Chapter 34
The Neuropeptide PACAP, a Potent Disease Modifier Candidate for Brain Stroke Treatment

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Abstract Despite years of intense research, acute ischemic stroke remains a leading cause of death and long-term disabilities worldwide. Although preclinical studies lead to the identification of over 1000 potential neuroprotective compounds, the current treatments for brain ischemia only rely on clot thrombolysis through injection of recombinant-tissue plasminogen activator (r-tPA) or mechanical revascularization, which benefit to less than 10 % of stroke victims due to a narrow therapeutical time window and side effects. Consequently, there is a crucial need for the identification of new molecules and the development of other strategies that could target later phases of the pathophysiological cascade of mechanisms following stroke. Indeed, because stroke initiates a complex series of pathophysiological events evolving both in time and location, putative therapeutic molecules need to be effective on several of the biochemical processes evoked by stroke. Pituitary adenylate cyclase-activating polypeptide (PACAP) has been reported to decrease infarct volume and improve functional recovery in several models of global and focal brain ischemia. The unique particularity of PACAP relies on its ability to act on various pathological processes of cerebral ischemia. PACAP can counteract excitotoxicity,

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inhibit apoptosis, reduce oxidative stress, modulate inflammation, and promote brain repair mechanisms. Nevertheless, due to several limitations, the pertinence of a potential therapeutical use of PACAP is still under investigation to successfully ensure the bench to bedside continuum.

Keywords PACAP • Brain stroke • Ischemia • Neuroprotection • Therapeutical drug • Inflammation

Abbreviations

AEPO	Asialo-erythropoietin
AIF	Apoptosis inducing factor
AIS	Acute ischemic stroke
BBB	Blood brain barrier
CBF	Cerebral blood flow
CNS	Central nervous system
CRMP2	Collapsing response mediator protein 2
DPP-IV	Dipeptidyl peptidase IV
EPO	Erythropoietin
GHRH	Growth hormone-releasing hormone
GPx-1	Glutathione peroxidase-1
HIF-1α	Hypoxic inducible factor-1α
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
i.v.	Intravenous
IL	Interleukin
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
NOS2	Nitric oxide synthase 2
PACAP	Pituitary adenylate cyclase-activating polypeptide
PEG	Polyethylene glycol
PLA	Polylactic acid
PLGA	Poly(lactic-co-glycolic acid) copolymer
PTS-6	Peptide transporter system 6
r-tPA	Recombinant-tissue plasminogen activator
SOD2	Superoxide dismutase-2
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide

Brain Stroke

Definition and Epidemiology

Stroke is a cerebrovascular accident caused by an abrupt reduction of cerebral blood flow (CBF) generating irreversible cerebral tissue damages. Although stroke can sometimes be due to intracerebral hemorrhage, the ischemic origin accounts for 87 % of the cases and results from the occlusion of an arterial cerebral vessel responsible for sudden focal neurological deficits. According to the World Health Organization, stroke encompasses the transient ischemic attack defined as neurological deficits disappearing spontaneously in less than 1 h without neither sequel nor brain imaging abnormalities, and the acute ischemic stroke (AIS) corresponding to clinical symptoms persisting beyond 24 h which will be in the scope of this review article.

In the United States, 795,000 Americans experience a new or recurrent stroke with 610,000 first-ever cases each year. It represents one stroke victim every 40 s and it is responsible for one death every 4 min. We assume that 6.6 million Americans already experienced a stroke event, an estimated stroke prevalence of 2.6 % on the 2009–2012 period. Stroke is considered as the third cause of death after heart diseases and cancer, the second cause of dementia after Alzheimer's disease, and the first cause of nontraumatic acquired disability in adults. The consequences after cerebral ischemia vary, but are often dramatic. Mortality rate was 20 % at 1 month and from 32 to 60 % after 3 years. While 25 % of patients recover without apparent sequel, 75 % of stroke survivors have various physical and psychological handicaps depending on the location and severity of the lesions. Among people bearing irreversible deficits, 30 % will not be autonomous in their daily activities and 40 % will not be able to resume their work. The management of stroke victims represents therefore a significant societal cost. In Europe, about 3–4 % of public health expenditures are dedicated to stroke, which represents a total of 64 billion € each year [1, 2]. The incidence and prevalence of stroke being closely associated with the aging of the population in industrialized countries, the projections for 2030 foresee a 20.5 % increase in prevalence of stroke in the United States [3]. Thus, it becomes obvious that stroke victims will represent a major public health and economic concerns.

Pathophysiology

Representing 2 % of the total body weight but consuming 20 % of cardiac output, the brain is particularly dependent on the continuous supply of oxygen and nutrients. The transient or permanent decrease in CBF observed in stroke results in 30 % of cases from occlusion of the middle cerebral artery (MCA) or one of its collaterals.

The consecutive energetic depletion initiates a complex series of pathophysiological events, which evolve in a spatiotemporal fashion and lead to a massive and progressive neurodegeneration responsible for disabling functional deficits [4].

From the spatial point of view, the hypoperfusion gradient from the periphery to the ischemic core delimits two distinct zones. The ischemic core, where energy supply is insufficient to allow cell survival, is the location of rapid and irreversible cell death, whereas the penumbra area, surrounding the ischemic core, is defined as an electrically silent but metabolically active tissue, thanks to the presence of arterial collaterals sufficient to support cell survival at least for a short period of time.

From the temporal point of view, the cascade of pathophysiological events responsible for the extension of the lesions, spreading from the ischemic core toward the penumbra, can be seen as a set of entangled processes taking place in two phases: an acute phase occurring during the first hours after the occlusion which mainly involves excitotoxic and oxidative stress processes, and a chronic phase developing over days and weeks after the stroke onset which includes apoptotic and inflammatory mechanisms. All these events lead to cerebral infarction whose size and location determine the nature and severity of the neurological deficits among stroke's victims.

