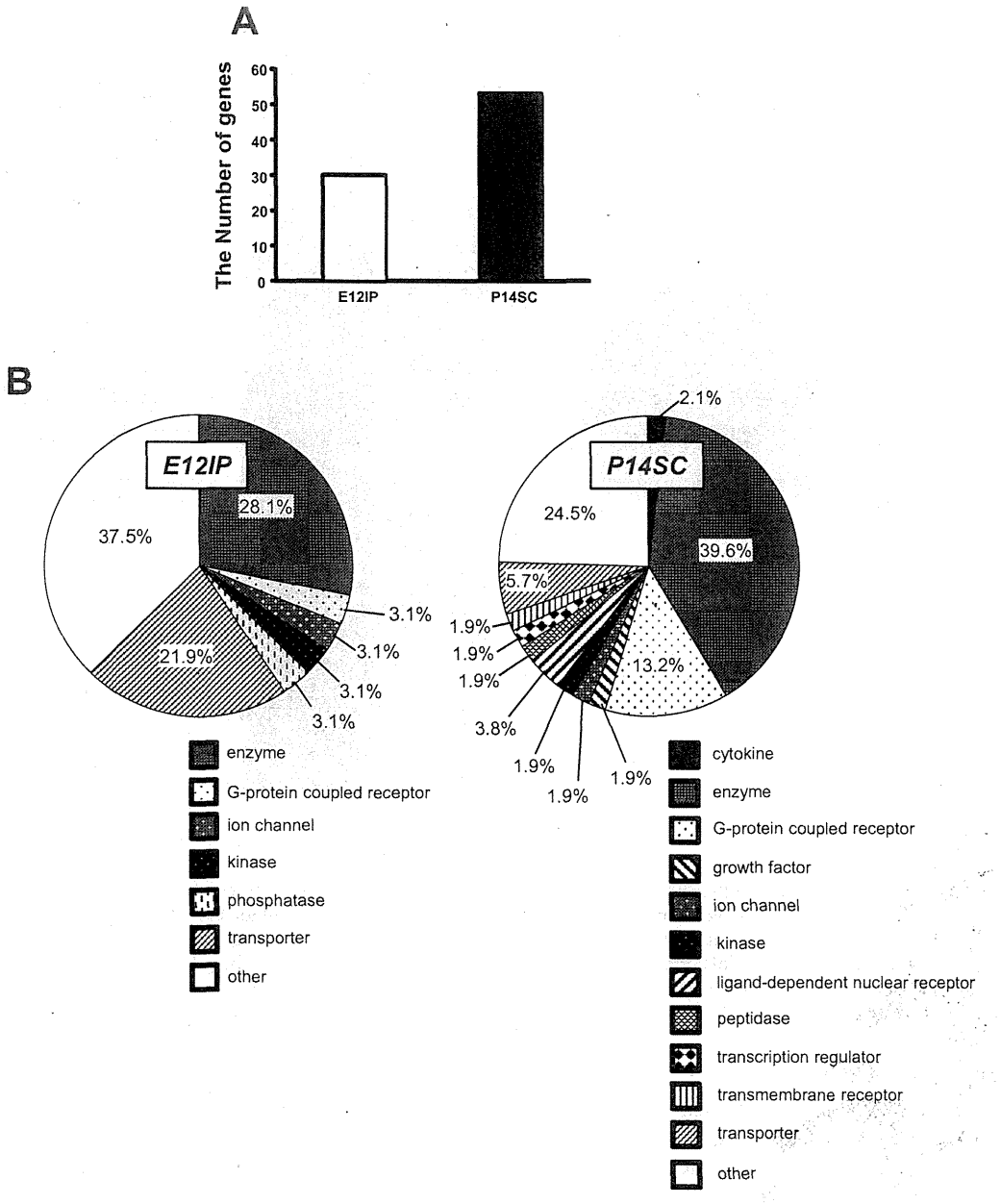

Fig. 3. Summary of the gene expression profiles of E12IP and P14SC rats (male). For both of E12IP and P14SC, total RNA of the amygdalae of 5w male rats (P33-37, N = 4) were analyzed with the microarray chip. Data were collected using Affymetrix GeneChip® Operating Software (GCOS) and analyzed using GeneSpring software. A. The numbers of significantly changed genes in E12IP and P14SC ($p < 0.05$, N = 4). B. Functional classification of the significantly changed genes by GeneSpring.

and-dependent nuclear receptor', 'peptidase', 'transcription regulator', and 'transmembrane receptor' appeared only in P14SC. As for categories common to E12IP and P14SC, their proportions were different from each other; e.g., 'transporters' accounted for 21.9% in E12IP but only 5.7% in P14SC.

Only two genes, Neu2 and Mt2a, exhibited the significant changes in the same direction in E12IP and P14SC

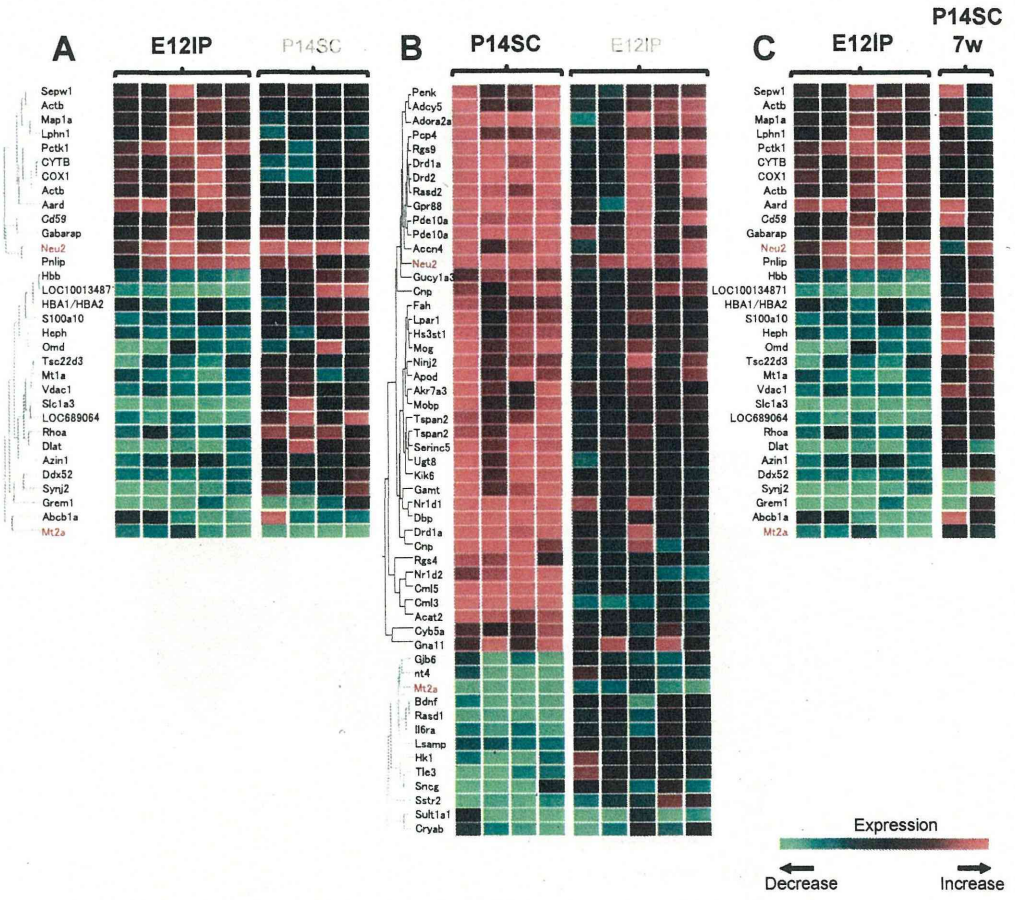

Fig. 4. Heat maps representing hierarchical clustering of the significantly changed genes of E12IP and P14SC. Each vertical column represents an individual sample, and each horizontal row represents a single gene. (N = 4 for P14SC and E12IP, N = 2 for P14SC7w) A. A heat map/hierarchical clustering of the significantly changed genes of E12IP with a heat map of the same genes of P14SC. Clustering was performed using the Benjamini and Hochberg FDR method. B. A heat map/hierarchical clustering of the significantly changed genes of P14SC with a heat map of the same genes of E12IP. C. A heat map of E12IP was compared with that of P14SC7w in which cRNA was extracted 7 w after VPA exposure at P14.

(red letters in Figs. 4A and B). The expression profile in E12IP was also different from that in P14SC7w (Fig. 4C), suggesting that the differences between E12IP and P14SC were not due to the varied length of time between the VPA exposure and the gene expression analysis. Precise gene lists for the heat maps of E12IP (Fig. 4A) and P14SC (Fig. 4B) are shown in Table 1 and Table 2, respectively. 'Behavior'-related genes were identified only in P14SC (Table 3). A larger number of genes were categorized as 'nervous system development and function', 'neurological disease' and 'psychological disorders' in P14SC than in E12IP.

Pathway analysis

The most significantly changed network (Fig. 5A [i]) was that linking 'cell death', 'cellular compromise', and 'neurological disease'. The hubs of this network were MYC, HTT, and CASP3, although the expression levels of these three genes remained unchanged. The second most significantly changed network was that linking 'cell death', 'neurological disease', and 'carbohydrate metabolism' (Fig. 5A [ii]). TNF, which plays an especially important role in cell death, was a highly interconnected node. In P14SC, three significant networks were identified (Fig. 5B [i]-[iii]). These networks are related to 'nucleic acid metabolism' (Fig. 5B [i]), 'cell signaling' (Fig. 5B

