted mice. Noticeably, the close proximity of Arg-1+ vessels and Arg-1+/GFP+ microglia/macrophage cells may suggest a local and direct involvement of M2-like microglia/macrophages in the neoangiogenic process. Altogether, these results tend to demonstrate that the PACAP-dependent functional recovery improvements rely on the redirection of the microglial response toward a M2-like phenotype. After stroke and in parallel of microglia activation, many inflammatory cells infiltrate the brain parenchyma. Initially, monocyte-derived macrophages and resident microglial cells were both considered as detrimental. However, more recently, studies demonstrated that these 2 cell populations are functionally distinct, engaged in nonredundant roles. More particularly, Gliem et al showed that the inflammatory Ly6C- CX3CR1+CCR2+ monocytes are protective after stroke by preventing hemorrhagic transformation and delaying clinical deterioration. This suggests that the reduction of the functional deficits observed after delayed PACAP delivery may result from an increased recruitment of inflammatory monocytes, exerting an activity of inflammation resolution. Nevertheless, because injection of M2-differentiated macrophages 4 days after stroke does not improve functional recovery in contrast to the transplantation of human microglial cells, and because delayed PACAP delivery modulates the tissular chemotactic response, we think that the improved functional recovery results more probably from the redirection of the microglial response toward a neuroprotective M2 phenotype.

Interestingly, in a murine model of permanent middle cerebral occlusion, Perego et al reported, 24 hours after ischemia, that the microglia/macrophages adopt a M2-like phenotype that progressively evolves toward a neurotoxic phenotype at day 7 post stroke. Because the kinetics of expression of endogenous PACAP after stroke by pyramidal cortical neurons (PACAP expression increases as soon as 6 hours after middle cerebral artery occlusion and peaks at 24 hours before disappearing) parallels with the evolution of the microglial response, the in vivo PACAP release can be part of an endogenous protective mechanism aimed at controlling the...
differentiation process of microglial cells, the loss of PACAP favoring the acquisition of a neurotoxic phenotype.

Finally, our study identifies the NFκBIA (IκB-α), C/EBP-β, and RBP-J transcriptional regulators as factors potentially mediating PACAP’s polarizing effects on microglia/macrophages (Figure IV in the online-only Data Supplement). The inhibition of nuclear factor-κB pathway has been already reported to mediate the anti-inflammatory properties of the neuropeptide.14,15 PACAP-dependent inhibition of IKK-β phosphorylation induces NFκBIA (IκB-α) stabilization, thereby decreasing nuclear factor-κB-p65 nuclear translocation. In macrophages, the neuropeptide PACAP induces through vasoactive intestinal peptide receptor 1 activation, cyclic adenosine monophosphate response element–binding protein (CREB) phosphorylation which in turn expresses the enzyme of C/EBP-β.15-18 Interestingly, CREB-C/EBP-β cascade has been reported to support the expression of M2 macrophage–specific gene expression.19 In association with the decrease of RBP-J activity, a factor recently associated with M1 differentiation processes in macrophages20; these results suggest that PACAP could induce a shift toward the M2 microglial phenotype through CREB and RBP-J regulation.

Conclusions

In conclusion, we show that even delayed PACAP local delivery can efficiently promote functional recovery after brain stroke. The PACAP-dependent neurological improvements are associated with the modulation of inflammation and, more precisely, of the microglia/macrophage responses. The ability to redirect the microglia/macrophage phenotype from a M1 phenotype toward a M2 response by PACAP delivery at 3 dpi highlights the enduring plasticity of microglial cells after the stroke onset. Further studies are required to evaluate the PACAP-dependent modulation of the Notch/RBP-J pathway in microglial cells and to decipher the mechanisms by which M2-like microglial cells can improve functional recovery after stroke. It seems important to evaluate the functional relationships among the different microglia populations and their roles in cerebral and neural plasticity. Nevertheless, our results confirm the importance of developing approaches designed to target the local inflammatory response to open new therapeutic windows for stroke treatment. In this context, immunomodulatory strategies capable of redirecting the microglial response toward the neuroprotective M2 phenotype could represent attractive options for stroke treatment and perhaps even more widely for neurodegenerative disease with inflammatory components.

