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Neuroprotection by endogenous and exogenous PACAP following stroke☆

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Abstract

We investigated the effects of PACAP treatment, and endogenous PACAP deficiency, on infarct volume, neurological function, and the cerebrocortical transcriptional response in a mouse model of stroke, middle cerebral artery occlusion (MCAO). PACAP-38 administered i.v. or i.c.v. 1 h after MCAO significantly reduced infarct volume, and ameliorated functional motor deficits measured 24 h later in wild-type mice. Infarct volumes and neurological deficits (walking faults) were both greater in PACAP-deficient than in wild-type mice, but treatment with PACAP reduced lesion volume and neurological deficits in PACAP-deficient mice to the same level of improvement as in wild-type mice.

A 35,546-clone mouse cDNA microarray was used to investigate cortical transcriptional changes associated with cerebral ischemia in wild-type and PACAP-deficient mice, and with PACAP treatment after MCAO in wild-type mice. 229 known (named) transcripts were increased (228) or decreased (1) in abundance at least 50% following cerebral ischemia in wild-type mice. 49 transcripts were significantly up-regulated only at 1 h post-MCAO (acute response transcripts), 142 were up-regulated only at 24 h post-MCAO (delayed response transcripts) and 37 transcripts were up-regulated at both times (sustained response transcripts). More than half of these are transcripts not previously reported to be altered in ischemia.

A larger percentage of genes up-regulated at 24 hr than at 1 hr required endogenous PACAP, suggesting a more prominent role for PACAP in later response to injury than in the initial response. This is consistent with a neuroprotective role for PACAP in late response to injury, i.e., even when administered 1 hr or more after MCAO. Putative injury effector transcripts regulated by PACAP include β-actin, midline 2, and metallothionein 1. Potential neuroprotective transcripts include several demonstrated to be PACAP-regulated in other contexts. Prominent among these were transcripts encoding the PACAP-regulated gene Ier3, and the neuropeptides enkephalin, substance P (tachykinin 1), and neurotensin.

Keywords: Pituitary adenylate cyclase activating polypeptide, PACAP; PACAP-deficient mouse; Middle cerebral artery occlusion, MCAO; Neurological severity score, NSS; Cerebral ischemia; Infarct volume; cDNA microarray; PACAP responsive gene; Neuroprotection; Neurotrauma

1. Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) was discovered as a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) family [1]. It is widely distributed in neurons of the brain and peripheral nervous system [2], and has multiple transmitter and trophic functions [3]. PACAP affects neuronal cell cycle exit during central nervous system formation [4], promotes neuronal differentiation in cultured rat sympathetic neuroblasts [5,6], differentially modulates proliferation of central and peripheral neuroblasts [7], stimulates neuritogenesis in PC12 cells [8,9] and regulates neuron-specific gene expression in human neuroblastoma cell lines [10]. PACAP prevents apoptotic cell death and protects cultured rat cortical neurons against glutamate-induced cytotoxicity [11], and dopaminergic neurons against 6-hydroxydopamine–induced cytotoxicity [12].

These properties of PACAP are consistent with a possible endogenous neuroprotective role after stroke or brain injury. In fact, PACAP has significant neurotrophic and neuroprotective

☆ Data deposition: The raw data from the expression profiling experiments reported in this paper have been deposited in the Gene Expression Omnibus database (accession no. GSE5902). The GSE5902 will be hyperlinked when it is released.

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effects in brain damage models *in vivo* [13,14] and *in vitro* [15–19]. PACAP prevents the ischemic death of rat CA1 neurons when given either intracerebroventricularly or intravenously in a model of transient global ischemia, even if administration is delayed for 24 h after the ischemic event [13]. PACAP can prevent loss of hippocampal neurons even after systemic administration presumably because it is a ligand of peptide transport system PTS-6, which transports it across the blood-brain barrier (BBB) at modest rates [20]. Systemic administration of PACAP also effectively reduces infarct volume in a rat model of focal ischemia when administration begins 4 h after MCAO [14]. In addition to its neuroprotective effects, PACAP is cardioprotective for cultured ischemic myocytes [21], attenuates reperfusion injury following ischemia of brain, kidney and lung [22–24], and is protective in endotoxemia *in vivo* [25], all suggesting an even more general role for PACAP in injury response.

Cerebral ischemia causes neuronal cell death in the areas where blood flow to the brain is permanently or transiently interrupted, and additional neuronal cell death (secondary damage) in immediately surrounding brain areas, due to altered extracellular ion concentrations, release of excitotoxic neurotransmitters such as glutamate, and elevated levels of toxic cytokines and generation of reactive oxygen species through inflammatory processes that begin shortly after an ischemic event [26,27]. No pharmacological treatment is available to prevent these post-ischemic events that occur as a consequence of the initial injury. Thus, investigation of PACAP's role in the prevention of secondary neuronal damage in ischemia is potentially of great importance.

Characterization of changes in gene expression that occur during stroke, and therapeutic intervention in stroke, can illuminate mechanisms of ischemic neuronal death and neurological dysfunction, or identify novel therapeutic targets in cerebral ischemia. The sequencing of the mouse and human genomes, growing databases for differential gene expression in different tissues and under different conditions in each species, and annotation of human and mouse mRNA transcripts, have contributed in concert to this process. Many genes have been reported to be differentially expressed and highly up-regulated in cerebral ischemia [28–38]. Some of the cognate encoded proteins may contribute to the pathogenesis of cerebral ischemia [39]. Microarray analysis offers a unique way to investigate changes in gene expression over time, identify the transitional transcriptome involved in a given process, and potentially evaluate the efficacy of treatments aimed at abating neurological deficits. This technique has been recently applied to identify genes associated not only with nervous tissue response to cerebral ischemia, but in related conditions such as spinal cord and traumatic brain injury in which secondary neuronal damage plays a key role in the long-term physiological outcome of the initial injury [40–43].

We report here that PACAP is neuroprotective in a mouse model of cerebral ischemic damage. We compare the therapeutic effects of exogenous PACAP in improvement in neurological function and reduction of infarct volume of the ischemic brain, with the effects of endogenous PACAP deficiency on exacerbation of ischemic damage and functional outcome of cerebral ischemia. Comparison of transcriptome alterations during ischemic insult in wild-type and PACAP-deficient mice provides a basis for identification of mRNA transcripts whose regulation by PACAP may be related to its neuroprotective effects. PACAP may act in part through the enhanced expression of other neuropeptides in ischemic cortex, including met-enkephalin, substance P, and neurotensin.

2. Materials and methods

2.1. Animals

A mouse strain deficient in the expression of PACAP was employed, as described previously [44]. Adult 129XC57BL6 PACAP -/- and +/+ F2 littermates were used in this study, and maintained with a standard 12-h light/dark cycle with humidity and temperature controlled at normal level, and water and food available ad libitum. All experiments were approved by the Animal Care and Use Committee of the National Institute of Mental Health Intramural Research Program.

2.2. Middle cerebral artery occlusion (MCAO)

Animals were anesthetized with 5% isoflurane for induction and 1.5% isoflurane for maintenance in a 30% O_2 and 70% N_2O gas mixture via a face mask. Body temperature was maintained in the normal range (36 °C–37.5 °C) with a heating pad during the operation. MCAO was performed as described [45]. Briefly, a 1.5-cm skin incision was placed between the left margin of the orbit and the tragus. The temporalis muscle was incised and retracted to expose the squamous portion of the temporal bone. A small burr hole (2 mm) was made with a high-speed microdrill through the outer surface of the semitranslucent skull over the visibly identified middle cerebral artery at the level of the inferior cerebral vein. The inner layer of the skull was removed with fine forceps, and the dura was opened with a 30-gauge needle. The left MCA was electrocauterized between the olfactory tubercle and the distal segment of the MCA (unilateral occlusion) using a small vessel cauterizer. The olfactory branch of the MCA, which was consistently present, was preserved. The coagulated MCA segment was then transected with microscissors to verify that the occlusion was permanent. The surgical site was closed with 4-0 monofilament nylon sutures. A single dose of antibiotics (Gentamicin, 2.5 mg/kg) was applied topically before closing the surgical sites, and 30% lidocaine cream was also applied to all surgical sites as an analgesic. After surgery, mice were returned to their cages and allowed free access to food and water. Duration of anesthesia did not exceed 30 min.

2.3. Drug administration

Administration of PACAP began 1 h after MCAO. PACAP (Phoenix Pharmaceuticals, Inc., Belmont, CA) was dissolved in 0.9% saline containing 0.1% BSA. For i.c.v. administration, a

27-gauge inner cannula was inserted into the guide cannula stereotaxically implanted in the right lateral ventricle 2–3 min before the 1 h drug administration time point following MCAO. 40 pmol of PACAP was infused through the inner cannula over 15 min, at a rate of 0.8 ml/h using a microprocessor syringe pump (Stoelting). The i.v. administration (0.75 nmol PACAP per mouse) was also at 1 h after MCAO through the jugular vein as a bolus. Control groups received 0.1% BSA in 0.9% saline as a bolus injection or infusion 1 h after MCAO under the same operative conditions as the PACAP-treated animals.