Despite years of intense research and preclinical identification of numerous potential neuroprotective compounds, the current treatments for brain ischemia only rely on clot thrombolysis through injection of recombinant-tissue plasminogen activator (r-tPA) or mechanical revascularization. However, these treatments benefit to less than 10 % of stroke victims due to a narrow therapeutical time window and side effects [5]. Consequently, there is a crucial need for the development of other strategies that could target the overall pathophysiological cascade of events following stroke in the acute and chronic phases.

The PACAPergic System, an Endogenous Mechanism for Cell Protection After Stroke

The PACAPergic System

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated by Arimura and his colleagues from ovine hypothalamus extracts on the basis of its capacity to stimulate adenylate cyclase activity [6]. This neuropeptide exists in two forms, with a 38-amino acid peptide (PACAP38) and a truncated 27-residue fragment (PACAP27). PACAP, which exhibits 68 % sequence identity with vasoactive intestinal peptide (VIP), was classified as a member of the secretin/glucagon/growth hormone-releasing hormone (GHRH) superfamily [7]. Interestingly, the biologically active region of PACAP, i.e., its N-terminal part, has been highly conserved during evolution, from fish to mammals, suggesting that this neuropeptide must exert essential biological functions.

PACAP binds to three different seven-transmembrane G protein-coupled receptors named by the International Union of Pharmacology PAC1, VPAC1, and VPAC2

receptors according to their respective affinity toward PACAP and VIP [8]. Indeed PAC1 receptor exhibits a high affinity for both PACAP isoforms and a much lower affinity for VIP [9] whereas VPAC1 and VPAC2 receptors show similar affinity for PACAP and VIP [7]. More detailed information on PACAP and its receptors has already been published and is available in other published reviews [7, 10].

Several studies have reported the wide distribution of PACAP and its receptors in the central nervous system (CNS) and in peripheral tissues, supporting the hypothesis that PACAP exerts important biological activities. In fact, in the developing brain, PACAP exerts neurotrophic effects by modulating cell proliferation [11, 12], enhancing neuronal differentiation [13, 14] and blocking apoptosis [15–17]. In peripheral organs, PACAP has a strong dilatory activity on the vascular system [18] and bronchi [19]. Moreover, in vitro and in vivo studies have revealed that PACAP acts as a potent regulator of the immune system, exerting a general anti-inflammatory effect on innate [20, 21] and adaptive responses [21–23]. Altogether, these data indicate that PACAP is a unique neuropeptide which acts through a complex interplay of cellular and molecular mechanisms to inhibit cell death, promote neurotrophic activities, and regulate inflammatory responses.

Endogenous PACAP Expression After Cerebral Ischemia

Several studies have shown that PACAP expression is rapidly upregulated after brain artery occlusion, suggesting that the neuropeptide may play a role in the early pathophysiological events of ischemia. In a permanent model of MCA occlusion (MCAO), Stumm et al. have identified a biphasic regulation of PACAP in rat cortical pyramidal cells. They demonstrated that PACAP mRNA level in the fronto-parietal cortex was increased by 95 % 6 h after artery occlusion but was reduced by 74 % 2 days after ischemia as compared with sham-operated animals [24]. A similar pattern of expression was also observed after transient MCAO in mice with an increase of PACAP mRNA by 251 % after 6 h [24], suggesting that the rapid upregulation of PACAP is independent of the stroke model. In accordance with this hypothesis, an increase of PACAP transcript is also observed in a mouse model of global ischemia, with a very strong elevation of PACAP mRNA expression in the granule cell layer of the dentate gyrus of the hippocampus 1 and 3 days after stroke [25]. According to these reports, PACAP mRNAs are essentially localized in cortical and pyramidal neurons [24, 25], suggesting that neurons are the main source of PACAP after stroke. Nevertheless, recent data revealed that in the penumbral region, PACAP upregulation is not restricted to neurons but can also be observed in astrocytes and endothelial cells. Interestingly, some blood-derived CD34+ cells may also produce the neuropeptide upon infiltration in ischemic brain regions and can contribute to the elevated PACAP expression observed in human brain samples from patients who died 1–3 days after stroke [26]. Finally, the importance of endogenous PACAP expression in stroke models has been highlighted in PACAP-deficient mice which exhibit increased infarct volumes and functional deficits when compared to their control littermates [27, 28].

Although the molecular mechanisms of PACAP upregulation are still unclear, it is likely that PACAP may be expressed in response to stress-related environmental factors appearing in ischemic conditions. Stumm et al. showed that PACAP upregulation is mediated by the activation of the NMDA receptor and excessive glutamatergic neurotransmission, a hallmark of the initial ischemic cascade [24]. Another mechanism by which PACAP expression may increase is through activation of the hypoxic inducible factor-1α (HIF-1α) transcription factor whose transcriptional activity is strongly enhanced in hypoxic conditions. Indeed, no increased PACAP expression can be observed in HIF-1α deficient mice or after chemical inhibition during experimental ischemia [26]. In parallel, Zhang et al. demonstrated that PACAP expression is upregulated in sensory neurons in a model of adjuvant-induced inflammation in rat paw, suggesting that inflammatory conditions stimulate PACAP expression in neuronal cells [29].

Thus, these observations lead to the conclusion that in pathological conditions, endogenous PACAP expression is triggered in response to stress-related cues such as cell death, tissue lesion, or local inflammation, suggesting that the PACAPergic system could be part of an endogenous mechanism of protection aimed to support cell survival and to modulate the inflammatory response.