Acknowledgments

We thank Tommy Seaborn for critically reading the article and Pascal Hilder for advices in behavioral studies.

Sources of Funding

Dr Brifault was a recipient of a doctoral fellowship from the Région Haute-Normandie. This study was supported by Institut National de la Santé et de la Recherche Médicale (U982), Rouen University, Plate-Forme de Recherche en Imagerie Cellulaire de Haute-Normandie, and the Interreg Peptide Research Network of Excellence project.

Disclosures

None.

References


Delayed Pituitary Adenylate Cyclase–Activating Polypeptide Delivery After Brain Stroke Improves Functional Recovery by Inducing M2 Microglia/Macrophage Polarization
Coralie Brifault, Marjorie Gras, Donovan Liot, Victor May, David Vaudry and Olivier Wurtz

Stroke. 2015;46:520-528; originally published online December 30, 2014;
doi: 10.1161/STROKEAHA.114.006864

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/46/2/520

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2015/01/26/STROKEAHA.114.006864.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Full title:
Delayed PACAP delivery after brain stroke improves functional recovery by inducing M2 microglia polarization

Authors:
Coralie Brifault, PhD a,b,c, Marjorie Gras, BS a,b,c, Donovan Liot, BS a,b,c, Victor May, PhD d, David Vaudry, PhD a,b,c and Olivier Wurtz, PhD a,b,c

SUPPLEMENTAL METHODS

Animals
Male 129Sv and CX3CR1+/GFP heterozygous C57BL/6 mice used in this study were bred in the animal facility of the University of Rouen (approval B.76-451-04), according to French legislation. Mice were housed in groups of four, under controlled temperature, in twelve hours day/night cycle with food and water available ad libitum. All procedures described conformed to the French recommendations for the care and use of laboratory animals, and were approved by the regional ethics committee under the authorization number N/01-07-09/15/07-12.

Focal permanent brain ischemia and cell transplantation
Electrocauterization of the middle cerebral artery: 12- to 16-weeks old male mice were anesthetized with isoflurane (5% for induction and 2% for maintenance). The duration of anesthesia did not exceed 30 min. The mice were kept warm with a heating pad and body temperature was monitored constantly with a rectal probe. Focal permanent cerebral ischemia was performed by electrocauterization of the right middle cerebral artery (MCA), 1. Sham-operated animals underwent the same surgery protocol without MCA electrocauterization.

Intracerebroventricular (i.c.v.) injections: 72 hours after permanent focal ischemia, the mice were subjected to unilateral i.c.v. injection (2 µL; AP = -0.10mm; Lat = -0.80mm; DV = -2.70mm from bregma) of 5 x 10^4 ES or ES-P cells, or saline.

Embryonic stem (ES) cells and PACAP-expressing ES (ES-P) cells
Murine embryonic stem cells from the 129Sv/Ev cell line and ES-P cell line were grown on gelatin-coated 10 cm culture dishes in serum-free ESGRO Complete Clonal Grade Plus medium (SF001-500P, Millipore). Before transplantation, the cells were recovered by Accutase treatment (SF006, Millipore), washed and resuspended in vehicle solution at the working density.