2.4. Neurological evaluation

Motor function and reflexes of the ischemic mice were evaluated at 1 h (prior to cannula implantation) and 24 h after MCAO using a neurological severity scoring system (NSS), which was modified from that used previously for mice suffering from closed head injury [46]. One point was given for either the lack of a tested reflex or for the inability to perform a given task. Thus, the magnitude of the NSS score is directly proportional to the severity of injury or impairment. The maximum achievable points are 13 at 1 h and 24 h. ΔNSS is the difference between NSS at 1 h (the earliest possible time of testing) and 24 h, and reflects the spontaneous recovery in motor function. It is therefore a useful parameter for the evaluation of the effects of drugs or of genetic manipulations on functional outcome in the MCAO model. Severity of injury scored at 1 h is a good predictor for the outcome at later times such as 24 h. Thus, an NSS score of 8–12 1 h after MCAO reflects a severe injury, a score of 5–7 indicates moderate injury and a score less than 5 corresponds to mild injury. This scoring system is useful for the evaluation of the neuroprotective effects of drugs in ischemic or traumatic brain injury models [46].

In the present experiment, another motor function task, the walking fault task was used to evaluate neurological deficits and recovery in all animals. This task has been shown to be effective in detecting fine motor coordination differences between injured and sham-operated animals [46]. The device consists of a narrow wooden beam 6 mm wide and 900 mm in length that is suspended 380 mm above a horizontal surface. The mouse was placed on one end of the beam, and the number of foot faults for the right hindlimb was recorded over 50 steps counted in either direction on the beam. Mice were allowed to walk across the beam two times for training purposes before MCAO. The difference between number of beam walking faults at 1 h and 24 h, namely ΔNWF (number of walking faults), reflects change in motor function after MCAO.

2.5. Assessment of infarct size

24 h after MCAO, the animals were deeply anesthetized with pentobarbital (65 mg/kg intraperitoneal injection), and then the brains were removed, frozen on dry ice, and cut into 20 μm sections in a cryostat at −20 ° C at 12 predetermined coronal levels (bregma +3.70 mm to −7.30 mm) at 0.4-mm intervals. Sections were postfixed in paraformaldehyde vapor and stained with cresyl violet. The infarcted areas of the brain slice were measured with the aid of the computer-based image analysis system NIH Image 1.62. The infarct volume was calculated by summing the infarct areas over affected brain slices and multiplying by slice thickness. Infarct volume was further corrected for edema volume as described earlier [47,48]. Mean infarct volumes in each group were compared by analysis of variance (ANOVA) followed by the Bonferroni/Dunn test to ascertain significance between groups.

2.6. RNA extraction, cDNA preparation and microarray analysis

1 h or 24 h following MCAO, animals were killed by decapitation, and the brain was removed quickly. Approximately 10 mg of ipsilateral (left) cortex was dissected from the MCA territory (ischemic zone) and submerged completely under 100 μl of RNAlater (Ambion, Austin, TX). The left cortex of normal (non-ischemic) mouse brain was used as a control for microarray labeling. RNA samples from the brains of both wild-type and PACAP-deficient mice were used in four microarray comparison groups. 5–6 chips were employed for each microarray comparison group. Total RNA was extracted with Trizol Reagent (Invitrogen) and purified using RNeasy Mini Kit (Qiagen, Valencia, CA). In the hybridization method employed, equal amounts (15–50 μg/15.5 μl) of total RNA obtained from brain tissue of different experimental groups were separately labeled with two different fluorescent dyes (Cy3 or Cy5) and applied on the same NIMH Mouse 36K cDNA microarray chip containing 13,217 distinct Entrez gene IDs (formerly known as LocusLink) and 15,888 distinct unigene IDs. The remainder of the elements are incompletely annotated IMAGE clones. Probe preparation and hybridization were performed as described previously [49]. In brief, total RNA (15–50 μg in 15.5 μl) was incubated with amine-modified random primer (2 μg/μl, 2 μl) and RNase inhibitor (5 units/μl, 1 μl) at 70 °C for 10 min. Primer-RNA solution was then incubated at 42 °C for 2 h with the reverse transcriptase mix containing 5× first-strand buffer, 50× aa-dUTP/dNTPs (25 mM dATP, 25 mM dGTP, 25 mM dCTP, 15 mM dTTP and 10 mM aminoallyl dUTP), 0.1 M DDT, and Superscript II reverse transcriptase. The cDNAs were labeled with NHS-ester Cy3 or Cy5 dye in the presence of 1 M sodium bicarbonate. Array slides placed in a hybridization chamber (Corning, Corning, NY) were incubated at 42 °C for 16–24 h, and successively washed with 0.5× SSC, 0.01% SDS, and 0.06× SSC at room temperature for 10 min each. Arrays were scanned with a GenePix 4000A scanner (Axon, Foster City, CA), and the resulting images were analyzed using IPlab (Fairfax, VA) and a FileMaker Pro 5 (Santa Clara, CA) relational database designed by Yidong Chen, NHGRI (see [50]). Following background subtraction and normalization, a calculated ratio of Cy3 to Cy5 signal intensities was used to define the relative increase or decrease of a particular transcript. Ratios were calculated only from those spots with a combined ratio quality above 0.3 [51].

2.7. Statistical analysis and gene annotation

Neurological function and infarct volume were compared between wild-type and PACAP-deficient groups using Student's t-test, and when appropriate, multiple comparison of means was performed using ANOVA with Bonferroni post-test analysis of significance. SAM (Significance Analysis of Microarrays) was used to identify transcripts significantly changed in expression compared to control in each data set by assimilating a set of gene-specific *t*-tests with the number of permutations set at 1000 and a fixed random seed for reproducible results [52,53]. As noted, elements with quality scores less than 0.3 were removed in advance [50]. Automatic imputation of missing data employed a nearest neighbor algorithm, and the adjustable threshold determining the number of genes called significant was set at a false discovery rate of around 10%, using 5–6 microarray slides for each comparison (1 h WT I/N, $n=6$ individual slides; 24 h WT I/N, $n=5$; 1 h PD, I/N, $n=6$; 24 h PD, I/N, $n=5$; 24 h IP/N, $n=5$, where in I/N, I is cortex tissue from ischemic side of brain, and N is cortex from sham-operated but non-MCAO control mice, and in IP/N, IP is cortex tissue from ischemic side of brain in PACAP-treated mice, and N is cortex from sham-operated but non-MCAO control mice. Each slide represented the hybridization of extracts from individual pairs of mice, one subjected to ischemia, and the other to sham surgical intervention. Transcripts significantly altered in expression (increased or decreased) following SAM analysis in each set were aligned against the complete list of genes in the array using Microsoft ACCESS, to compare the changes in different experimental conditions. Gene lists were further restricted to increases or decreases of 1.5-fold or greater.

2.8. Quantitative RT-PCR

cDNA microarray data were confirmed by quantitative two-step reverse transcriptase-polymerase chain reaction (Q-RT-PCR). mRNA levels for selected transcripts were quantified using the same cortical RNA samples employed for microarray analysis. Q-RT-PCR was conducted as described previously [44]. In brief, contaminating DNA was removed by treatment with RNase-free DNase I, and cDNA was reverse transcribed from 1 μg of total RNA using a SuperScript First Strand Synthesis System for RT-PCR (Invitrogen). Q-RT-PCR was performed in buffer containing either 1X PCR buffer II (Applied Biosystems, Foster City, CA, USA), 0.02% gelatin (Sigma G-1393), 1% Tween 20 (Sigma P-9416), 0.2 μM 5′-FAM-AAAA-NH link T-ROX-3′ probe (Biosource International, Camarillo, CA, USA), with 3.5 mM $MgCl_2$ (Applied Biosystems), 1.25 U/sample Taq gold polymerase (Applied Biosystems), 90 nM forward and reverse primers, or 2× SYBR Green Master Mix (SYBR Green I Dye, AmpliTaq® DNA polymerase, dNTPs with dUTP and optimal buffer components; Applied Biosystems, Foster City, CA, USA). Assays were run in triplicate on the Taq-Man 7700 Sequence Detection System (Applied Biosystems) under default conditions (AmpliTaqGold activation, 95 °C for 10 min; PCR, 40 cycles of 95 °C, 15 s and 60 °C, 1 min). The abundance of each transcript was determined relative to the standard 18S transcript using a TaqMan ribosomal RNA control reagent kit (Ribosomal RNA Control primers and VIC probe were obtained from Applied Biosystems). Samples in which RT was not added during the cDNA synthesis step yielded no detectable signals under these assay conditions. The primer sequences (5′ to 3′) for preproenkephalin 1 (pENK1) (Mm.2899), heat shock protein, 110 kDa (Hsp110), and MAP kinase kinase 2 (MAPKK2) (Mm.196584) were as follows:

pENK1: forward primer, 5′-CTGGGAGACCTGCAAGGATCT-3′
reverse primer, 5′-AGTGGCTCTCATCCTGTTTGCT-3′
probe, 6FAM-CCAGGCCCGAGTTCCCTTGGGTAMRA;
Hsp110: forward primer, 5′-CCAGGCTAAGCAGGCATACATT-3′
reverse primer, 5′-AACACTTTCGGTCGTTCCTCA-3′
MAPKK2: forward primer, 5′-CTGCCCAGTGGTGTGTTCAG-3′
reverse primer, 5′-TGGTTCATCAGCAGCTTCAGA-3′