Expression of PACAP Receptors After Stroke

If less data are available regarding expression of PACAP receptors after stroke, they also support the idea that the endogenous PACAPergic system contributes to a mechanism of protection initiated after brain injury. Indeed, from day 1 to day 3 after ischemia, a transient increase of the PAC1 mRNA expression has been reported in neurons at the level of the post-ischemic fronto-parietal cortex, primary olfactory cortex, and caudate putamen in models of focal cerebral ischemia [24, 30, 31]. Similar results are observed in a mouse model of global ischemia where numerous PAC1 positive neurons are detected in the hippocampus 1 day after transient MCAO. In contrast, transcriptomic analysis revealed a significant decrease of VPAC1 mRNA at day 3, while VPAC2 mRNA levels remained unchanged over time [32]. From day 3, the PAC1 receptor is no longer detectable in neurons but its expression gradually increases in astrocytes. Indeed, an increase of PAC1 mRNA was observed in astrocytes of the corpus callosum in a permanent MCAO model [24]. Nakamachi and collaborators have also reported an elevation of PAC1 mRNA in hippocampal astrocytes surrounding the damaged CA1 pyramidal 7 days after ischemia [32], suggesting that the PACAPergic system may play a role in the reactive astrogliosis occurring after brain injury.

Altogether, these data reinforce the hypothesis of a participation of the PACAPergic system in an endogenous protective mechanism through the targeting at different levels of many pathophysiological processes in order to promote cell survival.

PACAP's Therapeutical Potential in Stroke Models: Mechanisms of Action

Neuroprotective Action of Exogenous PACAP's Delivery After Ischemia

Since its discovery, numerous studies using different approaches have demonstrated the neuroprotective effects of PACAP against ischemic lesions in various animal models of brain ischemia (Table 34.1).

Table 34.1 Reported PACAP's neuroprotective effects in experimental models of cerebral ischemia

Species	Model	Route	Doses	Delay	Results	References
Rat	4VO	*i.c.v*	0.1–10 pmol/h	0 h	↓ Infarct volume	[31]
		i.v.	5 nmol/kg + 16 or 160 pmol/h	24 h	↓ Infarct volume; ↓ JNK-SAPK; ↑IL-6	
Rat	CA	*i.c.v.*	1 pmol/h	0 h	↓ Infarct volume	[34]
Rat	tMCAO	*i.v.*	5 nmol/kg + 160 pmol/h	4, 8 and 12 h	↓ Infarct volume ↓ Functional deficits	[36]
Rat	tMCAO	*i.v.*	20 nmol/kg + 160 pmol/h	4 h	↓ Infarct volume	[37]
Rat	pMCAO	*i.c.v.*	450 pmol	Pre-ischemic	↓ Infarct volume	[97]
Rat	CA	*i.c.v.*	1 pmol/h	Pre-ischemic	↓ Infarct volume ↓ TUNEL ↓ p38-JNK-SAPK	[33]
Rat	pMCAO	*i.c.v.*	0.25–4 µg	Pre-ischemic	↓ Infarct volume and functional deficits	[39]
			2 µg or 2 µg×7 days			
Rat	pMCAO	*i.c.v.*	2 µg	Pre-ischemic	↓ TUNEL	[43]
Mice (PACAP KO)	pMCAO	*i.c.v.*	1 pmol	0 h	↑ Infarct volume and functional deficits ↑ Cytochrome c; ↓ Bcl-2	[28]
Mice (IL-6 KO)	tMCAO	*i.v.*	5 nmol/kg + 16 pmol/h	0 h	No effect of PACAP	[28]
Mice (PACAP KO)	pMCAO	*i.v.*	0.75 nmol	1 h	↑ Infarct volume and functional deficits	[27]
		i.c.v.	40 pmol	1 h		

(continued)

Table 34.1 (continued)

Species	Model	Route	Doses	Delay	Results	References
Rat	tMCAO	*i.v.*	0.002–20 µg/kg	1 h	↓ Infarct volume and functional deficits ↓ TNF-α; TNFR1; iNOS; MIP-1α; NF-kB	[35]
Mice	pMCAO	*i.c.v.*	1 pmol	0 h	↑ IL-22; IGF-1; CTRAM	[38]
Mice	pMCAO	*i.c.v.*	1 pmol	0 h	↑ S100a5; IL-1β; Ccl2; IL-11; Cxcr2; FGF-21 and CRMP2 ↓ Prlr	[58]
Mice	pMCAO	*i.c.v.*	Constitutive secretion by ESC	3 Days	↓ Functional deficits ↓ Pro-inflammatory responses ↑ M2 microglia	[40]
Rat	tMCAO	*i.p.*	0.1, 1 or 10 µg/kg×5 days	4 h	↓ Infarct volume and functional deficits ↑ CD34$^+$ cells homing ↑ Angiogenesis and CBF	[26]

4VO: 4 vessels occlusion, *CA* cardiac arrest, *tMCAO* transient middle cerebral artery occlusion, *pMCAO* permanent cerebral artery occlusion, *KO* knock-out, *i.c.v.* intracerebroventricular, *i.v.* intravenous, *i.p.* intraperitoneal, *ESC* embryonic stem cells, *CBF* cerebral blood flow

The therapeutic benefit of exogenous PACAP administration for the treatment of cerebral ischemia was reported for the first time in 1996 in a rat model of transient global ischemia. A chronic intracerebroventricular (*i.c.v.*) infusion of PACAP, 1 pmol/h for 7 days, started immediately after induction of the ischemic lesion significantly reduced hippocampal neuronal cell death [31]. Other studies, using global ischemia models, confirmed that *i.c.v.* administration of PACAP before or during the ischemia induction correlates with a reduction of infarct volumes [33, 34]. Similarly, in animal models of transient focal cerebral ischemia, the intravenous (*i.v.*) injection of PACAP induces beneficial effects when administered before the induction of ischemia and up to 4 h after arterial occlusion, including a reduction of lesion volume and functional deficits [28, 35–37]. Finally, the therapeutic potential of PACAP was also confirmed in permanent models of cerebral ischemia, in which PACAP was administrated either *i.v.* or *i.c.v.* up to 1 h after stroke [27, 28, 38, 39].