Plasmid and ES-P cell line establishment (Figure S1)
Plasmid used for ES cell transfection: To allow a concomitant expression of the neuropeptide PACAP and enhanced Green Fluorescent Protein (eGFP) in ES cells, the full length cDNA encoding PACAP was cloned into the bicistronic vector of expression plRES2-eGFP (Invitrogen). Briefly, the full length cDNA encoding the neuropeptide PACAP was obtained by RT-PCR from RNA of brain marmoset using primers allowing insertion of a MluI and a EcoRV restriction site in the 5' and 3' ends, respectively, of the amplified product (700 bp): MluI-Forward: CTGACGCGTATGACCATGTGTAGCGGAG, EcoRV-Reverse: TTAGATATCCTACAAATAAGCTATTGGCG. The PCR product was cloned by TA-cloning into pGEM-T (Promega) and the sequence of PACAP cDNA verified by sequencing analysis. Then a MluI/EcoRV fragment was excised, subjected to blunt-ending and subcloned into EcoRI/BamHI blunt-ended sites of plRES2-eGFP to obtain the plRES2-eGFP/PACAP plasmid.
**PACAP-expressing ES cells line (ES-P cells) establishment:** To generate a stably transfected ES cell line designed to produce and release the neuropeptide PACAP concomitantly with the eGFP expression, 4.10^6 129Sv/Ev ES cells (Millipore, Ref CMTI-1) were nucleofected (AMAXA Nucleofector System, Lonza) with 4 µg of AfIII-linearized pRES2-eGFP/maPACAP plasmid. 48h after nucleofection, 0.5mg/ml genetecin (G-418, Sigma Aldrich) was added to the culture medium. After 14 days of selection, G-418 resistant-clones were isolated and selected for eGFP- and PACAP-expression. Then, one clone was further expanded, frozen and used for cell transplantation experiments.

**Characterization of PACAP expression**
**RT-PCR:** Conventional RT-PCR experiments were realized to survey the expression of PACAP encoding mRNA in transfected cells. Total RNAs were extracted from 1.10^6 cultured cells using Tri-reagent (Invitrogen), and isolated using Nucleospin RNA II kit (Macherey-Nagel) according to the manufacturer’s instructions. From each sample, 1.5 µg of total RNA was converted into single stranded cDNA using the ImPromII reverse transcriptase kit (Promega) with random primers (0.5µg/ml). The primer pair designed to amplify the PACAP was: sens 5' CTAAGGCGTATGACATGGTCAGCGAG-3' and anti-sens 5'-TTGATATCCTACAATAGCTATTCCGGC-3'. Amplification was performed on Verity™ 96-Well Thermal Cycler (Applied Biosystems). The PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

**Western blot analysis:** To validate the expression of the neuropeptide PACAP at the protein level in the ES-P cell line, Western blot analysis were performed on total protein extracts from ES and ES-P cells. Briefly, total proteins were extracted from cultured ES and ES-P cells, in a lysis buffer consisting of 1% triton X-100, 50mM Tris-HCl and 10mM EDTA. Cell lysates were centrifuged (12,000 rpm, 4°C) and precipitated at 4°C by addition of ice-cold 100% trichloroacetic acid. After centrifugation (12,000 rpm, 4°C) the pellets were washed twice with an alcohol/ether solution (30:70). Finally the pellets were resuspended in Laemmli buffer and denatured at 100°C for 5 min. Each sample was subjected to SDS-polyacrylamide gel (10%) electrophoresis. Then, the proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond, GE Healthcare). The membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 5% dry-milk for 2 hours at room temperature. Membranes were then incubated overnight at 4°C with PACAP38 antisemur (89083-3 antisemur, 1:2000; generous gift from Pr A. Arimura). After incubation with peroxydase-conjugated secondary antibodies, immunoblots were revealed by enhanced chemiluminescence (GE Healthcare). Standard dilutions (10µg-100ng) of synthetic PACAP38 peptide were used as control (generous gift from Pr. A. Fournier).

**Immunocytofluorescence on cultured cells:** To confirm the detectable expression of eGFP and PACAP, ES and ES-P cultured cells were rinsed in PBS and fixed in 4% PFA for 20 min at room temperature (RT) before permeabilization in PBS containing 0.3% Triton X-100. After blockade in PBS containing 10% normal donkey serum, cells were incubated with PACAP38 anti-serum (89083-3 antisemur, 1:200) for 1 hour at RT and then with Alexa Fluor 594-conjugated anti-rabbit IgG (Invitrogen) for 1 hr at RT. Cells were counterstained with 4', 6-diamo-2-phenylindole (DAPI, Sigma) to identify cellular nuclei. Following immunostaining, cells were mounted on glass slides using Mowiol® (Calbiochem) and viewed under a confocal laser scanning microscope TCS SP2 (Leica Microsystem).