3. Results

3.1. Effect of PACAP on neurological deficits and infarct volume

Mice were subjected to middle cerebral artery occlusion (MCAO), and treated with PACAP or saline 1 h later, with clinical neurological status evaluated by the modified neurological severity scoring system **(**NSS) and walking fault task at 1 h and 24 h, the same times at which cortical tissue was harvested for microarray analysis (Fig. 1). There was no difference in NSS at 1 h among non-PACAP treatment, PACAP treatment (i.v.) and PACAP treatment (i.c.v.) groups of both wild type and PACAP-deficient mice, indicating similar severity of the initial injury in mice among the six groups (Fig. 2a). Untreated PACAP-deficient mice showed a significant decrease in motor recovery (i.e., decreased ΔNWF) 24 h after cerebral ischemia, versus untreated wild-type mice (Fig. 2c). This suggests that endogenous PACAP affects the extent of neurological damage, or functional recovery from ischemic insult, in mice following MCAO. The effect of exogenous PACAP on neurological functional improvement 24 h after MCAO is shown in Fig. 2b and c. A significant neurological functional recovery induced by PACAP administered either i.v. or i.c.v. 1 h after MCAO was apparent as an increased ΔNSS (Fig. 2) and ΔNWF (Fig. 2) in PACAP-treated mice following MCAO, compared to untreated mice following MCAO. Improvement in neurological function, and significant reduction in infarct volume, occurred after either i.v. or i.c.v. PACAP administration (Fig. 2). The reduction was greater in PACAP-deficient (34%) than in wild-type (19%) mice, consistent with the greater damage in PACAP-deficient mice without PACAP treatment, and suggesting that endogenous PACAP provides neuroprotection against cerebral ischemic insult in mouse brain.

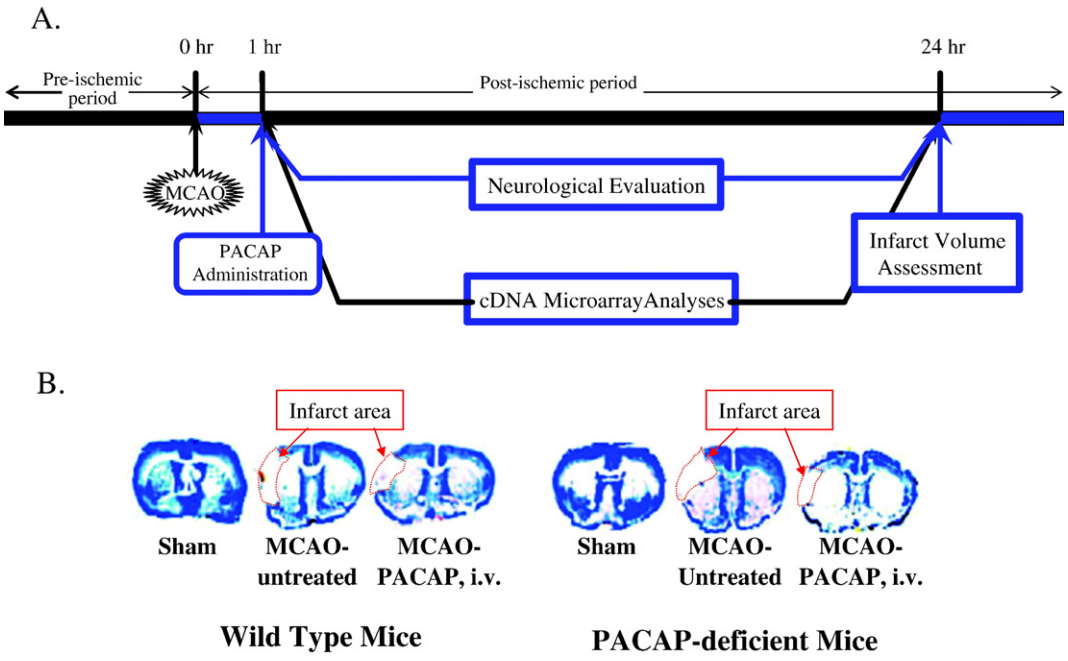

Fig. 1. MCAO in the mouse. A. Experimental flowchart of the cerebral ischemia protocol. Animals were subjected to MCAO or sham surgery at 0 hr; PACAP or saline was administered at 1 hr; neurological evaluations and microarray analyses were performed at 1 hr and 24 hr; infarct volume was assessed at 24 hr after MCAO. B. Coronal sections of brain slices fixed with formaldehyde and stained with cresyl violet, showing the effects of PACAP (i.v. injection, 1 hr post-MCAO) on infarct volume in both wild type and PACAP-deficient mice 24 h following MCAO. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

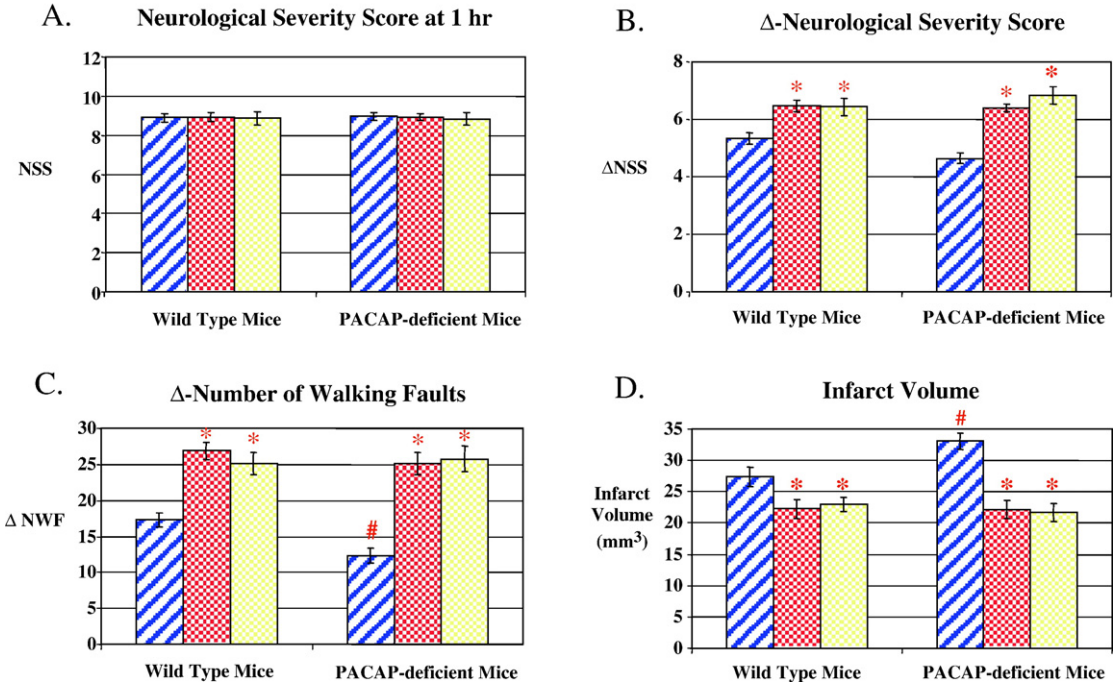

Fig. 2. Effect of endogenous PACAP and PACAP treatment on neurological and neuropathological outcomes after MCAO. A. Initial neurological severity score at 1 hr, n = 8), B. Change in neurological severity score at 24 hr (*: $p < 0.01$ compared to corresponding untreated group, n = 8), C. Change in number of walking faults from 1 to 24 hr (#: $p < 0.05$ compared to the untreated wild-type group; *: $p < 0.05$ compared to the corresponding untreated control group, n = 7), and D. Infarct volume at 24 hr (#: $p < 0.05$, compared to untreated wild type mice, n = 7). Blue bars, MCAO alone; red bars, MCAO with i.v. PACAP; yellow bars, MCAO with i.c.v. PACAP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

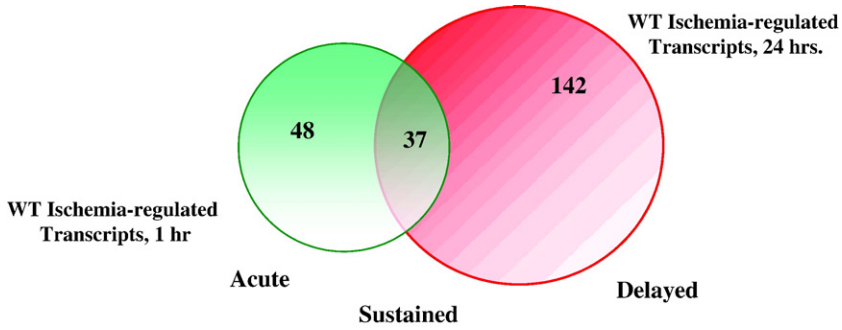

Fig. 3. Changes in the cerebrocortical transcriptome 1 and 24 h after MCAO. Venn diagram depicts transcripts up-regulated at 1 and 24 h post-MCAO, and divides transcripts into those up-regulated only at 1 h (acute), at both 1 and 24 h (sustained) and only at 24 h (delayed). Named transcripts, i.e. those with designated gene name and associated gene i.d. (LocusLink) designation, in each category are listed in Tables 1–3.