These data highlight that PACAP is a potent neuroprotective agent when administered during the acute phase of brain ischemia, specifically within the first hours after

artery occlusion. Nevertheless, a study also reports that a delayed delivery of PACAP beyond the acute phase can also improve functional recovery 7 and 14 days after stroke. Such effect was obtained using a stem cell-based strategy to deliver PACAP in the vicinity of the infarct area 3 days after the stroke onset in a mouse model of permanent MCAO and indicates that PACAP may represent an interesting therapeutical candidate to counteract the delayed pathophysiological processes occurring during the chronic phase of cerebral ischemia and therefore constitutes a novel alternative for stroke treatment in a currently unexploited therapeutical time window [40].

Antiapoptotic Role of PACAP

The impairment of blood supply after vessel occlusion in a brain territory initiates a pathophysiological cascade of events leading to a massive neuronal loss. If cell death responsible for ischemic lesions has firstly been considered only of necrotic nature, it is now established that part of the cells also die by apoptosis [41]. Apoptotic processes preferentially develop within the penumbra and participate in the extension of the lesions from the infarct core toward the ischemic penumbra, therefore representing a potentially important therapeutic target [42]. Several in vitro and in vivo studies on ischemia report that the protective effect of PACAP is based on its well-recognized antiapoptotic properties as illustrated in rats by a 50 % reduction of apoptotic cell death after *i.c.v.* administration of PACAP (2 μg) immediately before permanent MCA occlusion [43].

The antiapoptotic property of the neuropeptide relies on its ability to interfere at multiple levels of the neurotoxic cascade that ends to cell apoptosis. The O_2 and glucose withdrawal impairs the activity of critical ATP-dependent ionic pumps such as ATP-dependent Na^+/K^+ and Ca^{2+} pumps disrupting ionic homeostasis and transmembrane gradients resulting in a massive release of excitatory amino acids, especially glutamate [44]. Lucas and Newhouse in 1957 and then Olney in 1969 have established a correlation between extracellular glutamate concentration and cytotoxicity, and shown that glutamate neurotoxicity is due to its excitatory effect, leading to the concept of excitotoxicity [45–47]. The binding of glutamate to its ionotropic NMDA, AMPA, and kainate receptors increases the permeability of these channel receptors to Cl^-, Na^+, and Ca^{2+} ions. Furthermore, the intracellular accumulation of Ca^{2+} triggers molecular events leading to the activation of apoptotic processes. At high concentration (above 10 nM), PACAP inhibits glutamate receptors transmission, from NMDA- as well as AMPA-type receptors [48, 49] and increases glial glutamate uptake and metabolism in astrocytes by promoting the expression of GLT-1, GLAST transporters, and glutamine synthetase [50]. Thus, PACAP can interfere in a first step with this initial excitotoxic cascade by modulating glutamate signaling (Fig. 34.1).

Consecutively to energetic depletion and associated excitotoxicity, the increased cytosolic Ca^{2+} concentration initiates the uncoupling of the mitochondrial oxidative phosphorylation, leading to deleterious free radicals production. In oxidative stress
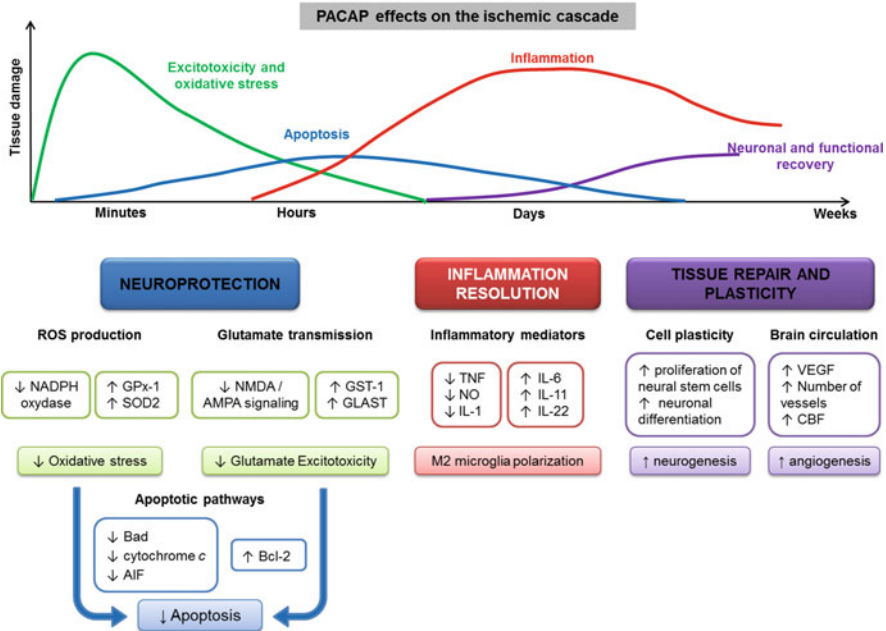
Fig. 34.1 Summary of PACAP's neuroprotective effects during acute ischemic stroke. PACAP acts on the different physiopathological events of the ischemic cascade developing in the early as well as the late phase of brain stroke. During the acute phase of ischemia, PACAP prevents neuronal cell death directly by regulating the caspase-dependent apoptotic pathways and indirectly by reducing oxidative stress and glutamate excitotoxicity. Moreover, PACAP affects the local inflammatory response in both phases of ischemia by modulating the balance between pro- and anti-inflammatory mediators and promoting the redirection of the microglial cell response toward a neuroprotective M2 phenotype. In the late phase, the PACAP-dependent redirection of the microglial response combined to the neurotrophic properties of the peptide on neural progenitors amplifies the post-ischemic neurogenesis and neoangiogenesis processes improving the endogenous tissue repair and functional recovery

