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DNA Microarray-Bioinformatics Based Analysis of PACAP38 Neuroprotective Effect on Ischemic Brain Suggests Differential Molecular Signaling Pathways Involved in GSK3β and CRMP2 Regulation

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ABSTRACT

Our group has been systematically experimenting with the permanent middle cerebral artery occlusion (PMCAO) mouse model revealing large number of genes differentially expressed in the ischemic brain, hemispheres and targeting specific tissues in the ischemic core and penumbra brain regions after pituitary adenylate-cyclase activating polypeptide (PACAP) intervention. Utilizing a high throughput DNA microarray-based whole mouse genome analysis on the Agilent platform by two-color/dye-swap approach for genome-wide transcriptome datasets, the neuroprotective effects of the PACAP38 (1 pmol) treatment is being revealed step by step in the PMCAO model mouse brain. This report revisits the large volume of data (dye-swap based gene inventory) specifically obtained from the ischemic core and penumbra regions of the ischemic brain by filtering it over the brain hemispheres data and healthy control brain in conjunction with Ingenuity Pathway Analysis (IPA) analysis. In conjunction with targeted Western analysis of a potential key player in PACAP-mediated neuroprotection, namely CRMP2, previously identified by us using 2-DGE analysis and other researchers, these combined results suggest a role for PACAP in regulating CRMP2 function through GSK3β and upstream signaling networks as revealed by the bioinformatics analysis of specific genome data at 6 h post-treatment with PACAP38. A schematic and hypothetical model showing genes potentially involved behind neuroprotective function of PACAP38 in the ischemic brain early on is presented.

Keywords: PACAP38, Ischemic core and penumbra, Mice, DNA microarray, CRMP2

INTRODUCTION

Pituitary adenylate-cyclase activating polypeptide (PACAP) [1-3] has neuroprotective functions especially under the condition of brain ischemia [4-18]. Based on these evidences, some from our own group using the permanent middle cerebral artery occlusion (hereafter referred to as PMCAO) mouse model [6,7,11-16] and that from other researchers in stroke models [17-22] this neuropeptide was suggested to be a potential therapy in counteracting effects of brain ischemia or stroke.

It is to be noted that our group's research uses the intraluminal filament technique-based PMCAO model over the transient MCAO, which results in reperfusion injury, and is avoided in our research model. To address a question on whether PACAP38 treatment has indeed an effect on the brain infarction size (in mice), previous researches have quantitatively/qualitatively (by 2, 3, 5-triphenyltetrazolium

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chloride, TTC, staining) demonstrated that PACAP38 treatment improved brain infarction compared to a control 0.9% saline [6,19,12; and references therein]. Thus, previous results demonstrated PACAP38 efficacy for neuroprotection of the brain following an ischemic insult in mice.

A unique aspect of our group's PACAP38 research is the strategy involving a large-scale DNA microarray-based [23] high-throughput omics analysis on the PMCAO model brain hemispheres and regions, over a period of time to step by step create an inventory of genes (transcriptome database, freely accessible on the NCBI GEO database - http://www.ncbi.nlm.nih.gov/geo/info/linking.html, under accession numbers - GSE28201, GSE37565, GSE62884, GSE 67421) [12-16]. These gene lists and expression profiles have revealed their modulation in the ischemic brain with or without PACAP38 treatment at two different time points of 6 and 24 h post-treatment, providing the first inventory of the mouse ischemic brain transcriptome [12-14]. All the above researches resulted in a vast gene database resource on novel molecular factors and potential mechanisms that might underlie the neuroprotective effects of PACAP using animal models, and that may be used to give new insight into the potential therapeutic use of PACAP38 and/or its analogs [6, 7, 11-17,19-22]. For example, Tamas and colleagues have indicated in their review that the role of PACAP as a neurotrophic factor has now been validated and the data suggesting an important function of PACAP in neuronal regeneration promises its use as a therapeutic agent in injuries to the nervous system [22]. Rivnyak and co-workers have recently reviewed the effects of PACAP on the transcriptome and proteome in the brain [18].

PACAP38 effect comprises of numerous and interlinked molecular genetic factors and/or pathways as revealed from all these studies. As a next step we have narrowed down to specifically dissected brain regions in the ischemic brain, namely the ischemic/infract core (hereafter abbreviated IC) and the penumbra (hereafter abbreviated P or ischemic P, IP, when looking at the ischemic region) to analyze these gene inventories and get new insight into PACAP38 effect (**Figure 1**).