3.2. Ischemia-induced alterations in mouse cortex

To identify expression patterns of transcripts altered by ischemia in mouse cortex and the possible roles of specific genes in cerebral ischemia, up- or down-regulated transcripts were examined under the four conditions described in Materials and methods: I/Nwt 1 h, I/Nwt 24 h, I/Npd 1 h, and I/Npd 24 h, where wt indicates wild-type (PACAP +/+) and pd indicates PACAP-deficient (PACAP -/-) mice. 227 known (named) transcripts were elevated or decreased in ischemia. These fell into three classes: (1) ischemia-altered acute transcripts; (2) ischemia-altered sustained transcripts; and (3) ischemia-altered delayed transcripts (Fig. 3). In addition, exogenous PACAP-dependent, ischemia-regulated transcripts were identified as those altered (up- or down-regulated) by PACAP treatment in ischemic cortex (IP/N) more than in ischemic cortex without treatment (I/N), and endogenous PACAP-dependent ischemia regulated transcripts as those controlled (up- or down-regulated) in normal cortex by ischemia (I/Nwt), but no longer regulated by ischemia in PACAP-deficient cortex (I/Npd).

3.3. Transcripts regulated at 1 h after MCAO: acute MCAO transcripts

The transcripts up-regulated by 1 h, but no longer up-regulated 24 h after ischemia (acute transcripts), include several classical markers for the ischemic response, such as prostaglandin endoperoxide synthase 2 (Ptgs2), the heat-shock protein 40 (Hsp40) homologs Dnajb5 and Dnajc3, and activity regulated cytoskeletal-associated protein (arc) (Table 1). Transcripts up-regulated acutely fell into one or more of the following categories. *Immediate early genes and transcription factors* included Egr1, Egr3, Ets2, Fosb, Fosl2, Klf9, and Nr4a1; *signal transduction-related transcripts* included the protein phosphatases Dusp4, Dusp6, Dusp14, and Ptpn5, the protein kinase Plk2, the MAPK kinase kinase Map3k10, phosphodiesterase Pde1b, and the signal modulating proteins Ppp1r1b, Rgs2, and Rheb; *calcium signaling pathway or calcium binding components* included Dusp4, Dusp6, Ppp1r1b, and protocadherin 17; and *receptors and transmembrane proteins* included Fcrl5 and Tmem55b. The *cAMP-related transcripts* Arpp19, Cap2, Pde1b and Expo1 were all up-regulated by 1 h after ischemia, with all but Expo1 regulated only acutely.

Of the 48 known genes regulated acutely by ischemia, most were PACAP-dependent, i.e., not elevated in PACAP-deficient mouse cortex at 1 h. Prominent among known genes regulated acutely in a PACAP-independent manner were the immediate early genes Egr1, Egr3, and Fosb, the transcription factor Klf9 and the GTPase activating protein, Rgs2 (Table 1). It is noteworthy that Egr1, Klf9 and Rgs2 are elevated upon PACAP stimulation of PC12-G cells [54] and are PACAP-dependent transcripts in the adrenal medulla (Samal, Hamelink and Eiden, unpublished observations) where PACAP is the major slow transmitter [44]. Since these transcripts are elevated in ischemia even in PACAP-deficient mouse cortex, their expression might be under the control of other slow transmitters among the more heterogeneous chemical synapses in the brain.

3.4. Transcripts regulated at both 1 and 24 h after MCAO: sustained MCAO transcripts

There were 37 MCAO-induced transcripts designated as sustained, i.e., elevated at least 1.5-fold at both 1 and 24 h (Table 2). These include brain-specific angiogenesis inhibitor 1-associated protein 2 (Baiap2), calsyntenin 3 (Clstn3), the RNA binding cold shock domain containing protein 2 (Csdc2), several heat shock proteins (Hsp a1b, 110, and a5), dual-specificity phosphatase-like 15 (Dusp15), the metabotropic glutamate receptor Grm7, G-protein coupled receptor 88 (Gpr88), Gadd45g, Gas2l1, interferon regulatory factor 1 (Irf1), Mt2, Penk1, tachykinin 1 (Tac1) and villin 2 (Vil2). Hsp110, metallothionein 2 and villin 2 were prominent transcripts not under control of endogenous PACAP (up-regulated at 24 h in both wild-type and PACAP-deficient mice). Several sustained transcripts required endogenous PACAP for up-regulation at 1 h but not 24 h, including the aldehyde reductase Akr1a4, Hspa1b and a5, procollagen IIIα1and ubiquitin C (Table 2). The remainder were PACAP-dependent, consistent with PACAP's role as an emergency response peptide that sustains a neuroprotective response. Notable are PEnk and Tac1, both implicated as activity-regulated transcripts at the adrenomedullary synapse [55,56], and as

Table 1
Acute transcripts and their PACAP-dependence

Gene ID	Gene symbol	Gene name	WT1 SAM mean	WT1 SAM-SD	PD1 SAM Mean	PD1 SAM-SD
81489	Dnajb1	DnaJ (Hsp40) homolog	1.70	0.18	1.85	0.21
13653	Egr1	Early growth response 1	1.69	0.11	1.49	0.10
13655	Egr3	Early growth response 3	1.98	0.10	1.85	0.26
74155	Errfi1	ERBB receptor feedback inhibitor 1	2.21	0.14	2.05	0.22
14282	Fosb	FBJ osteosarcoma oncogene B	1.63	0.12	2.21	0.24
16601	Klf9	Kruppel-like factor 9	1.75	0.12	1.84	0.18
434047	LOC434047	Similar to Heat shock cognate 71 kDa	1.70	0.21	1.63	0.19
19653	Rbm4	RNA binding motif protein 4	1.85	0.23	2.14	0.27
19735	Rgs2	Regulator of G-protein signaling 2	2.69	0.24	2.03	0.15
11472	Actn2	Actinin alpha 2	1.71	0.14		
11838	Arc	Activity regulated cytoskeletal-associated protein	1.56	0.11		
59046	Arpp19	cAMP-regulated phosphoprotein 19	1.60	0.20		
105171	Arrdc3	Arrestin domain containing 3	1.51	0.15		
67252	Cap2	Adenylate cyclase-associated protein, 2	1.53	0.05		
56323	Dnajb5	DnaJ (Hsp40)	1.60	0.08		
19107	Dnajc3	DnaJ (Hsp40) homolog	1.51	0.16		
56405	Dusp14	Dual specificity phosphatase 14	1.64	0.10		
319520	Dusp4	Dual specificity phosphatase 4	1.70	0.14		
67603	Dusp6	Dual specificity phosphatase 6	1.61	0.14		
13803	Enc1	Ectodermal-neural cortex 1	1.52	0.17		
23872	Ets2	E26 avian leukemia oncogene 2, 3′ domain	1.57	0.14		
329693	Fcrl5	Fc receptor-like protein 5	2.17	0.56		
14284	Fosl2	fos-like antigen 2	1.51	0.15		
278795	Gm705	Gene model 705, (NCBI)	1.63	0.24		
15387	Hnrpk	Heterogeneous nuclear ribonucleoprotein K	1.71	0.14		
64294	Itm2c	Integral membrane protein 2C	1.58	0.12		
16476	Jun	Jun oncogene	1.85	0.39		
380928	Lmo7	LIM domain only 7	1.56	0.19		
74365	Lonrf3	LON peptidase N-terminal domain and ring finger 3	1.60	0.13		
76690	Map3k10	Mitogen activated protein kinase kinase kinase 10	1.64	0.25		
78818	Mbnl2	MKIAA4072 protein	1.74	0.18		
53324	Nptx2	Neuronal pentraxin 2	1.74	0.11		
15370	Nr4a1	Nuclear receptor subfamily 4, group A, member 1	1.53	0.19		
64011	Nrgn	Neurogranin	1.67	0.27		
67405	Nts	Neurotensin	1.56	0.19		
219228	Pcdh17	Protocadherin 17	1.54	0.13		
18574	Pde1b	Phosphodiesterase 1B	1.52	0.15		
71853	Pdia6	Protein disulfide isomerase associated 6	1.54	0.09		
67245	Peli1	Pellino 1	1.52	0.15		
20620	Plk2	Polo-like kinase 2 (Drosophila)	1.80	0.13		
19049	Ppp1r1b	Protein phosphatase 1	1.65	0.27		
19225	Ptgs2	Prostaglandin-endoperoxide synthase 2	1.85	0.17		
19259	Ptpn5	Protein tyrosine phosphatase, non-receptor type 5	1.65	0.14		
30939	Pttg1	Pituitary tumor-transforming 1	1.51	0.13		
19744	Rheb	RAS-homolog enriched in brain	1.66	0.23		
72124	Seh1l	SEH1-like	1.56	0.21		
219024	Tmem55b	Transmembrane protein 55b	1.78	0.32		
22139	Ttr	Transthyretin	2.10	0.31		

Values are the SAM means and standard deviations as described in Materials and methods. Values not shown did not achieve significance at FDR = 10% following SAM analysis.

neurotransmitters previously implicated in the brain neuronal and glial response to ischemia [57,58].