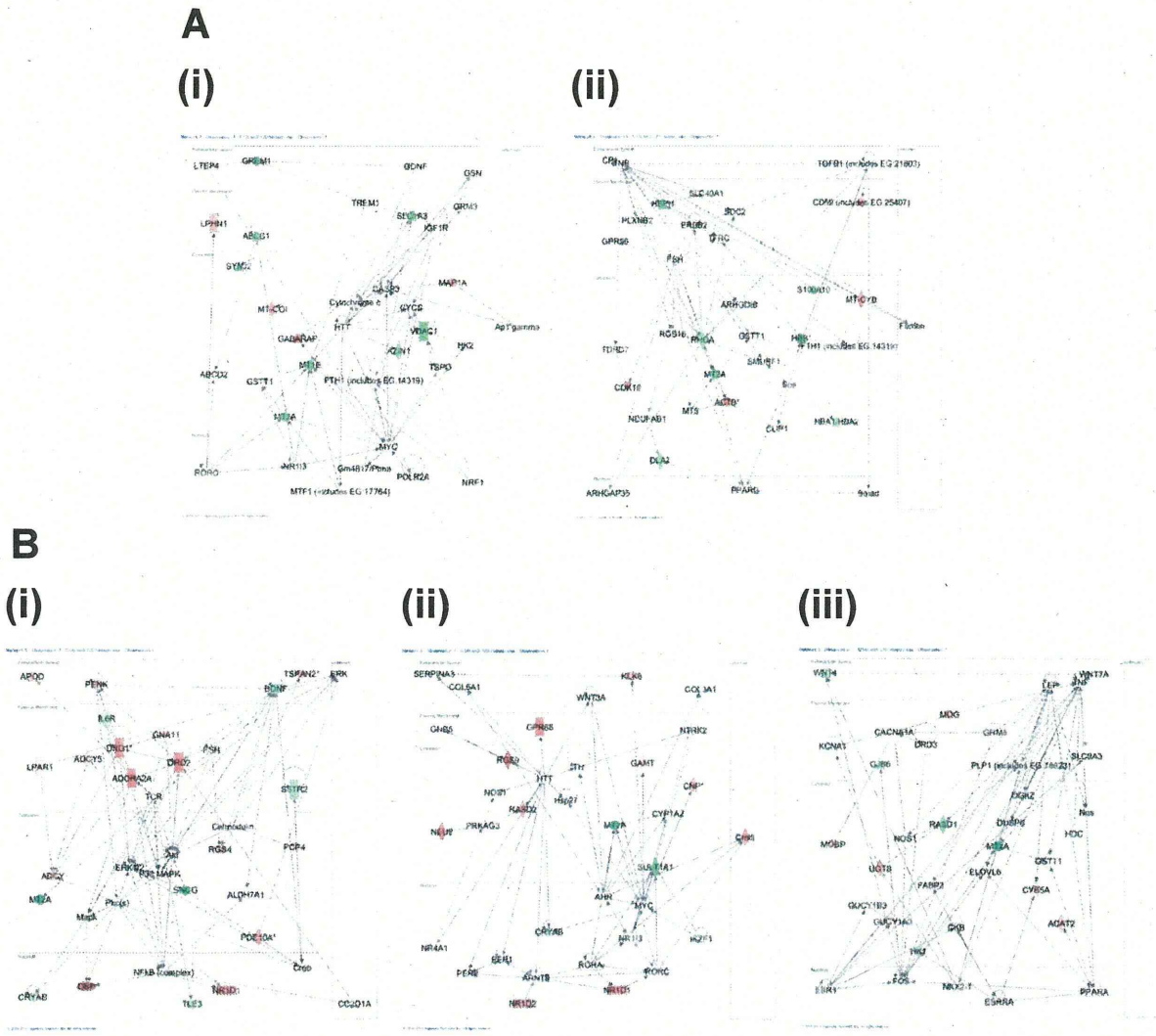

Fig. 5. IPA networks generated with significantly changed genes of E12IP and P14SC. A. The first (i) and second (ii) most significantly altered networks in E12IP. The most significantly changed network linked 'cell death', 'cellular compromise', and 'neurological disease'. The second most significantly changed network linked 'cell death', 'neurological disease', and 'carbohydrate metabolism'. B. The first (i), second (ii), and third (iii) most significantly changed networks in P14SC. These networks are related to 'nucleic acid metabolism' (i), 'cell signaling' (ii), and 'neurological disease' (iii).

[ii]), and 'neurological disease' (Fig. 5B [iii]). One notable aspect of these networks that was not observed in the E12IP networks is the alterations in the expression levels of nuclear genes such as DBP (transcription regulator), NR1D1 (nuclear receptor), and NR1D2 (nuclear receptor).

In conclusion, there are little similarities in the gene expression profiles between the two rat models for autism and regressive autism produced by pre- and post-natal exposures to VPA respectively. It is considered that that gene expression changes per se in the amygdala may be an important cause for impaired social behavior and enhanced anxiety, rather than expression changes of particular genes.

Table 1. The list of genes which changed significantly in E12IP

ID	Symbol	Entrez Gene name	Type(s)
Increase			
1389956_a_at	MT-COI	cytochrome c oxidase subunit I	enzyme
1367996_a_at	LPHN1	latrophilin 1	G-protein coupled receptor
1367882_at	MAP1A	microtubule-associated protein 1A	other
1388159_at	MT-CYB	cytochrome b	enzyme
1367929_at	CD59 (includes EG:25407)	CD59 molecule, complement regulatory protein	other
1398836_s_at	ACTB	actin, beta	other
1368127_at	NEU2	sialidase 2 (cytosolic sialidase)	enzyme
1398835_at	ACTB	actin, beta	other
1370326_at	CDK16	cyclin-dependent kinase 16	kinase
1370804_at	GABARAP	GABA(A) receptor-associated protein	transporter
1367593_at	SEPW1	selenoprotein W, 1	enzyme
1368554_at	PNLIP	pancreatic lipase	enzyme
1370459_at	C8orf85	chromosome 8 open reading frame 85	other
Decrease			
1387197_at	OMD	osteomodulin	other
1371245_a_at	HBB	hemoglobin, beta	transporter
1371130_at	SLC1A3	solute carrier family 1 (glial high affinity glutamate transporter), member 3	transporter
1368971_a_at	SYNJ2	synaptojanin 2	phosphatase
1371102_x_at	LOC100134871	beta globin minor gene	other
1369113_at	GREM1	gremlin 1	other
1371237_a_at	MT1E	metallothionein 1E	other
1368533_at	HEPH	hephaestin	transporter
1368588_at	DDX52	DEAD (Asp-Glu-Ala-Asp) box polypeptide 52	enzyme
1367771_at	Tsc22d3	TSC22 domain family, member 3	other
1386909_a_at	VDAC1	voltage-dependent anion channel 1	ion channel
1388271_at	MT2A	metallothionein 2A	other
1370464_at	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	transporter
1367553_x_at	HBB	hemoglobin, beta	transporter
1386890_at	S100A10	S100 calcium binding protein A10	other
1370575_a_at	AZIN1	antizyme inhibitor 1	enzyme
1370130_at	RHOA	ras homolog gene family, member A	enzyme
1388194_at	DLAT	dihydrolipoamide S-acetyltransferase	enzyme
1388608_x_at	HBA1/HBA2	hemoglobin, alpha 1	transporter

ID, symbol, entrez gene name, type of each gene were shown based on IPA software database.

Table 2. The list of genes which changed significantly in P14SC

ID	Symbol	Entrez Gene name	Type(s)
Increase			
1386904_a_at	CYB5A	cytochrome b5 type A (microsomal)	enzyme
1387897_at	CNP	2',3'-cyclic nucleotide 3' phosphodiesterase	enzyme
1370048_at	LPAR1	lysophosphatidic acid receptor 1	G-protein coupled receptor
1368092_at	FAH	fumarylacetoacetate hydrolase (fumarylacetoacetase)	enzyme
1368506_at	RGS4	regulator of G-protein signaling 4	other
1368298_at	ADCY5	adenylate cyclase 5	enzyme
1370834_at	HS3ST1	heparan sulfate (glucosamine) 3-O-sulfotransferase 1	enzyme
1368154_at	GUCY1A3	guanylate cyclase 1, soluble, alpha 3	enzyme
1368145_at	PCP4	Purkinje cell protein 4	other
1368253_at	GAMT	guanidinoacetate N-methyltransferase	enzyme
1387822_at	GNA11	guanine nucleotide binding protein (G protein), alpha 11 (Gq class)	enzyme
1368263_a_at	MOBP	myelin-associated oligodendrocyte basic protein	other
1367949_at	PENK	proenkephalin	other
1370206_at	ACCN4	amiloride-sensitive cation channel 4, pituitary	ion channel
1386979_at	SERINC5	serine incorporator 5	transporter
1368104_at	TSPAN2	tetraspanin 2	other
1372462_at	ACAT2	acetyl-CoA acetyltransferase 2	enzyme
1398257_at	MOG	myelin oligodendrocyte glycoprotein	other
1398258_at	APOD	apolipoprotein D	transporter
1368105_at	TSPAN2	tetraspanin 2	other
1368384_at	KLK6	kallikrein-related peptidase 6	peptidase
1370541_at	NR1D2	nuclear receptor subfamily 1, group D, member 2	ligand-dependent nuclear receptor
1370693_a_at	CNP	2',3'-cyclic nucleotide 3' phosphodiesterase	enzyme
1368121_at	AKR7A3	aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)	enzyme
1368438_at	PDE10A	phosphodiesterase 10A	enzyme
1368858_at	UGT8	UDP glycosyltransferase 8	enzyme
1370372_at	RASD2	RASD family, member 2	enzyme
1370816_at	NR1D1	nuclear receptor subfamily 1, group D, member 1	ligand-dependent nuclear receptor
1368478_at	DRD1	dopamine receptor D1	G-protein coupled receptor
1370669_a_at	PDE10A	phosphodiesterase 10A	enzyme
1387874_at	DBP	D site of albumin promoter (albumin D-box) binding protein	transcription regulator
1388176_at	Cml5	camello-like 5	enzyme
1368135_at	NINJ2	ninjurin 2	other
1368127_at	NEU2	sialidase 2 (cytosolic sialidase)	enzyme
1368479_at	DRD1	dopamine receptor D1	G-protein coupled receptor

Table 2. (Continued)

ID	Symbol	Entrez Gene name	Type(s)
Increase			
1387241_at	GPR88	G protein-coupled receptor 88	G-protein coupled receptor
1368708_a_at	DRD2	dopamine receptor D2	G-protein coupled receptor
1368300_at	ADPRA2A	adenosine A2a receptor	G-protein coupled receptor
1368500_a_at	RGS9	regulator of G-protein signaling 9	enzyme
1370991_at	Cml3/Gm4477	camello-like 3	enzyme
Decrease			
1370550_at	LSAMP	limbic system-associated membrane protein	other
1370026_at	CRYAB	crystallin, alpha B	other
1386987_at	IL6R	interleukin 6 receptor	transmembrane receptor
1368641_at	WNT4	wingless-like MMTV integration site family, member 4	cytokine
1387169_at	TLE3	transducin-like enhancer of split 3 (E(sp 1) homolog, Drosophila)	other
1368577_at	GJB6	gap junction protein, beta 6, 30kDa	transporter
1386929_at	HK1	hexokinase 1	kinase
1368782_at	SSTR2	somatostatin receptor 2	G-proein coupled receptor
1368677_at	BDNF	brain-derived neurotrophic factor	growth factor
1387908_at	RASD1	RAS, dexamethasone-induced 1	enzyme
1370019_at	SULT1A1	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	enzyme
1398245_at	SNCG	synuclein, gamma (breast cancer-specific protein 1)	other
1388271_at	MT2A	metallothionein 2A	other

ID, symbol, entrez gene name, type of each gene were shown based on IPA software database.

Table 3. The list of genes which changed significantly in E12IP and P14SC were categorized to groups based on their functions using IPA software