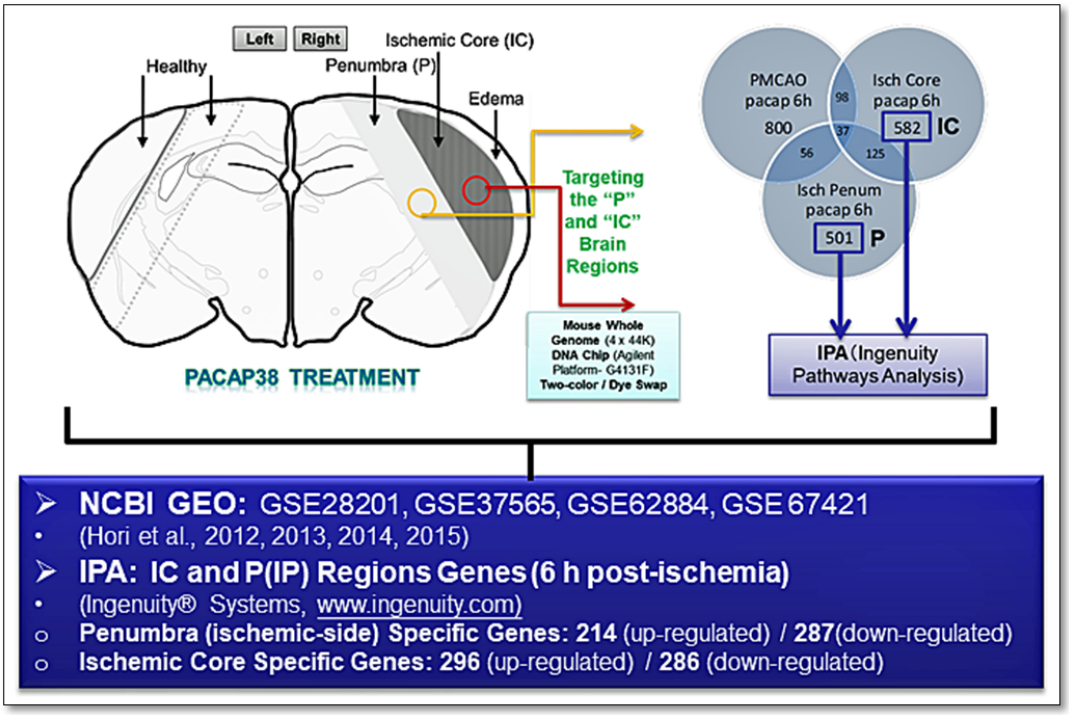

Figure 1. The experimental strategy to screen for genes that are influenced by PACAP38 differentially and specifically in the brain regions of ischemic core (IC) and penumbra (P/IP) of the ischemic brain in permanent middle cerebral artery occlusion (PMCAO) mouse model.

Using the DNA microarray data under the mentioned GEO accessions, Ingenuity Pathway Analysis (IPA) analysis was performed on first, the obtained up and down-regulated genes in IC and P(IP) at 6 h and 24 h post-treatment with PACAP38, and second, on the PACAP38 specifically up (red)- and down(green)-regulated genes in the IC and P(IP) at 6 h. The brain cross-section sketch has been used in our previous papers too, and here shows a composite figure with specifically targeted core and penumbra regions

Our recent hypothesis is that targeting the P and IC regions in the ischemic brain would give information on the unique expressions of genes involved in modulating the neuroprotective effect of PACAP38 early on (i.e., 6 h post ischemia) in the ischemic insult. The 6 h time period has been specifically examined in our current analysis of gene expressions in order to know what signaling process occur at this time point, which is the acute phase following PMCAO. As generally believed, during the stroke onset (acute phase) ischemic injury causes a blockage of nutrients via deprivation of blood and there is irreversible damage to the ischemic core; and compared to the 24 h chronic phase where there may be some remodeling signaling in the penumbra (IP). Therefore, our experiment examining the effect of PACAP38 intervention at the 6 h time point is critical to see and seek out the signaling factors working in the IC and P(IP), and how the remodeling via PACAP38 is occurring to cause improvements in the brain in both the IC and the P(IP). Therefore, at present the 24 h time point (chronic phase) has not been examined in the present analysis.

Thus, this current report, first, puts forward a bioinformatics-based evidence to explain the nature of the genes expressed in specifically dissected P(IP) and IC regions in relation to recovery effect of PACAP38 treatment early on (6 h post ischemic insult), in PMCAO mouse model. Second, based on the bioinformatics analyses and gene functions and pathways unravelled during this study, targeted protein analysis was performed using immunoblotting with specific antibodies to further support the role of the previously identified collapsing response mediator protein 2 (CRMP2) [24], and other related up-stream potential target molecules for the proposed neuroprotective mechanisms behind PACAP38 action in mice brain ischemia.

MATERIALS & METHODS

DNA microarray data usage and ingenuity pathway analysis (IPA)

The present study uses raw whole gene expression data from previously conducted experiments in our laboratory and unpublished data (but deposited in the NCBI GEO database: GSE28201, GSE37565, GSE62884, GSE 67421; Figure 1) [12-16]. All the animal research (animal care and experimental procedures) were previously performed with the relevant guidelines and regulations which were approved by the Institutional Animal Care and Use Committee of Showa University (School of Medicine), Tokyo, Japan. PMCAO model mice were generated and brain regions dissected and processed for DNA microarray analysis as described previously, including PACAP38 injection/treatment [6,12-14]. Utilizing these gene expression datasets, the biological function and network analyses were performed using IPA (Ingenuity® Systems, www.ingenuity.com). Concurrent to the previous experiments, a new analysis was performed using the IPA tool [Content version: 23814503 (Release Date: 2015-03-22), QIAGEN]. The dataset from the DNA microarray which includes the differentially expressed (≧/≦ 1.5/0.75-fold) genes and their corresponding fold change values, was uploaded as an Excel spread sheet into the IPA tool. To create gene data (bio-functions, canonical pathways and upstream regulators), the genes were overlaid onto a global molecular network that was developed from information that was contained in the ingenuity knowledge base. The functional analysis identified the biological functions that were most significant to the dataset (p-value <0.05) according to a right-tailed Fisher's exact test.

Extraction of total soluble protein

Total protein was extracted from approximately 50 mg sample powders (each P/IP and IC brain region; n=6 each, and sample powders were prepared from pooled brain regions that were extremely small in size and quantity due to the microdissection form the mentioned sampling regions of the brain) using a lysis buffer that contained Thiourea and Tris (LB-TT) for extraction of brain proteins [13, 25]. Composition of slightly modified LB-TT was as follows: 7 M (w/v) urea, 42 g; 2 M (w/v) Thiourea, 15.2 g; 4 % (w/v) CHAPS, 4.0 g; 18 mM (w/v) Tris-HCl (pH 8.0), 1.8 mL; 14 mM (w/v) Trizma base, 169.5 mg; 0.2 % (v/v) Triton X-100, 0.2 mL; 50 mM (w/v) DTT, 771.5 mg; 1 % (v/v) pH 3-10 Ampholyte, 1 mL; and two EDTA-free proteinase inhibitor (Roche) tablets in a total volume of 100 mL at room temperature (RT). Protein extraction was initiated by quickly adding 1 mL of LB-TT to the 2 mL microfuge tube containing sample powder following removal from the -80°C deep freezer, and immediately mixed by vortexing (at full speed using a Lab mixer, Iwaki, Tokyo, Japan) for 1 min at RT. Protein solution in LB-TT was incubated at RT for 30 min with mixing by vortexing (30 s) and sonication (30 s in a water-bath type sonicator) for a total of 5 times. Insoluble protein pellet and/or debris were pelleted by centrifugation at 18,500 g for 15 min at 20ºC using a high-speed refrigerated micro centrifuge (MX-150, TOMY, Tokyo, Japan). Clear supernatant (around 900 µL) was transferred to a new 1.5 mL microfuge tube and stored at -80°C as the total soluble protein. Protein concentration was determined with a Coomassie Plus™ Protein Assay Kit (PIERCE, Rockford, IL, USA) using bovine serum albumin (BSA) as a standard and a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Western blot analysis