**Behavioral testing**
**Neurological Severity Score (NSS):** Post-traumatic neurological impairment was analyzed using a mouse NSS, as previously described 2. The score was determined on the basis of 10 individual tasks evaluating motor function, alertness, and physiologic behavior. A maximal NSS of 10 points indicates severe neurologic dysfunction whereas a score of zero is achieved by healthy uninjured mice.

**Hole board test:** This test allowed an evaluation of the exploratory behavior and motor coordination of mice 3. At the beginning of single trial, each mouse was placed in the center of the hole board. The time spent walking, the number of head-dips and the number of stumbles were assessed over a 5-min...
period. The stumble frequency (number of stumbles per minute of walking) was calculated as an index of motor coordination, the walking time and the head-dips frequency (number of head-dips per minute of walking) as an index of exploratory behavior. Behavioral tests were performed during the light phase of the circadian cycle by a blinded experimenter.

**Histopathological studies**

At 7 and 14 dpi, animals were deeply anesthetized with pentobarbital and euthanized by transcardiac perfusion with 0.1 M PBS followed by 4% paraformaldehyde (PFA) in PBS. Brains were collected and post-fixed for 48h in 4% PFA. Coronal slices of 30µm-thick were cut using a vibrating blade microtome VT1200S (Leica Microsystems). The ischemic area was measured using a thionin-based morphometric analysis on a series of 30µm-thick coronal brain slices at a regular interval of 180µm. Images of thionin-stained slices were acquired on a Leica Z6 APO Zoom macroscope (Leica Microsystem). After image alignment using Autoaligner software (Bitplane), the 3D volumes were reconstructed using IMARIS software version 6.4.2 (Bitplane). The total volume of the ischemic lesion was calculated by the “indirect method”, which corrects for brain oedema. Data were expressed as percentage values of the lesioned versus contralateral hemisphere. The Oedema/Atrophy index was calculated by subtracting the total volume of the ipsilateral hemisphere from the contralateral hemisphere and expressed as a percentage of the contralateral.

**Immunohistochemistry analysis**

Microglial cells presenting the M2 phenotype were identified on the basis of the specific marker Arginase-1 expression. Briefly, free-floating sections at the level of maximal infarct area were immunoblocked with 5% donkey serum and 5% BSA in PBS containing 0.3% Triton-X100. Brain sections were incubated overnight at 4°C with goat antibody directed against mouse liver Arg-1 (Abcam, Ref. ab60176) at 4°C overnight, followed by incubation with donkey anti-goat secondary Alexa 568 conjugated antibody (Invitrogen, Ref. A11057). All sections were mounted on glass slides using Mowiol® based mounting medium (Calbiochem) and viewed under a confocal laser scanning microscope TCS SP5 (Leica Microsystem).

**Morphological analysis of microglial cells**

Morphometric measurements of cell shape were performed on GFP expressing microglia of CX3CR1<sup>+/GFP</sup> transgenic mice in three consecutive sections where the infarct lesion was maximal. Morphological parameters characterizing microglial activation such as the number of microglia per mm<sup>2</sup> in the peri-infarct area, the length of the dendrites (µm), the number of dendrites per cell and the soma size (µm<sup>2</sup>) were assessed using IMARIS software (Bitplane).

**Radioimmunoassay**

PACAP38 radioimmunoassays were performed essentially as before by double-antibody immunoprecipitation. The spent culture medium was acidified, recycled 3 times onto Sep-Pak minicolumns, washed with 0.1% trifluoroacetic acid (TFA) and eluted with 80% acetonitrile/0.1% TFA. The eluates were dried under reduced pressure and resuspended in 100 mM sodium phosphate buffer, pH 7.4 containing 0.5% bovine serum albumin and 0.3 mg/ml PMSF. The PACAP38 antiserum VT027 was directed against the amidated C-terminal peptide and using a 1:200,000 working dilution, the assay had a 12 – fold range with an assay midpoint of 9 fmol.