E12IP			P14SC	
p-value	Molecules	Category	P14SC	p-value
		Behavior	RGS9,GJB6,RASD2,DBP,BDNF,PDE10A,IL6R,CNP,SNCG,DRD2,PCP4,DRD1,NR1D1,LPAR1,ADCY5,PENK,ADORA2A	2.16E-07-1.85E-02
1.02E-02-1.02E-02	RHOA	Cell Cycle	CRYAB,SSTR2,WNT4	4.65E-03-1.85E-02
6.33E-06-3.04E-02	ABCB1,CD59,MT2A,RHOA,ACTB,GREM1,VDAC1,MT1E	Cell Death	CRYAB,BDNF,IL6R,RGS4,UGT8,DRD2,HK1,LPAR1,SSTR2,ADCY5,MT2A,ADORA2A,MOG	3.03E-04-2.31E-02
2.56E-03-4.46E-02	CD59,HBB,MT2A,RHOA,SLC1A3,VDAC1	Cell Morphology	HK1,LPAR1,DRD1,MT2A,BDNF,IL6R,GNA11,KLK6,RGS4,SNCG,DRD2,ADORA2A	4.59E-04-2.31E-02
4.93E-02-4.93E-02	CD59,HBA1/HBA2,VDAC1	Cell Signaling	RGS9,LPAR1,SSTR2,DRD1,BDNF,ADCY5,PDE10A,IL6R,GNA11,RGS4,DRD2,ADORA2A	9.12E-05-2.07E-02
6.3E-05-2.54E-02	CD59,MT2A,RHOA,SLC1A3,GREM1,GABARAP,VDAC1,LPHN1,MT1E,S100A10	Cell-To-Cell Signaling and Interaction	RASD2,GUCY1A3,BDNF,GNA11,CNP,IL6R,RGS4,KLK6,SNCG,DRD2,PCP4,DRD1,ADCY5,MT2A,SULT1A1,PENK,ADORA2A,MOG,NINJ2	2.34E-07-2.31E-02
6.33E-06-4.27E-02	SYNJ2,CD59,MT2A,RHOA,HBA1/HBA2,VDAC1,LPHN1,MT1E	Cellular Assembly and Organization	RGS9,HK1,CRYAB,LPAR1,DRD1,MT2A,BDNF,GNA11,CNP,SNCG,DRD2,MOG	4.65E-03-2.31E-02
4.67E-04-4.46E-02	Tsc22d3,HBB,MT2A,RHOA,HBA1/HBA2,SLC1A3,	Cellular Development	NR1D1,DRD1,MT2A,BDNF,SERINC5,IL6R,RGS4,UGT8,DRD2	5.55E-04-2.31E-02
1.32E-04-4.27E-02	CD59,MT2A,RHOA,HBA1/HBA2,VDAC1,LPHN1,MT1E	Cellular Function and Maintenance	CRYAB,DRD1,BDNF,CNP	4.65E-03-2.31E-02
4.67E-04-2.07E-02	ABCB1,MT2A,RHOA,ACTB,GREM1,GABARAP,VDAC1,MT1E	Cellular Growth and Proliferation	GJB6,CRYAB,GUCY1A3,BDNF,IL6R,GNA11,KLK6,RGS4,SNCG,DRD2,RASD1,SSTR2,LPAR1,NR1D1,MT2A,PENK,WNT4,ADORA2A,MOG	3.03E-04-2.17E-02
2.56E-03-4.77E-02	CD59,RHOA,LPHN1,S100A10	Cellular Movement	GUCY1A3,BDNF,GNA11,CNP,IL6R,RGS4,KLK6,SNCG,DRD2,NR1D1,SSTR2,DRD1,LPAR1,PENK,ADORA2A,MOG	6.76E-05-1.85E-02
1.67E-05-2.54E-02	Tsc22d3,MT2A,RHOA,GREM1,VDAC1,MT1E	Connective Tissue Development and Function		
5.12E-03-4.27E-02	HBB,RHOA,PNLIP	Developmental Disorder	CRYAB,GJB6,SSTR2,DRD1,GUCY1A3,BDNF,IL6R,GNA11,WNT4,RGS4,DRD2	2.02E-05-2.31E-02
7.67E-03-7.67E-03	ABCB1	DNA Replication, Recombination, and Repair	GUCY1A3,BDNF,RGS4,DRD2,ADORA2A	1.28E-02-1.28E-02
2.56E-03-4.27E-02	ABCB1	Drug Metabolism	DRD1,BDNF,SULT1A1,GNA11,RGS4,SNCG,DRD2,ADORA2A	2.12E-05-1.85E-02
2.56E-03-4.77E-02	HBB,RHOA,GREM1	Embryonic Development	GJB6,LPAR1,BDNF,WNT4,KLK6,DRD2	1.17E-03-1.85E-02
2.56E-03-2.56E-03	ABCB1	Endocrine System Development and Function	BDNF,SULT1A1,GNA11,WNT4,ADORA2A	2.1E-04-2.31E-02
2.25E-02-2.25E-02	MT1E,S100A10	Endocrine System Disorders	ACAT2,SSTR2,DRD1,IL6R,DRD2	2.02E-05-2.31E-02
4.52E-02-4.52E-02	RHOA	Gene Expression	DBP,BDNF,IL6R,RGS4,KLK6,NR1D2,DRD2,RASD1,NR1D1,DRD1,LPAR1,TLE3,WNT4,ADORA2A,MOG	1.38E-02-1.39E-02

Table 3. (Continued)

E12IP			P14SC	
p-value	Molecules	Category	P14SC	p-value
1.29E-03-4.27E-02	ABCB1,HBB,MT2A,ACTB,PNLIP,MT1E	Genetic Disorder	ACCN4,RGS9,CRYAB,GJB6,RASD2,ACAT2,BDNF,GNA11,KLK6,NR1D2,DRD1,ADCY5,MT2A,WNT4,LSAMP,MOG,GUCY1A3,DBP,PDE10A,CNP,IL6R,TSPAN2,RGS4,NEU2,SNCG,DRD2,GPR88,FAH,PCP4,LPAR1,NR1D1,MOBP,SSTR2,SULT1A1,PENK,TLE3,CYB5A,ADORA2A,APOD	4.1E-09-2.31E-02
2.56E-03-4.27E-02	ABCB1,RHOA,MT1E	Inflammatory Disease	CRYAB,BDNF,IL6R,DRD2,MOG	4.65E-03-2.31E-02
2.56E-03-4.27E-02	CD59,RHOA,MT1E,S100A10	Inflammatory Response	GNA11,IL6R,PENK,CNP,ADORA2A,MOG	9.29E-03-2.31E-02
2.56E-03-4.4E-02	SYNJ2,ABCB1,MT2A,DLAT,RHOA,MT1E,PNLIP,S100A10	Lipid Metabolism	ACAT2,DBP,BDNF,SERINC5,GNA11,RGS4,UGT8,DRD2,LPAR1,DRD1,SSTR2,SULT1A1,WNT4,ADORA2A,MOG,APOD	2.1E-04-2.31E-02
6.33E-06-4.93E-02	ABCB1,CD59,HBA1/HBA2,CDK16,SLC1A3,HEPH,LPHN1,HBB,MT-CYB,MT2A,RHOA,GABARAP,VDAC1,MT1E,PNLIP,S100A10	Molecular Transport	ACAT2,GUCY1A3,BDNF,PDE10A,GNA11,IL6R,RGS4,UGT8,SNCG,DRD2,HK1,LPAR1,SSTR2,DRD1,MT2A,ADCY5,WNT4,ADORA2A,APOD	1.11E-05-2.31E-02
3.99E-03-4.27E-02	MT2A,RHOA,HBA1/HBA2,SLC1A3,GABARAP,VDAC1,LPHN1,MT1E	Nervous System Development and Function	GJB6,RASD2,GUCY1A3,DBP,BDNF,SERINC5,CNP,GNA11,RGS4,UGT8,SNCG,DRD2,PCP4,DRD1,NR1D1,LPAR1,ADCY5,MT2A,PENK,ADORA2A,MOG,NINJ2	1.59E-05-2.31E-02
3.79E-05-3.78E-02	MT2A,ACTB,SLC1A3,MT1E,S100A10	Neurological Disease	RGS9,CRYAB,RASD2,GJB6,BDNF,GNA11,DRD1,MT2A,ADCY5,LSAMP,MOG,GUCY1A3,DBP,PDE10A,CNP,IL6R,RGS4,SNCG,DRD2,GPR88,PCP4,LPAR1,MOBP,SSTR2,NR1D1,PENK,ADORA2A,APOD	1.43E-09-2.31E-02
2.64E-02-2.64E-02	MT2A,MT1E	Psychological Disorders	CRYAB,BDNF,PDE10A,CNP,RGS4,DRD2,PCP4,MOBP,DRD1,SSTR2,MT2A,LSAMP,ADORA2A,MOG,APOD	1.57E-04-2.01E-02
6.33E-06-4.4E-02	ABCB1,AZIN1,HBA1/HBA2,SLC1A3,SYNJ2,HBB,MT2A,RHOA,DLAT,VDAC1,PNLIP,MT1E,S100A10	Small Molecule Biochemistry	RGS9,ACAT2,BDNF,SERINC5,GNA11,HK1,DRD1,MT2A,ADCY5,WNT4,MOG,DBP,GUCY1A3,PDE10A,IL6R,RGS4,UGT8,SNCG,DRD2,SSTR2,LPAR1,GAMT,SULT1A1,CYB5A,ADORA2A,APOD	1.11E-05-2.31E-02
2.56E-03-2.79E-02	CD59,Tsc22d3,HBB,RHOA,SLC1A3,GREM1	Tissue Development	GJB6,CRYAB,GUCY1A3,BDNF,GNA11,CNP,IL6R,KLK6,RGS4,DRD2,GAMT,DRD1,LPAR1,TLE3,WNT4,ADORA2A,MOG,NINJ2	4.59E-04-2.31E-02
3.43E-04-2.79E-02	CD59,HBB,MT2A,RHOA,SLC1A3,GREM1,MT1E,S100A10	Tissue Morphology	CRYAB,ACAT2,GUCY1A3,BDNF,IL6R,DRD2,GAMT,DRD1,SSTR2,LPAR1,WNT4,ADORA2A,MOG	8E-04-2.31E-02

Behavior'-related genes were identified only in P14SC. Additionally a larger number of genes were categorized to 'nervous system development and function', 'neurological disease', and 'psychological disorders' in P14SC than in E12IP.

ACKNOWLEDGMENT

This work was supported by Grants-in-Aid from the Food Safety Commission of Japan (No. 1003).

REFERENCES

Blackford, J.U. and Pine, D.S. (2012): Neural substrates of childhood anxiety disorders: a review of neuroimaging findings. Child Adolesc. Psychiatr. Clin. N. Am., **21**, 501-525.

Etkin, A. and Wager, T.D. (2007): Functional neuroimaging of anxiety: a meta-analysis of emotional processing in PTSD, social anxiety disorder, and specific phobia. Am. J. Psychiatry., **164**, 1476-1488.

Markram, K., Rinaldi, T., Mendola, D.L., Sandi, C. and Markram, H. (2008): Abnormal fear conditioning and amygdala processing in an animal model of autism. Neuropsychopharmacology, **33**, 901-912.

Neuhaus, E., Beauchaine, T.P. and Bernier, R. (2010): Neurobiological correlates of social functioning in autism. Clin. Psychol. Rev., **30**, 733-748.

Schneider, T. and Przewtocki, R. (2005): Behavioral alterations in rats prenatally exposed to valproic acid: Animal model of autism. Neuropsychopharmacology, **30**, 80-89.

Schneider, T., Ziolkowska, T., Gieryk, T., Tyminska, T. and Przewlocki, R. (2007): Prenatal exposure to valproic acid disturbs the enkephalinergic system functioning, basal hedonic tone, and emotional responses in an animal model of autism. Psychopharmacology, **193**, 547-555.

Schneider, T., Roman, A., Basta-Kaim, A., Kubera, M., Budziszewska, B., Schneider, K. and Przewtochi, R. (2008): Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. Psychoneuroendocrinology, **33**, 728-740.

Wagner, G.C., Reuhl, K.R., Cheh, M., McRae, P. and Halladay, A.K. (2006): A new neurobehavioral model of autism in mice: Pre- and postnatal exposure to sodium valproate. J Autism Dev. Disord., **36**, 779-793.

Yochum, C.L., Dowling, P., Reuhl, K.R., Wagner, G.C. and Ming, X. (2008): VPA-induced apoptosis and behavioral deficits in neonatal mice. Brain Res., **1203**, 126-132.

Yochum, C.L., Bhattacharya, P., Patti, L., Mirochnitchenko, L. and Wagner, G.C. (2010): Animal model of autism using GSTM1 knockout mice and early post-natal sodium valproate treatment. Behav. Brain Res., **210**, 202-210.

Cell Mol Neurobiol
DOI 10.1007/s10571-012-9869-4

ORIGINAL RESEARCH

Secretion of Matrix Metalloproteinase-9 from Astrocytes by Inhibition of Tonic $P2Y_{14}$-Receptor-Mediated Signal(s)

Manao Kinoshita · Kaoru Nasu-Tada · Kayoko Fujishita · Kaoru Sato · Schuichi Koizumi

Received: 12 July 2012 / Accepted: 20 July 2012

Abstract Glial cells have various important roles in regulation of brain functions. For such events, extracellular nucleotides/P2 receptors have central roles. Although there have been huge amount of literature about activation of P2 receptors and glial functions, little is known about what happens in glia or the brain if glial P2 receptor is inhibited. Here we show that the inhibition of P2 receptors in astrocytes, the most abundant glial cells and cause a constitutive release of nucleotides, resulted in secretion of metalloproteinase-9 (MMP-9), a metal-dependent endopeptidase that degrades extracellular matrix molecules and is important in regulation of brain remodeling. When cultured astrocytes were treated with apyrase (ecto-nucleotidase), reactive blue 2 (P2 receptor antagonist), and pertussis toxin, they secreted MMP-9, suggesting that Gi-coupled P2Y receptor-mediated signals constitutively suppress the production of MMP-9. Among Gi-coupled P2Y receptors, we found that an inhibition of $P2Y_{14}$ receptor, a receptor for nucleotide-sugars such as UDP-glucose, is responsible for the production of MMP-9 by pharmacological and molecular biochemical analysis. As for the mechanisms, the inhibition of $P2Y_{14}$ receptors resulted in the release of tumor necrosis factor (TNF)-α which then acted on astrocytes to induce MMP-9. Taken together, our results suggest that the constitutive releases of nucleotide-sugars in astrocytes should play an important role in maintaining the normal status of the cell, through Gi-coupled $P2Y_{14}$ receptors, and when the signal is removed, the cells start to release TNF-α, which then acts on astrocytes in a feedback fashion to boost MMP-9 synthesis and secretion.