SDS-PAGE (mini-gel) was carried out on the separated proteins as per standard protocols proteins [13, 25]. The well separated proteins were electro transferred onto a polyvinyldifluoride [PVDF (Trans-Blot Turbo Midi PVDF, 0.2 µM, Transfer Packs kit; Cat. no. 170-4157)] using the Trans-Blot Turbo Transfer System (Bio-Rad; StandardSD protocol; 25V, 1.0 A, 30 min). Following the transfer of proteins on the PVDF membrane (confirmed by visualizing

all the 10 colored molecular mass standards), the membrane was incubated in 25 mL of 5 % blocking solution (Block-Ace powder, Cat. no. UK-B80, DS Pharma, Osaka, Japan; Yukizirushi, Sapporo, Hokkaido, Japan) for 1 h under constant slow shaking at RT. Blocking solution was prepared by dissolving 4 g powder in 80 mL 1X TTBS [10X TTBS: NaCl, 80 g; 1 M Tris-HCl, pH 7.5, 200 mL; Tween-20, 5 mL]. Western blotting and detection was carried out using the Immun-Star WesternC Chemiluminescent Kit (Cat. no. 170-5070, Bio-Rad) following the manufacturer's instructions. Blocking solution was decanted and the membrane was washed once in 1X TTBS (5 min), followed by incubation in 25 mL of primary antibody solution [PAS; 1 μL rabbit anti-CRMP2 phophoT514 protein antibody (Cat. no. ab62478; 100 mg, 2 mg/mL; Abcam, www.abcam.co.jp; anti-CRMP2 phophoSer522 and anti-CRMP2 phophoThr555] for 1 h, as above. The membrane was then washed with 25 mL of 1X TTBS for five times. The last TTBS wash was decanted and the membrane was incubated in 25 mL of secondary antibody solution [SAS; 0.5 μL of Amersham, ECL anti-rabbit IgG, HRP linked species-specific whole antibody (from Donkey); Cat. no. NA 934; GE Healthcare] for 1 h with slow shaking at RT. The 1X TTBS wash step was repeated five times. For blot development, the luminol/enhancer and peroxide buffer solutions were mixed in a 1:1 ratio (1 mL: 1 mL; one membrane volume) and spread over the membrane and incubated at RT for 5 min. Excess solution was drained by touching one end of the membrane on a KimWipe paper towel and the signal (cross-reacting protein bands) was visualized using the ChemiDoc XRS+imaging system (Bio-Rad). The Western blot analysis was repeated at least three times, and representative data from one blot is shown.

RESULTS AND DISCUSSION

Specifically up- and down-regulated genes in ischemic core and penumbra following PACAP38 treatment and their IPA

IPA-based bioinformatics analysis provided a number of IC-specific genes: 241 (up-regulated)/229 (down-regulated) and also P(IP)-specific genes: 181 (up-regulated)/236 (down-regulated) at 6 h post-ischemia following the injection of PACAP38 **(Figure 1)**. The 6 h early time point was targeted because of early visualization of the brain regions and ischemia (also by TTC staining which shows the ischemic region in the brain) in the brain along with numerous gene expression changes [12-16] and the very early treatments for stroke given within 4 to 6 h of the stroke. Keeping in mind the hypothesis that PACAP38 should be effective by its nature as a neuroprotective peptide/agent on the cells (or neurons) by direct action or indirect action through modulating the cells through activating the release of neuroprotective factors. Thus the authors wished to see if the narrowed down microarray-based transcriptome contains these hints and whether they can be delineated in the IC and P(IP) regions, on the very early time point (acute phase) of 6 h post-ischemia. Remarkable differences were seen upon performing IPA and generating the bio-functions, canonical pathways and upstream regulators for both IC **(Figures 2, 3 and 4)** and P(IP) **(Figures 5, 6 and 7)**.

Both IC and the P(IP) regions are affected by the PACAP38 injection in various gene bio-functions and pathways and upstream targets; a greater number of gene categories are being affected in the IC by PACAP38. In the IC gene categories of bio function: lipid metabolism, small molecule biochemistry, gene expression, cell to cell signaling and interaction, others (down-regulated) to up-regulated genes in cellular development to embryonic development and cell morphology are seen **(Figure 2)**; In the IC, gene functions can be sub-divided mainly into antithrombotic, ant-inflammation and which are expected if PACAP38 is acting to suppress the spread of the ischemia. This is not the case in the P(IP) region. In the case of bio-functions, PACAP38 appears to show a general decrease in the categories of the nervous system development and function (sensation and nociception functions), cell cycle, tissue development and cellular growth and proliferation; all these are related to reduction in cellular proliferation function in the P(IP) region **(Figure 5)**. This can be expected as the penumbra (IP) is supposed to be the site or boundary between active recovery (IC, under PACAP38) and the presumably healthy regions. However, we find that an increase in the gene category of endocrine system disorders with benign thyroid disease as the annotated function is seen in the P(IP) **(Figure 5)**. For example, one of the prominent genes is CTLA4 which suppresses the T-cells sending them turn-off signals in case of chronic infections [26]. This anti-inflammation function of CTLA4 would be required for the P(IP) region and which should not be actively producing a defensive response by the T cells. Similarly, the TTR gene is being up-regulated and which along with BDNF has been shown to have neuroprotective effects [15, 27-29]. However, it is to be noted that TTR and especially BNDF may have different functions depending on the cells and conditions and that is why we see it (BDNF) being reduced in the annotated function of sensation.