3.5. Transcripts regulated only by 24 h after MCAO: delayed MCAO transcripts

Delayed transcripts (regulated by ischemia in wild-type mice only by 24 h after MCAO) were the most numerous (Fig. 3; Table 3). 142 known transcripts were up-regulated and only one was significantly down-regulated in this category. Transcripts up-regulated in both wild-type and PACAP-deficient mice include several structural proteins, GFAP, vimentin, moesin and β-actin, previously implicated in response to ischemia [59,60], and a transcription factor, Stat3 also previously implicated in ischemic and injury responses [60]. Other PACAP-independent transcripts previously increased in ischemia are cathepsin C, Hmox1, Mt1, and secreted phosphoprotein 1 (osteopontin).

Many of the delayed transcripts were PACAP-dependent (Table 3) and include both transcripts already associated with

Table 2
Sustained transcripts and their PACAP dependence

Gene ID	Gene symbol	Gene name	WT1 SAM mean	WT1 SAM-SD	WT24 SAM mean	WT24 SAM-SD
232370	Clstn 3	Calsyntenin 3	2.94	0.33	2.77	0.21
15505	**Hsp110**	**Heat shock protein 110**	**2.05**	**0.28**	**1.80**	**0.14**
17750	**Mt2**	**Metallothionein 2**	**1.63**	**0.10**	**2.35**	**0.13**
22350	**Vil2**	**Villin 2**	**1.37**	**0.05**	**1.70**	**0.11**
105501	Abhd4	Abhydrolase domain containing 4	1.39	0.09	1.54	0.17
58810	**Akr1a4**	**Aldo-keto reductase family 1**	**1.63**	**0.15**	**1.63**	**0.18**
108100	Baiap2	BAI1-associated protein 2	1.73	0.20	1.55	0.14
27267	Cars	Cysteinyl-tRNA synthetase	1.47	0.15	1.52	0.20
12465	Cct5	Chaperonin subunit 5 (epsilon)	1.40	0.05	1.51	0.09
12825	**Col3a1**	**Procollagen, type III, alpha 1**	**1.71**	**0.15**	**1.70**	**0.16**
105859	Csdc2	Cold shock domain containing C2, RNA binding	1.73	0.22	2.00	0.15
13179	Dcn	Decorin	1.46	0.06	1.94	0.07
83768	Dpp7	Dipeptidylpeptidase 7	1.70	0.14	1.66	0.09
252864	Dusp15	Dual specificity phosphatase-like 15	1.54	0.19	1.34	0.10
107035	Fbxo38	F-box protein 38	1.64	0.13	1.82	0.17
69754	**Fbxo7**	**F-box only protein 7**	**1.56**	**0.16**	**1.28**	**0.06**
23882	Gadd45g	Growth arrest and DNA-damage-inducible, gamma	1.94	0.11	1.49	0.15
78926	Gas2l1	Growth arrest-specific 2 like 1	1.62	0.18	1.64	0.11
14590	Ggh	Gamma-glutamyl hydrolase	1.52	0.12	1.52	0.13
218963	**Gm1821**	**Gene model 1821, (NCBI)**	**1.61**	**0.13**	**1.66**	**0.18**
14694	Gnb2l1	Guanine nucleotide binding protein (G protein), beta polypeptide 2 like 1	1.50	0.11	2.08	0.18
64378	Gpr88	G-protein coupled receptor 88	2.10	0.31	1.50	0.23
108073	Grm7	Glutamate receptor, metabotropic 7	1.88	0.19	1.93	0.20
15127	Hbb	Hemoglobin beta chain complex	2.64	0.76	2.15	0.24
15511	**Hspa1b**	**Heat shock protein 1B**	**2.30**	**0.48**	**1.84**	**0.20**
14828	**Hspa5**	**Heat shock 70kD protein 5 (glucose-regulated protein)**	**2.35**	**0.25**	**1.61**	**0.13**
15937	Ier3	Immediate early response 3	1.47	0.13	1.62	0.19
16362	Irf1	Interferon regulatory factor 1	1.58	0.17	1.64	0.12
245049	Myrip	Myosin VIIA and Rab interacting protein	1.62	0.20	1.71	0.16
228961	Npepl1	Aminopeptidase-like 1	1.50	0.11	1.63	0.19
14827	**Pdia3**	**Protein disulfide isomerase associated 3**	**1.55**	**0.19**	**1.56**	**0.10**
18619	Penk1	Preproenkephalin 1	2.45	0.50	1.89	0.26
71726	Smug1	Single-strand selective monofunctional uracil DNA glycosylase	1.70	0.23	1.89	0.14
21333	Tac1	Tachykinin 1	2.02	0.29	1.61	0.32
75909	Tmem49	Transmembrane protein 49	1.70	0.05	1.66	0.18
22190	**Ubc**	**Ubiquitin C**	**1.68**	**0.13**	**1.59**	**0.13**
103573	Xpo1	Exportin 1, CRM1 homolog (yeast)	1.64	0.18	1.74	0.13

Values are the SAM mean and standard deviations as described in Materials and methods. Values not shown did not achieve significance at FDR = 10% in SAM. Values in **bold** are transcripts that were also elevated in PACAP-deficient mice at 1 or 24 hr post-MCAO (i.e. PACAP-independent transcripts).

ischemic response and others demonstrated here for the first time. Cathepsins D and L, C1q, lipocalin 2, Mapk1 (Erk1), S100, Timp2 and tubulin [61] are in the former category, while annexin 2 and Ddr1 are in the latter.

3.6. Transcripts up-regulated in MCAO normalized by treatment with PACAP

Several of the transcripts up-regulated at 24 h in MCAO, in both wild-type and PACAP-deficient mice, are potential injury effector transcripts involved in the dissemination of secondary neuronal damage following stroke. To examine the hypothesis that the ameliorative effects of PACAP on lesion volume and motor deficits after MCAO might be at the level of suppression of such transcripts, we examined the MCA territory of mice subjected to MCAO at 24 h, following treatment with PACAP (i.v.) at 1 h, compared to non-ischemic cortex (IP/N). Transcript representation in this set of arrays was lower than in the four array sets examined initially, with approximately 16,000 spots yielding quality indices above 0.3 in the six hybridizations performed. Nevertheless, there were some prominent PACAP-independent targets affected. Exogenous PACAP also exerted significant effects in reversing expression of ischemia-altered sustained transcripts. For example several transcripts were up-regulated significantly more in wild-type mice treated with PACAP than in untreated wild-type mice subjected to MCAO and were not up-regulated at all in PACAP-deficient mice subjected to MCAO. These included alanyl-tRNA synthetase (Aars), ATP-binding cassette sub-family F member 3 (Abcf3), ARP10 actin-related protein 10 homolog (Actr10), branched chain aminotransferase 1 (Bcat1), coatomer protein complex subunit alpha (Copa), COP9 homolog subunit 4 (Cops4), DnaJ homolog subfamily C member 10 (Dnajc10), Eukaryotic translation initiation factor 3 subunit 8 (Eif3s8), Myelination associated SUR4-like protein (Elovl6), G-protein signalling modulator 1 (Gpsm1), Glycophorin A (Gypa), H3 histone family 3B (H3f3b), Hemoglobin alpha adult chain 1 (Hba-a1), Heterogeneous nuclear ribonucleoprotein K (Hnrpk), Heat