Keywords Astrocytes · Nucleotide-sugar · MMP-9 · P2 receptor

Manao Kinoshita, Kaoru Nasu-Tada and Kayoko Fujishita contributed equally to this work.

M. Kinoshita · K. Fujishita · S. Koizumi (✉)
Department of Neuropharmacology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan
e-mail: skoizumi@yamanashi.ac.jp

K. Nasu-Tada · K. Sato
Division of Pharmacology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan

S. Koizumi
Japan Science and Technology Agency, Core Research for Evolutional Science and Technology, Tokyo 102-0076, Japan

Introduction

Astrocytes function as a multifunctional player that controls synaptic transmission (Haydon 2001), migration of neuroblasts (Kaneko et al. 2010), synaptogenesis (Franke et al. 2012; Tsai et al. 2012), and several remodeling events (Hayakawa et al. 2012). As for the brain remodeling, matrix metalloproteinases (MMPs), metal-dependent endopeptidases that degrade various substrates such as extracellular matrix (ECM) have central roles for making spaces (Lu et al. 2008; Hsieh et al. 2010; Murata et al. 2012). They are divided into sub-groups including gelatinase (MMP-2 and -9), collagenases (MMP-1, -8, and -13), stromelysins (MMP-3, -10, and -11), and others (MMP-7 and -12) (Nagase and Woessner 1999). Although MMPs function under physiological circumstances, their basal expression level is low in the normal CNS (Noble et al. 2002). They are, however, dramatically upregulated during various brain diseases, injuries, and inflammation, and are therefore thought to be crucial for pathological or recovery

Published online: 08 August 2012

events in the CNS. For example, brain ischemia results in an aberrant increase in MMP-9 that causes neuronal death (Gu et al. 2005) and disruption of the blood–brain barrier (BBB) function by degrading tight junctional proteins (Rosenberg et al. 1998) (Yang et al. 2006) (Takata et al. 2011). On the other hand, the upregulation of MMP-9 after stroke is also involved in the matrix remodeling that permits axonal and dendritic extension (Reeves et al. 2003), migration of neuroblast cells (Lee et al. 2006) and vessel formation (Zhao et al. 2006). In addition, MMP-9 is also reported to be involved in the clearance of amyloid β-proteins (Deb et al. 2003) (Yan et al. 2006). Thus, they are probably capable of acting both hazardously and protectively for the brain function depending on the circumstances. Astrocytes appear to be important as a source for MMPs in these events, but the mechanisms underlying the MMP-9 production in astrocytes have received only limited attention so far.

Astrocytes are a sub-type of glial cells abundantly found in the brain tissue, outnumbering the neurons by tenfold. In addition to their classical roles such as the physical support of neurons, astrocytes serve as active regulators of brain functions, i.e., they communicate with microglia and even neurons (Haydon 2001; Nedergaard et al. 2003). Apparently, astrocytes can dynamically control communication between neurons at synapses (Koizumi et al. 2003; Newman 2003), and may also regulate synaptogenesis (Slezak and Pfrieger 2003). The so-called "gliotransmitters" play important roles in such a dynamic communication, and extracellular nucleotides such as ATP and UTP have a central role as gliotransmitters. It is now widely recognized that ATP (Guthrie et al. 1999) (Cotrina et al. 1998), UTP (Lazarowski et al. 1997; Homolya et al. 2000), and even nucleotide-sugars such as UDP-glucose (Lazarowski et al. 2003a) are released from astrocytes by various mechanisms. Interestingly, astrocytes release ATP (Koizumi et al. 2003), UTP and UDP-glucose (Lazarowski et al. 2003a) even spontaneously, the biological significance of which is intriguing but not understood at all.

Astrocytes constitutively release ATP, which strongly suggests that the cells constitutively receive the ATP (or its degradation products), acting on the P2 receptors in a feedback fashion. It is well-known that, after stroke, damaged or injured cells release (or leak) a large amount of ATP, which subsequently causes the long-lasting upregulation of ecto-ATPases (Braun et al. 1998). Ecto-ATPases are the enzymes that degrade extracellular ATP, and their sustained upregulation would decrease the extracellular ATP concentration, leading to the reduction of the constitutive ATP-mediated signals in astrocytes.

In this study, we investigated the effects of the tonic P2 receptor-mediated signals in astrocytes in relation to MMP-9 production. To suppress the tonic signal(s), we blocked a part of the putative, constantly incoming signaling pathway(s) by using apyrase, reactive blue 2 (RB-2), and pertussis toxin (PTX) in vitro. Here, we demonstrate that all these treatments stimulate astrocytes to release MMP-9, indicating that inhibition of Gi-coupled P2 receptors is involved in the production of MMP-9. We also show that $P2Y_{14}$ receptor is the responsible P2 receptor for the MMP-9 production by a small interference RNA (siRNA) strategy. Furthermore, we show that the MMP-9 production is dependent on the release of TNF-α in astrocytes. Thus, our study shows a novel role of the constitutively released nucleotides/nucleotide-sugars in astrocytes, i.e., $P2Y_{14}$ receptors are constitutively stimulated, which may contribute to maintaining the homeostasis of the cells in the CNS by inhibiting TNF-α, and the subsequent production of MMP-9.

Materials and Methods

Preparation of Rat Primary Cell Cultures

The study was conducted in accordance with guidelines for the care and use of animals of University of Yamanashi. All experiments were performed under protocols approved by the university animal care and use committee. Mixed glial cultures were derived from the cerebral cortex of neonatal Wistar rats (Japan SLC, Hamamatsu, Japan). In brief, the rat cortices were separated from the meninges, minced, treated with trypsin and DNAse (Roche, IN), and then centrifuged to remove dead cells. The pellet was resuspended in DMEM (Gibco, NY), filtrated and cultured in medium with 5 % fetal bovine serum (Gibco) and 5 % fetal horse serum (Gibro). After 7–10 days of incubation, the mixed glial culture was placed on a shaker and shaken overnight at 37 °C to detach non-astrocytic cells from the astrocyte monolayer. Adherent astrocytes were detached by exposure to trypsin/EDTA (Gibco) and then plated on 6-cm dishes and cultured in DMEM 5 % fetal bovine serum and 5 % fetal horse serum. Astrocytes were used within 2–3 days after preparation (sub-culturing) and at about 60–70 % confluency.

Gelatin Zymography

Astrocytes were stimulated with appropriate reagents in serum-free medium at 37 °C. The tissue culture supernatant was collected and mixed with Lamelli sample buffer excluding β-mercaptoethanol. Samples were resolved by SDS-PAGE containing 0.2 g/ml gelatin (Sigma-Aldrich Japan, Tokyo, Japan and Wako Pure Chemical, Osaka, Japan) at 150–200 V (c.v.) for 5 h at 4 °C. After the SDS-PAGE, the native gel was washed with Triton-X 2.5 % for

30 min twice, then incubated in the activation buffer [100 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$] for 24 h at 37 °C. Detection was performed using Coomassie R-250 (Bio-Rad Japan, Tokyo, Japan) and the gel was dried in a gel dryer (Bio-Rad Japan).

Western Blotting

Astrocytes were lysed in lysis buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 % NP-40, 1 % SDS, 5 mM EDTA, protease inhibitor cocktail (Calbiochem, CA)] and mixed with Lamelli sample buffer. Cell lysates were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Japan). The membrane was blocked with 0.05 % TBS-Tween 5 % skimmed milk (Wako Pure Chemical) for 2 h at room temperature. Then the membrane was probed with primary antibody rabbit anti-rat MMP-9 (Chemicon CA, 1:2000 dilution). The antibody was detected using horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare Biosciences, Tokyo, Japan, 1:1000 dilution) and visualized with an ECL system (GE Healthcare Biosciences).

Immunocytochemistry

Astrocytes were cultured in an eight-well, LABTEK chamber (NUNC, Naperville, IL, USA) that was coated with poly-L-lysine, and cultured in DMEM 5 % fetal bovine serum and 5 % fetal horse serum were cultured for 24 h at 37 °C. After stimulation in serum-free DMEM, the cells were fixed in 3.7 % formaldehyde in PBS for 5 min and then washed with PBS. Then the cells were permeabilized with 0.1 % Triton-X in PBS for 5 min, washed again with PBS, and then blocked for 30 min with ACE blockace (Dainihon-Sumitomo Pharma, Osaka, Japan) with 3 % goat serum at room temperature. To visualize MMP-9 and astrocytes, the cells were immunostained with primary antibodies, mouse anti-MMP-9 (Diichi Fine Chemical, Toyama, Japan, 2 μg/ml) and rabbit anti-glial fibrillary acidic protein (GFAP; Chemicon, 1:100 dilution), and detection antibodies, Alexa-Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, OR, 1:1,000 dilution) and Alexa-Fluor 546-conjugated goat anti-rabbit IgG (Molecular Probes, 1:1,000 dilution, and observed under a fluorescent microscope.

Transfection of Astrocytes with $P2Y_{14}$ siRNA

Astrocytes were plated onto six-well plates at 1.7×10^5–1×10^6 cells per well and cultured in DMEM 10313 for 24 h. Then, the astrocytes were transfected with 0.5 nM $P2Y_{14}$ siRNAs or 0.5 nM negative control siRNA (Sigma) using HiPerfect transfection reagent according to the manufacturer's instructions (Qiagen). Three combinations of $P2Y_{14}$ siRNA were tested, with each siRNA targeting a different region of $P2Y_{14}$ mRNA. $P2Y_{14}$ siRNAs ($P2Y_{14}$ #1 sense strands: GCUUUGACAGGUACUAUAATT and UUAUAGUACCUGUCAAAGCT; $P2Y_{14}$ #2 sense strands: CACUAACAGUCCAGAAUGATT and UCAUUCUGGA CUGUUAGUGTT; $P2Y_{14}$ #3 sense strands: CCGUUUAGA GAAGUCUUGATT and UCAAGACUUCUCUAAACG GTT) were designed and synthesized by Sigma Genosys siRNA Service. All siRNAs for $P2Y_{14}$ receptors, i.e., #1-#3 siRNAs, could knockdown $P2Y_{14}$ receptors (assessed by quantitative PCR, data not shown), but among them, $P2Y_{14}$ #1 siRNA most potently inhibited the expression of $P2Y_{14}$ receptors. We therefore chose and used $P2Y_{14}$ #1 siRNA throughout the experiments.

Ca^{2+} Imaging in Single Astrocytes

Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in single cells were measured by the fura-2 method. In brief, astrocytes were cultured in an eight-well flexiperm chamber coated with poly-L-lysine. Two days after transfection with $P2Y_{14}$ siRNA, the culture medium was replaced with balanced salt solution (BSS) of the following composition (in mM): NaCl 150, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.2, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) 25, and D-glucose 10 (pH 7.4). Cells were incubated with fura-2 acetoxymethyl ester (fura-2-AM, Invitrogen) at room temperature in BSS for 45 min, and then the cells were washed with BSS and further incubated with BSS for 30 min. The coverslips were mounted on an inverted epifluorescence microscope (TE2000-U, Nikon) equipped with a 75-W xenon lamp and band-pass filters of 340 and 380 nm wavelengths. Image data were recorded by a charged-coupled device (CCD) camera (Orca-ER, Hamamatsu Photonics, Hamamatsu, Japan). Data processing was carried out using AQUACOSMOS 2.6 (Hamamastu Photonics) and MS Excel software. All data are expressed as changes in the fura-2 fluorescence ratio (ΔF340/F380) against time. Each experiment was repeated at least six times.

Cytokine Detection

Multiple proinflammatory cytokines were simultaneously detected in the cell supernatant using a commercially available enzyme-linked immunosorbent assay (ELISA)-based cytokine protein array (RayBio™ Rat Cytokine Array I, RayBiotech, Norcross, GA). TNF-α that was released into the tissue culture supernatant from astrocytes was quantitatively measured using an Endogen Rat TNF-α ELISA kit (Pierce Biotechnology, Inc., Rockford, IL).