Second, in the canonical pathways, IL1 signaling, dopamine receptor signaling, acute phase response signaling, CDK5 signaling, and cAMP-mediated signaling show a decrease in the P(IP) region **(Figure 6)** compared to the opposite gene functions in the IC **(Figure 3)**. Anti-inflammation and blood coagulation are the main events occurring in the P(IP) region following PACAP38 treatment, especially with the cAMP-mediated pathway having an effect on the blood coagulation. Considering the fact that the region around the core is healthy, it would not be desirable to have a higher level of blood flow in response to the ischemic insult. In the case of the IC region, we see that Gα12/13 signaling function,

IC 6 h: Top Bio Functions

Categories	Diseases or Functions Annotation	p-Value	Predicted Activation State	Activation z-score	Molecules	Molecules
Cell Morphology	shape change of axons	3.26E-03	Increased	2.13	CHRNA1,CXCL12,DCC,GRASP,NTRK1,PLAT,SLIT2,ULK2	8
Embryonic Development, Organ Development, Organismal Development, Skeletal and Muscular System Development and Function, Tissue Development	formation of muscle	3.80E-03	Increased	2.22	ALOX12,ALX4,CAV3,CTNNB1,CXCL12,F2,FHL2,IGF1R,IHH,ISL1,ITGB1BP2,JAG1,LAMA1,Meg3,MESP1,MSTN,MSX1,MYOM1,SGCB,SHH,SRPK3,STIM1,TBX5,WFIKKN1	24
Cellular Development, Cellular Growth and Proliferation, Embryonic Development, Organ Development, Organismal Development, Skeletal and Muscular System Development and Function, Tissue Development	formation of muscle cells	7.16E-03	Increased	2.60	CAV3,F2,FHL2,IGF1R,LAMA1,MESP1,MSTN,MYOM1,SGCB,STIM1,TBX5,WFIKKN1	12
Lipid Metabolism, Small Molecule Biochemistry	binding of lipid	5.39E-03	Decreased	-2.35	APOH,CAMP,ESR1,F2,NF2,SLPI,VDR	7
Gene Expression	activation of DNA endogenous promoter	2.15E-03	Decreased	-2.21	ALX4,ANXA4,BCL11A,BCL9,BRWD1,CTNNB1,DDX58,DMBX1,EGR4,EHF,ELL3,ESR1,ESRRB,FHL2,FOSB,FOXD1,FOXE1,HIRA,HOXA2,IHH,IKZF2,ISL1,JAG1,KCNH8,KDM2B,Maml2,MED17,MESP1,MSX1,NANOG,NFAT5,NFATC2IP,NFE2,NHLH1,NKX2-1,NKX3-1,OLIG2,PHF14,PTCH1,RAI1,Rsl1 (includes others),RUNX3,SALL4,SERPINF2,SFPQ,SFRP2,SHH,SOX3,SOX5,SUFU,SUV39H2,TBX22,TBX5,TEAD1,TNFSF8,TP73,VDR,ZBTB20,ZFAT,Zfp748	60
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Inflammatory Response, Tissue Development	adhesion of blood platelets	1.08E-02	Decreased	-2.19	APOH,C3,CD226,F2,SLIT2	5
Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	activation of granulocytes	7.94E-03	Decreased	-2.16	C3,CXCL6,F2,IL1RN,LTF,MPO,SERPINF2,SLPI	8
Organismal Survival	mortality	3.57E-03	Decreased	-2.14	C3,DGCR8,IDO1,MMP9,MSTN,OPRM1,Pmaip1,PTCH1,SLPI,STIM1,TREM1	11
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	migration of phagocytes	2.09E-04	Decreased	-2.13	ADORA2A,ASB2,C3,CAMP,CCR7,CTSG,CXCL12,CXCL6,F2,FHL2,IL12B,IL1RN,ITGAV,MMP9,MPO,RPTOR,RUNX3,SERPINC1,SLIT2,SLPI	20
Organismal Survival	perinatal death	1.30E-02	Decreased	-2.08	ALX4,ATP1A3,BCL11A,CHRNA1,COL12A1,CXCL12,CYP26A1,DCC,EC	32
Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking, Inflammatory Response	migration of Langerhans cells	2.38E-04	Decreased	-2.00	C3,CCR7,ITGAV,RPTOR,SLIT2	5
Immunological Disease, Organismal Injury and Abnormalities	formation of granuloma	6.43E-03	Decreased	-2.00	IL12B,IL1RN,MMP9,TREM1	4

Figure 2. The bio-functions of the identified annotated genes in PACAP38 specifically up (red) - and down(green)-regulated genes in the ischemic core (IC) at 6 h. Ingenuity Pathway Analysis (IPA) analysis was performed.

IC 6 h: Top Canonical Pathways

Ingenuity Canonical Pathways	-log(p-value)	Ratio	z-score	Molecules
Sperm Motility	7.29E-01	4.63E-02	2.00	PLA2G4E,PDE4A,PDE1C,PLCZ1,SLC16A10
Synaptic Long Term Depression	7.88E-01	4.58E-02	1.63	IGF1R,PLA2G4E,RYR1,GUCY2D,GRID2,PLCZ1
Calcium Signaling	4.98E-01	3.64E-02	1.63	TRDN,RYR1,CHRNA1,ASPH,NFAT5,RAP2A
cAMP-mediated signaling	2.68E-01	2.86E-02	1.63	ADORA2A,PDE4A,CHRM3,PDE1C,CHRM2,OPRM1
Glioblastoma Multiforme Signaling	4.28E-01	3.50E-02	1.34	WNT2B,IGF1R,NF2,CTNNB1,PLCZ1
Coagulation System	1.79E+00	1.14E-01	1.00	SERPINF2,PLAT,SERPINC1,F2
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	1.08E+00	5.56E-02	1.00	NANOG,WNT2B,CTNNB1,SALL4,BMP15,ZFP42
RhoA Signaling	6.35E-01	4.27E-02	-0.45	IGF1R,ARHGAP6,LPAR4,SEPT6,ARHGAP12
Leukocyte Extravasation Signaling	1.09E+00	4.81E-02	-0.82	CXCL12,ARHGAP6,CLDN17,MMP9,CTNNB1,BTK,TEC,ARHGAP12,MMP28
LXR/RXR Activation	1.08E+00	5.56E-02	-0.82	IL1RN,C3,MMP9,APOH,IL1RL2,SERPINF2
Gα12/13 Signaling	6.65E-01	4.39E-02	-1.34	CTNNB1,BTK,LPAR4,TEC,F2

Figure 3. The canonical pathways of the identified annotated genes in PACAP38 specifically up (red)-and down (green)-regulated genes in the ischemic core (IC) at 6 h. Ingenuity Pathway Analysis (IPA) analysis was performed.