**Gene expression analysis**

**Real time PCR:** Real time PCR experiments were performed to evaluate the level of mRNA expression of the pro-inflammatory TNF-α cytokine as well as the anti-inflammatory IL-10 cytokine and the neuroprotective Ym-1 protein in the different experimental groups. Mice were killed at 7 and 14 dpi and brains were immediately frozen on dry ice. Total RNAs from contiro- and ipsilateral hemispheres were extracted from a series of 40µm-thick brain slices cut on a cryostat cryomicrocut 3050S (Leica Microsystems) spaced by 340µm, using Tri-reagent (Sigma) and Nucleospin RNAII kit (Macherey-
Nagel) according to the manufacturer’s protocol. From each sample, 1.5 µg of total RNA was converted into single stranded cDNA using the ImPromII reverse transcriptase kit (Promega) with random primers (0.5µg/ml). Real time PCR experiments were performed and monitored by ABI Prism 7500 Sequence Detection System (Life Technologies). The primer pairs used were: for GAPDH, sens 5’-TCCCATTCCTCCACCTTTGA-3’, anti-sens 5’-CAGGAAATGAGCTTCCACAAAGTTG-3’; for TNF-α, sens 5’-AGGGCTGCCCAGCTACGTGC-3’, anti-sens 5’-CGGCAGAGGGAGTTGACTT-3’; for IL-10, sens 5’-AGAAGTGCCACCCAGAACAC-3’, anti-sens 5’-CTCTTCACCTGCTCCACTG-3’; and for Ym-1 sens 5’-TTTCTCCAGTGACCCATCCTT-3’, anti-sens 5’-TCTGGGTACAAGATCCCTGAA-3’. Mouse glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA was used as control. Relative expression between a given sample and a reference sample was calculated using the 2-ΔΔCt method, where ΔCt is the difference in the Ct values for the target gene and the reference gene.

Tagman Openarray: To gain a deeper understanding of PACAP-dependent modulation of the inflammatory responses, we performed a transcriptomic analysis based on TaqMan® OpenArray® Gene Expression platform. Briefly, mRNA extracted from ipsilateral hemispheres of Saline, ES and ES-P cell injected mice (n=3 for each group) were reverse transcribed into cDNA and loaded into TaqMan® OpenArray® Mouse Inflammation Panel plate (Life Technologies, Ref. 4475393) consisting of 632 gene targets selected for their involvement in inflammatory response. Real time PCR and quantitative gene expression were performed on a QuantStudio 12K Flex Real-Time PCR System (Life Technologies) and calculated RQ and p-values exported to Excel software (Microsoft).

Bioinformatic analysis: A file of RQ values calculated from comparisons between ES-P cell group and ES cell group were submitted to bioinformatic analysis through Ingenuity Pathway Analysis (IPA) software tools (Ingenuity® Systems, www.ingenuity.com). We defined parameters for IPA analysis such that only genes with a fold change greater or lesser than 2 would be considered; the requested knowledge database included all species and analysis was based only on direct interactions between genes. IPA was then used to identify network interactions between genes, regulated cellular and molecular functions and upstream regulators based on an extensive manually curated database of published gene interactions. A z-score was calculated to establish statistically significant predictions of activation or inhibition for a range of transcriptional regulators, and all calculations were linked to published findings accessible through the Ingenuity Knowledge Base. A z-score > 2 corresponds to a predicted activated function while a z-score < -2 reflects a predicted downregulated function.

Statistical analysis
All values were expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc.). For behavioral tests, lesion volumes and mRNA expression levels, one-way ANOVA followed by post-hoc Tukey’s test for multiple comparisons were performed, after confirming the normal distribution of the datasets by Kolmogorov-Smirnov test. For statistical analysis of Arg-1+ cell numbers, Kruskal-Wallis analysis followed by Dunn’s post hoc test were performed. Statistical significance was set at p<0.05.
**SUPPLEMENTAL TABLE I**

**Supplement Table I:** List of the top five modulated molecular and cellular functions and their respective predicted activation status after ES-P cells transplantation, 7 days after stroke.