Statistical Analysis

Experimental results are expressed as mean ± SEM. Statistical analysis was performed using Student's *t* test. One way analyses of variance (ANOVA) followed by Tukey test were applied for multiple comparisons. The differences between means were considered to be significant when the p values were less that 5 %.

Results

Treatment with Apyrase, RB-2 or PTX Induced Astrocytes to Release MMP-9 into the Culture Medium In Vitro

Proteins of the MMP family are involved in the breakdown of the ECM in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as in arthritis and metastasis. MMP-2 and MMP-9 are also known as gelatinase A and B and, as the name suggests, those enzymes degrade gelatin among other substrates. Astrocytes were stimulated with apyrase (20 U/ml), RB-2 (10 μM) or PTX (1 μg/ml) for 24 h at 37 °C, and the culture supernatants were analyzed by gelatin zymography or by western blotting (Fig. 1b). The results in Fig. 1 indicate that MMP-2 is constitutively expressed and released by astrocytes and none of the treatments changed the pattern of its expression (Fig. 1b). Most MMPs are secreted as inactive pro-proteins which are activated when cleaved by extracellular proteinases, and the results in Fig. 1 showed both the proenzyme (64 kDa) and the active form (60 kDa) of MMP-2. MMP-9, on the other hand, is known to be expressed at very low levels by quiescent cells including astrocytes (Noble et al. 2002), and this agreed with our control result, but its expression was markedly increased by the treatment with apyrase, RB-2 and PTX (Fig. 1). Here again, both the proenzyme (91 kDa) and active form (83 kDa) were observed. We checked the possible gelatinase activity of the three reagents by gelatin zymography, and we confirmed that none of them possessed any gelatinase activity (data not shown). Treatment of atrocytes with apyrase, RB-2 and PTX for 12 h also upregulated mRNAs for MMP-9 (Fig. 1a). Next, the time-course of the MMP-9 release by apyrase (20 U/ml) treatment was studied by gelatin zymography or western blotting (Fig. 1c). The tissue culture supernatants were left undiluted for the gelatin zymography, whereas they were concentrated for the western blotting analysis. MMP-9 started to appear after 12 h of incubation with apyrase and it lasted until 24 h of incubation. The upregulation of MMP-9 was also confirmed immunocytochemically (Fig. 1d). Astrocytes cultured on a coverglass were stimulated with apyrase (20 U/ml) for 5–24 h at 37 °C, fixed, permeabilized, and then MMP-9 and GFAP were visualized by immunocytochemical detection. Almost no expression of MMP-9 was seen in the unstimulated astrocytes (Fig. 1d, control). MMP-9 staining became clear after 5 h of apyrase stimulation, and strong expression was observed at 14 h. The expression somewhat peaked off and a decreased level of expression was seen at 24 h of incubation.

Pharmacological Examination Suggested that Gi-Coupled P2Y Receptor is Strongly Associated with the MMP-9 Release From Astrocytes

The results so far demonstrated that apyrase, RB-2, and PTX induced MMP-9 production from astrocytes. Apyrase degrades various nucleotides, RB-2 is a potent inhibitor of some P2X and P2Y families and PTX is a Gi inhibitor. These results indicate a link between MMP-9 production and the metabolism of extracellular nucleotides. It is now widely known that astrocytes and possibly other cells constitutively release ATP, other nucleotides including nucleotide-sugars (Lazarowski et al. 2000; Koizumi et al. 2003; Lazarowski et al. 2003a), and therefore it is highly plausible that some act in an autocrine fashion on their P2 receptors. Combining that idea with our results so far, it is speculated that blocking some of the constitutive P2 receptor-mediated signals, which was mimicked here by apyrase, RB-2 and PTX, lead to MMP-9 production. Based on this hypothesis, we did a pharmacological analysis to identify the responsible P2 receptor subtype that is involved in MMP-9 production. Firstly, astrocytes were treated with suramin (300 μM), pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS) (100 μM), or brilliant blue G (BBG) (10 μM) for 24 h at 37 °C and the undiluted tissue culture supernatants were analyzed by gelatin zymography (Fig. 2a). Suramin and PPADS inhibit both P2X receptors and some P2Y receptors, whereas BBG mostly inhibits $P2X_7$. The results in Fig. 2a showed that none of the P2X family members seemed to influence the MMP-9 production. In additional, we studied the effect of adenosine (Fig. 2a), since the treatment of apyrase normally results in an increase in extracellular adenosine and astrocytes are known to possess adenosine receptors. As shown in Fig. 2a, adenosine (100 μM) treatment did not result in MMP-9 generation, and therefore the involvement of P2X and adenosine receptors is possibly negligible in this case. We also examined whether ATP and other major nucleotides induce MMP-9 production (Fig. 2b). As the result shows, neither ATP (100 μM), ADP (100 μM), ATPγS (100 μM), UTP (100 μM), nor UDP (100 μM) induced the synthesis of the enzyme. Here again,

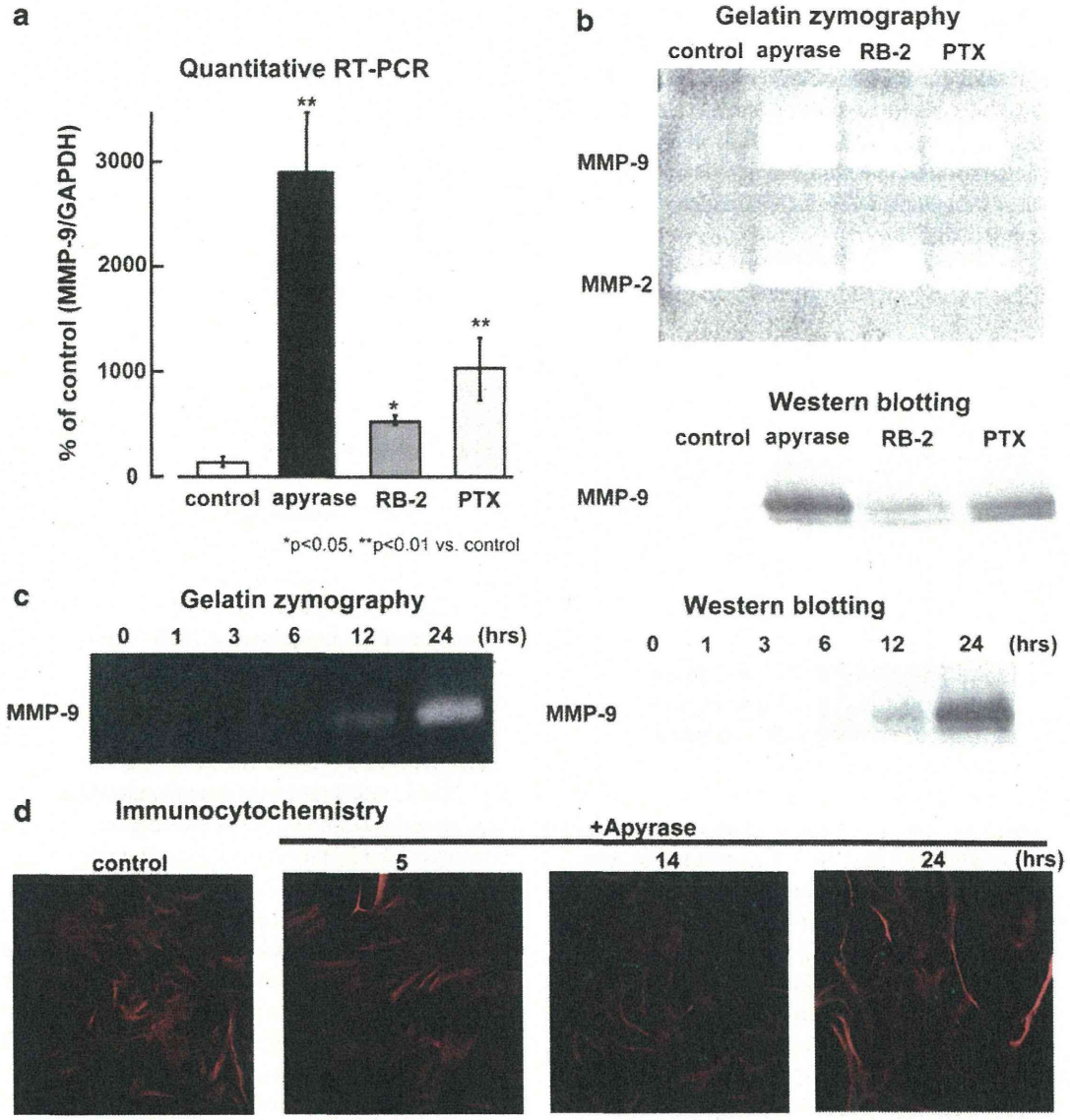

Fig. 1 Release and production of MMP-2 and MMP-9 in cultured astrocytes. **a** Astrocytes were stimulated with apyrase (20 U/ml), RB-2 (10 μM) or PTX (1 μg/ml) for 12 h at 37 °C, and then total RNA was extracted for quantitative RT-PCR. The expression of mRNA for MMP-9 was normalized by GAPDH and expressed as % of control. $*p < 0.05$, $**p < 0.01$ versus control. **b** To confirm the release of MMP-9, gelatin zymography and western blotting were performed. Astrocytes were treated with apyrase (20 U/ml), RB-2 (10 μM) or PTX (1 μg/ml) for 24 h at 37 °C, and then the culture supernatants were either undiluted or concentrated and analyzed by gelatin zymography or by western blotting, respectively. In contrast to MMP-9, astrocytes release MMP-2 constitutively into the culture medium. **c** Astrocytes were treated with apyrase (20 U/ml) and the time-course of the MMP-9 release was studied by gelatin zymography or western blotting. Here again, the tissue culture supernatants were left undiluted for the gelatin zymography but were concentrated for the western blotting analysis. The data represent three independent experiments. **d** Astrocytes were stimulated with apyrase (20 U/ml) for 5, 14, or 24 h at 37 °C, and the presence of MMP-9 in the culture medium was studied immunocytochemically. *Red* and *green* are anti-GFAP and anti-MMP-9 stainings, respectively. The basal expression of MMP-9 was very low in astrocytes without stimulation (control). It started to appear after 5 h of incubation with apyrase. Strong expression was seen at 14 h and it lasted until 24 h. The data represent three independent experiments (Color figure online)

the MMP-2 level remained at the basal level. Since PTX treatment also resulted in high production of MMP-9, we examined the involvement of $P2Y_{12}$ and $P2Y_{13}$ receptors, both of which are linked to Gi, by using their specific antagonists, ARC-69931 and ARC-66096 (Fig. 2c). The results demonstrated that neither $P2Y_{12}$ nor $P2Y_{13}$ receptors

seemed to be involved. We also treated cells with forskolin, which is a potent adenylate cyclase activating agent. If the MMP-9 release depends on the activation of PKA, this treatment with forskolin would also cause the cells to produce MMP-9. The result, however, showed no increase, suggesting that PKA is irrelevant to this case.