conditions, such as after hydrogen peroxide exposure [17], lipopolysaccharide-induced dopaminergic neurotoxicity [51] or controlled cortical impact [52], PACAP reduces the production of reactive oxygen species by inhibiting the activity of NADPH oxidase and inducing the expression of Glutathione Peroxidase-1 (GPx-1) and Superoxide Dismutase-2 (SOD-2) antioxidant enzymes. Moreover, in a model of global ischemia, PACAP counteracts hippocampal CA1 neuronal death consecutive to oxidative DNA damages *via* induction of apurinic/apyrimidinic endonuclease 1 DNA repair enzyme [53]. Therefore, it appears that PACAP's anti-apoptotic effect results not only from the modulation of glutamatergic transmission but also depends on a powerful antioxidant activity, two of the initial processes responsible for the activation of the apoptotic cascade (Fig. 34.1).

Besides the inhibition of these initial steps of the neurotoxic cascade, the investigation of the mechanisms involved in the protective action of PACAP revealed its

ability to inhibit directly the caspase-dependent mitochondrial apoptotic pathway. Western blot analysis performed in PACAP-deficient mice reported a rapid decrease, within 6 h after permanent focal ischemia, of Bcl-2 expression levels in the mitochondrial fraction and a concomitant cytosolic accumulation of cytochrome c release [28]. PACAP can also regulate the expression of other members of the Bcl-2 family. In particular, in an in vitro model of cortical neurons submitted to oxygen/glucose deprivation, PACAP promotes cell survival through the phosphorylation and therefore the inactivation of Bad [24]. Similarly, the neuroprotective effect of PACAP against monosodium glutamate-induced retinal degeneration is mediated by inhibition of pro-apoptotic signaling pathways and relies on the ability of PACAP to phosphorylate Bad but also to decrease cytochrome c and apoptosis inducing factor (AIF) release [54] (Fig. 34.1).

Altogether, these observations demonstrate that PACAP has a potent antiapoptotic activity due to combined inhibitory actions on the initiation phase as well as the effector phase of the apoptotic pathway.

Immunomodulatory Effects of PACAP

The post-ischemic inflammation is a complex process initiated early after the stroke onset, sustained over weeks, exerting both detrimental and beneficial roles and controlled by microglia, a unique immunocompetent cell population in the brain parenchyma. A correlation between the neuroprotective effect of PACAP and its immunomodulatory role was first proposed in 1998 by Shioda and coworkers. In a rodent model of global cerebral ischemia, *i.c.v.* infusion of PACAP for 48 h before ischemia causes an increase in interleukin (IL)-6 levels in the cerebrospinal fluid of animals correlated to a decrease of brain lesions [34]. Immunofluorescence studies highlighting IL-6 production in PAC1-expressing cortical neurons and absence of PACAP-mediated neuroprotection in IL-6 knock-out mice following MCAO suggest that PACAP's neuroprotective properties rely on the PACAP-dependent IL-6 production (Fig. 34.1). Although the mechanisms underlying the protective effects of PACAP through the production of IL-6 are not clearly identified to date, such results illustrate the close link between modulation of the inflammatory response and neuroprotection.

Microarray-based transcriptomic studies showed in a mouse model of permanent focal ischemia that *i.c.v.* administration of the neuropeptide PACAP immediately after MCA occlusion modulates the expression of numerous genes encoding inflammatory mediators 6 and 24 h after PACAP injection. Among these genes, *Il-22* and *Il-11* were found upregulated after PACAP treatment whereas *tumor necrosis factor (tnf)* was downregulated [38] (Fig. 34.1). Similarly, *i.v.* administration of very low quantity of PACAP (0.02 μg/kg) in a rat model of transient ischemia negatively regulates the expression of pro-inflammatory compounds such as TNF-α, the nitric oxide synthase (NOS)-2, the chemokine MIP-1α, and the transcription factor NF-kB (Fig. 34.1). Importantly, the modulation of the local inflammatory response

seems to correlate with a reduction in lesion volume and neurological deficits in animals suggesting that the anti-inflammatory properties of the neuropeptide PACAP could account for a large part of its neuroprotective effects [35]. Interestingly, these results have been confirmed in a model of delayed PACAP delivery 3 days after the stroke onset [40]. In this study, the observed modulation of the local inflammatory response after delayed PACAP delivery reveals a PACAP-dependent shift of the inflammatory response toward a resolutive phenotype, supporting cell survival and tissue repair, that correlates with decreased neurological deficits in animals. This PACAP-dependent skewing of the inflammatory response relies, at least partly, on direct or indirect neuropeptide's effects on microglial cells (Fig. 34.1). Indeed, we report a PACAP-dependent polarization of microglial cell phenotype toward a neuroprotective M2-like phenotype as illustrated by an increased number of Arginase-1 positive microglial cells in the peri-infarct zone [40].

Altogether, these studies highlight that therapeutical strategies aimed at targeting the inflammatory process in the early phase as well as in the late phase of stroke and more specifically by skewing the microglial response toward a neuroprotective response rather than simply inhibiting inflammation could improve functional recovery in stroke victims.

Neurotrophic Properties of PACAP

PACAP exerts other important biological activities capable of interfering with the delayed pathophysiological events following stroke. More particularly, PACAP could mobilize different endogenous mechanisms of tissue repair including modulation of inflammation but also processes such as neurogenesis and angiogenesis, which in concert improve tissue homeostasis, neuroplasticity, and neuronal functions.