<table>
<thead>
<tr>
<th>Category</th>
<th>Functions Annotation</th>
<th>p-Value</th>
<th>Predicted Activation State</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organismal Survival</td>
<td>organismal death</td>
<td>3.71E-21</td>
<td>Decreased</td>
</tr>
<tr>
<td>Cell Death and Survival</td>
<td>cell survival</td>
<td>9.34E-23</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>cell viability</td>
<td>1.26E-21</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>apoptosis of macrophages</td>
<td>8.39E-15</td>
<td>Decreased</td>
</tr>
<tr>
<td>Cellular Function and Maintenance</td>
<td>flux of Ca^{2+}</td>
<td>2.26E-17</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>flux of ion</td>
<td>7.75E-18</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>phagocytosis</td>
<td>1.90E-17</td>
<td>Increased</td>
</tr>
<tr>
<td>Organismal Development</td>
<td>development of blood vessel</td>
<td>1.28E-23</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>proliferation of endothelial cells</td>
<td>4.62E-13</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>endothelial cell development</td>
<td>1.50E-13</td>
<td>Increased</td>
</tr>
<tr>
<td>Cellular Movement</td>
<td>cell movement</td>
<td>1.42E-52</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>chemotaxis</td>
<td>1.45E-41</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>migration of cells</td>
<td>2.87E-53</td>
<td>Increased</td>
</tr>
</tbody>
</table>
Supplemental Figure I: Establishment and characterization of a PACAP expressing ES cell line (ES-P cells). After stable nucleofection and selection of 129Sv/Ev embryonic stem cells, one clone was isolated, expanded and further characterized. The expression at mRNA and protein levels of the neuropeptide PACAP and its release were monitored by confocal imaging (A.), RT-PCR (B.), Western blotting (C.) and radioimmunoassay (D.). A. Only ES-P cells show detectable eGFP fluorescence and PACAP expression (Blue, DAPI; Green, eGFP; Red, PACAP). B. ES-P cells express transgene derived PACAP encoded mRNA as detected by RT-PCR experiments. The amplicon size is indicated on the left. C. Total protein extracts are obtained from cultured stem cells and analyzed by western blot. The neuropeptide PACAP is detected in ES-P cell extracts only. Serial dilutions of PACAP peptide are loaded on the gel as standards. D. The neuropeptide PACAP is efficiently released in the supernatant of ES-P cells cultures as assessed by RIA detection. E. Transplanted ES and ES-P cells differ in their tumorigenic potential at 4 weeks post-ischemia. The release of PACAP from ES-P cells could reduce the tumorigenic potential of ES cells.
**Supplemental Figure II:** Transplanted GFP\(^+\) ES-P or ES cells do not express Arg-1 one week after stroke. ES-P cells or ES cells were i.c.v. injected three days after pMCAO in C57Bl/6 mice. One week after stroke, brain tissue slices were labeled for the M2 phenotypical marker Arg-1 (Red) and nuclei (DAPI). Scale bar: 10 µm.
**Supplemental Figure III:** Peri- and intra-infarct localization of stem cells 4 days after transplantation. 7 dpi, i.e. 4 days post-transplantation, the ES and ES-P cells can be mainly detected in the peri-infarct and infarct zone showing their migration in the lesioned hemisphere. The bottom panels represent a zoomed image of the upper panels. GFP+ stem cells (green) and DAPI+ nuclei (blue) are visualized. Scale bars, 100µm.
Supplemental Figure IV: Transcriptional factors potentially mediating PACAP’s polarizing effects on microglial cells. These transcriptional factors were identified by IPA analysis based on the experimentally observed regulations of their known target genes.
SUPPLEMENTAL REFERENCES