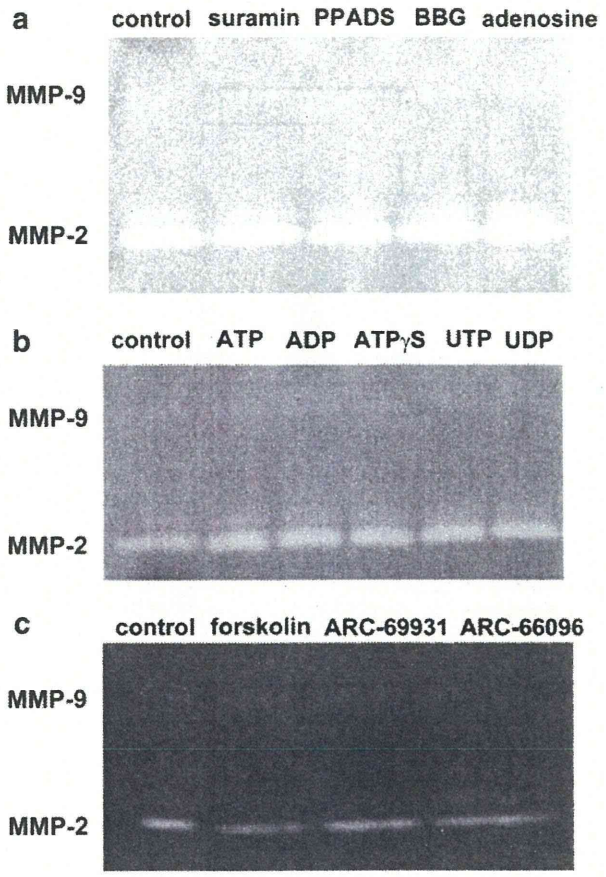

Fig. 2 Pharmacological study of the production of MMP-9 in astrocytes. **a** Astrocytes were treated with suramin (300 μM), PPADS (100 μM), BBG (10 μM) and adenosine (100 μM) for 24 h at 37 °C to study the effects of various P2 receptors on MMP-9 generation. Suramin, PPADS, BBG, apyrase, and adenosine neither induced the release of MMP-9 nor inhibited the production of MMP-2. **b** Astrocytes were treated with ATP (100 μM), ADP (100 μM), ATP-γS (100 μM), UTP (100 μM), or UDP (100 μM) for 24 h at 37 °C to examine the effect of various agonists to P2 receptors on MMP-9 production. Stimulation with ATP, ADP, ATP-γS, UTP or UDP neither induced MMP-9 release nor influenced MMP-2 production. **c** Astrocytes were treated with forskolin (10 μM), ARC-69931 (10 μM) or ARC-66096 (10 μM) for 24 h at 37 °C to investigate the involvement of PKA, $P2Y_{12}$ or $P2Y_{13}$ receptors in the production of MMP-9. Forskolin, ARC-69931 and ARC-66096 neither induced the release of MMP-9 nor inhibited the production of MMP-2. The undiluted tissue culture supernatants were analyzed by gelatin zymography. The data represent three independent experiments

$P2Y_{14}$ Receptor Knockdown in Astrocytes Promoted Expressions MMP-9

In addition to $P2Y_{12}$ and $P2Y_{13}$ receptors, $P2Y_{14}$ receptors are known to be coupled to Gi. Identification of the physiological functions of the $P2Y_{14}$ receptor has been difficult to establish (Brautigam et al. 2008; Dovlatova et al. 2008), in part due to the lack of selective high affinity antagonists for this receptor. To verify that the loss of signals to the $P2Y_{14}$ receptor leads to the production of MMP-9, we knocked down the $P2Y_{14}$ receptor by siRNA. As shown in Fig. 3a, western blot analysis revealed that the $P2Y_{14}$ receptor siRNA (0.5 nM), but not negative control siRNA (NC siRNA) (0.5 nM), reduced proteins of the $P2Y_{14}$ receptor in astrocytes, which was summarized in Fig. 3b.

Activation of $P2Y_{14}$ receptors resulted in increased $[Ca^{2+}]i$ in cells (Fumagalli et al. 2003; Skelton et al. 2003), and UDP-glucose is reported as one of the most potent agonist of the $P2Y_{14}$ receptor (Abbracchio et al. 2003). To evaluate the function of $P2Y_{14}$ receptors, we measured changes in $[Ca^{2+}]_i$ in single astrocytes in response to UDP-glucose (100 μM). Astrocytes were treated with either negative control siRNA (0.5 nM) or $P2Y_{14}$ receptor siRNA (0.5 nM), and then incubated further for 48 h. As shown in Fig. 3c, UDP-glucose produced increases in $[Ca^{2+}]i$ in naive and control siRNA-treated astrocytes. The UDP-glucose-evoked elevation in $[Ca^{2+}]i$ was almost halved in $P2Y_{14}$ siRNA-treated astrocytes (Fig. 3d).

Astrocytes were treated with negative control siRNA (0.5 nM) or $P2Y_{14}$ receptor siRNA (0.5 nM) for 72 h (in Fig. 3e) or 108 h (in Fig. 3f), and then MMP-9 mRNA and protein were quantitatively measured by quantitative RT-PCR and western blotting, respectively as described in "Materials and Methods". As shown in Fig. 3e, f, $P2Y_{14}$ receptor siRNA, but not negative control siRNA, significantly enhanced the expression of MMP-9 mRNA and MMP-9 protein.

Several Cytokines were Released from Astrocytes in Response to Apyrase, RB-2 or PTX Stimulation

Several stimuli such as cytokines are known to cause MMP-9 production from astrocytes. Therefore, there was a possibility that apyrase, RB-2 or PTX induced the release of some mediators, which then worked on astrocytes themselves to produce MMP-9. To examine this possibility, we stimulated astrocytes with apyrase, RB-2, and PTX for 24 h at 37 °C and studied their tissue culture supernatants by a RayBio Cytokine array (Table 1). Cytokine-induced neutrophil chemoattractant (CINC)-3 and tumor necrosis factor (TNF)-α were not detected in the control culture supernatants, but their expression dramatically increased after apyrase, RB-2 and PTX treatment

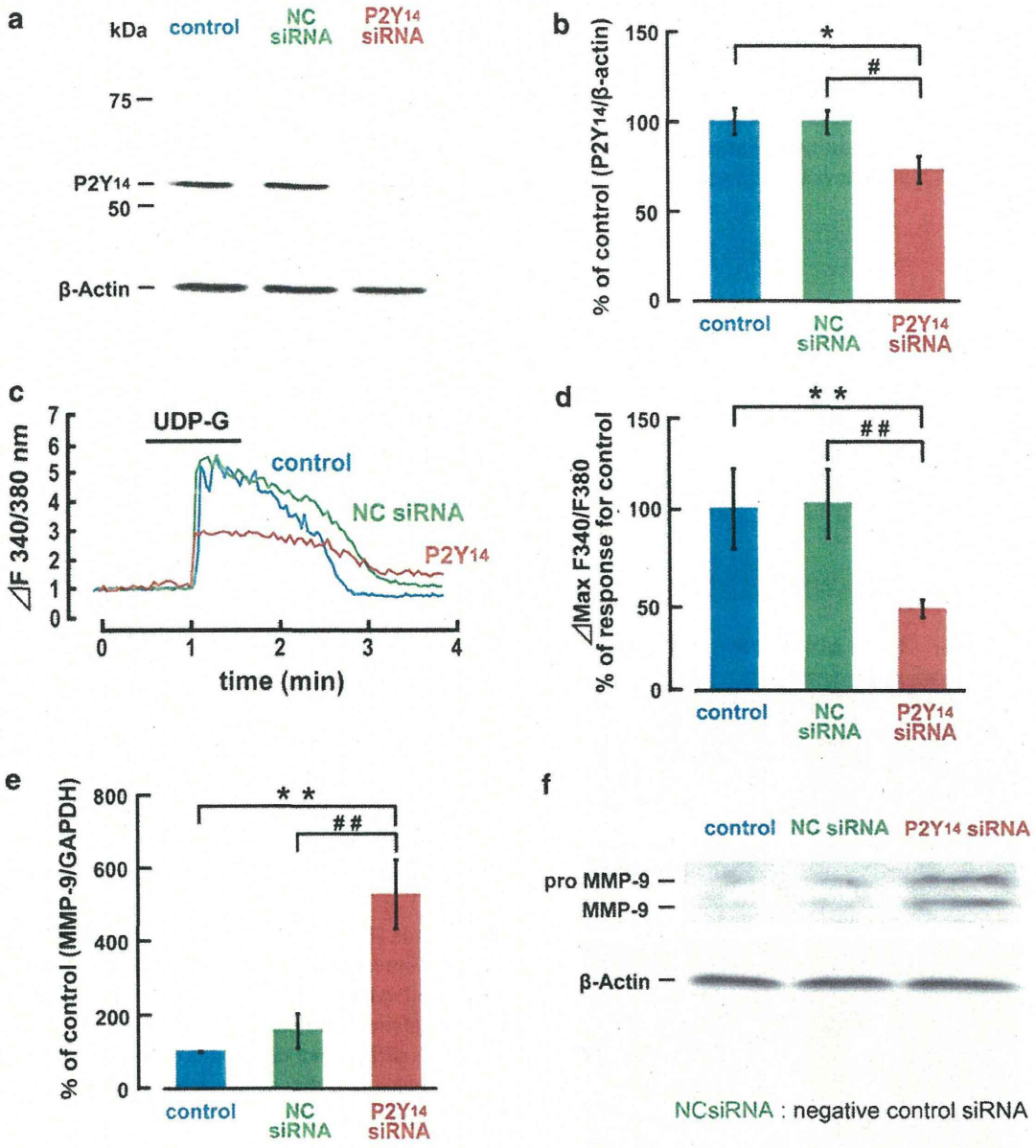

Fig. 3 Inhibition of $P2Y_{14}$ receptors are responsible for the induction of MMP-9 in astrocytes. **a** Knock down of $P2Y_{14}$ receptors by $P2Y_{14}$ siRNA in astrocytes. Astrocytes were treated with negative control siRNA (NC siRNA) (0.5 nM) or $P2Y_{14}$ siRNA (0.5 nM) for 48 h, and then western blotting was performed to detect $P2Y_{14}$ receptor proteins. NC siRNA had no effect wherease $P2Y_{14}$ siRNA significantly reduced the $P2Y_{14}$ receptors, which was summarized on **b**. $^{*}p < 0.05$ versus control, $^{\#}p < 0.05$ versus NC siRNA. **c** Decrease in $P2Y_{14}$ receptor-mediated responses by $P2Y_{14}$ siRNA. The $P2Y_{14}$ receptor agonist UDP-glucose (UDP-G)-evoked increase in $[Ca^{2+}]i$ was analyzed in control, NC siRNA- or $P2Y_{14}$ siRNA-treated astrocytes. Changes in $[Ca^{2+}]i$ were measured by the fura-2 method and were expressed as the ΔF340/F380 ratio. The UDP-G-evoked elevations in $[Ca^{2+}]i$ in control and NC siRNA-treated astrocytes were almost the same, whereas those in $P2Y_{14}$ siRNA-treated astrocytes were decrease by about 50 % (summarized in **d**). $^{**}p < 0.01$ versus control, $^{\#\#}p < 0.01$ versus NC siRNA. **e**, **f** Knock down of $P2Y_{14}$ receptors in astrocytes resulted in increases in mRNA (**e**) and protein (**f**) of MMP-9 in astrocytes. Astrocytes were treated with NC siRNA (0.5 nM) or $P2Y_{14}$ siRNA (0.5 nM) for 12 h, and then, total RNA was extracted for quantitative RT-PCR. Expression of MMP-9 was normalized by GAPDH and expressed as % of control. Data are mean ± SEM of triplicate measurements. The same experiment was repeated thrice and similar results were obtained. $^{**}p < 0.01$ versus control, $^{\#\#}p < 0.01$ versus NC siRNA (**e**). For the measurement of MMP-9 proteins, astrocytes were treated with each siRNA for 96 h, and then cells were lysed for Western blotting analysis

especially for TNF-α. RB-2 induced only a slight increase in CINC-3. Tissue inhibitory metalloproteinase (TIMP)-1 and monocyte chemoattractant protein (MCP)-1 were produced by unstimulated astrocytes in vitro and the strong increase in their expression was observed especially for MCP-1 (Table 1).