Following stroke, neurogenesis processes, which promote the generation of new parenchymal cells from neural stem cells and progenitor cells, are increased in the subventricular zone and in the dentate gyrus of the hippocampus. However, only 0.2 % of dead neurons are efficiently replaced by newborn cells [55], which is insufficient to achieve a complete functional recovery. Although there is no direct demonstration of a beneficial effect of PACAP in the neurogenic processes in in vivo models of ischemia, evidence from in vivo and in vitro studies using neural cells indicates that PACAP, due to its neurotrophic properties, may represent a promising pharmacological tool to improve such mechanisms. Indeed, besides an in vitro pro-mitogenic effect of PACAP on various cell types including neural stem/progenitor cells [56, 57], *i.c.v.* PACAP administration in naive mice as well as delayed PACAP delivery 3 days after ischemia (unpublished observations) increase the number of BrdU-positive cells in the subventricular zone and in the dentate gyrus [12, 56], indicating that PACAP enhances in vivo adult neurogenesis. Although the molecular mechanisms involved are not clearly identified so far, the pro-mitogenic effect of PACAP seems mediated through the PAC1 receptor which is expressed in the neurogenic areas [56].

Confirming further the potential of PACAP to enhance neurogenesis, several studies also report that PACAP signaling can guide stem/progenitor cell differentiation toward a neuronal phenotype. In particular, in the presence of PACAP, embryonic stem (ES) cells grown as embryoid bodies present an increased number of neuritic extensions compared to control cells, reflecting the neurogenic effect of the neuropeptide in the earliest stages of development. Furthermore, electrophysiological data demonstrate that PACAP enhances the amplitude of outward currents in these embryoid bodies-derived cells after 2 weeks of treatment [13], illustrating the functionality of the newly formed neurons. Furthermore, recent work revealed that in a murine stroke model, PACAP increases the expression of the *collapsing response mediator protein 2* (CRMP2), known to participate in axonal growth and neuronal differentiation, suggesting that PACAP could promote neuroregeneration through CRMP2 induction [58]. The fact that migration and differentiation of neural cells contribute to the mechanisms associated with endogenous neurogenesis to improve functional recovery [59, 60] suggests that PACAP could enhance post-stroke neurogenesis and consequently improve neurological and functional outcomes (Fig. 34.1).

Another mechanism that plays a critical role in improving long-term recovery after stroke is the reestablishment of a functional microvasculature in the ischemic penumbra. Indeed, neuronal function is critically dependent on regulation of CBF [61]. Among the numerous factors regulating angiogenesis and vascular maturation, the vascular endothelial growth factor (VEGF) appears to be a key player. In rodents, VEGFc and its receptors are overexpressed during the first 7–10 days after transient MCA occlusion and would be involved in post-ischemic revascularization processes [62, 63]. Previous investigations have demonstrated that PACAP stimulates VEGF production in rat pituitary folliculostellate cells [64], suggesting that it may play an important role in angiogenesis. Such pro-angiogenic properties of PACAP have recently been confirmed in a transient model of MCAO in rat where intraperitoneal (*i.p.*) injections of PACAP for 5 consecutive days after MCA ligation lead to a higher cerebral microvascular perfusion and to an increased number of cerebral blood vessels in the penumbral area compared with control animals, as visualized with FITC-dextran. Moreover, a significant elevation of CBF in the ischemic cortex of PACAP-treated animals was observed 1 week after ischemia [26], confirming that PACAP may be considered as a potent angiogenic factor after stroke (Fig. 34.1).

PACAP Therapeutical Potential for Stroke Treatment: Limitations and Possibilities

All the above-mentioned observations indicate that PACAP could be useful as a therapeutic agent to reduce cell loss and promote neurological and functional recovery after ischemic stroke. However, its clinical use may be compromised by a poor bioavailability, a short half-life, and potential side effects as discussed below.

Time Window for PACAP Administration

Although PACAP represents a very interesting candidate for stroke treatment, its ability to induce tissue protection and functional recovery has mainly been investigated early after administration of the peptide, i.e., within the first 4 h after the stroke onset [33, 35, 36, 38]. It should be remembered that a narrow time window of only 4.5 h is one of the most important limitations of the current available treatments for brain ischemia which are only given to about 10 % of stroke victims. As a consequence, the development of therapeutical strategies with enlarged time window is a priority and in this context, our recent findings of a beneficial effect of PACAP 3 days after the stroke onset offer new avenues for the use of PACAP as a therapeutic drug with unexploited therapeutical window that could benefit to a larger number of stroke patients [40].

PACAP Bioavailability and Administration Route

Numerous studies using rodent models of cerebral ischemia demonstrate promising results after an intravenous injection of PACAP, as illustrated in Table 34.1. In human however, strategies based on a systemic administration of pharmacological agents are restricted by the difficulties to access the damaged cerebral tissues especially after cerebrovascular disruption. Interestingly, while the passage of numerous molecules is restricted by the blood brain barrier (BBB), PACAP38 crosses the BBB *via* the specific peptide transporter system 6 (PTS-6) [65]. Moreover, in two different models of brain ischemia, one caused by cardiac arrest and the other by MCAO, a rapid transient increase of PACAP influx into the brain within 4 h after *i.v.* injection has been shown [66, 67]. Although the exact mechanisms involved in the observed influx of PACAP have not been directly demonstrated, these changes of PACAP permeability are more likely attributable to an enhanced PTS-6 activity across the BBB rather than the ischemia-induced increased BBB permeability. This ability of PACAP to rapidly enter the brain may account for the neuroprotective effects observed early after the stroke onset [35–37]. Nevertheless, treatment with the native peptide starting 8 or 12 h after occlusion did not result in reduced infarct or improve functional recovery [35–37], which could be explained by the decreased entry of PACAP beginning 6 h after ischemia [66]. Thus it appears that while intravenous PACAP injection at early time points after ischemia may represent a relevant approach for neuroprotection, after the first 4 h, *i.v.* PACAP injections do not reach efficiently the brain parenchyma to promote neurological improvement and functional recovery. Thus, alternative strategies to improve the delivery of PACAP in the vicinity of the infarct area, such as stem cell-expressing PACAP injected 3 days after stroke [40], may be of great interest. Moreover, by enhancing the rate of entry of PACAP in the CNS, neuroprotection would be achieved with lower doses of the peptide, leading to a reduction of the adverse side effects associated with an *i.v.* injection.