P(IP) 6 h: Top Bio Functions

Categories	Diseases or Functions Annotation	p-Value	Predicted Activation State	Activation z-score	Molecules	Molecules
Endocrine System Disorders	benign thyroid disease	4.84E-03	Increased	2.00	CD28,CTLA4,GLIS3,HOXA3,IL4,TRHR,TTR	7
Nervous System Development and Function	sensation	1.14E-03	Decreased	-2.65	ADCYAP1,AQP1,B3GNT3,BDNF,FAM19A4,IL1A,MME,NF1,NTF3,P2RY1,PROK2,TRPM3,TRPV4	13
Cell Cycle	cell cycle progression	3.41E-03	Decreased	-2.50	ACVRL1,ADCYAP1,APBB2,CCNB3,CCNO,CD28,CDK1,CENPC,CENPE,CITED2,CTLA4,CUL7,DNAJB4,DTL,EP300,Ifi202b,IFNB1,IKZF3,IL12A,IL1A,IL2RB,IL4,JUN,KRT18,KRT8,LEF1,NEK2,NF1,NTF3,PADI4,PRKAR2A,PRKCD,PTHLH,PTPRO,RARB,ROCK2,SHB,SKA1,SPHK1,STAT5B,TCF7L2,TGFA,TNFSF10,TPP2,TRIM25,WNT3A,ZIC1	47
Nervous System Development and Function	nociception	5.84E-03	Decreased	-2.24	ADCYAP1,AQP1,B3GNT3,BDNF,FAM19A4,IL1A,MME,P2RY1,PROK2,TRPM3,TRPV4	11
Tissue Development	growth of epithelial tissue	3.58E-04	Decreased	-2.03	ACVRL1,ADAMTS2,AIMP1,BCL11B,BDNF,C5AR1,CD36,CITED2,COL8A1,EP300,FGF6,IL1A,IL4,JUN,KCNJ2,KCNK2,KDF1,KLF10,MYOF,NF1,NRP1,NTS,PRKCD,PRLR,PROK2,PTHLH,REG3G,SLC8A1,SPHK1,TCF7L2,TGFA,THBS4,TNFRSF25,TNFSF10,TRIM24,TYRP1,WNT3A	37
Cellular Growth and Proliferation	proliferation of cells	8.19E-03	Decreased	-2.02	ACVRL1,ADAMTS2,ADCYAP1,AGO4,AIMP1,APBB2,AQP1,B3GNT3,BCL11B,BDNF,C5AR1,CACNA1S,CCDC88A,CCKAR,CCNO,CD28,CD36,CDCP1,CDK1,CITED2,CLDN1,CLEC4G,CNTN2,COL14A1,COL8A1,CR2,CTC1,CTLA4,CUL7,CUX2,DACH1,DES,DNAJB4,DTL,EML4,ENTPD1,EP300,EP400,FGF21,FGF6,FOXA2,FOXC2,GLI3,GML,GPR6,GRAP2,HOXA3,Ifi202b,IFNB1,IFNK,IKZF3,IL12A,IL1A,IL20RB,IL2RB,IL4,INSL6,JUN,KALRN,KCNA5,KCNJ2,KCNJ5,KCNK2,KDF1,KL,KLF10,KRT8,LEF1,MAP4K1,MID1,MUSK,MYOF,NDUFAB1,NEK2,NF1,NPSR1,NPTX2,NR2E3,NRP1,NT5E,NTF3,NTS,OTX2,OXTR,P2RY1,PADI4,PAX5,PHLDA1,PKP2,PPL,PPP1R1B,PRKAR2A,PRKCD,PRLR,PROK2,PRPH,PTHLH,PTPRO,RARB,RASGRP2,RASSF4,REG3G,RERG,ROCK2,RPS6KA5,RXFP2,SATB1,SETDB1,SHB,SLC43A1,SLC8A1,SOCS6,SOD1,SPHK1,STAT5B,SULT2A1,TBC1D8,TCF7L2,TGFA,THBS4,TNFRSF25,TNFRSF9,TNFSF10,TNN,TPP2,TRIM24,TRIM25,TTLL4,TTR,TYRP1,VCAN,VIPR2,VTI1A,WNT3A,ZIC1	135

Figure 4. The upstream regulators of the identified annotated genes in PACAP38 specifically up(red)-and down(green)-regulated genes in the ischemic core (IC) at 6 h. Ingenuity Pathway Analysis (IPA) analysis was performed.