Table 1 Cytokines released from astrocytes in response to apyrase, RB-2 or PTX stimulation

Released cytokine	Control	Ayprase	RB-2	PTX
CINC-3	0	0.30 ± 0.10	0.07 ± 0.04	0.160 ± 0.05
TNF-α	0	0.39 ± 0.14	0.26 ± 0.13	0.34 ± 0.12
TIMP-1	0.22 ± 0.05	0.58 ± 0.10	0.23 ± 0.05	0.24 ± 0.06
MCP-1	0.14 ± 0.09	0.86 ± 0.42	0.52 ± 0.15	0.59 ± 0.17

Integrated density values [IDV, (average intensity) times (total area)] were determined for each individual cytokine dot. Cytokine dot intensities were normalized to HRP-positive controls on each membrane. Mean data for each cytokine spot are expressed as protein ratios (each cytokine specific IDV divided by HRP-positive control IDV. Data are mean ± SEM of three separate experiments

TNF-α, but not MCP-1, Induced MMP-9 Release from Aastrocytes In Vitro

Based on the above findings, we decided to test the effect of TNF-α and MCP-1 on astrocytes in the context of MMP-9 release. Astrocytes were treated with TNF-α (5, 10, 50 ng/ml) or with MCP-1 (50, 100, 1,000 ng/ml) for 24 h at 37 °C. The tissue culture supernatants were concentrated and studied by western blotting (Fig. 4a). Astrocytes started to release MMP-9 when stimulated by TNF-α at 50 ng/ml and above. On the other hand, MCP-1, despite its marked augmentation in release by these three stimuli, was not accompanied by any sign of MMP-9 release (Fig. 4a). TNF-α increased mRNAs for MMP-9 in a concentration-dependent fashion over a concentration range from 0.3 to 30 ng/ml with an ED50 value of ~3 ng/ml (Fig. 4b).

To further investigate the role of TNF-α as an important mediator between the inhibition of $P2Y_{14}$ receptors and MMP-9 production in astrocytes, the cells were treated with either negative control siRNA (NC siRNA) (0.5 nM) or $P2Y_{14}$ receptor siRNA (0.5 nM) for 60 h, and then TNF-α that was released into the tissue culture supernatant from astrocytes was quantitatively measured using a Rat TNF-α/TNFSF1A Immunoassay kit. Knockdown of $P2Y_{14}$ receptors resulted in a significant increase in TNF-α (Fig. 4c). The production of TNF-α from astrocytes was also observed when cells were treated with apyrase, RB-2 or PTX for 24 h, which was confirmed by western blotting (Fig. 4d). Finally, to prove the necessity of TNF-α for the production of MMP-9 in astrocytes, we treated astrocytes with anti-TNF-α neutralization antibody (3 μg/ml) before and during apyrase stimulation for 12 h at 37 °C, and then MMP-9 mRNA was quantified by real-time RT-PCR (Fig. 4e). Upregulation of MMP-9 mRNA was reduced by anti-TNF-α neutralization antibody in a concentration-dependent manner.

Discussion

MMP-9 in Astrocytes

MMP-9 is a major enzyme that degrades gelatin, collagen IV, V, and XI, elastin, vitronectin, myelin basic protein, vascular proteins, and many other substrates (Dzwonek et al. 2004). In the normal CNS, MMP-9 expression is very limited. Noble et al. (2002) observed only a limited basal expression of MMP-9 in the meninges and motorneurons of the uninjured spinal cord. Several lines of evidence, however, indicate that MMP-9 expression is largely elevated in astrocytes, microglia/macrophages, and hippocampal cells during CNS injuries and diseases (Backstrom et al. 1996; Cuzner et al. 1996; Gottschall and Deb 1996; Liu et al. 1998; Noble et al. 2002). Although there are some reports suggesting a protective role of MMP-9 (Lelongt et al. 2001; Dewil et al. 2005), a large body of work has described its role as deleterious, including studies with MMP-9 knockout mice or with MMP-9 inhibitors (Asahi et al. 2000; Asahi et al. 2001; Jiang et al. 2001). Since MMP-9 is also capable of degrading vascular ECM and endothelial occludins and claudins (Yang et al. 2006), its aberrant expression often causes the disruption of the BBB (Rosenberg 1995; Rosenberg et al. 1995; Mun-Bryce and Rosenberg 1998), leading to the abnormal migration of leukocytes and other inflammatory cells across the BBB into the CNS and resulting in CNS tissue injuries. Since the brain capillaries are surrounded by astrocytic endfeet, the secretion of MMP-9 from astrocytes may greatly affect the BBB function. With regard to the astrocytic MMP-9, previous studies have already shown that various cytokines and other stimuli can induce astrocytes to express MMP-9 (Gottschall and Yu 1995; Deb and Gottschall 1996; Arai et al. 2003; Deb et al. 2003; Lee et al. 2003; Hsieh et al. 2004; Leveque et al. 2004; Wu et al. 2004). In this study, we demonstrate a novel mechanism in which $P2Y_{14}$ receptor-mediated signaling plays an important role in the initiation of MMP-9 production/secretion in astrocytes.

The Importance of the Constitutive Release of Nucleotides/Nucleotide-Sugars from Astrocytes and Activation of Gi-Coupled $P2Y_{14}$ Receptors

During pathological conditions such as ischemia or epilepsy, cells release (or leak) a large amount of ATP, other nucleotides or even nucleotide-sugars into the extracellular space. In addition, cells release these nucleotides in response to various physiological stimuli. In the CNS, astrocytes are known to be a dominant source of extracellular nucleotides by which they communicate with each other. Interestingly, astrocytes or astrocytomas release ATP

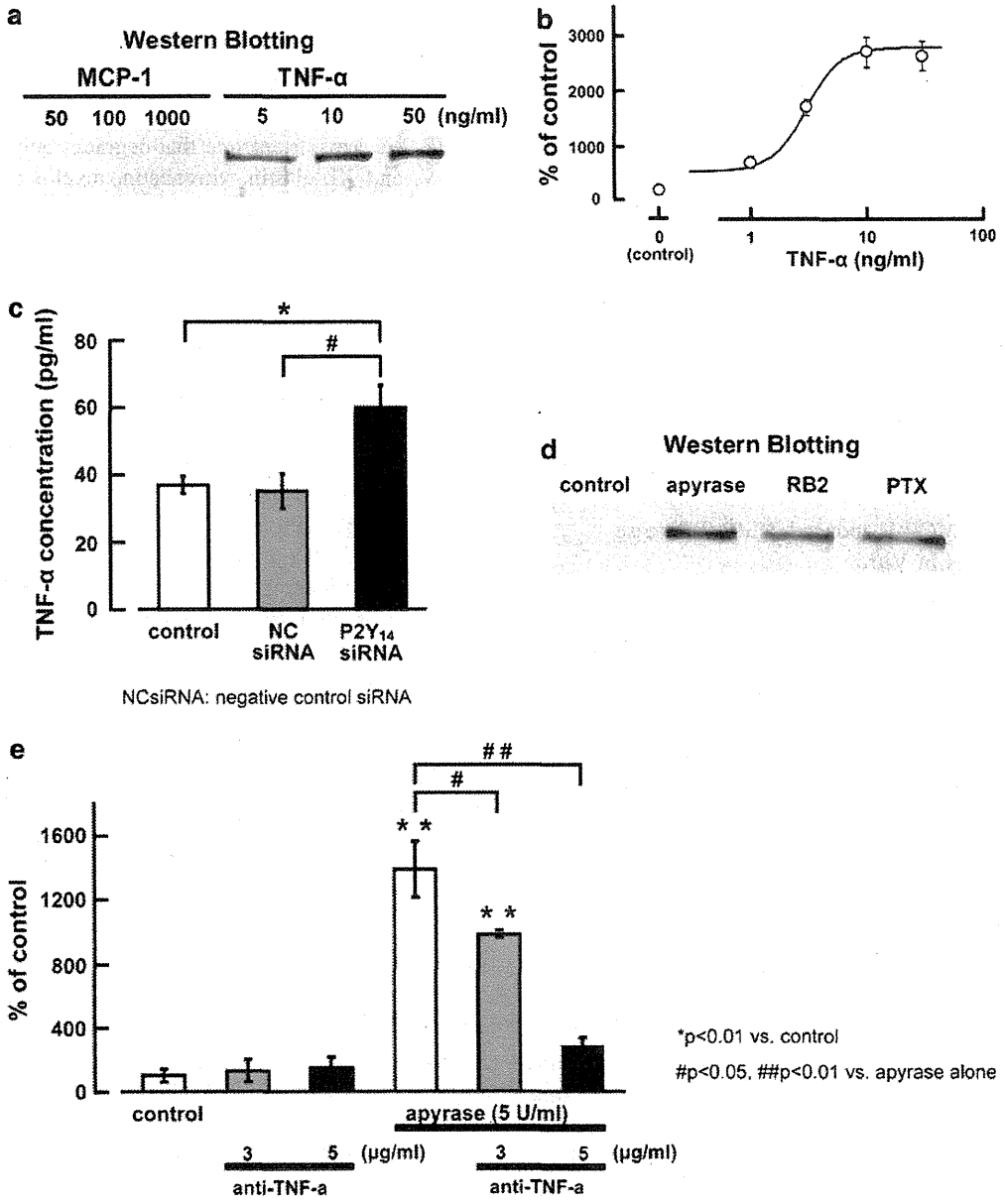

Fig. 4 MMP-9 induction by the inhibition of $P2Y_{14}$ receptors was mediated by TNF-α in astrocytes. **a** Astrocytes were treated with MCP-1 (50, 100, 1,000 ng/ml) or with TNF-α (5, 10, 50 ng/ml) for 24 h at 37 °C. The tissue culture supernatants were concentrated and studied by western blotting. TNF-α but not MCP-1 released MMP-9 in astrocytes. The data show representative results obtained form 3 independent experiments. **b** Concentration-dependency of TNF-α-evoked MMP-9 production was studied by quantitative RT-PCR. Astrocytes were stimulated with various concentrations of TNF-α for 12 h at 37 °C, and then total RNA was extracted for quantitative RT-PCR. Data are mean ± SEM of three independent experiments. **c** Inhibition of $P2Y_{14}$ receptors by siRNA resulted in release of TNF-α in supernatants. Astrocytes were treated with negative control sirRNA (NC siRNA) (0.5 nM) or $P2Y_{14}$ siRNA (0.5 nM) for 60 h, and then TNF-α that was released into the medium was measured using an Endogen Rat TNF-α ELISA kit. **d** Treatment of astrocytes with the ATP-degrading enzyme apyrase (20 U/ml), the P2 receptor antagonist RB2 (10 μM) or PTX (1 μg/ml) for 24 h at 37 °C resulted in the release of TNF-α into the supernatants. The release of TNF-α was assessed by western blotting. **e** Induction of MMP-9 by apyrase was inhibited by anti-TNF-α antibody. Astrocytes were stimulated with apyrase (5 U/ml) with and without anti-TNF-α neutralization antibody (3 and 5 μg/ml) before and during apyrase for 12 h, and then total RNA was extracted for quantitative RT-PCR analysis. Data are mean ± SEM of triplicate measurements. The same experiments were repeated 3 times and similar results were obtained. *Asterisks* show significant difference in MMP-9 production between control and apyrase-treated astrocytes ($^{**}p < 0.01$ vs. control). $^{\#}$ and $^{\#\#}$ show significant difference in MMP-9 production between apyrase-treated and apyrase plus anti-TNF-α-antibody-treated astrocytes ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. apyrase alone)

(Koizumi et al. 2003), UTP (Lazarowski et al. 2000),s and nucleotide-sugars such as UDP-glucose (Lazarowski et al. 2003a, b) constitutively, suggesting that astrocytes and adjacent cells receive P2 receptor-mediated signals in a tonic fashion. To investigate the function that the constitutive release of nucleotides or nucleotide-sugars from astrocytes may possess, we deprived astrocytes of the tonic P2 receptor-mediated signals by treating the cells with apyrase, RB-2, or PTX, and found that the treatments caused astrocytes to release enormous amounts of MMP-9. The data revealed the importance of the constitutive, basal release of nucleotides or nucleotide-sugars from astrocytes to maintain a "normal" status.