PACAP Stability

In the systemic circulation, PACAP, as most natural peptides, is rapidly degraded by plasmatic enzymes including carboxypeptidase and endopeptidase, greatly reducing its biological half-life and therefore restricting its use as a therapeutical agent. Furthermore, in vitro and in vivo studies revealed that PACAP is rapidly metabolized by dipeptidyl peptidase-IV (DPP-IV), a ubiquitous aminopeptidase that releases dipeptides from the N-terminal fragment of diverse peptides. Thus, after intravenous injection to mice, the half-life of PACAP in plasma is less than 2 min [68]. In human, a case study in an elderly male patient estimated PACAP blood half-life between 5 and 10 min [69], while in healthy volunteers PACAP exhibits a mean plasma half-life of 3.5 ± 1.3 min [70]. Moreover, the cleavage of PACAP by DPP-IV generates two metabolites PACAP(3-38) and PACAP(5-38) [68] that behave as PAC1 antagonists [71], reducing further the biological effects of PACAP. As a consequence, obtaining an effective therapeutical dose of PACAP in the ischemic territory may be hampered by this low metabolic stability.

Potential Side Effects

PACAP and its receptors are widely distributed throughout the CNS and peripheral tissues. Such a large distribution implies that this neuropeptide exerts numerous biological activities [7], some of them being potential side effects when injected systemically, therefore preventing the safe therapeutic use of the peptide. For instance, PACAP and its receptors are expressed in the cardiovascular system [72–74]. Considering that cerebral ischemia results from vascular impairment, potential effects of PACAP on the cardiovascular system have to be considered very carefully. In fact, in healthy volunteers, intravenous infusion of PACAP (10 pmol/kg/min) for 20 min significantly increases heart rate [70]. As PACAP was reported to be a potent and long-lasting vasodilator, this tachycardia probably results from the peripheral vasodilatation caused by the peptide [75, 76]. It has also been showed that PACAP in a dose-dependent manner can cause tachycardia, bradycardia, and auricular fibrillation [77], various events that may lead to the occurrence of ischemic stroke. Moreover, it is worth noting that in human, all subjects who received an *i.v.* injection of PACAP experienced a sustained skin flushing on the face and chest [70] and some of them were subjected to headache [78]. Because such physiological responses may impede the use of PACAP as a safe therapeutical drug for stroke treatment, the development of approaches to limit PACAP side effects represents an important goal. As discussed below, different strategies may be considered to counteract the limitations associated with PACAP poor bioavailability and side effects, including the design of stable analogs of PACAP with improved selectivity and the development of strategies to enhance its specific brain delivery.

Development of Stable Analogs

One possibility to increase PACAP metabolic stability is to develop PACAP stable analogs presenting a reduced sensitivity to plasmatic endopeptidase and DPP-IV. In this attempt, chemical modifications at potential peptidase cleavage sites into the peptide sequence can drastically improve its resistance to enzymatic degradation. For instance, the sequence of PACAP contains two potential cleavage sites for plasmatic endopeptidases represented by two dibasic pairs Arg^{14}-Lys^{15} and Lys^{20}-Lys^{21} [79]. Therefore, the replacement of the Lysine amino acid by an Alanine residue confers an increased resistance to enzymatic digestion. Similarly, N-terminal acetylation of the peptide abolished its degradation by DPP-IV. Structure-activity relationship studies led to the development of two metabolically stable PACAP analogs, i.e., acetyl-[Ala^{15}, Ala^{20}]PACAP38-propylamide and acetyl-PACAP27-propylamide that exhibit a complete resistance to DPP-IV degradation and an increased plasmatic half-life while remaining highly active [79]. It has been shown in a rat model of transient cerebral ischemia that PACAP38 and its analog acetyl-[Ala^{15}, Ala^{20}] PACAP38-propylamide, when administered intravenously at very low dose (0.02 μg/kg), present similar potency and act through same mechanisms to reduce neuronal cell loss and functional deficits [35]. These results indicate that picomolar doses of PACAP and analogs are sufficient to exert maximal neuroprotective activities in case of stroke and it is tempting to speculate that at such low doses, side effects associated with the systemic administration of PACAP would be considerably reduced.

Nevertheless, PACAP analogs have also shown detrimental effects in animal stroke models. As a matter of fact, the administration of a VPAC2 agonist worsens neuronal damages after stroke [80]. Therefore, development of Ac-[Phe(pI)^6, Nle^{17}]PACAP(1-27) which activates PAC1 and VPAC1 receptors but not VPAC2 could be a new tool to reduce PACAP side effects [81]. Actually, in an in vivo model of Parkinson disease, this analog was as potent as PACAP to restore tyrosine hydroxylase expression and to modulate the inflammatory response in the *substantia nigra* but had significantly less effects on mean arterial pressure when compared to PACAP [82].

Enhancement of Brain Delivery

Another possible approach to counteract the limitations associated with the plasmatic instability of PACAP and its potential peripheral side effects is the development of strategies aiming at enhancing the specific delivery of PACAP within the ischemic brain.