Table 3
Delayed Transcripts and Their PACAP-dependence

Gene ID	Gene symbol	Gene name	WT24 SAM mean	WT24 SAM-SD	PD24 SAM mean	PD24 SAM-SD
11461	Actb	Actin, beta, cytoplasmic	1.83	0.24	1.78	0.13
11504	Adamts1	A disintegrin-like and metallopeptidase	1.47	0.06	1.7	0.09
71994	Cnn3	Calponin 3, acidic	2	0.09	1.81	0.04
13032	Ctsc	Cathepsin C	1.99	0.16	2.12	0.25
14580	Gfap	Glial fibrillary acidic protein	7.2	0.36	6.73	0.2
15368	Hmox1	Heme oxygenase (decycling) 1	2.02	0.15	1.73	0.08
18826	Lcp1	Lymphocyte cytosolic protein 1	1.63	0.14	1.92	0.08
14728	Lilrb4	Leukocyte immunoglobulin-like receptor	1.71	0.14	1.53	0.03
23947	Mid2	Midline 2	1.69	0.3	1.68	0.12
17698	Msn	Moesin	2.35	0.25	2.34	0.27
17748	Mt1	Metallothionein 1	1.69	0.25	1.64	0.07
18458	Pabpc1	Poly A binding protein, cytoplasmic 1	1.54	0.11	1.6	0.04
20750	Spp1	Secreted phosphoprotein 1	2.66	0.22	3.79	0.16
20848	Stat3	Signal transducer and activator of transcription 3	1.68	0.11	1.63	0.08
22041	Trf	Transferrin	2.15	0.25	2.92	0.25
22352	Vim	Vimentin	3.57	0.3	3.73	0.03
11475	Acta2	Actin, alpha 2, smooth muscle, aorta	1.73	0.2		
11465	Actg1	Actin, gamma, cytoplasmic 1	1.69	0.22		
12306	Anxa2	Annexin A2	1.65	0.18		
11772	Ap2a2	Adaptor protein complex AP-2, alpha 2 subunit	1.89	0.11		
11815	Apod	Apolipoprotein D	1.95	0.18		
11829	Aqp4	Aquaporin 4	1.54	0.11		
11837	Arbp	Acidic ribosomal phosphoprotein P0	1.54	0.16		
54208	Arl6ip1	ADP-ribosylation factor-like 6 interacting protein 1	1.5	0.2		
27053	Asns	Asparagine synthetase	1.5	0.18		
75302	Asxl2	Additional sex combs like 2 (Drosophila)	1.57	0.08		
50770	Atp11a	ATPase, class VI, type 11A	1.5	0.07		
11949	Atp5c1	ATP synthase, gamma polypeptide 1	1.61	0.08		
67126	Atp5e	ATP synthase, epsilon subunit	1.72	0.37		
20877	Aurkb	Aurora kinase B	1.64	0.17		
12010	B2m	Beta-2 microglobulin	1.5	0.23		
67239	Bxdc1	Brix domain containing 1	1.58	0.09		
12260	C1qb	Complement component 1	1.59	0.13		
12330	Canx	Calnexin	1.53	0.18		
52502	Carhsp1	Calcium regulated heat stable protein 1	1.61	0.17		
12419	Cbx5	Chromobox homolog 5 (Drosophila HP1a)	1.65	0.25		
12462	Cct3	Chaperonin subunit 3 (gamma)	1.52	0.13		
12466	Cct6a	Chaperonin subunit 6a (zeta)	1.68	0.1		
12468	Cct7	Chaperonin subunit 7 (eta)	1.55	0.12		
12505	Cd44	CD44 antigen	1.52	0.07		
12508	Cd53	CD53 antigen	1.88	0.24		
12512	Cd63	Cd63 antigen	1.62	0.22		
29876	Clic4	Chloride intracellular channel 4 (mitochondrial)	1.54	0.16		
12785	Cnbp1	Cellular nucleic acid binding protein 1	1.52	0.07		
12842	Col1a1	Procollagen, type I, alpha 1	1.83	0.07		
12848	Cops2	Constitutive photomorphogenic homolog, subunit 2	1.29	0.09		
78885	Coro7	Coronin 7	1.57	0.17		
12387	Ctnnb1	Catenin	1.54	0.07		
13033	Ctsd	Cathepsin D	1.52	0.14		
13039	Ctsl	Cathepsin L	1.54	0.18		
72584	Cul4b	Cullin 4B	1.52	0.06		
67789	Dalrd3	DALR anticodon binding domain containing 3	1.5	0.21		
226414	Dars	Aspartyl-tRNA synthetase	1.56	0.08		
13190	Dct	Dopachrome tautomerase	2.65	0.69		
12305	Ddr1	Discoidin domain receptor family, member 1	1.58	0.13		
13627	Eef1a1	Eukaryotic translation elongation factor 1 alpha 1	1.75	0.13		
13628	Eef1a2	Eukaryotic translation elongation factor 1 alpha 2	1.55	0.16		
13681	Eif4a1	Eukaryotic translation initiation factor 4A1	1.58	0.11		
13806	Eno1	Enolase 1, alpha non-neuron	1.63	0.13		
319945	Flad1	Flavin adenine dinucleotide synthetase, homolog	1.6	0.14		
192176	Flna	Filamin, alpha	1.7	0.19		
55935	Fnbp4	Formin binding protein 4	1.51	0.17		
14325	Ftl1	Ferritin light chain 1	1.73	0.27		
69823	Fyttd1	Forty-two-three domain containing 1	1.58	0.08		

Table 3 (*continued*)

Gene ID	Gene symbol	Gene name	WT24 SAM mean	WT24 SAM-SD	PD24 SAM mean	PD24 SAM-SD
15081	H3f3b	H3 histone, family 3B	1.58	0.07		
15121	Hba	Hemoglobin alpha chain complex	1.84	0.15		
15129	Hbb-b1	Hemoglobin, beta adult major chain	2.36	0.13		
319162	Hist3h2a	Histone 3, H2a	1.5	0.21		
15384	Hnrpab	Heterogeneous nuclear ribonucleoprotein A/B	1.61	0.13		
15519	Hspca	Heat shock protein 1, alpha	1.53	0.07		
66141	Ifitm3	Interferon induced transmembrane protein 3	1.52	0.16		
16590	Kit	Kit oncogene	1.51	0.2		
16819	Lcn2	Lipocalin 2	1.98	0.22		
269629	Lhfpl3	Lipoma HMGIC fusion partner-like 3	1.55	0.11		
433923	LOC433923	Similar to ADP/ATP translocase 2	1.53	0.11		
544939	LOC544939	Similar to Tubulin beta chain (T beta-15)	1.54	0.15		
545369	LOC545369	Similar to 60S ribosomal protein L4 (L1)	1.51	0.07		
16956	Lpl	Lipoprotein lipase	1.7	0.16		
26413	Mapk1	Mitogen activated protein kinase 1	1.55	0.26		
17181	Matn2	Matrilin 2	1.57	0.1		
17210	Mcl1	Myeloid cell leukemia sequence 1	1.58	0.13		
17433	Mobp	Myelin-associated oligodendrocytic basic protein	1.59	0.11		
121022	Mrps6	Mitochondrial ribosomal protein S6	1.57	0.23		
66377	Ndufc1	NADH dehydrogenase	1.6	0.25		
18111	Nnat	Neuronatin	1.61	0.13		
18115	Nnt	Nicotinamide nucleotide transhydrogenase	1.62	0.2		
76303	Osbp	Oxysterol binding protein	1.6	0.07		
18453	P4hb	Prolyl 4-hydroxylase, beta polypeptide	1.64	0.13		
18516	Pbx3	Pre B-cell leukemia transcription factor 3	1.63	0.33		
170677	Pcdh21	Protocadherin 21	1.65	0.16		
66425	Pcp4l1	Purkinje cell protein 4-like 1	1.67	0.22		
30052	Pcsk1n	Proprotein convertase subtilisin/kexin type 1 inhibitor	1.64	0.2		
231887	Pdap1	PDGFA associated protein 1	1.62	0.17		
18611	Pea15	Phosphoprotein enriched in astrocytes 15	1.59	0.17		
70998	Phf6	PHD finger protein 6	1.73	0.09		
18685	Phtf1	Putative homeodomain transcription factor 1	1.73	0.11		
18767	Pkia	Protein kinase inhibitor, alpha	1.77	0.13		
51792	Ppp2r1a	Protein phosphatase 2	1.57	0.17		
19073	Prg1	Proteoglycan 1, secretory granule	1.5	0.1		
56351	Ptges3	Prostaglandin E synthase 3 (cytosolic)	1.63	0.2		
19277	Ptpro	Protein tyrosine phosphatase, receptor type, O	1.93	0.4		
19288	Ptx3	Pentraxin related gene	1.64	0.16		
216344	Rab21	RAB21, member RAS oncogene family	1.65	0.19		
215449	Rap1b	RAS related protein 1b	1.51	0.07		
104433	Rnu108	RNA, U108 small nucleolar	1.54	0.12		
27367	Rpl3	Ribosomal protein L3	1.52	0.12		
67891	Rpl4	Ribosomal protein L4	1.6	0.08		
20055	Rps16	Ribosomal protein S16	1.6	0.35		
66481	Rps21	Ribosomal protein S21	1.54	0.21		
27050	Rps3	Ribosomal protein S3	1.55	0.06		
20115	Rps7	Ribosomal protein S7	1.57	0.08		
16785	Rpsa	Ribosomal protein SA	1.58	0.1		
81910	Rrbp1	Ribosome binding protein 1	1.5	0.11		
20202	S100a9	S100 calcium binding protein A9 (calgranulin B)	1.54	0.15		
19018	Scand1	SCAN domain-containing 1	1.66	0.06		
20971	Sdc4	Syndecan 4	1.54	0.16		
103080	Sept10	Septin 10	1.57	0.21		
75747	Sesn3	Sestrin 3	1.54	0.13		
20382	Sfrs2	Splicing factor, arginine/serine-rich 2 (SC-35)	1.73	0.18		
108052	Slc14a1	Solute carrier family 14	1.76	0.08		
20511	Slc1a2	Solute carrier family 1	1.73	0.17		
11739	Slc25a4	Solute carrier family 25	1.55	0.16		
17254	Slc3a2	Solute carrier family 3	1.53	0.18		
69178	Snx5	Sorting nexin 5	1.58	0.11		
67437	Ssr3	Signal sequence receptor, gamma	1.52	0.12		
72003	Synpr	Synaptoporin	1.72	0.23		
24001	Tiam2	T-cell lymphoma invasion and metastasis 2	1.59	0.09		
21858	Timp2	Tissue inhibitor of metalloproteinase 2	1.74	0.24		