Our experiments using RB-2 and PTX strongly suggested that the cultured astrocytes constitutively receive signals through Gi-coupled P2Y receptors to suppress MMP-9 expression (Figs. 1, 2). Among the P2Y receptors, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ are known to be coupled to Gi (Chambers et al. 2000; Hollopeter et al. 2001; Marteau et al. 2003). Our data with $P2Y_{12}$ and $P2Y_{13}$ inhibitors (ARC-69931 and ARC-66096) indicated that these two receptors are not responsible for the MMP-9 production from astrocytes (Fig. 2). However, knockdown of $P2Y_{14}$ receptors by siRNA clearly showed that $P2Y_{14}$ receptors are responsible for suppression of MMP-9 production. The $P2Y_{14}$ receptor, originally known as KIAA001(Chambers et al. 2000), is a unique receptor that is activated by nucleotide-sugars such as UDP-glucose, UDP-galactose, UDP-glucuronic acid, and UDP-*N*-acetylglucosamine. The $P2Y_{14}$ receptor is a relatively broadly expressed receptor that is prominently associated with immune and inflammatory cells. In the CNS, the $P2Y_{14}$ receptor is specifically expressed in astrocytes (Moore et al. 2003) and microglia (Bianco et al. 2005). Interestingly, in addition to ATP and UTP, nucleotide-sugars such as UDP-glucose are constitutively released from various cells including astrocytoma cells (Lazarowski et al. 2003a), suggesting that UDP-glucose should be an autocrine signal that inhibits MMP-9 production via $P2Y_{14}$ receptors in astrocytes. It has recently been reported that the rat-$P2Y_{14}$ receptor, but not the human-$P2Y_{14}$ receptor, is potently activated by UDP, as well (Fricks et al. 2008). We used rat astrocytes in this study and, therefore, should consider pyrimidine nucleotides, i.e., UDP as an additional autocrine molecule that activates $P2Y_{14}$ receptors in rat astrocytes.

So, what is the physiological relevance of the decrease in extracellular nucleotides/nucleotide-sugars or the inhibition of $P2Y_{14}$ receptors? One possibility is that they mimic the upregulation of ecto-nucleotidases. Various ecto-nucleotidases are ubiquitously expressed in all eukaryotic cells including astrocytes, and they hydrolyse extracellular nucleosides, nucleotides and nucleotide-sugars. Braun et al. (1998) have reported the sustained upregulation of extracellular ATP hydrolysis after rat brain ischemia. It is well-known that the release or the leakage of intracellular ATP causes a sharp increase in the extracellular ATP concentration after brain ischemia or epilepsy. Such a sustained upregulation of ecto-nucleotidases after ischemia, however, would finally result in a much lower concentration of extracellular nucleotides or nucleotide-sugars. Alternatively, the treatment with apyrase, RB-2, PTX, or $P2Y_{14}$ siRNA in our experiments might mimic the down regulation of $P2Y_{14}$ receptors.

Mechanisms Underlying MMP-9 Secretion by Removal of $P2Y_{14}$ Receptor-Mediated Signals

It has already been reported that astrocytes secrete MMP-9 in response to various stimuli including interleukin-1 (IL-1) (Gottschall and Yu 1995) (Wu et al. 2004), β-amyloid (Deb and Gottschall 1996; Deb et al. 2003), lipopolysaccharide (LPS) (Gottschall and Yu 1995; Lee et al. 2003; Hsieh et al. 2004) and TNF-α (Gottschall and Yu 1995; Leveque et al. 2004). In accordance with these previous reports, in this study, TNF-α stimulated the secretion of MMP-9 from astrocytes (Fig. 4). In our study, it should be noted that the production/secretion of TNF-α was triggered by the knockdown of $P2Y_{14}$ receptors. When the suppression is disturbed, TNF-α is then quickly synthesized and released, and then this cytokine acts on astrocytes in a feedback fashion and, subsequently, MMP-9 starts to be produced and released. Our present findings show that MMP-9 production induced by apyrase was significantly inhibited by anti-TNF-α antibody (Fig. 4e) would strongly support this idea. The high expression of MMP-9 and TNF-α observed here suggests that the elevated expression of these two molecules seen in many neuronal diseases and injuries may be partly due to the same mechanism. It is plausible that $P2Y_{14}$ receptor is constitutively activated, balancing and the intracellular environment to maintain the "control" situation, but when such a signal is withdrawn, the balance collapses and the cell interprets this as a "danger" signal.

In summary, our results suggest that the constitutive release of nucleotides or nucleotide-sugars from astrocytes plays an important role in maintaining the normal status of the cell, possibly through the Gi-coupled $P2Y_{14}$ receptor, and when the signal is removed from astrocytes, the cells start to release TNF-α, which then acts on astrocytes in a feedback fashion to boost MMP-9 synthesis and secretion. Our results are the first, to our knowledge, to suggest the existence of a putative signal from P2 receptors that is constitutively switched on in order to maintain the cell's apparently "normal" or "basal" status.

Acknowledgments We thank Koizumi's lab members for helpful comments and/or discussion. We also thank Dr. T. Kawanishi for kindly supplying us some reagents. ARC-69931 was from Astra-Zeneca. This study was partly supported by CREST, a grant from Takeda Science foundation, Sumitomo Foundation, a Grant-in-Aid for Scientific Research (A) and on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan, a Grant-in-Aid from Food Safety Commission Japan.

References

Arai K, Lee SR, Lo EH (2003) Essential role for ERK mitogen-activated protein kinase in matrix metalloproteinase-9 regulation in rat cortical astrocytes. Glia 43:254–264

Asahi M, Asahi K, Jung JC, del Zoppo GJ, Fini ME, Lo EH (2000) Role for matrix metalloproteinase 9 after focal cerebral ischemia: effects of gene knockout and enzyme inhibition with BB-94. J Cereb Blood Flow Metab 20:1681–1689

Asahi M, Wang X, Mori T, Sumii T, Jung JC, Moskowitz MA, Fini ME, Lo EH (2001) Effects of matrix metalloproteinase-9 gene knockout on the proteolysis of blood–brain barrier and white matter components after cerebral ischemia. J Neurosci 21:7724–7732

Backstrom JR, Lim GP, Cullen MJ, Tokes ZA (1996) Matrix metalloproteinase-9 (MMP-9) is synthesized in neurons of the human hippocampus and is capable of degrading the amyloid-beta peptide (1–40). J Neurosci 16:7910–7919

Bianco F, Fumagalli M, Pravettoni E, D'Ambrosi N, Volonte C, Matteoli M, Abbracchio MP, Verderio C (2005) Pathophysiological roles of extracellular nucleotides in glial cells: differential expression of purinergic receptors in resting and activated microglia. Brain Res Brain Res Rev 48:144–156

Braun N, Zhu Y, Krieglstein J, Culmsee C, Zimmermann H (1998) Upregulation of the enzyme chain hydrolyzing extracellular ATP after transient forebrain ischemia in the rat. J Neurosci 18: 4891–4900

Brautigam VM, Dubyak GR, Crain JM, Watters JJ (2008) The inflammatory effects of UDP-glucose in N9 microglia are not mediated by P2Y14 receptor activation. Purinergic Signal 4:73–78

Chambers JK, Macdonald LE, Sarau HM, Ames RS, Freeman K, Foley JJ, Zhu Y, McLaughlin MM, Murdock P, McMillan L, Trill J, Swift A, Aiyar N, Taylor P, Vawter L, Naheed S, Szekeres P, Hervieu G, Scott C, Watson JM, Murphy AJ, Duzic E, Klein C, Bergsma DJ, Wilson S, Livi GP (2000) A G protein-coupled receptor for UDP-glucose. J Biol Chem 275:10767–10771

Cotrina ML, Lin JH, Alves-Rodrigues A, Liu S, Li J, Azmi-Ghadimi H, Kang J, Naus CC, Nedergaard M (1998) Connexins regulate calcium signaling by controlling ATP release. Proc Natl Acad Sci USA 95:15735–15740

Cuzner ML, Gveric D, Strand C, Loughlin AJ, Paemen L, Opdenakker G, Newcombe J (1996) The expression of tissue-type plasminogen activator, matrix metalloproteases and endogenous inhibitors in the central nervous system in multiple sclerosis: comparison of stages in lesion evolution. J Neuropathol Exp Neurol 55:1194–1204

Deb S, Gottschall PE (1996) Increased production of matrix metalloproteinases in enriched astrocyte and mixed hippocampal cultures treated with beta-amyloid peptides. J Neurochem 66: 1641–1647

Deb S, Wenjun Zhang J, Gottschall PE (2003) Beta-amyloid induces the production of active, matrix-degrading proteases in cultured rat astrocytes. Brain Res 970:205–213

Dewil M, Schurmans C, Starckx S, Opdenakker G, Van Den Bosch L, Robberecht W (2005) Role of matrix metalloproteinase-9 in a mouse model for amyotrophic lateral sclerosis. NeuroReport 16:321–324

Dovlatova N, Wijeyeratne YD, Fox SC, Manolopoulos P, Johnson AJ, White AE, Latif ML, Ralevic V, Heptinstall S (2008) Detection of P2Y(14) protein in platelets and investigation of the role of P2Y(14) in platelet function in comparison with the EP(3) receptor. Thromb Haemost 100:261–270

Dzwonek J, Rylski M, Kaczmarek L (2004) Matrix metalloproteinases and their endogenous inhibitors in neuronal physiology of the adult brain. FEBS Lett 567:129–135

Franke H, Verkhratsky A, Burnstock G, Illes P (2012) Pathophysiology of astroglial purinergic signalling. Purinergic Signal 8:629–657

Fricks IP, Maddileti S, Carter RL, Lazarowski ER, Nicholas RA, Jacobson KA, Harden TK (2008) UDP is a competitive antagonist at the human P2Y14 receptor. J Pharmacol Exp Ther 325:588–594

Fumagalli M, Brambilla R, D'Ambrosi N, Volonté C, Matteoli M, Verderio C, Abbracchio MP (2003) Nucleotide-mediated calcium signaling in rat cortical astrocytes: role of P2X and P2Y receptors. Glia 43:218–230

Gottschall PE, Deb S (1996) Regulation of matrix metalloproteinase expressions in astrocytes, microglia and neurons. Neuroimmunomodulation 3:69–75

Gottschall PE, Yu X (1995) Cytokines regulate gelatinase A and B (matrix metalloproteinase 2 and 9) activity in cultured rat astrocytes. J Neurochem 64:1513–1520

Gu Z, Cui J, Brown S, Fridman R, Mobashery S, Strongin AY, Lipton SA (2005) A highly specific inhibitor of matrix metalloproteinase-9 rescues laminin from proteolysis and neurons from apoptosis in transient focal cerebral ischemia. J Neurosci 25:6401–6408

Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB (1999) ATP released from astrocytes mediates glial calcium waves. J Neurosci 19:520–528

Hayakawa K, Pham LD, Katusic ZS, Arai K, Lo EH (2012) Astrocytic high-mobility group box 1 promotes endothelial progenitor cell-mediated neurovascular remodeling during stroke recovery. Proc Natl Acad Sci USA 109:7505–7510

Haydon PG (2001) GLIA: listening and talking to the synapse. Nat Rev Neurosci 2:185–193

Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 409:202–207

Homolya L, Steinberg TH, Boucher RC (2000) Cell to cell communication in response to mechanical stress via bilateral release of ATP and UTP in polarized epithelia. J Cell Biol 150:1349–1360

Hsieh HL, Yen MH, Jou MJ, Yang CM (2004) Intracellular signalings underlying bradykinin-induced matrix metalloproteinase-9 expression in rat brain astrocyte-1. Cell Signal 16:1163–1176

Hsieh HL, Wang HH, Wu WB, Chu PJ, Yang CM (2010) Transforming growth factor-beta1 induces matrix metalloproteinase-9 and cell migration in astrocytes: roles of ROS-dependent ERK- and JNK-NF-kappaB pathways. J Neuroinflamm 7:88

Jiang X, Namura S, Nagata I (2001) Matrix metalloproteinase inhibitor KB-R7785 attenuates brain damage resulting from permanent focal cerebral ischemia in mice. Neurosci Lett 305:41–44

Kaneko N, Marin O, Koike M, Hirota Y, Uchiyama Y, Wu JY, Lu Q, Tessier-Lavigne M, Alvarez-Buylla A, Okano H, Rubenstein JL, Sawamoto K (2010) New neurons clear the path of astrocytic processes for their rapid migration in the adult brain. Neuron 67:213–223