P(IP) 6 h: Top Canonical Pathways

Ingenuity Canonical Pathways	-log(p-value)	Ratio	z-score	Molecules
Thrombin Signaling	3.12E-01	2.70E-02	2.00	PRKCD,MYL4,ADCY5,ROCK2,GNAL
Wnt/β-catenin Signaling	1.66E+00	5.45E-02	1.41	JUN,FZD10,LEF1,DKK2,MAP4K1,EP300,TCF7L2,WNT3A,RARB
PPAR Signaling	1.15E+00	5.62E-02	1.34	JUN,IL1A,EP300,STAT5B,CITED2
CXCR4 Signaling	7.79E-01	4.03E-02	1.34	JUN,PRKCD,MYL4,ADCY5,ROCK2,GNAL
Phospholipase C Signaling	3.53E-01	2.79E-02	1.34	GRAP2,PRKCD,MYL4,ADCY5,CALML5,EP300
Calcium Signaling	1.27E+00	4.85E-02	1.13	TNNC1,MYL4,CHRNB4,CALML5,SLC8A1,ATP2A1,EP300,PRKAR2A
PPARα/RXRα Activation	9.32E-01	4.24E-02	1.13	JUN,CD36,ADCY5,EP300,LPL,STAT5B,PRKAR2A
Sperm Motility	8.83E-01	4.63E-02	1.00	PRKCD, ,CALML5,PLA2R1,PRKAR2A
Mouse Embryonic Stem Cell Pluripotency	6.99E-01	4.30E-02	1.00	FZD10,LEF1,TCF7L2,WNT3A
Glioblastoma Multiforme Signaling	5.49E-01	3.50E-02	1.00	PRKCD,FZD10,LEF1,NF1,WNT3A
ILK Signaling	5.92E-01	3.49E-02	0.82	JUN,RPS6KA5,MYL4,LEF1,EP300,KRT18
ERK/MAPK Signaling	5.64E-01	3.41E-02	0.82	RPS6KA5,PRKCD,RAPGEF4,EP300,HSPB3, PRKAR2A
Dopamine-DARPP32 Feedback in cAMP Signaling	2.77E+00	7.10E-02	0.63	DRD1,KCNJ2,PRKCD,ADCY5,CACNA1S,CALML5, ATP2A1,EP300,KCNJ5,PPP1R1B,PRKAR2A
Androgen Signaling	1.30E+00	5.61E-02	0.45	JUN,PRKCD,CALML5,EP300,GNAL,PRKAR2A
α-Adrenergic Signaling	1.24E+00	5.95E-02	0.45	PRKCD,ADCY5,CALML5,SLC8A1,PRKAR2A
eNOS Signaling	9.33E-01	4.48E-02	0.45	PRKCD,CHRNB4,ADCY5,CALML5,AQP1,PRKAR2A
CREB Signaling in Neurons	6.28E-01	3.59E-02	0.45	PRKCD,ADCY5,CALML5,EP300,GNAL,PRKAR2A
Endothelin-1 Signaling	4.26E-01	3.09E-02	0.45	JUN,PRKCD,ADCY5,GNAL,PLA2R1
Prolactin Signaling	2.05E+00	8.33E-02	-0.45	JUN,PRKCD,PRLR,EP300,SOCS6,STAT5B
PCP pathway	1.73E+00	8.06E-02	-0.45	JUN,FZD10,RSPO3,ROCK2,WNT3A
Nitric Oxide Signaling in the Cardiovascular System	1.07E+00	5.32E-02	-0.45	PRKCD,CACNA1S,CALML5,ATP2A1,PRKAR2A
p38 MAPK Signaling	8.71E-01	4.59E-02	-0.45	RPS6KA5,IL1A,MAP4K1,EP300,HSPB3
GNRH Signaling	7.09E-01	4.03E-02	-0.45	JUN,PRKCD,ADCY5,EP300,PRKAR2A
P2Y Purigenic Receptor Signaling Pathway	1.16E+00	5.17E-02	-0.82	JUN,PRKCD,ADCY5,EP300,P2RY1,PRKAR2A
Growth Hormone Signaling	1.07E+00	5.97E-02	-1.00	RPS6KA5,PRKCD,SOCS6,STAT5B
Aryl Hydrocarbon Receptor Signaling	6.54E-01	3.85E-02	-1.00	JUN,IL1A,EP300,HSPB3,RARB
iCOS-iCOSL Signaling in T Helper Cells	6.36E-01	4.04E-02	-1.00	GRAP2,CD28,CALML5,IL2RB
Renin-Angiotensin Signaling	5.61E-01	3.74E-02	-1.00	JUN,PRKCD,ADCY5,PRKAR2A
HMGB1 Signaling	5.19E-01	3.57E-02	-1.00	JUN,IL12A,IL1A,IL4
Role of NFAT in Regulation of the Immune Response	4.43E-01	3.14E-02	-1.00	JUN,XPO1,CD28,CALML5,GNAL
Gαs Signaling	1.80E+00	6.67E-02	-1.13	DRD1,VIPR2,ADCY5,RAPGEF4,C0,Htr5b,PRKAR2A
cAMP-mediated signaling	2.20E+00	5.71E-02	-1.16	DRD1,PDE12,VIPR2,ADCY5,RAPGEF4,CALML5, PDE7B,EP300,Htr5b,GNAL,PDE10A,PRKAR2A
Melanocyte Development and Pigmentation Signaling	1.26E+00	6.02E-02	-1.34	RPS6KA5,ADCY5,EP300,TYRP1,PRKAR2A
CD28 Signaling in T Helper Cells	8.83E-01	4.63E-02	-1.34	JUN,GRAP2,CD28,CALML5,CTLA4
CDK5 Signaling	1.51E+00	6.32E-02	-1.63	DRD1,ADCY5,PPP1R1B,BDNF,GNAL,PRKAR2A
Cardiac β-adrenergic Signaling	1.88E+00	6.35E-02	-1.89	PDE12,ADCY5,CACNA1S,PDE7B,SLC8A1,ATP2A1,PDE10A,PRKAR2A
Dopamine Receptor Signaling	9.52E-01	5.41E-02	-2.00	DRD1,ADCY5,PPP1R1B,PRKAR2A
Acute Phase Response Signaling	2.60E-01	2.55E-02	-2.00	JUN,IL1A,SOCS6,TTR
IL-1 Signaling	1.15E+00	5.62E-02	-2.24	JUN,ADCY5,IL1A,GNAL,PRKAR2A

Figure 5. The bio-functions of the identified annotated genes in PACAP38 specifically up(red)- and down(green)-regulated genes in the penumbra (P/IP) at 6 h. Ingenuity Pathway Analysis (IPA) analysis was performed.

IC 6 h: Upstream Regulators

Upstream Regulator	Fold Change	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap	Target molecules in dataset
MKL1		transcription regulator	Inhibited	-2.39	4.43E-04	CAMP,CTSG,CXCL6,EYA2,LTF,MMP9,Ngp,P2RX1,SLC35D3,SLPI
MKL2		transcription regulator	Inhibited	-2.24	3.89E-04	CAMP,CTSG,CXCL6,EYA2,LTF,Ngp,P2RX1,SLC35D3,SLPI

Figure 6. The canonical pathways of the identified annotated genes in PACAP38 specifically up(red)- and down(green)-regulated genes in the penumbra (P/IP) at 6 h. Ingenuity Pathway Analysis (IPA) analysis was performed.