(continued on next page)

Table 3 (*continued*)

Gene ID	Gene symbol	Gene name	WT24 SAM mean	WT24 SAM-SD	PD24 SAM mean	PD24 SAM-SD
17112	Tm4sf1	Transmembrane 4 superfamily member 1	1.64	0.33		
66676	Tmed7	Transmembrane emp24 protein transport domain containing 7	1.52	0.11		
27279	Tnfrsf12a	Tnf receptor superfamily, member 12a	2.11	0.62		
233833	Tnrc6a	Trinucleotide repeat containing 6a	1.51	0.17		
64930	Tsc1	Tuberous sclerosis 1	1.69	0.14		
52808	Tspyl2	TSPY-like 2	1.94	0.1		
22143	Tuba2	Tubulin, alpha 2	1.7	0.16		
22151	Tubb2a	Tubulin, beta 2a	1.61	0.17		
73710	Tubb2b	Tubulin, beta 2b	1.54	0.18		
22154	Tubb5	Tubulin, beta 5	1.58	0.16		
67951	Tubb6	Tubulin, beta 6	1.52	0.16		
22327	Vbp1	von Hippel-Lindau binding protein 1	1.51	0.27		
22388	Wdr1	WD repeat domain 1	1.55	0.12		
241494	Zfp533	Zinc finger protein 533	1.71	0.15		

Values are the SAM mean and standard deviations as described in Materials and methods. Values not shown did not achieve significance at FDR = 10% in SAM.

shock factor binding protein 1 (Hsbp1), 5-hydroxytryptamine receptor 2B (Htr2b), Multiple coagulation factor deficiency 2 (Mcfd2), Myeloid ecotropic viral integration site-related gene 1 (Mrg1), Procollagen C-endopeptidase enhancer 2 (Pcolce2), Protein phosphatase 1 catalytic subunit gamma isoform (Ppp1cc), Peroxiredoxin 2 (Prdx2), Ribosomal protein S6 (Rps6), Splicing factor arginine/serine-rich 7 (Sfrs7), Sphingosine phosphate lyase 1 (Sgpl1), Spermidine synthase (Srm), T-cell lymphoma invasion and metastasis 1 (Tiam1), Tomoregulin-1 (Tmeff1), Ubiquitin protein ligase E3A transcript variant 2 (Ube3a), and WD repeat domain 6 (Wdr6). Transcripts either up- or down-regulated in both wild-type and PACAP-deficient mice at 24 h after MCAO, whose expression was normalized in PACAP-treated wild-type mice 24 h after MCAO included β-actin (Actb), Activity regulated cytoskeletal-associated protein (Arc), Calponin 3 (Cnn3), Early growth response 1 (Egr1), Heat shock protein 110 (Hsp110), RING finger protein; Fxy2 (Mid2), Metallothionein 1(Mt1), Poly A binding protein (Pabpc1), and thioredoxin reductase 1, (Txnrd1).

3.7. Gene expression verification by quantitative RT-PCR

In order to confirm the changes in gene expression determined by cDNA microarray analysis, three genes (pENK1, Hsp110, and Mapkk2) with varying expression profiles were chosen for Q-RT-PCR analysis. Table 4 shows changes in mRNA levels of selected transcripts measured by Q-RT-PCR, for transcripts demonstrated by SAM analysis of microarray data (Tables 1–3) to be up-regulated by MCAO in a sustained, PACAP-dependent manner (Penk1), a sustained PACAP-independent manner (Hsp110), and to be unregulated by MCAO in cortex (Mapkk2). In each case, RT-PCR analysis confirmed the direction and PACAP-dependence of transcriptional regulation, although RT-PCR revealed larger fold changes than did microarray analysis, consistent with previous comparisons of these two methods of quantifying changes in mRNA abundance [50].

Table 4
RT-PCR Verification of transcript alterations in MCAO

Gene name	Time (h)	Comparison	Wild-type	PACAP-def
Preproenkephalin 1 (Penk1)	1 hr	I/N	2.32±0.46 *	1.53±0.35
	24 hr	I/N	3.48±0.22 *	1.04±0.06
Heat shock protein, 110 kDa (Hsp 110)	1 hr	I/N	2.64±0.35	2.57±0.20
	24 hr	I/N	2.59±0.26	2.48±0.22
MAP kinase kinase 2 (Mapkk2)	1 hr	I/N	1.43±0.13	1.26±0.10
	24 hr	I/N	1.75±0.58	1.04±0.49

Values represent the mean±s.e.m. of at least three individual samples of each condition, in which I/N represents $\delta\delta Ct_{I/N} = \delta Ct_I$ minus δCt_N, where δCt_I = Ct (transcript)$_I$-Ct(18S rRNA)$_I$ and δCt_N = Ct(transcript) $_N$-Ct(18S rRNA) $_N$, and Ct = cycle number at threshold.
* $p < 0.05$, Student's two-tailed t-test.

4. Discussion

4.1. Role of PACAP in limiting ischemic damage and its functional sequelae in the mouse

Earlier studies have confirmed that the NSS is a useful parameter for assessing the therapeutic effects of drugs in the ischemic mouse [46,62]. In this study, the attainment of only partial spontaneous recovery of NSS in untreated mice subjected to MCAO served as a basis for testing pharmacological intervention with exogenous PACAP treatment, and the effects of endogenous PACAP deficiency. A significant decrease in ΔNWF in PACAP-deficient mice after cerebral ischemia suggested an important role for endogenous PACAP in neuroprotection after stroke. Exogenous PACAP significantly attenuated neurological deficits and improved motor function in wild-type mice (increased ΔNSS by 25% and increased ΔNWF by 36%) and in PACAP-deficient mice (increased ΔNSS by 39% and increased ΔNWF by 51%). Increased ΔNSS and ΔNWF in the injured mice treated with PACAP defined a significant neurological functional recovery induced by PACAP administered either i.v. or i.c.v. in MCAO-injured mice, especially in PACAP-deficient mice. The more significant improvement by PACAP treatment in the MCAO-injured PACAP-deficient mice indicates a requirement for endogenous PACAP in the brain, for maintenance of homeostatic neuronal signaling, neuronal survival and neuroprotection against cerebral ischemia.

Infarct volume in the injured brain is a precise criterion for judging the severity and extent of cerebral ischemia. It is well

accepted that the extent of permanent ischemic injury is not defined immediately following arterial occlusion, but rather expands over time [27]. The efflux of potentially harmful elaborated products from ischemic cells into surrounding non-ischemic areas is one mode of amplification of injury in focal stroke. After reduction in blood flow by MCAO, both neurons and astrocytes in the ischemic core are depleted of cellular energy metabolites, lose the ability to regulate their ion content, and finally die during the resultant ischemic infarction [63]. In the present study, a highly significant increase in infarct volume was observed in PACAP-deficient mice following MCAO. PACAP-deficient mice may be more vulnerable to brain damage in cerebral ischemia due to the absence of PACAP-dependent neuroprotective mechanisms against decreased blood flow. Exogenous PACAP as well has significant effect in reducing infarct volumes produced by MCAO in the injured brain. The overall 19% infarct volume reduction represents more than 85% of the maximum salvage that has been achievable in the injured brain after MCAO [35,64–67]. The PACAP-induced ischemic tolerant or neuroprotective state may be achieved in brain by a combination of different factors including metabolic inhibition, and cytokine mobilization and antagonism [28]. In addition, PACAP's actions as a vasodilator and neurotrophic factor may be helpful to reduce post-ischemic edema formation and MCAO-induced inflammation reaction.

4.2. Ischemia-altered acute transcripts—indicators of initial response to stroke

Ischemia-induced secondary neuronal damage is likely to be progressive, and to depend on the altered expression of many transcripts induced by ischemic insult [68,69]. These transcriptional and translational events are potentially modifiable by post-ischemic interventions. Altered gene expression is a pronounced feature of ischemic cerebral injury and affects proteins with different function, including intracellular signaling, inflammation or stress response, cell survival, apoptosis, cytoskeleton biogenesis, metabolism and protein synthesis [35,67]. Ischemia-induced transcriptional changes likely contribute to both behavioral deficits and pathological consequences of brain injury [70,71]. Therefore, identification of ischemia-altered gene expression and determination of the gene expression associated with the recovery or impairment of neurological functions following cerebral ischemia will help illuminate the pathophysiology of cerebral ischemia and also provide therapeutic targets for intervention in stroke.