Intranasal Delivery

During the last few decades, the nasal route for drug delivery into the brain has been extensively investigated. The "nose-to-brain" delivery is of great interest because this route is noninvasive and can deliver drugs directly into the brain, bypassing the

BBB which is impermeable for a high percentage of molecules. Additionally, such approach avoids rapid metabolism in the systemic circulation as well as peripheral side effects, therefore representing a promising hope for counteracting the limitations associated with the intravenous injection of PACAP. Several therapeutic proteins such as granulocyte-colony stimulating factor, transforming growth factor-beta, erythropoietin (EPO), and insulin-growth factor-1 have been successfully delivered into the brain of rodents in different models of stroke [83–85], indicating that intranasal drug delivery is a promising method to specifically provide therapeutical compounds into the ischemic brain. Moreover, different formulations including simple drop (nanogel) or nasal spray (insoluble powder or emulsions) can be designed to maximize the convenience and comfort of patients. Interestingly, nasal application of PACAP can reach the brain rapidly and was effective to slow down Alzheimer's disease-like pathology in a mice transgenic model [86]. Noteworthy, such administration of the peptide has also been reported in human and did not affect heart rate and blood pressure in patients [86], indicating that using the "nose-to-brain" route for PACAP delivery can efficiently avoid the side effects of PACAP on the cardiovascular system.

Colloidal Carriers

The use of colloidal carriers including liposomes and nanoparticles could represent another interesting avenue for a selective and prolonged delivery of PACAP and its derivatives into the ischemic brain. Colloidal carriers are drug vectors, particles, or vesicles in the nanometer size range used for the transport of loaded drugs to a target site. These systems are biocompatible, nontoxic, and biodegradable and therefore represent a promising tool for a clinical use. They can carry various therapeutic agents (hydrophilic, lipophilic, and amphoteric drug molecules) which are generally encapsulated, entrapped, adsorbed, or chemically attached to the carrier surface [87]. These encapsulation systems offer the advantage to protect drug molecules from enzymatic degradation in the blood circulation, significantly increasing their bioavailability. The biocompatibility and longevity of such particles can be achieved through surface modifications involving a coating of biodegradable polymers such as chitosan, PLGA (poly(lactic-co-glycolic acid) copolymer), PLA (polylactic acid), and PEG (polyethylene glycol) [88]. In particular, PEGylation of drug-loaded liposomes is very efficient to enhance the longevity of particles in the systemic bloodstream [89]. Such surface modifications are also used to achieve tissue specificity. For example, the biopolymer polysorbate, PLGA, and PEG have been successful in delivering therapeutics into the brain parenchyma [90, 91]. Other ligands can also be added to specifically target the brain including insulin, transferrin, lactoferrin, glutathione, and apolipoprotein [92]. It is worth mentioning that such nanocarrier formulations also exist for nose-to-brain delivery [93].

Colloidal vectors, in particular polymeric nanoparticles and liposomes, have been used with success to transport therapeutically relevant agents into the brain parenchyma in various animal models of brain ischemia. For instance, in a transient model of MCAO, chitosan-nanoparticles loaded with the basic fibroblast growth

factor and/or with an inhibitor of caspase-3 have been administrated systematically just before the induction of ischemia. In this study the authors found that nanoparticles rapidly transport the active molecules to the brain parenchyma in their active form, which results in reduced infarct volume and functional impairment [94]. PEGylated liposomes administered within 3 h after the stroke onset also accumulate in the ischemic penumbral area in a time-dependent manner with a peak of accumulation at 24 h [95]. Hence, the long-lasting accumulation of the nanocarriers in the vicinity of the infarct area could lead to a prolonged drug delivery and an increased therapeutic efficacy of PACAP. Supporting this hypothesis, asialo-erythropoietin (AEPO, a metabolite of EPO) loaded liposomes present higher protective effect compared to native AEPO [95]. Furthermore, magnetic resonance imaging studies revealed that 100 nm-liposomes, injected intravenously 1 or 2 h after permanent MCAO in rats, accumulated in the ipsilateral parenchyma [96]. These results raise the possibility that liposome-based strategies may be possible even in the absence of reperfusion and could therefore benefit to patients who are not eligible for r-tPA treatment. All these observations indicate that application of such technologies to PACAP could not only enhance its therapeutic efficacy to improve neurological and functional ischemia-induced deficits, but also enable a reduction of the dose of peptide which must be administered to achieve a therapeutic response, thus minimizing its adverse side effects. Remarkably, encapsulation systems may also present the advantage of targeting PACAP delivery at the cellular level. As discussed previously a possible mechanism underlying the beneficial effect of PACAP during cerebral ischemia relies on its immunoregulatory activity on microglial cells. Thus, incorporating molecules specifically recognized by microglial cells in the surface of liposomes or nanoparticles would be of great interest. For instance, it would be interesting to evaluate the therapeutical potential of PACAP-loaded particles coated with molecules known to activate microglia phagocytic function without triggering a pro-inflammatory program such as the agonist of the TREM2 receptor Hsp60 [97].

Altogether, these approaches represent alternative strategies for the stabilization and development of pharmaceutical formulation of PACAP and its derivatives to benefit from its neuroprotective properties while overcoming its limitations in the context of brain stroke.

Conclusion

In conclusion, there is now clear evidence that PACAP exerts a potent neuroprotective activity and improves functional recovery in various models of cerebral ischemia. PACAP acts at very low doses on the main pathophysiological events developing in the early and in the late phases of brain stroke, making PACAP a potential therapeutical candidate for stroke treatment even in still unexploited therapeutical windows. Interestingly, several pathophysiological processes modulated by PACAP in stroke models are known to be involved in many other neurodegenerative

diseases among which Parkinson's disease, multiple sclerosis, spinal cord injury, or Alzheimer's disease, suggesting that PACAP could represent also a novel alternative for the treatment of these neurological troubles. Nevertheless, because of the wide distribution of PACAP and its receptors, a systemic delivery of the peptide would have many peripheral actions responsible for numerous side effects limiting the PACAP's therapeutical use. Therefore, it is now necessary to develop selective analogs and efficient route of administration to specifically and locally target the signaling mechanisms and cell responses relaying the protective effects of PACAP without affecting peripheral organs.

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