P(IP) 6 h: Upstream Regulators

Upstream Regulator	Fold Change	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap	Target molecules in dataset
STAT5B	-1.56	transcription regulator	Inhibited	-2.06	3.62E-05	Cox8b,CSRP3,CUX2,DGAT2,IL2RB,IL4,MYL4,PAX5,PRLR,STAT5B, TNFRSF25,TNNC1,TRIM24,UCP1
WISP2		growth factor	Activated	2.24	1.50E-03	CLDN1,JUN,KRT18,KRT8,LAMB3

Figure 7. The upstream regulators of the identified annotated genes in PACAP38 specifically up(red)- and down(green)-regulated genes in the penumbra (P/IP) at 6 h. Ingenuity Pathway Analysis (IPA) analysis was performed.

leukocyte extravasation signaling, LXR/AXR signaling is being decreased. These decreased functions can be sub-divided into anticoagulation, anti-inflammation. The G12/13 family is well known for its involvement in cell proliferation and morphology, and PACAP38 might be function to prevent cell proliferation till the recovery process is best suited for new cell/neuron growth. Interestingly, the anti-inflammation response function might require a reduction in the fatty acid synthesis, and this is what we find (IL1RN) in the IC region. On the other hand, Wnt/β-catenin signaling may be more pronounced in the P(IP) region as compared to the IC region where it may both be induced and suppressed.

Thirdly, in the case of identified upstream regulators **(Figures 4 and 7)**, we can see that proliferation (STAT5B) is reduced in the P(IP) region compared to the growth factor (WISP2) which is activated. These play active roles in the central nervous system and their modulation by PACAP38 suggests PACAP38 recruitment of these molecular factors and pathways for aiding the recovery process in the P(IP) region. Taken together, these different gene functions being differentially expressed under PACAP38 injection in the IC and P(IP) regions of the ischemic brain are evidences of a specific and different PACAP38 action in the IC and P(IP), in light of its neuroprotective role in the ischemic brain. Further, in the IC region, as upstream regulators, MKL1 and MKL2 can be seen along with their target molecules, and where both are inhibited **(Figure 4)**. The MKL/myocardin-like protein 1 or myocardin-related transcription factor A (MRTF-A) produces a protein which is a key regulator of smooth muscle cell differentiation. Interestingly, the protein has other functions, including in the intracerebral hemorrhagic stroke and a role in maintaining cerebral small vessel (blood) integrity [30]. This makes for an important finding for inhibited MLK1/MRTF-A function also in neuroprotective functions under PACAP38 treatment. In the P(IP), two upstream regulators, STAT5B (signal transducer and activator of transcription-5, inhibited) and WISP2 (Wnt-1 inducible signaling pathway protein-2, activated) were identified **(Figure 7)**. Both these are now know to be involved in the ischemic insult signaling cascades. For example, the STAT5B has been shown to contribute to the erythropoietin-mediated neuroprotection against hippocampal neuronal death after transient global cerebral ischemia [31], whereas, the WISP2, a member of the connective tissue growth factor and nephroblastoma overexpressed gene family of matricellular proteins is critical in growth factor mediated cell proliferation [32,33]. In summary, the identification of these three (MKL1, STAT5B and WISP2) factors in the ischemic brain regions supports the proposed PACAP-mediated neuroprotective function.

Six-hour post ischemic response in the penumbra might involve cell cycle suppression in conjunction with CRMP2 function

Previously, we had shown that the CRPM2 [24] protein is accumulated during the ischemia following PACAP38 treatment, especially at 6 h post-ischemia, and we also showed that the additional band observed in our experiments might be not a degradative product but having some role in neuroprotection following PACAP38 treatment [14,15]. Recently, using brain-specific *Crmp2* knockout (cKO) mice it was shown that these mice not only display molecular, cellular, structural and behavioral deficit but also that a loss of *Crmp2* in the hippocampus leads to reduced long-term potentiation, abnormal NMDA receptor composition, aberrant dendrite development and defective synapse formation in CA1 neurons, and a knockdown of *crmp2* specifically in newborn neurons results in stage-dependent defects in their development during adult hippocampal neurogenesis [34]. This recent paper [34] provides the strongest evidence to date on the importance of CRMP2 in axonal growth/neurogenesis, and we attempted to link our gene data with CRMP2 action, and which is presented as a model in **Figure 8** (upper panel). The identified (DNA microarray-based gene expressions) up-stream up-regulated (in red arrowheads) and down-regulated (in green arrowheads) molecules are marked in the diagram, which is linked to the phosphorylation-dephosphorylation of the CRMP2 protein, in our model (**Figure 8**, lower panel). PACAP38 influences the regulation of other gene functions as indicated, especially those acting to suppress the GSK3β-pathway; it may be possible that CRMP2 action is enhanced in the penumbra (IP).