Our study revealed an increase in the expression of a number of ischemia-associated transcripts reported in earlier studies. These comprise three categories, i.e., acute, delayed and sustained. They include annexin A2 (Anxa2), aquaporin 4 (Aqp4), activity-regulated cytoskeletal-associated protein (Arc), β2 microglobulin (B2m), C1qb, CD44 antigens (CD44, CD53, CD63, Cd9), cathepsins C and L, decorin (Dcn), DnaJ (Hsp40) homolog, Dnajb1, Egr1, FosB, Fosl2, Gadd45g, GFAP, Hsp110, Hspa5, Ier3, Irf1, Jun, moesin (MSN), Mt1, MTt, Penk1, Ptgs2, Rgs2, Spp1, Stat3, Timp2 and vimentin (Vim). In all, about 25% of the ischemia-altered transcripts detected in our study have been seen earlier [28–38].

Several of the transcripts regulated 1 h after MCAO are immediate early genes (IEGs). Egr1, Egr3, Fos and Jun are known to control signaling by inducing the expression of multiple downstream gene products [72]. Most of the IEGs induced by MCAO are PACAP-independent, including Egr1, even though this IEG is up-regulated by PACAP in PC12 cells [54]. In the more heterogeneous synaptic environment of the brain, Egr-1 transcription may be controlled by PACAP at only a minority of synapses, and PACAP-dependent regulation of this IEG is therefore not evident in the middle cerebral artery territory after stroke. Some prominent IEGs however, are PACAP-dependently regulated in stroke, including Fosl2 and Jun.

4.3. Ischemia-altered sustained transcripts—contributions to post-ischemic pathophysiological events and secondary brain injury and regulation by PACAP

We identified 37 (Table 2) named transcripts that were altered by ischemia in a sustained fashion, all of them up-regulated and none down-regulated. Eleven of these were also induced by ischemia in PACAP-deficient mice, among them calsyntenin 3 (Clstn3), heat shock protein 110 (Hsp110), heat shock protein 1b (Hspa1b), heat shock 70 kD protein 5, (Hspa5), metallothionein 2 (Mt2), and villin 2 (Vil2).

The remainder of the sustained transcripts were not regulated in PACAP-deficient mouse cortex after MCAO, indicating their dependency on endogenous PACAP for full expression. While it seems unlikely that all of these transcripts are directly affected by PACAP, the neuropeptide may act to trigger cell survival cascades leading to subsequent gene expression.

Proenkephalin A may be neuroprotective, based on its up-regulation in wild-type but not PACAP-deficient mice following MCAO. Given the role of PACAP in regulation of enkephalin in glial cells [73,74] and the proposed developmental role of enkephalin in gliogenesis in brain [75], it may be of interest to examine stroke-related injury in enkephalin knock-out compared to wild-type C57Bl/6 mice.

We have also identified several genes up-regulated by MCAO in both wild-type and PACAP-deficient mice and suppressed by PACAP treatment following MCAO. This pattern of regulation is consistent with an injury-effector function for these transcripts, since exogenous PACAP treatment is associated with improved neurological and neuropathological outcome after stroke. Hsp70 and Hsp110 are two transcripts whose regulation fits this pattern, and whose cognate proteins may be involved in effecting injury rather than protection in stroke.

The heat shock proteins may be valuable marker genes for further examination of PACAP analogs useful in stroke. This is especially important in considering that exogenous PACAP itself can induce gene transcription in both physiologically appropriate ways, by acting at synaptic receptors where PACAP is normally present, and pharmacologically, at VPAC receptors normally controlled by VIPergic transmission but at which exogenous PACAP may exert important effects.

Post-ischemic pathophysiological and histopathological changes and secondary brain damage (such as edema formation, neuronal cell death and subsequent neurological dysfunction, etc.) occur mainly within 24 h in the brain following MCAO. Therefore, sustained transcripts as a group may induce post-ischemic pathophysiological changes and secondary brain damage in the ischemic cortex, or limit and control harmful processes and outcomes of ischemic stroke. Pharmacological treatment targeting the sustained transcripts must therefore be tailored towards the suppression of the former, and enhanced expression of the latter, to be helpful in preventing secondary damage and controlling neurodegenerative processes triggered by cerebral ischemia in the injured brain.

4.4. Ischemia-altered delayed transcripts—targets relevant to mechanisms of PACAP action in amelioration of stroke damage

The delayed transcripts are a potentially important target not only for investigating the underlying mechanisms and pathophysiology of ischemic insults, but also for designing future therapeutic strategies for the treatment of stroke, since these are potentially reversible within the window of intervention available clinically. 24 h after MCAO, 142 delayed known transcripts were regulated by ischemia in normal mice. Most of these transcripts were PACAP-dependent, and their expression could contribute to the differences in infarct volume and Δ-NWF between wild-type and PACAP-deficient mice. In contrast, each of the PACAP-independent transcripts encodes a candidate injury-effector or neuroprotective protein not controlled by PACAP, and therefore within the purview of combination therapy that might increase the salvage potential of PACAP treatment in stroke. Glial fibrillary acidic protein (Gfap), moesin (Msn), metallothionein 1 (Mt1), transferrin (Trf) and vimentin (Vim) were all up-regulated two-fold or greater in both wild-type and PACAP-deficient mice, and distinguishing between injury-effector and neuroprotective roles for their cognate proteins is an important criterion for further development of complementary combinatorial approaches in treatment of secondary neuronal damage in stroke. Secreted phosphoprotein 1 is an additional important molecule in this regard because its secretion makes it an attractive indicator or marker protein in translational studies focused on combinatorial or adjunct therapeutic approaches.

4.5. Relationships between PACAP-dependent transcripts and injury effector versus neuroprotective transcripts in ischemic cortex

The ischemic response involves increased expression of transcripts encoding proteins that act within injury-effector pathways, and those acting within neuroprotective pathways that represent a counter-response to injury. PACAP may attenuate ischemic insult in mouse brain through inhibiting expression of proteins deleterious for neuronal cell survival, or inducing genes that encode intrinsically neuroprotective proteins. These can be distinguished in the final analysis only empirically, in transgenic mice or those treated with specific protein antagonists and agonists. However, potential, or candidate, effector and protector transcripts can be identified on the basis of their regulation under conditions that correlate with overall pathological and functional improvement or deterioration. For example, reversal of the direction of regulation of a given transcript during ischemia by treatments or conditions that ameliorate ischemic damage would identify it as an injury-effector transcript, while reversal of its regulation by conditions that exacerbate ischemic damage would identify it as a candidate injury-dependent neuroprotective transcript. In the present case, the best candidates in each category are those whose regulation is inversely affected by endogenous PACAP deficiency and treatment with exogenous PACAP. Transcripts in the first category would likely include those up-regulated by MCAO in both wild-type and PACAP-deficient mice, whose MCAO-dependent up-regulation is blocked by treatment with exogenous PACAP. Calponin 3, Hsp 110, metallothionin 1, and thioredoxin reductase may be examples of such transcripts. Transcripts in the second category would likely include those up-regulated by MCAO in wild-type but not PACAP-deficient mice, whose expression remains up-regulated (or is up-regulated further) by PACAP treatment. The neuropeptides enkephalin, substance P, and neurotensin may be direct PACAP-dependent neuroprotective response mediators by this criterion. Combining microarray analysis, measurement of functional outcomes, and transcriptional modulation by pharmacological agents in mice deficient in other genes besides PACAP will provide a basis for distinguishing additional injury-effector and neuroprotective targets for more precise therapeutic targeting.

The anatomical specificity of PACAP-regulated expression may determine the final contributions of a protein, especially another neuropeptide, to the PACAP responses in stroke. The tachykinin A system, for example, may contribute to secondary neuronal damage, or its amelioration, depending on enhanced tachykinin neurotransmission across glutamatergic excitatory synapses, or GABAergic inhibitory ones, in cortex [58]. The induction by PACAP of three major endogenous neuropeptide systems in ischemic cortex may also be a clue to the large number of globally regulated, PACAP-dependent transcripts found here by microarray analysis in the MCA territory after MCAO.

Further meta-analysis of MCAO-induced transcripts modulated by various treatments for stroke will identify overlapping and complementary mechanisms of action as a prelude for the efficient design of combination therapy for stroke. For example, determining whether PACAP's neuroprotective effects are mediated through inhibition of glutamate-induced excitotoxicity [76–78], cytokine generation [18], or other independent mechanisms based on microarray analyses of glutamate- versus cytokine-oriented stroke therapeutics will help to place PACAP within the general armamentarium of potential treatments for secondary neuronal damage following stroke.

Finally, many specific PACAP target transcripts identified by microarray analysis were not discussed in this report, because they are currently of unknown biological function. Identification and analysis of these specific PACAP target transcripts in ischemic cortex, in conjunction with the analysis of known

(named) transcripts reported here, should be a productive focus for further understanding of signal transduction pathways related to PACAP neuroprotection in the brain, and the development of novel PACAP-related stroke therapy.

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