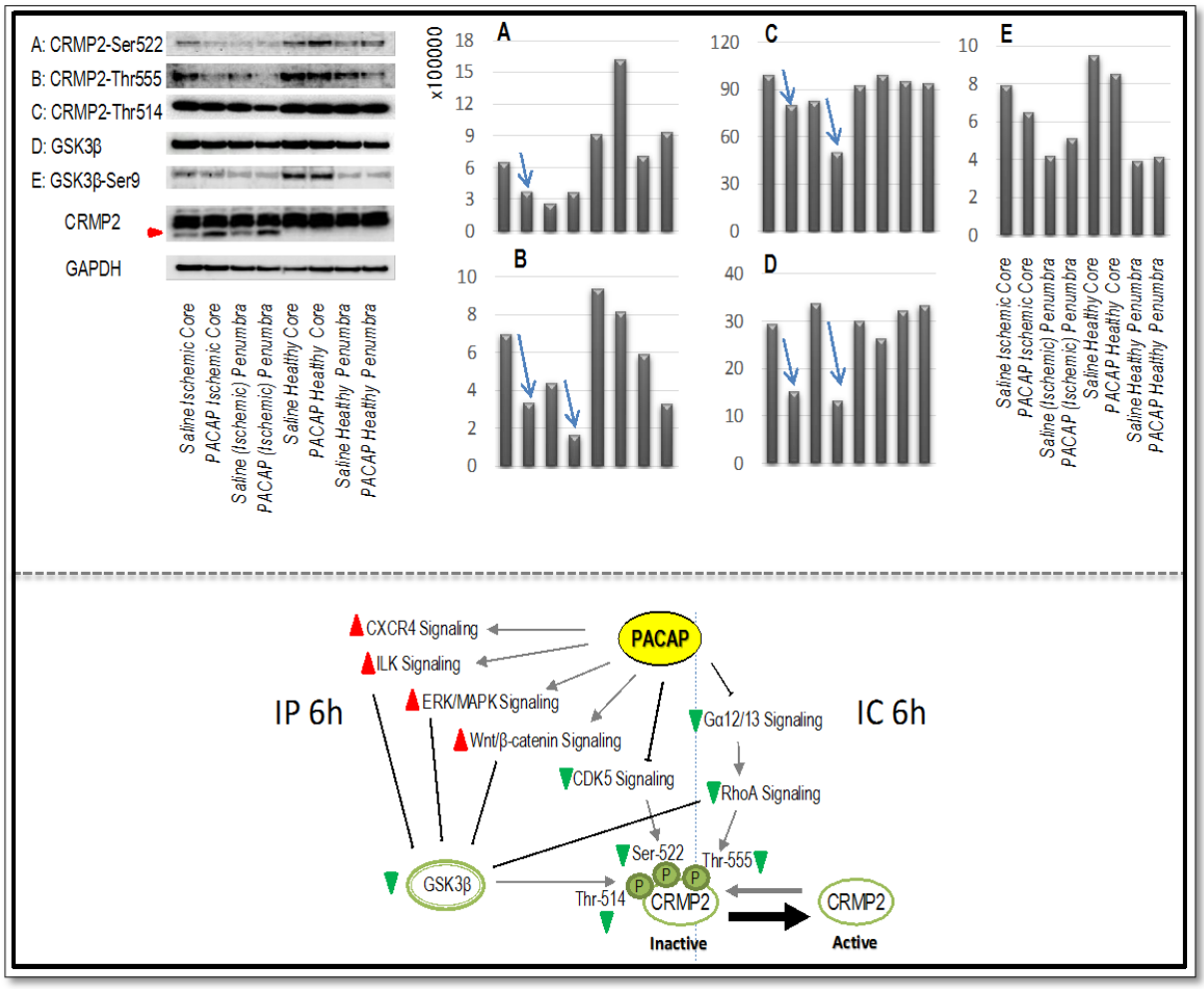

Figure 8. Schematic model of PACAP38 affected molecular factors (pathways) identified through the IPA analyses of ischemic core (IC) and penumbra (P/IP) in the penumbra early on (6 h) following the ischemic insult (A) and CRMP2 dephosphorylation leading to the active form by PACAP38 treatment (B). Western blot analysis of the phosphoCRMP2 proteins (Thr-514, Ser-522, and Thr-55) cross-reacting proteins in P/IP and IC samples reveal the much-decreased phosphorylation in the P/IP over the IC over no change in the healthy tissues with or without PACAP38 treatment.

Total protein extraction, separation, Western blotting and image analyses procedures are detailed in the Methods section

Our combined data also focuses on the short form of CRMP2 which could play an important role in nerve regeneration upon treatment by PACAP38. A study by Rogemond et al. [35] looked at a new short isoform of 58 kDa, expressed during brain development, and derived from C-terminal processing of the CRMP2B subtype. Those authors demonstrated its presence in the nuclear fraction of brain extract and showed that this short CRMP2 induces neurite outgrowth inhibition in neuroblastoma cells and suppressed axonal growth in cultured cortical neurons [35]. They went on to suggest that the post-transcriptionally processed and nuclear localized CRMP2 may be key in the regulation of neurite outgrowth in brain development. Rogemond et al. [35] study shows a link with our finding of CRMP2 involvement and its post-translational modified state among the signaling pathways identified via the IPA analysis in IC and IP regions. Dephosphorylation of CRMP2 by PACAP38 was confirmed by Western blotting. As a result of using the CRMP2 antibodies of Ser522, Thr555 and Thr514, it was confirmed that the addition of PACAP38 promoted the dephosphorylation (**Figure 8**, upper panel-A, B, C). RhoA signaling, CDK5 signaling pathways are involved in CRMP2 dephosphorylation (Ser522, Thr555, Thr514), and gsk3β [36]. The phosphorylation of Gsk3β by PACAP was confirmed by Western blotting. Gsk3β is known to be inactivated by phosphorylation (GSK3β-Ser9). As a result of Western blotting, it was confirmed that the expression level of Gsk3β was reduced by PACAP38, but it was not confirmed that phosphorylation (GSK3β-Ser9) was promoted. As a result of the IPA pathway analysis,

PACAP38 suppressed RhoA Signaling and its upstream Gα12/13 Signaling in the 6 h IC, and suppressed CDK5 Signaling in 6h IP, as well as CXCR4 signaling, ILK signaling, ERK/MAPK signaling, Wnt/β-activation of catenin signaling was predicted. As shown in **Figure 8** (lower panel) from these results, in the IC 6 h scenario RhoA signaling and its upstream Gα12/13 signaling dephosphorylates CRMP2-Thr555 and suppresses GSK3β, and in the IP 6 h scenario the CDK5 signaling dephosphorylated CRMP2-Ser522. It was suggested that CXCR4 Signaling, ILK Signaling, ERK/MAPK Signaling, and Wnt/βcatenin signaling are involved in GSK3β suppression and CRMP2-Thr514 dephosphorylation.

CONCLUSION

Using the unique datasets of specifically up and down-regulated genes in the IC and P(IP) regions post PACAP38 treatment, we have provided new insight into how PACAP38 might act in the ischemic brain of mice. The datasets have been obtained from the highly credible and reproducible Agilent Gene Expression Microarray platform wherein we utilized the two-color and dye-swap approach (Agilent Technologies). Our results suggest how the PACAP38 might be affecting the recovery of the ischemic region by targeting the adjacent P(IP) region signalling pathways and this neurogenesis or new nerve growth would be critical for the recovery from the ischemic insult and therein to recovery. However, the above discussion based on the obtained bioinformatics gene analysis (IPA) will have to be proved further in wet-lab experiments and downstream analyses in new studies. It is our view that a bioinformatics strategy to dissect out genes specifically to the ischemic and adjoining regions will be most helpful in not only pinpointing gene candidates potentially involved in mediating the neuroprotective effect of PACAP38 but also support the reasons behind the current therapy for stroke in human patients. A recently published paper utilized metabolomics, the third high-throughput technique in the omics arsenal, to suggest the involvement of small molecule metabolites in brain injury and functional recovery. Their study [37] combined with transcriptomic and proteomic data (recently reviewed by [18]) imply that continued research and interlinking big data (omics) will be critical to truly understand the brain stroke and mechanisms therein, which would then lead to not only diagnostic markers but also therapeutic drugs.

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